Molecular Characterization of the Induction of Cell Cycle Inhibitor p21 in Response to Inhibition of the Mitotic Kinase Aurora B

Untersuchungen zur Induktion des Zellzyklusinhibitors p21 nach Inhibition der Mitotischen Kinase Aurora B

Doctoral thesis for a doctoral degree
at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg,
Section, Biomedicine

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Date of Public Defence: ……

Date of Receipt of Certificates: ……

Substantial parts of this thesis were published in following two articles:

1) Geeta Kumari, Tanja Ulrich, Michael Krause, Florian Finkernagel and Stefan Gaubatz. “Induction of p21CIP1 and cell cycle arrest after inhibition of Aurora B kinase is attributed to aneuploidy and reactive oxygen species.” J Biol Chem. 2014 April 29; doi: 10.1074/jbc.M114.555060.

2) Geeta Kumari*, Tanja Ulrich*, and Stefan Gaubatz. "A role for p38 in transcriptional elongation of p21CIP1 in response to Aurora B inhibition." Cell Cycle. 2013 Jul 1;12(13):2051-60. doi: 10.4161/cc.25100. Epub 2013 Jun 6. *These authors contributed equally to this work.

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1 Introduction

1.1 The mammalian cell cycle and its regulation

The mammalian cell cycle consists of temporally distinct phases that include DNA replication (S-phase) and cell division or mitosis (M-phase) separated by two gap phases (G1 and G2-phase), which allow time for DNA repair and replication errors (Fig. 1). G1 phase (occurring between M-phase and S-phase) is a critical stage during cell cycle, as during this phase the cell is responsive to various metabolic, stress and extracellular signals and the critical decision to enter S-phase (in other words, to cross the restriction point, R) is made, which commits the cell for the rest of the cell cycle (Pardee 1974). The next gap phase G2 is between S-phase and M-phase, which monitors the completion of DNA replication and genomic integrity before the cell starts dividing. The final phase of the

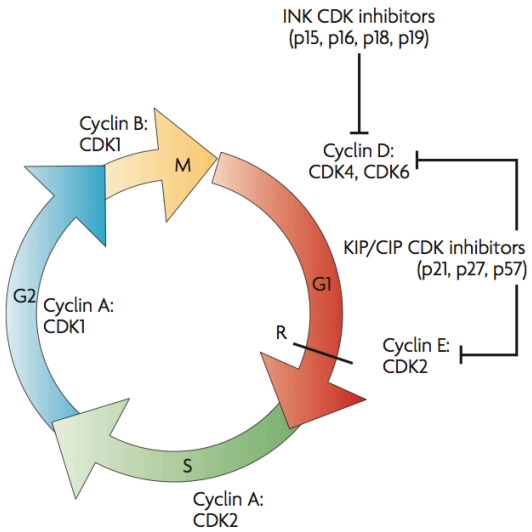

Figure 1: The eukaryotic cell cycle and its regulation by cyclin-CDK complexes and CDK inhibitors (CKIs).
Figure taken from (Dehay and Kennedy 2007).

cell cycle, M-phase consists of mitosis (division of nucleus) and cytokinesis (division of cytoplasm). Mitosis is further divided into five distinct phases, prophase, prometaphase, metaphase, anaphase and telophase. During prophase the chromosomes condense and centrosomes move apart towards opposite spindle poles. Subsequently nuclear envelope breakdown occurs. During prometaphase, kinetochores (molecular structures at the centromeres of the chromosomes) capture the microtubules originating from both spindle poles. By the time the cell enters metaphase, all the chromosomes are aligned at the metaphase plate in equatorial plane. During anaphase the sister chromatids move towards the opposite poles of the cell. Telophase is comprised of reformation of nuclear

envelope around the daughter chromosomes at poles and chromosome decondensation. Finally, formation of a contractile ring at the midbody separates the parent cell into two daughter cells by the process of cytokinesis (Norbury and Nurse 1992).

The orderly progression of mammalian cell cycle is regulated by a family of serine/threonine kinases known as cyclin dependent kinases (CDKs), which form active hetrodimeric complexes with cyclins (Morgan 1997). For example during early G1, CDK4 and CDK6 form active complex with cyclin D, during late G1 CDK2 forms complex with cyclin E1 and E2, during S phase CDK2 is activated by cyclin A1 and A2, CDK1 controls entry into M-phase along with cyclin A and finally during M-phase CDK1 forms complex with cyclin B (Fig. 1) (Malumbres and Barbacid 2009). Besides being controlled by the fluctuating levels of cyclins during the cell cycle, CDK activity is also controlled by CDK inhibitors (CKIs). There are two families of CKIs, the INK4 family (composed of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$) which inhibit CDK4 and CDK6 and hence only affect G1-S transition and CIP/KIP family (composed of $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) which affect the binding of cyclins D, E, A with their respective CDKs and hence affect both G1-S and G2-M transitions (Sherr and Roberts 1999).

In addition to being regulated by CDKs, the coordinated progression of cell cycle is also controlled by various other kinases, for example, checkpoint kinases (Chk), Polo like kinases (Plk) and Aurora family of kinases. Checkpoint kinases (Chk1 and Chk2) are activated in response to DNA damaging agents and provide cells time to repair the damage by inducing cell cycle arrest (Bartek and Lukas 2003). Plk1 is required for CDK1/Cyclin B activation, centrosome maturation, and spindle assembly as well as cytokinesis (Petronczki et al. 2008). Aurora kinases are implicated in mitosis and meiosis and play a key role in faithful segregation of the diploid content of genome into two daughter cells (see section 1.3).

Genomic integrity is maintained by three major checkpoints during cell cycle, the G1-S checkpoint, G2-M checkpoint and spindle assembly checkpoint. G1-S checkpoint is activated due to DNA damage or DNA replication stress and is mediated via ATM/ATR-Chk2(Chk1)-p53-p21 pathway, which arrests cells in G1 phase of the cell cycle. Any unrepaired damage in previous S/G1 phase or DNA damage in G2 phase activates the G2-M checkpoint, which inhibits the activity of cyclin B-CDK1 complex and hence prevents entry into mitosis until the damage is repaired (Kastan and Bartek 2004). Spindle assembly checkpoint (SAC) is activated in presence of unattached kinetochores during metaphase to anaphase transition which targets the anaphase promoting complex (APC/C) and prevents cell cycle progression until all the chromosomes are accurately

bioriented which in turn ensures accurate segregation of genome (Musacchio and Salmon 2007).

1.2 Transcriptional regulation during cell cycle

Transcription during cell cycle generates the molecular components required for various essential processes (e.g. DNA replication, chromosome segregation etc.) and replenishes the proteins degraded during cell cycle progression and cell division. To regulate the expression of genes in a periodic manner, the process of transcription is intricately regulated and coupled to post-translational regulation during cell cycle and this whole mechanism is highly conserved across metazoans (Whitfield et al. 2002; Rustici et al. 2004; Jensen et al. 2006). Of all the cyclically regulated proteins in cell, the most important ones are cyclins, which along with CDKs regulate the expression of a large number of genes at critical transitions along with E2F transcription factors (Koepp et al. 1999; Murray 2004). Transcription mainly occurs during G1-to-S, G2-to-M and M-to-G1 transition, of which transcription during M-to-G1 phase transition is the least explored (in humans) while during G1-to-S transition is the most studied. This is because of the important role of G1-S transition in regulating the 'restriction point' during the G1 phase of the cell cycle, which is deregulated in most of the cancers and is mainly regulated by E2F/pRb pathway (Weinberg 1995; Sherr 1996). E2F are a family of transcription factors whose target gene expression is regulated by pocket proteins (pRb, p130 and p107) (Dimova and Dyson 2005; Heuvel and Dyson 2008). Some of the E2F family members function as transcriptional activators (E2F1, E2F2 and E2F3A) whereas others function as transcriptional repressors (E2F3B, E2F4-8). However recent evidences suggest that they can switch their function from activation to repression and vice-versa (Chong et al. 2009; Lee et al. 2011; Weijts et al. 2012).

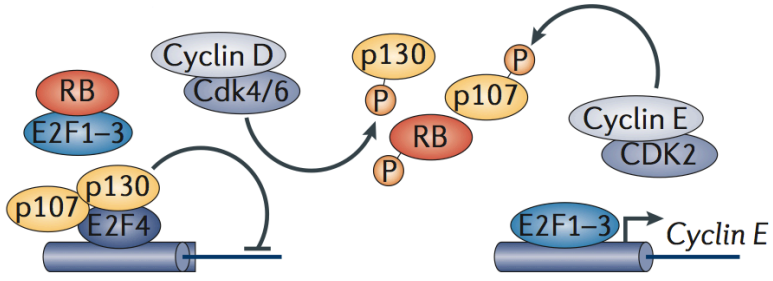

Figure 2: G1-S control by E2F-pocket protein complexes.
Figure adapted from (Bertoli et al. 2013).

pRb binds to activator E2Fs and repressor E2Fs (E2F4 and E2F5) are bound by p130 and p107 to repress transcription during early G1 (Takahashi et al. 2000). Phosphorylation of pocket proteins by G1 cyclin-CDKs dissociates them from their respective E2F partners and this in turn causes dissociation of repressive E2Fs from promoters allowing the activator E2Fs to bind to these promoters and hence activate target gene expression required for G1-S transition (Fig. 2) (Takahashi et al. 2000; Balciunaite et al. 2005). Once cells pass through this restriction-point, they initiate DNA replication and enter into S phase.

RNA Polymerase II (RNAPII) performs transcription of all the coding genes in eukaryotes. RNAPII catalytic core (composed of 12 subunits), associates with general transcription factors (GTFs) such as TATA binding protein (TBP), TBP associated factors, TFIIB, TFIIE, TFIIF and TFIIH at the promoters of genes to regulate their expression (Hahn 2004). Transcription by RNAPII is regulated by phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNAPII (Phatnani and Greenleaf 2006). This phosphorylation of CTD is in turn regulated during the cell cycle as various CTD kinases are members of the cyclin-dependent kinase (cdk) superfamily, including $p34^{cdc2}$ (cdk1), cdk7, cdk8, and cdk9. Cdk7, a component of the general transcription factor TFIIH phosphorylates CTD at serine 5 and serine 7 and is required for promoter clearance (Akhtar et al. 2009). Cdk8 functions as a part of mediator complex to phosphorylate CTD at serine 5 (Galbraith, Donner, and Espinosa 2010). Cdk9 functions as a part of PTEFb (positive transcription elongation factor) complex to phosphorylate CTD at serine 2 and converts it into elongating form (Price 2000). $p34^{cdc2}$ phosphorylates CTD to inhibit transcription in vitro in yeast (Gebara et al. 1997).

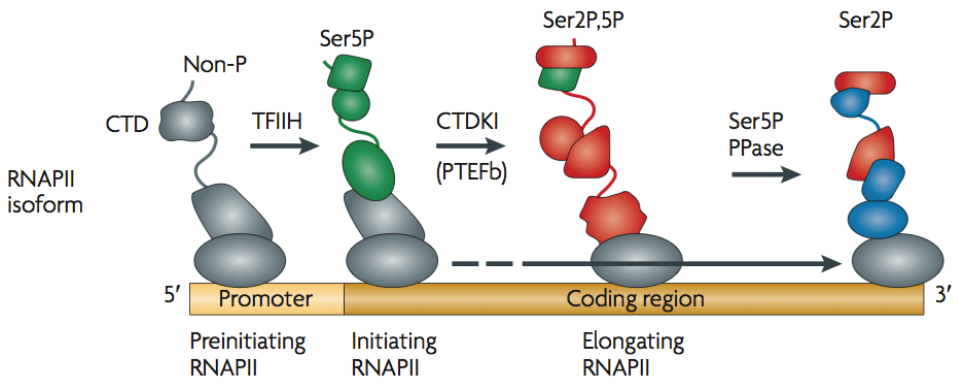

Figure 3: Differential phosphorylation of CTD of RNA Polymerase II during transcription cycle.
Figure taken from (Sutherland and Bickmore 2009).

Hence, cyclin-CDK complexes are the connecting link between cell cycle and RNAPII transcription. Phosphorylation state of CTD dictates the transcriptional stage (pre-initiation, initiation and elongation) of RNAPII (Fig. 3) (Egloff et al. 2012).

Besides phosphorylating RNAPII at CTD, the cyclin-CDK complexes can phosphorylate transcription factors themselves. For example, cyclinA-Cdk2 phosphorylates E2Fs decreasing their DNA binding ability and hence transcription (Krek et al. 1994). Thus the transcription machinery and cell cycle are intricately related and regulated by cyclin-CDK complexes through phosphorylation of pRb, E2F and CTD of RNAPII (Dynlacht 1997; Bregman et al. 2000).

1.3 Aurora kinases and there role in cancer

The Aurora family of kinases was initially discovered in *Drosophila melanogaster*, where two family members are present. In yeast there is only one representative of this family known as Ipl1p, whereas mammals have three family members, Aurora A, Aurora B and Aurora C. In case of mammals, the three family members share around 70 % homology in their C-terminal catalytic domains (Fig. 4). The mammalian family members are closely related to AGC (cAMP-dependent, cGMP-dependent, protein kinase C) family of serine/threonine kinases and share a common consensus phosphorylation motif ([R/K]x[S/T]Φ, in which x can be any amino acid and Φ is a hydrophobic residue) (Gold et al. 2006; Alexander et al. 2011). Their expression levels peaks during mitosis, during which each member has a distinct subcellular localization and function, ensuring that the full complement of genome is divided equally to future generations.

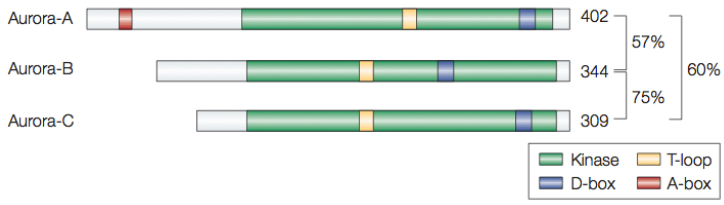

Figure 4: Domain structure of Aurora family of kinases.
Schematic representation of domain structure of human Aurora A, B and C. There size is represented (in amino acid numbers) on right and the numbers in percentage indicate sequence identities. The kinase domains are in green while the activating T-loops are shown in red. The destruction box (D-box, blue) and the D-box activating domain (DAD, or A-box, red) are responsible for degradation of Aurora A but not for Aurora B and C. A-box is absent in Aurora B and Aurora C. Figure taken from (Keen and Taylor 2004).

Despite the striking similarity in the catalytic domains of the three members of the Aurora family in mammals, they have different localizations and functions during cell cycle progression (for details see Fig. 5). Aurora A localizes to centrosomes and mitotic spindle and has a function in centrosome maturation and separation as well as in spindle assembly (Hirota et al. 2003; Barr and Gergely 2007). It's inhibition leads to mitotic arrest. On the other hand Aurora B localizes on chromosomes and midbody and is required for spindle assembly checkpoint and cytokinesis (Carmena and Earnshaw 2003; Vader 2006). Inhibition of Aurora B leads to a catastrophic mitosis, leading to G1 arrest and cell death by apoptosis.

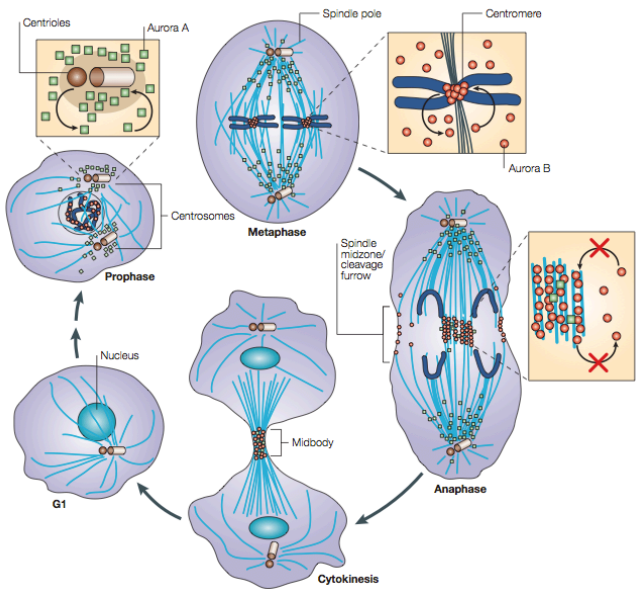

Figure 5: Subcellular localizations of Aurora kinases A and B during mitosis.
The schematic shows the relative localizations of Aurora A and Aurora B during mitosis. During prophase Aurora A (in green) is concentrated at centrosomes, and Aurora B (in red) localizes along the chromosome arms. By metaphase, Aurora A is on microtubules near the spindle poles while Aurora B is at kinetochores near to centromeres. As the cells enter Anaphase, Aurora A is on the polar microtubules and Aurora B becomes highly concentrated at the spindle midzone at the cell cortex near the site of the cleavage furrow. Finally during cytokinesis both the kinases are concentrated at the midbody and there levels drop as the cell exits mitosis. Figure taken from (Carmena and Earnshaw 2003).

Information about Aurora C remains scarce. It is mainly expressed in male germline and can take over the function of Agurora B in its absence by associating with the chromosomal passenger complex (CPC) (Sasai et al. 2004; Avo Santos et al. 2011; Fernandez-Miranda et al. 2011). Recently it was shown that overexpression of Aurora C causes degradation of Aurora B (Lin et al. 2014).

1.3.1 Role of Aurora B in cell cycle

Aurora B has various functions during the cell cycle as a part of the chromosomal passenger complex (CPC) (Ruchaud et al. 2007; Carmena et al. 2012). CPC consists of the enzymatic component Aurora B, a scaffold protein inner centromere protein (INCEP) and two non-enzymatic subunits Survivin and Borealin. The protein stability of each subunit of CPC depends on protein-protein interactions within the complex, hence knockdown of any of the components or chemical inhibition results in a similar phenotype in wide range of organisms (Adams et al. 2001; Biggins and Murray 2001; Lens et al. 2003; Gassmann 2004; Vader et al. 2006; Klein et al. 2006; Xu et al. 2009; Kelly et al. 2010). Aurora B has three important functions during the cell cycle:

1) Chromosome condensation - At the beginning of mitosis, in prophase Aurora B mediates sister chromatid separation by causing cohesin dissociation from chromosome arms (Giménez-Abián et al. 2004; Dai et al. 2006; Nishiyama et al. 2013), loading of Condensin I complex on the chromosome arms (Lipp et al. 2007), or by phosphorylating H3. Histone H3 is a substrate of Aurora B, which gets phosphorylated at Ser10 during mitosis and is a widely used mitotic marker (Crosio et al. 2002; Hirota et al. 2005). H3S10 is necessary for chromosome condensation in *Drosophila* (Giet and Glover 2001), but in humans this modification is implicated in chromosome condensation as well as dissociation of HP1 from chromatin during mitosis (Hirota et al. 2005; Crosio et al. 2002). In mammalian cells an additional phosphorylation at Ser28 is required for chromosome condensation (Goto et al. 2002).
2) Spindle assembly checkpoint - The spindle assembly checkpoint (SAC) prevents the metaphase to anaphase transition until all the chromosomes are accurately bi-oriented (Musacchio and Salmon 2007). During pro-metaphase and metaphase Aurora B becomes highly concentrated at inner centromeres and performs ‘error correction’ by regulating microtubule-kinetochore attachments through phosphorylation of a number of substrates like, MCAK (Lan et al. 2004), KNL1/Mis12/Ndc80 complex (Welburn et al. 2010; Chan et al. 2012) and Mps1 (Biggins 2001; Santaguida et al. 2011; Saurin et al. 2011). Aurora B resolves syntelic attachments and converts them to amphitelic attachments (for details see Fig. 6). Aurora B generates unattached kinetochores which are sensed by SAC and hence keeps this mitotic checkpoint active until all the incorrect attachments are resolved (Ditchfield et al. 2003; Hauf et al. 2003; Nezi and Musacchio 2009; Maldonado and Kapoor 2011).

3) Cytokinesis – The final stage of cell cycle is cytokinesis, which generates two daughter cells by formation of actomyosin ring at the cell equator. Cytokinesis requires proper functioning of the centralspindlin complex [composed of GTPase activating protein (GAP) MgcRacGAP and a kinesin, MKLP1] (Zhao and Fang 2005; Neef et al. 2006) whose activity in turn is regulated by Aurora B (Minoshima et al. 2003; Touré et al. 2008; Guse et al. 2005; Douglas et al. 2010). Besides this, Aurora B also phosphorylates other cytoskeletal proteins, like vimentin (Goto et al. 2003), myosin II regulatory light chain (Murata-Hori et al. 2000), desmin and GFAP (Kawajiri et al. 2003) required for abscission of the cell at correct time (Norden et al. 2006; Ozlu et al. 2010).

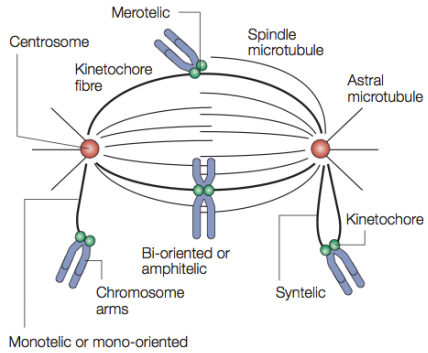

Figure 6: Chromosome bi-orientation at metaphase plate.
Schematic representation of different types of kinetochore-microtubule attachments during metaphase. Correctly bi-oriented chromosomes show amphitelic attachment (with the sister kinetochores attached to opposite poles, lower center). Mal-oriented chromosomes show monotelic/mono-oriented attachment (only one kinetochore is attached to one pole, lower left), merotelic attachment (one kinetochore is attached to both poles, upper center) or syntelic attachments (both kinetochores are attached to the same pole, lower right). Figure taken from (Keen and Taylor 2004).

Although Aurora B mainly functions in mitosis, a recent study reported it's function in interphase where Aurora B phosphorylates and mediates degradation of p53 (Gully et al. 2012). Mice in which one allele of Aurora B is disrupted develop tumors, signifying the importance of Aurora B in maintaining genome integrity (Fernandez-Miranda et al. 2011).

1.3.2 Regulation of Aurora B kinase function

Aurora B is highly regulated during the cell cycle to execute an orderly and timely phosphorylation of its substrates. The mechanisms that regulate Aurora B are discussed below:

- Phosphorylation- In order to have kinase activity, Aurora B must be phosphorylated at a key threonine residue (T232) in its T-loop. This is accomplished by auto-phosphorylation induced by a conformational change upon interaction of Aurora B with the C-terminal IN-box of INCEP (Yasui et al. 2004; Sessa et al. 2005). Further the phosphorylation of substrates by Aurora B is counteracted by antagonistic phosphatases protein phosphatase 1 (PP1) and protein phosphatase 2A during mitosis (Liu et al. 2010; Foley et al. 2011).
- Localization- Substrate specificity of Aurora B during mitosis is determined by its differential localization which is dictated by its interaction with various protein partners. For example, two non-enzymatic proteins Survivin and Borealin along with INCEP, target Aurora B to different sites such as chromosome arms and inner centromere by docking to these sites and hence function as 'passenger proteins' during cell division (Vader et al. 2006).
- Ubiquitin mediated proteolysis- Aurora B is targeted for degradation by the E3 ubiquitin ligase, anaphase-promoting complex/cyclosome (APC/C). APC/C mediates proteasomal degradation of Aurora B by ubiquitylation in conjunction with cdc20 homolog 1 (Cdh1) as the cells exit mitosis to ensure G1 cells have very low Aurora B protein levels (Stewart and Fang 2005).

1.3.3 Role of Aurora B kinase in cancer and inhibitors against Aurora B

Cancer is a disease whose characteristic features include chromosomal rearrangements and aneuploidy. As Aurora B has key functions in spindle assembly, mitotic checkpoint and chromosome segregation, it is reported to be deregulated in a wide range of cancers for example, NSCLC (non small cell lung cancer), colon and pancreatic cancer to name a few (Bischoff et al. 1998; Adams et al. 2001). Both the activity as well expression levels of Aurora B are increased in cancer and this is associated with poor prognosis as well. Also ectopic expression of Aurora B has been reported to cause transformation of cells in culture (Ota et al. 2002). This extensive correlation of Aurora B with cancer provides the basis for its importance as a target for chemotherapy (Keen and Taylor 2004; Girdler 2006; Gully et al. 2010). A variety of chemical inhibitors against Aurora B have been developed so far, some of the examples being ZM447439 (Ditchfield et al. 2003), Hesperadin (Hauf et al. 2003), VX680 (Harrington et al. 2004) and AZD1152 (Mortlock et

al. 2007). These chemical compounds are competitive inhibitors of the ATP binding site of Aurora B.

ZM447439 is a quinazoline derivative and was initially thought to inhibit both Aurora A and B. But further in vitro studies revealed that it had 20 times more specificity towards Aurora B and the cellular phenotypes were consistent with Aurora B inhibition (Ditchfield et al. 2003).

AZD1152, a member of 5-acetanilide-substituted 3-aminopyrazoles series is a highly selective Aurora B kinase chemical inhibitor (IC50 of 0.37 nM for Aurora B in contrast to 1368 nM for Aurora A). It is a dihydrogen prodrug which is highly soluble in pH adjusted aqueous solutions and undergoes rapid conversion to active form AZD1152-HQPA in vivo (Mortlock et al. 2007; Wilkinson et al. 2007).

1.4 The cellular stress response and its relevance for cancer therapy

Cellular stress response is a defense mechanism elicited when cells are challenged with adverse conditions such as damage to DNA/protein, hypoxia, metabolic constraints, oxidative stress or oncogene activation. The severity and duration of stress determine the output, which could be either cell survival or cell death (by apoptosis, necrosis or autophagy) (Kültz 2005; Fulda et al. 2010). In addition to integrating the extracellular and intracellular stimuli, the cellular stress response also determines the efficacy and outcome of a chemotherapeutic regimen. The p53 tumor suppressor pathway and mitogen activated protein kinase pathway (MAPK) standout in this respect.

1.4.1 The p53 tumor suppressor pathway

Central to regulation of most of the stress signaling in a cell and mutated/deregulated in more than 50 % human tumors is p53, a tumor suppressor protein also known as “the guardian of genome” (Lane 1992; Vogelstein et al. 2000). p53 is a transcription factor which prevents tumor development by causing G1 cell cycle arrest, apoptosis and senescence under adverse stressful conditions by activation of a number of target genes including p21, GADD45, BAX and 53BP1 (Fig. 7) (Vousden and Prives 2009; Bieging et al. 2014). Some of the p53 functions are also independent of its transcriptional activity as summarized in Fig. 7 (Moll et al. 2005; Sengupta and Harris 2005; Suzuki et al. 2009). Elegant recent findings demonstrate that other functions of p53 in DNA repair, regulation of metabolism and oxidative stress also contribute to its tumor suppressive functions (Brady et al. 2011; Li et al. 2012; Valente et al. 2013) besides its classical functions in preventing tumor growth by cell cycle arrest, apoptosis and senescence known so far.

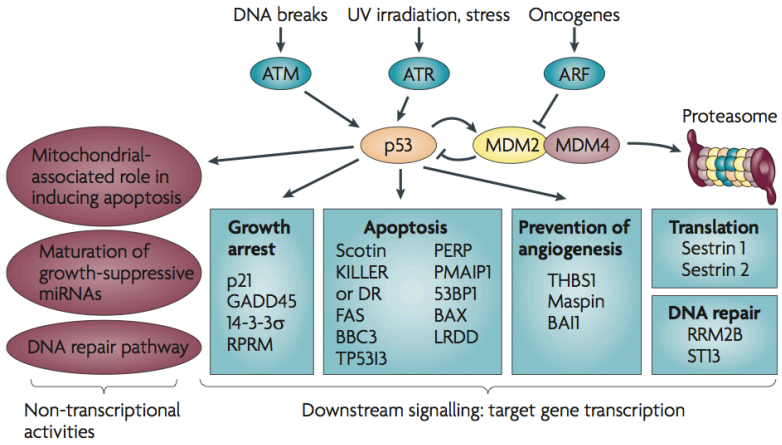

Figure 7: p53 signaling.
Figure taken from (Brown et al. 2009).

The half-life of p53 protein is very short under unstressed conditions as it is continuously degraded by the E3 ubiquitin ligase MDM2 through proteasome-mediated degradation (Momand et al. 1992; Kubbutat et al. 1997). In response to a wide range of stress (DNA damage, hypoxia, damage to mitotic spindle, heat shock, oncogenes or unfolded proteins), various signaling proteins phosphorylate p53, preventing its interaction with MDM2 and hence its degradation. This is followed by other modifications (acetylation and methylation) of p53 and it's binding as a homotetramer to its binding site (p53BS1 and 2) on the target gene promoters resulting in their induction (Vousden and Lane 2007; Riley et al. 2008; Kruse and Gu 2009).

The p53 signaling is deregulated in tumors by three common mechanisms, (a) mutations in DNA binding domain of p53 and hence preventing its binding to DNA, (b) mutations that prevent the proper folding and oligomerization of p53 and (c) overexpression of p53 regulatory proteins such as MDM2. Hence, not surprisingly all these mechanisms are being currently explored in clinic for effective cancer therapy (Muller and Vousden 2013; Hoe et al. 2014).

1.4.2 Mitogen activated protein kinase pathway (MAPK pathway)

The mitogen-activated protein kinases (MAPKs) are a family of stress kinases that serve to integrate the signals from a number of environmental and cellular stimuli to activate cellular responses. Of all the kinases in the eukaryotic genome (around 518 in humans) MAPKs are involved in most of the signaling, which is highly conserved from yeast to mammals (Qi and Elion 2005). Each MAPK cascade is composed of three tiers of kinases MAPKKK (MAP3K, MAPK-kinase-kinase), MAPKK (MAP2K, MAPK-kinase) and MAPK and kinases in each tier phosphorylate and activate the members of next tier (Fig. 8). In

humans, the MAPKs are composed of four subfamilies; (1) ERKs (extracellular signal-regulated kinases), (2) JNK/SAPK (c-Jun N-terminal related kinases or stress activated protein kinases), (3) p38-MAPK, (4) ERK5/big MAPK-1 (BMK1), classified by the MAPK at the end of the phosphorylation cascade (Raman et al. 2007). Signaling by ERK is activated by growth factors and results in cell growth and differentiation (Shaul and Seger 2007). JNK and p38-MAPK signaling is mainly activated by inflammatory cytokines, environmental stress as well as genotoxic stress and they contribute to cell cycle regulation, cell differentiation, apoptosis and inflammation (Wagner and Nebreda 2009). Growth factors as well as cellular stress activate the ERK5 cascade leading to angiogenesis, anti-apoptosis, cell proliferation and differentiation (Wang and Tournier 2006).

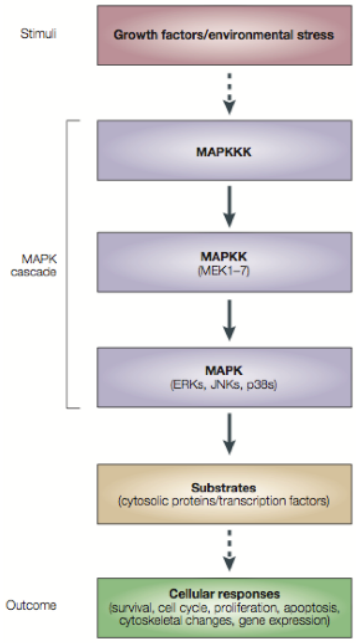

Figure 8: General cascade of MAPK pathways.
Figure taken from (Kumar et al. 2003).

The p38 MAPK family is composed of four family members: MAPK11 (p38α), MAPK12 (p38β), MAPK13 (p38γ) and MAPK14 (p38δ), which differ in their expression profiles, substrate specificity and sensitivity towards chemical inhibitors such as SB202190 and BIRB796. p38α is ubiquitously expressed in most cell types, whereas the expression of other isoforms is more restricted to certain tissues (e.g. p38β in brain, p38γ skeletal muscle, p38δ in endocrine glands). p38α, the mammalian MAPK orthologue of Hog1 (the osmosensing MAPK of *Saccharomyces cerevisiae*) is the most extensively characterized

isoform among all (Cuadrado and Nebreda 2010).

p38 MAPK, a serine/threonine kinase can function both as a tumor suppressor as well oncogene depending on the intensity and duration of stress, the cell type and cross talk with other signaling pathways. For example it halts cell cycle progression in response to DNA damage and other environmental insults (Bulavin et al. 2001), but can also induce angiogenesis under hypoxic conditions (Pages 2000). p38 MAPK has various physiological functions such as myogenic differentiation, keratinocyte differentiation and cell migration (Wu et al. 2000; Efimova 2003; Rousseau et al. 1997). p38 MAPK regulates cell cycle checkpoints at G0, G1/S and G2/M transitions during the cell cycle. By regulating the cyclin levels (cyclin A or D1), phosphorylation of retinoblastoma protein (pRb) and phosphorylation of p53 (Ser33 and Ser46), p38 has and effect on G1/S transition (Ambrosino and Nebreda 2001; Bulavin et al. 1999; Sanchez-Prieto et al. 2000). G2/M checkpoint activated by various stress stimuli is controlled by p38 MAPK through activation of MAPKAP-K2, which phosphorylates Cdc25B and Cdc25C causing their translocation into cytoplasm (Mikhailov et al. 2005; Manke et al. 2005). Besides this, p38 MAPK also plays an important role in gene expression control (Nadal et al. 2011). Regulation of p38 MAPK is mainly by dual phosphorylation, auto-phosphorylation, phosphatases, and scaffold proteins (Kyriakis and Avruch 2001; Ge 2002; Keyse 2000; Owens and Keyse 2007). Due to the critical relevance of p38 MAPK pathway in proliferation control and apoptosis, it is deregulated in array of cancer types and is a attractive clinical target (Wagner and Nebreda 2009).

1.5 Aneuploidy and cancer

Chromosomal instability (CIN), a term assigned jointly for aneuploidy (numerical/whole-chromosome alterations) and structural chromosome alterations (translocations/deletions/insertions) is a hallmark of cancer (Mertens et al. 1994; Mertens et al. 1997; Lengauer et al. 1998; Weaver and Cleveland 2007). There are different mechanisms, which can generate aneuploidy as summarized in Fig. 9; (a) Mitotic checkpoint defects - Compromised SAC (due to loss or gain of individual components such as BUBR1), can cause abrupt entry into anaphase despite the presence of unattached kinetochores resulting in daughter cells with a gain or loss of chromosome(s). In fact a number of cancers have been reported to have mutated or altered expression and gene silencing (by methylation) of SAC components (Wang et al. 2004; Kops et al. 2005; Park et al. 2007; Haruta et al. 2008). (b) Cohesion defects - Inability to separate sister chromatids during mitosis due to defects in components of the molecular machinery that keeps the sister chromatids attached (e.g. separase, cohesin, securin), also contributes to aneuploidy. Aneuploid cancers show a high correlation with somatic

mutations in the protein components required for attachment of sister chromatids (Barber et al. 2008; Zhang et al. 2008). (c) Merotelic attachments - Defects to resolve merotelic attachments during mitosis often result in misseggregations and lagging chromosomes resulting in aneuploidy (Cimini 2008). Merotelic attachments arise due to an increase in number of centrosomes or and increased stability of kinetochore-microtubule attachments (Ganem et al. 2009; Bakhoum et al. 2009). Cancer cells also show high frequency of merotelic attachments (Cimini et al. 2001; Cimini 2008).

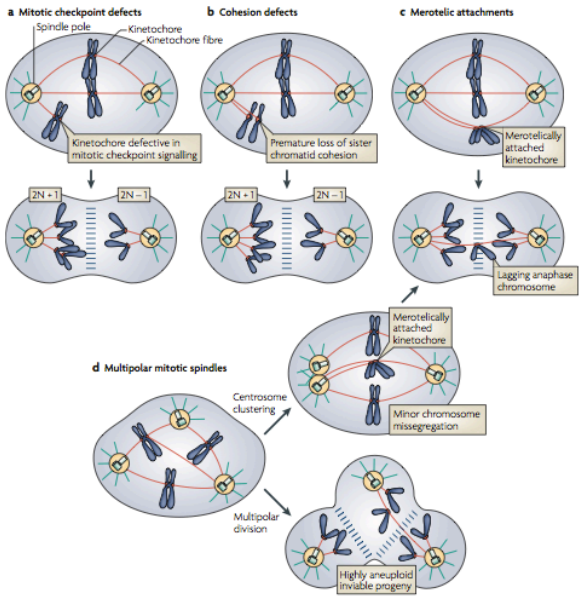

Figure 9: Different mechanisms that generate aneuploidy during mitosis.
For details see text. Figure taken from (Holland and Cleveland 2009).

(d) Multiploar mitotic spindles – Multipolar spindles arising due to multiple centrosomes allow the cells to undergo division, but often result in merotelic attachments and hence aneuploidy (Brinkley 2001; Nigg 2002; Silkworth et al. 2009). Centrosome amplification occurs in primary human tumors and is highly correlated with CIN (Pihan et al. 2003; Nigg 2006).

Extensive studies in yeast and mammalian studies implicate that aneuploidy reduces the fitness and generates a stressed state (Torres et al. 2007; Williams et al. 2008). The numerical change in chromosome number reflects into transcriptome and proteome of the aneuploid cells. Aneuploid yeast cells display a specific gene signature known as ‘’Environmental Stress Response’’ (ESR) (Torres et al. 2007; Pavelka et al. 2010). Disturbed protein balance due to aneuploidy activates the ubiquitin-proteasome pathway and chaperone pathways, which relives the protein burden on the cell by causing their degradation. This also generates a metabolic and energetic stress on the cell (Torres et

al. 2010; Stingele et al. 2012; Oromendia and Amon 2014). As aneuploidy is associated with a specific stress response and cancer cells have developed adaptations to tolerate it, aneuploidy might be an interesting target for clinic.

Aneuploidy is one of the 'hallmarks' of cancer as it is associated with around 90 % solid tumors and more than 50 % hematopoietic cancers in humans (Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer, 2014). But aneuploidy is not an accurate predictor of tumor susceptibility in mice models of mitotic checkpoint dysfunction and it is also associated with reduced proliferation rates of yeast and mammalian cells under in vitro conditions. For instance, mice which are prone to aneuploidy (due to mutations of various components of SAC), develop spontaneous tumors very late (>18 months) and only a fraction of aneuploid mice develop spontaneous tumors (Holland and Cleveland 2009), trisomic MEFs have proliferation defects and do not immortalize or undergo immortalization quite late (Williams et al. 2008) and haploid yeast strains with an extra chromosome do not proliferate (Torres et al. 2007). This discrepancy between observed strong association of aneuploidy with cancer (which grow rapidly) and adverse effects of aneuploidy on growth rate and a poor correlation with tumor susceptibility in mice is termed as 'aneuploidy paradox' (Sheltzer and Amon 2011). The most suitable explanation for this is the differences in the extracellular environments and genetic context of the tumor cells in comparison to the cells grown in culture. Under culture conditions, mammalian and yeast cells are selected for growing fast, whereas tumor cells are continuously adapting to the varying intracellular and extracellular conditions resulting in slower growth rate, which might provide additional advantages, such as acquisition of additional mutations that help them to ameliorate the imbalances in proteome due the aneuploid karyotype resulting in a more aggressive phenotype (Araujo et al. 2007; Anjomshoaa et al. 2009; Torres et al. 2008; Torres et al. 2010). In summary, aneuploidy can suppress or promote tumorigenesis depending on the cell type and genetic background (Weaver and Cleveland 2007; Holland and Cleveland 2009; Gordon et al. 2012; Holland and Cleveland 2012).

1.6 Objectives of thesis

The fact that Aurora B kinase is overexpressed in a variety of cancers and has enzymatic kinase activity (enabling it to be inhibited by chemical compounds) makes it an attractive target for cancer therapy. Currently two Aurora B inhibitors AZD1152 and BI811283 are in phase III and phase II clinical trials respectively (Marzo and Naval 2013). More detailed understanding of the cellular signaling pathways regulated by Aurora B is required to explain the side effects as well as to provide biomarkers of response, for better evaluation of these chemical inhibitors in clinic. Aims of this thesis were:

1) To study the mitotic stress signaling pathways activated due to Aurora B inhibition using two small molecule inhibitors of Aurora B (ZM447439 and AZD1152-HQPA) as chemical tools.
2) To further study the therapeutic implications of Aurora B inhibitors in combination therapy for treatment of cancer.

2 Materials and Methods

2.1 Materials

2.1.1 Chemical stocks and reagents

Unless specified, commonly used chemicals were purchased from AppliChem, Roth, Invitrogen, Invivogen or Sigma with analysis quality.

Chemical	**Stock concentration**
Agarose	Ready to use
AICAR (AMPK activator) (Biomol)	20 mM in DMSO
Ammonium persulfate (APS)	10 % in H_2O
AZD1152-HQPA (Aurora B kinase inhibitor) (Selleckchem)	10 mM in DMSO
BIRB796 (p38 MAP kinase inhibitor) (Selleckchem)	10 mM in DMSO
Bovine serum albumin (BSA)	20 mg/ml in H_2O
BrdU	10 mg/ml in 1X PBS
Cycloheximide	10 mg/ml in H_2O
Doxorubicin	1.7 mM
DMSO	Ready to use
dNTPs	2 mM dATP, dCTP, dGTP, dTTP each
DTT	1 M in H_2O
Ethidium bromide	10 mg/ml in H_2O
H2DCF-DA (Molecular Probes)	20 mM in anhydrous DMSO
Hoechst 33258	10 mg/ml in H_2O
Hoechst 33342	Ready to use
ImmuMount (Shandon)	Ready to use
KU5593 (ATM kinase inhibitor) (Selleck)	10 mM in DMSO
Low melting agarose	Ready to use
Luminol	250 mM in DMSO
MitoSox Red (Life Technologies)	5 mM in DMSO
p-Coumaric acid	90 mM in DMSO
PMSF (Phenylmethylsulphonyl-fluoride) (Roche)	10 mg/ml in isopropanol
Polybrene (Hexadimethrine bromide)	4 mg/ml in H_2O
Ponceau S solution	0.1 % Ponceau S in 5 % acetic acid
Propidium iodide (PI)	1 mg/ml in H_2O

Protease Inhibitor Cocktail Sigma	ready to use
Proteinase K	10 mg/ml in 50 mM Tris pH 8.0/ 1 mM $CaCl_2$
Protogel 30 % (Biozym)	Ready to use
Random Primer (Roche)	500 mg/ml in H_2O
RNase A	10 mg/ml in 10 mM Tris-HCl pH 7.4, 150 mM NaCl
Sodium dodecyl sulfate (SDS)	20 % (w/v) in H_2O
SB202190 (p38 MAP kinase inhibitor)	10 mM in DMSO
Tetramethylethylenediamine (Temed) 99 %	Ready to use
Trizol/Trifast (total RNA isolation reagent) (Peqlab/Thermo)	Ready to use
Thymidine	200 mM in H_2O
VE821 (ATR kinase inhibitor) (Tinib-Tools)	10 mM in DMSO
ZM447439 (Aurora kinase Inhibitor) (Enzo)	10 mM in DMSO
4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt (Tiron) (superoxide anion scavenger)	100 mM in H_2O
17AAG (HSP90 inhibitor) (Selleckchem)	20 mM in DMSO

2.1.2 Antibiotics

Antibiotic	**Stock concentration**	**Final Concentration**	**Use for cell line**
Ampicillin	100 mg/ml	100 µg/ml in LB-medium	DH5α (E-coli)
Blasticidin	10 mg/ml	10 µg/ml in DMEM	U2OS-EcoR-Neo
Puromycin	10 mg/ml	2 µg/ml in DMEM	U2OS-EcoR-Neo

2.1.3 Enzymes

Enzymes	**Company**
Absolute QPCR SYBER Green Mix	ThermoFisher
DNase I, RNase free	Roche
Fast Alkaline Phosphatase (1 U/µl)	Fermentas
M-MLV-RT Transcriptase (200 U/µl)	ThermoFisher
Pfu DNA Polymerase (2.5 U/µl)	Promega
Phusion High Fidelity DNA Polymerase (2 U/µl)	Finnzymes
Restriction Endonucleases	New England Biolabs (NEB), Fermentas
RiboLock RNase-Inhibitor (40 U/µl)	Fermentas
T4-DNA Ligase (400 U/µl)	New England Biolabs (NEB)

2.1.4 Molecular kits and Protein/DNA markers

Kits	Company
Jetstar Gel Extraction kit	Genomed
GeneRuler^{TM} DNA Ladder	Fermentas
Plasmid Midi-/Maxi-preps kit	Invitrogen
PageRuler^{TM} Prestained Protein Ladder	Fermentas
QIAquick PCR purification kit	Qiagen

2.1.5 Devices

Device	Company
Agarose gel electrophoresis system	Peqlab
Bioruptor	Diagenode
Centrifuges	Eppendorf (5417R and 5415D)
	Heraeus (Megafuge 1.0R)
FACS	Beckman Coulter (Cytomics FC500)
Incubators	Heraeus
	Nunc
Microscopes	Confocal (Nikon Eclipse Ti)
	Fluorescence (Leica DMI 6000B)
Mx3000 qPCR	Agilent technologies
Nanodrop	Theromo Scientific (Nanodrop 2000)
SDS-PAGE Gel Electrophoresis system	BIO-RAD

2.1.6 Buffers

2.1.6.1 General buffers

5X DNA Loading buffer
- 15 % Ficoll
- 0.05 % Bromophenol blue
- 0.05 % Xylene cyanol
- 0.05 M EDTA

0.5 M EDTA pH 8.0
- 0.5 M EDTA
- adjust pH to 8.0 with NaOH pellets

2X HBS
- 280 mM NaCl
- 1.5 mM Na_2HPO_4
- 50 mM HEPES-KOH, pH 7.05

Miniprep Solution S1
50 mM Tris-HCl, pH 8.0
10 mM EDTA
100 µg/ml RNase A

Miniprep Solution S2
200 mM NaOH
1 % SDS

Miniprep Solution S3
3.1 mM Potassium Acetate
adjust pH to 8.0 with glacial acetic acid

10X PBS
130 mM NaCl
3 mM KCl
64 mM Na_2HPO_4
15 mM KH_2PO_4
adjust pH to 7.4 with HCl

50X TAE buffer
200 mM Tris base
250 mM glacial acetic acid
500 mM EDTA, pH 8.0

10X TE
100 mM Tris-HCl, pH 7.5
10 mM EDTA

20X SSC
3 M NaCl
0.3 M Na-Citrate
adjust pH to 7.0 with NaOH

2.1.6.2 Buffers for whole cell lysates

TNN buffer
50 mM Tris-HCl, pH 7.5
120 mM NaCl
5 mM EDTA
0.5 % NP-40
10 mM $Na_4H_2PO_7$
2 mM Na_3VO_4
100 mM NaF
PIC (Sigma) 1:500 (added freshly)

Bradford Solution

50 mg Coomassie Brilliant Blue G
23.75 ml ethanol
50 ml 85 % (v/v) ortho-phosphoric acid
add to 500 ml H_2O
filter twice

2.1.6.3 Buffers for immunoblotting

4X Upper stock for SDS gels

33 g Tris
10 ml SDS (20 %)
add to 500 ml H_2O, adjust to pH 6.8

4X Lower stock for SDS gels

90.85 g Tris
10 ml SDS (20 %)
add to 500 ml H_2O, adjust to pH 8.8

Acrylamide buffer for SDS-gels (Protogel)

30 % (w/v) acrylamide
0.8 % (w/v) N,N'-methylenbisacrylamide

Blotting buffer (1X)

0.6 g Tris
2.258 g Glycin
150 ml methanol
add to 1 l H_2O

Blocking solution

3 % (w/v) milk powder in 0.05 % TBST, or
5 % (w/v) milk powder in 0.1 % TBST (for cell signaling antibodies)

Electrophoresis sample buffer (ESB) (3X)

300 mM Tris-HCl, pH 6.8
15 mM EDTA
150 mM DTT
12 % (w/v) SDS
15 % (w/v) glycerol
0.03 % (w/v) bromophenol blue

0.15 M NaCl

Ponceau S

0.1 % Ponceau S
5 % glacial acetic acid

SDS running buffer (10X)

144 g Glycin
30 g Tris
10 g SDS
add to 1 l H_2O

Substrate solution

10 ml 100 mM Tris-HCl, pH 8.5
50 µl 250 mM luminol
22 µl 90 mM p-coumaric acid
3 µl 30 % H_2O_2

TBST

0.05 % Tween 20 in 1X TBS, or
0.1 % Tween 20 in 1X TBS

2.1.6.4 Buffers for Chromatin Immunoprecipitation (ChIP)

Cell lysis buffer

5 mM PIPES, pH 8.0
85 mM KCl
0.5 % NP-40
PIC 1:500 (added freshly)
PMSF 1mM (added freshly)

Nuclei lysis buffer

50 mM Tris-HCl, pH 8.1
10 mM EDTA
1 % SDS
PIC 1:500 (added freshly)
PMSF 1mM (added freshly)

IP Dilution buffer

0.01 % SDS
1.1 % Triton
1.2 mM EDTA
16.7 mM Tris-HCl, pH 8.2
167 mM NaCl
PIC 1:500 (added freshly)
PMSF 1mM (added freshly)

LiCl wash buffer

0.25 M LiCl
0.5 % NP-40
0.5 % DOC
1 mM EDTA

10 mM Tris-HCl, pH 8.0
PIC 1:500 (added freshly)
PMSF 1mM (added freshly)

Elution buffer

50 mM Tris-HCl, pH 8.0
1 % SDS
10 mM EDTA

2.1.6.5 Buffers for flow cytometry (FACS)

Sodium citrate

38 mM in 1X PBS

2.1.6.6 Buffers for immunofluorescence

PSP

15 g paraformaldehyde
10 g sucrose
add to 500 ml in 1X PBS, stored at -20ºC

PBST

0.1 % Triton-X-100
500 ml 1X PBS, stored at 4ºC
and
0.2 % Triton-X-100
500 ml 1X PBS, stored at 4ºC

Blocking solution

5 % BSA in 1X PBS

2.1.6.7 Buffers for centromere Fluorescence in-situ hybridization (FISH)

Wash buffer I

0.4X SSC
0.3 % NP-40

Wash buffer II

2X SSC
0.1 % NP-40

2.1.6.8 Staining solution

Crystal violet

0.1 % crystal violet in 20 % ethanol

2.1.7 Antibodies

2.1.7.1 Primary antibodies

Antibody against	Catalog number	Origin	Application and dilution	Company
α-tubulin	T6074	Mouse monoclonal	WB 1:10,000 IF 1:200	Sigma
Aurora B	ab2254	Rabbit polyclonal	WB 1:1000	Abcam
β-actin	sc-47778	Mouse monoclonal	WB 1:5000	Santa Cruz
B-Myb (LX015.1)	none	Mouse monoclonal	WB 1:5	(Tavner et al., 2007)
BrdU-FITC	347583	Mouse monoclonal	IF 1:10	BD Bioscience
Cyclin-A (BF683)	sc-239	Mouse monoclonal	WB 1:1000	Santa Cruz
E2F-1 (C-20)	sc-193	Rabbit polyclonal	WB 1:1000	Santa Cruz
HA	MMA-101P	Mouse monoclonal	WB 1:1000 IF 1:100	HISS
IgG	I5006	Mouse monoclonal	ChIP 2 µg	Sigma
p21 (C-19)	sc-397	Rabbit polyclonal	WB 1:1000	Santa Cruz
p27	610241	Mouse monoclonal	WB 1:1000	BD Transduction Laboratories™
p38	#9212	Rabbit monoclonal	WB 1:1000	Cell signaling
p53 (DO-1)	sc-126	Mouse monoclonal	WB 1:5000 ChIP 3 µg	Santa Cruz

pH3	06-570	Rabbit polyclonal	WB 1:1000	Millipore
Phospho-ATM/ATR substrates	#2851	Rabbit monoclonal	WB 1:1000	Cell signaling
Phospho-Chk1 (Ser345)	#2348	Rabbit monoclonal	WB 1:1000	Cell signaling
Phospho-Chk2 (Thr68)	#2661	Rabbit monoclonal	WB 1:1000	Cell signaling
Phosho-Histone H2A.X (Ser139)	#2577	Rabbit monoclonal	WB 1:1000	Cell signaling
Phospho-p38	#4511	Rabbit monoclonal	WB 1:1000	Cell signaling
Phospho-Ser2-RNA Polymerase II	ab5095	Rabbit polyclonal	ChIP 3 µg	Abcam
pRb	sc-50	Rabbit polyclonal	WB 1:1000	Santa Cruz
RNA Polymerase II	sc-899	Rabbit polyclonal	ChIP 3 µg	Santa Cruz
SV40 Large T (Pab 108)	sc-148	Mouse monoclonal	WB 1:1000	Santa Cruz

2.1.7.2 Secondary antibodies

Antibody	Company	Application and dilution
anti-mouse HRP conjugated	GE Healthcare	WB 1:5000
anti-Protein A HRP conjugated	BD Biosciences	WB 1:5000
anti-rabbit HRP conjugated	Invitrogen	WB 1:5000
anti-mouse Alexa 488	Invitrogen	IF 1:500
anti-rabbit Alexa 594	Invitrogen	IF 1:500

2.1.8 Beads

Dynabeads Protein G	Life Technologies
Monoclonal Anti-HA Agarose Conjugate Clone HA-7	Sigma

2.1.9 Plasmids

2.1.9.1 Plasmids for overexpression

Internal number	Plasmid name	Description
210	pBabe-puro	Empty vector control for retroviral transfections
746	pBabe-H2B-GFP	GFP control for retroviral transfections
934	pBabe-puro-LargeT antigen-WT	Retroviral expression of Large T antigen (wild type)
1277	pBabe-puro-HA-p38alpha	Retroviral expression of HA-p38alpha
1279	pBabe-puro-HA-p38beta	Retroviral expression of HA-p38beta
1399	pBabe-puro-HA-mElongin A	Retroviral expression of mouse Elongin A
1400	pBabe-puro-LargeT-K1 mutant	Retroviral expression of Large T K1 mutant
1401	pBabe-puro-LargeT-Δ434-444 mutant	Retroviral expression of Large T Δ434-444 mutant

2.1.9.2 Plasmids for RNA knockdown

Internal number	Plasmid name	Description
652	pMSCV480-Blasticidin	Empty vector control for retroviral transfections
679	pMSCV480-shp53-Blasticidin	Retroviral expression of shp53

2.1.10 Primers

Primer oligonucleotides were purchased from Metabion or MWG.

2.1.10.1 Primers for cloning

Internal number	Sequence (5' to 3')	Target gene	Directionality
SG1785	gg**ggatcc**ATGGCGGCGGAGTC	Mouse Elongin A	Forward
SG1786	ggg**ctcgag**TTATCGCCGGGAGAATC		Reverse

Restriction sites (BamHI GGATCC / XhoI CTCGAG) are underlined.

2.1.10.2 Primers for quantitative real time PCR

All primers are for human sequences, unless indicated.

Internal number	Sequence (5' to 3')	Target gene	Directionality
SG572	GGTACTGAAGTCCGGGAACC	CCNA2	Forward
SG573	GAAGATCCTTAAGGGGTGCAA		Reverse
SG628	TCACTGTCTTGTACCCTTGTGC	p21	Forward
SG629	GGCGTTTGGAGTGGTAGAAA		Reverse
SG645	GCCCAATACGACCAAATCC	GAPDH	Forward
SG646	AGCCACATCGCTCAGACAC		Reverse
SG771	AGGCCTTGGAACTCAAGGAT	p53	Forward
SG772	CCCTTTTTGGACTTCAGGTG		Reverse
SG1511	GACTCCAAGCGCGAAAAC	MDM2	Forward
SG1512	GGTGGTTACAGCACCATCAGT		Reverse
SG1630	GATGGCCCAGAAGGAGAACT	Aurora B	Forward
SG1631	AGGCTCTTTCCGGAGGACT		Reverse
SG1632	CAGTTCTGCTCTAGGTGGAAGTC	TNFSF7	Forward
SG1633	AGGAAGAAGCGTTCGAGAGA		Reverse
SG1634	TTTGCCATCCAGAACAAGC	ATF3	Forward
SG1635	CATCTTCTTCAGGGGCTACCT		Reverse
SG1636	AGAGGAGGAAAGGCAATGAAG	SORC3	Forward
SG1637	TTGGTTGAGAGCATTAAACAGTG		Reverse
SG1638	GGGCCGTTACCCCTACATTA	SESN1	Forward
SG1639	TTCACTAAGTAGGAGCACTG		Reverse
SG1648	TACTGACCCCACCTGAGCA	FDXR	Forward
SG1649	TCGACTCTGCCTCAGTACACC		Reverse
SG1650	AAGGCACCTCTGAGAACTTCA	SERPINE1	Forward
SG1651	CCCAGGACTAGGCAGGTG		Reverse
SG1652	TTCACCCAAGTGGTGCAG	ANK1	Forward
SG1653	CTCATCCGTGAATTGCTCCT		Reverse
SG1656	CCGGATACTCACGCCAGA	GDF15	Forward
SG1657	AGAGATACGCAGGTGCAGGT		Reverse

SG1662	TTCCGTCCGCTAGGAGTCT	BLM	Forward
SG1663	GACGTTCTAGTTGCTCCTGTAGATT		Reverse
SG1666	CGACGTTATTCTGATCTCACCA	MCM3	Forward
SG1667	CAAGGGGATTGTTCTCCTCA		Reverse
SG1668	AGTAGGTGCTTGGCGGTTC	RFC3	Forward
SG1669	CACAGTAGATAACACGTGGCAAA		Reverse

2.1.10.3 Primers for Chromatin Immunoprecipitation

Internal number	Sequence (5' to 3')	Target	Directionality
SG540	GGCAGCAAGAGTCACTCCA	GAPDH2 promoter	Forward
SG541	TGTCTCTTGAAGCACACAGGTT		Reverse
SG1585	CTGTGGCTCTGATTGGCTTT	p53 binding site 1 (p21 promoter)	Forward
SG1586	CTCCTACCATCCCCTTCCTC		Reverse
SG1670	TATATCAGGGCCGCGCTG	p21 gene (-20)	Forward
SG1671	GGCTCCACAAGGAACTGACTTC		Reverse
SG1672	CCAGGAAGGGCGAGGAAA	p21 gene (+507), p21 primary transcript	Forward
SG1673	GGGACCGATCCTAGACGAACTT		Reverse
SG1675	CGTGTTCGCGGGTGTGT	p21 gene (+182)	Forward
SG1676	CATTCACCTGCCGCAGAAA		Reverse
SG1677	CCTCCCACAATGCTGAATATACAG	p21 gene (+8566)	Forward
SG1678	AGTCACTAAGAATCATTTATTGAGCACC		Reverse
SG1679	CCTGGCTGACTTCTGCTGTCT	p21 gene (+7011), p21 primary transcript	Forward
SG1680	CGGCGTTTGGAGTGGTAGA		Reverse
SG1683	TCTGTCTCGGCAGCTGACAT	p21 gene (+11443)	Forward
SG1684	ACCACAAAAGATCAAGGTGAGTGA		Reverse

2.1.11 siRNA sequences

siRNA oligos were purchased from MWG.

siRNA against	Sequence (5' to 3')	Target/Reference
ctrl	UGGUUUACAUGUCGACUAA	non targeting
Aurora B	AACGCGGCACUUCACAAUUGA	Human Aurora B, Lampson et al., 2005
pRb	Dharmacon smart pool	Human Retinoblastoma protein

2.1.12 Cell lines, cell culture media and transfection reagents

2.1.12.1 Media and additives for mammalian cell culture

DMEM (4.5 g Glucose/L-Glutamine)	Gibco®, Life Technologies
Fetal calf serum (FCS)	Gibco®, Life Technologies
OptimeM	Gibco®, Life Technologies
Penicillin/Streptomycin (10 U/µl each)	Cambrex/ Lonza
TrpLE™ Express	Gibco®, Life Technologies
Trypsin EDTA (200 mg/ml)	Gibco®, Life Technologies

2.1.12.2 Composition of media for soft agar assay

10X DMEM (20 % FCS) (50 ml)

10X DMEM	10ml
1 M Sodium bicarbonate, autoclaved	1.85 ml (3.7 %)
FCS	10 ml (20 %)
200 mM Glutamax	5 ml (20 mM)
D-gluc (dehydrated)	450 mg
Penstrep	0.5 ml
H_2O	22.65 ml

1.4 % low melting agarose, autoclaved — for base layer

0.7 % low melting agarose, autoclaved — for top layer

2.1.12.3 Human cell lines and media

All cell lines were cultured in DMEM media with 10 % FCS and 1 % Penstrep.

Cell line	Description	Reference
HCT116-WT	Human colorectal carcinoma tumor cell line (wild type p53 and p21)	(Brattain et al. 1981)
HCT116-p21-/-	Human colorectal carcinoma tumor cell line (p21 null)	(Waldman et al. 1995)
HCT116-p53-/-	Human colorectal carcinoma tumor cell line (p53 null)	(Bunz, 1998)
PlatE	Retroviral packaging cell line for generating stable cell lines by retroviral infection	(Morita et al. 2000)
U2OS	Human osteosarcoma tumor cell line	(Ponten and Saksela 1967)
U2OS-EcoR-neo	U2OS cells with ecotropic receptor for retroviral infection (neomycin resistance cassette)	Created in lab by stably expressing ecotropic receptor (neomycin resistance) in U2OS cells

2.1.12.4 Transfection reagents and cell lines

Transfection reagent	Cell line	Purpose
Lipofectamine RNAi Max	U2OS	siRNA transfection
Calcium phosphate	U2OS	Plasmid transfection
Calcium phosphate	PlatE	Plasmid transfection

2.1.12.5 Bacterial strains

E.coli DH5α- competent cells for transformation of plasmid DNA

2.1.12.6 Media for bacterial cell culture

Luria Bertani (LB) Agar 40 g powder in 1 l H_2O, autoclaved
Luria Bertani (LB) Medium 25 g powder in 1 l H_2O, autoclaved

2.2 Methods

2.2.1 Mammalian cell culture

2.2.1.1 Passaging of cells

Eukaryotic cells were cultivated in a tissue culture incubator at 37ºC with 5 % CO2. For passaging, cells were washed once with PBS and incubated with TrypLE Express (HCT116-WT, p21-/-, p53-/- cells) or Trypsin/EDTA (U2OS and PlatE cells) for a few minutes at 37ºC. The detached cells were resuspended in media and plated on new cell culture dishes.

2.2.1.2 Freezing and thawing of cells

To freeze cells, cells on 10 cm dishes were trypsinized and transferred into a 15 ml falcon tube with 10 ml fresh media. Cells were then pelleted by centrifugation for 3 min at 1200 rpm, the supernatant was discarded and the cells were resuspended in 1 ml ice-cold freeze medium (DMEM media containing 10 % DMSO) and transferred into cryotubes. Cells were stored at -80ºC for short term or in liquid nitrogen for long term.
For thawing cells, cells were quickly thawed in a 37ºC water bath. The cell suspension was mixed with 9 ml fresh medium and centrifuged for 3 min at 1200rpm. The supernatant was discarded and the pellet was resuspended in 10 ml fresh medium and seeded into 10 cm dishes.

2.2.1.3 Counting cells

Cell counting was performed using a Neubauer Chamber. The number of cells per ml in suspension was calculated using the following formula:
Cells/ml = (Cells counted/ number of counted large squares) x 10^4

2.2.1.4 Treatment of cells with reagents

All treatments were done 24 h after seeding the cells. Before treatment, the cells were fed with fresh media.

AMPK activator	Cells were treated with 200 µM AICAR for various time points.
ATM kinase inhibitor	Cells were pretreated with 5 µM or 10 µM KU5593 for 2 h before any further treatments.
ATR kinase inhibitor	Cells were pretreated with 0.1 µM or 1 µM VE821 for 2 h

	before any further treatments.
Aurora kinase inhibitors	Cells were treated with various concentrations of ZM447439 or AZD1152-HQPA for different time points.
BrdU	To label cells in S-phase cells were treated with 15 µg/ml BrdU for 2 h before fixation.
Doxorubicin	To induce DNA damage cells were treated with 1 µM Doxorubicin for 6 h or 24 h.
Hsp90 inhibitor	Cells were treated with 8 nM 17AAG for various time points.
p38 MAP kinase inhibitors	Cells were pretreated with 10 µM SB202190 or 1 µM BIRB796 for 2 h before any further treatments.
Thymidine	For cell synchronization in G1/S phase, cells were treated with 2.5 mM thymidine for 24 h.

2.2.1.5 Synchronization of U2OS cells by thymidine

For synchronization at the G1/S border, U2OS cells at 50 % confluency (seeded 24 h before) were treated with 2.5 mM thymidine for 24 h. Then, the cells were released into cell cycle by washing three times with PBS and feeding with fresh media.

2.2.1.6 Determination of cell cycle phases by Flow Cytometry

Cells in different cell cycle phases were measured by propidium iodide FACS (PI FACS). For this, cells were harvested by trypsinization and centrifuged at 1200 rpm for 5 minutes at 4ºC. Then, the pellet was washed once with ice cold PBS and the cells were fixed over night in 1 ml 80 % ethanol at -20ºC. Before measurement, cells were pelleted by centrifuging for 10 minutes at 1000 rpm followed by washing once with PBS at 4ºC. The cells were then resuspended in 500 µl 38 mM sodium citrate and 25 µl RNAse A (10 mg/ml) for 1 h at 37ºC. After this, the cells were stained with 15 µl PI (1 mg/ml) and then measured by FACS.

2.2.1.7 Transient transfection

2.2.1.7.1 Plasmid transfection with Calcium phosphate

PlatE cells were transfected using calcium phosphate. 30 µg of plasmid DNA was mixed with 50 µl of 2.5 M $CaCl_2$ and with H_2O to a final volume of 500 µl. In a 15 ml falcon tube, DNA/$CaCl_2$ mixture was added drop wise to 500 µl of 2X HBS. This solution was added slowly to the cells. After 18-24 h of incubation, cells were washed once with PBS and fed with fresh medium. 24 hours later, the virus supernatant produced was harvested for cell

infection (see section 2.2.1.6).

2.2.1.7.2 siRNA transfection with Lipofectamine RNAi Max

U2OS cells were transfected with 10-45 nM siRNA using Lipofectamine RNAi Max (Life Technologies). Before starting, cells (seeded 24 h before) were fed with fresh media without penicillin and streptomycin. siRNA was diluted in Optimem to a final volume of 250 µl (for 6 well) or 500 µl (for 6 cm dishes), mixed gently by pipetting once up and down and incubated for 5 minutes. In a separate tube, 2.5 µl Lipofecatime RNAi Max was diluted in OptimeM medium to a final volume of 250 µl (for 6 well), mixed gently by pipetting once up and down and incubated for 5 minutes (for 6 cm dishes 5 µl Lipofectamine RNAi Max was diluted to a final volume of 500 µl). Then, the siRNA/OptimeM mix was added gently to Lipofectamine RNAi Max/OptimeM mix, and mixed gently by pipetting once up and down. This complex was incubated for 20 minutes and then added gently dropwise to cells. After 24 h, cells were fed with fresh media. The cells were harvested after 48-72 h of transfection, and processed for RNA or protein analysis.

2.2.1.8 Retroviral infection of cells

For production of ecotropic viral supernatant, platE cells were transiently transfected with the plasmid of interest using calcium phosphate (see section 2.2.1.5.1). 36-48 h after transfection, the virus supernatants were harvested, filtered (0.45 µm pore size), mixed with 10 µg/ml polybrene and added to the cells (U2OS-EcoR cells seeded 18-20 h before). 24 h after infection, the cells were washed once with PBS and fed with fresh medium and selection was started 48 h after infection.

2.2.1.9 Immunofluorescence staining

For immunofluorescence staining, cells were plated on cover slips in 6-well plates. After washing once with PBS cells were fixed with PSP for 10 min at RT. Cells were then washed twice with PBS, permeabilized in PBS/0.2 % Triton-X-100 for 5 min at RT and washed once in PBS/0.1 % Triton-X-100 (PBST). Unspecific staining was minimized by blocking for 30 min with 3 % BSA (in PBS). The cells were washed 3 times in PBS (p21-staining) or 5 mM $MgSO_4$ (in PBS for BrdU-staining) and incubated with the primary antibody diluted in PBS or 5mM $MgSO_4$ (in PBS with DNAase for BrdU staining) for 1 h in a humidified chamber. The coverslips were then washed three times (3 minutes each) with PBS and incubated with the secondary antibody for 30 min in a humidified chamber. After washing with PBS for 3 times (3 minutes each) cells were stained with Hoechst 33258 (1:1000 in PBS) for 1 minute, washed once with PBS and mounted on glass slides

with ImmuMount.

2.2.1.10 ROS detection

For the detection of ROS (reactive oxygen species), cells grown on coverslips were washed once with warm PBS and incubated with 10 µM 2'-7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Molecular Probes) in PBS. After 10 minutes at 37°C, the H2DCF-DA solution was removed and the cells were incubated for 10 minutes with complete medium at 37°C. Cells were washed again with warm PBS and fixed in 4 % formalin. Nuclei were counterstained with Hoechst 33258. For detection with MitoSox Red, cells grown on coverslips were washed once with warm PBS and incubated for 10 minutes with 5 µM MitoSox Red (Molecular Probes) in PBS at 37°C. Cells were washed three times with PBS and nuclei were counterstained with Hoechst 33342 for 20 minutes. The coverslips were then washed once with PBS and mounted on glass slides. Intracellular ROS levels were visualized using an inverted microscope.

2.2.1.11 Centromere fluorescence in situ hybridisation (FISH)

Cells were trypsinized and centrifuged for 3 minutes at 1000 rpm at room temperature. The cell pellet was resuspended in 5 ml 0.8 % sodium citrate and incubated at 37ºC for 30 min. After a further centrifugation step of 3 minutes at 1000 rpm at room temperature, cells were fixed in 5 ml 3:1 methanol:glacial acetic acid solution (freshly prepared) and stored O/N at -20ºC. The fixed cells were dropped onto a slide and air-dried for 3 days (or at least for 24 h). After this, the sample slides were pretreated in 2X SSC/0.5 % NP-40 pH 7.0 at 37ºC for minutes and then dehydrated in 70 %, 85 %, and 100 % ethanol for 1 minute each. Then the slides were left to air dry at least for 30 minutes. After this 5µl of probe [5X, SE7(D7Z1)/8(D8Z1), Kreatech] against chromosome 7 (red) and chromosome 8 (green) was applied, covered with glass coverslip, sealed with fixogum and left in dark for 2 minutes. Then the sample and probe were denatured on a hot plate at 75ºC for 5 minutes in dark followed by overnight incubation at 37ºC in a humidified chamber in dark. The next day, after removing the coverslips the slides were first washed in preheated wash buffer I at 72ºC for 2 min, then washed in wash buffer II at room temperature for 1 minute. After a dehydration series (70 %, 85 %, 100 % ethanol) for 1 min each, slides were air dried and then counterstained with DAPI, and covered with coverslip.

2.2.1.12 Colony forming assay

U2OS and HCT116-WT cells were plated in a very low density on 10 cm dishes (6000 cells) or 6 well plates (700 cells/well) and treated with different drugs (ZM447439,

SB202190, AICAR, 17AAG). The cells were fed with fresh media and drug every 3 days. After 14 days, cells were fixed for 10 min with 4 % PFA, washed with tap water and air-dried. Then the cells were stained with 0.1 % crystal violet staining solution for 20 min, washed with tap water and air dried again.
For quantification, the crystal violet stain was extracted with 5 ml 10 % acetic acid by shaking for 20 minutes at room temperature. Then the extracted dye was diluted 1:4 in dH_2O and absorbance was measured at 590 nm (using 10 % acetic acid as reference).

2.2.1.13 Soft agar assay

HCT116 cells seeded in 6 cm dishes were treated with ZM447439 and AICAR. Three days later, $1x10^4$ cells were transferred to 2 ml DMEM containing 0.35 % low-gelling agarose (containing the respective drugs) and seeded in triplicate into six-well plates containing a 2-ml layer of solidified 0.7 % agarose in complete medium. After 13 days, the number of foci was scored.

2.2.2 Molecular methods

2.2.2.1 RNA isolation

Total RNA was isolated from cells by using the RNA isolation reagent Trizol/Trifast (Peqlab). After removing the medium, 1 ml Trifast was added onto the cell culture plate and cells were collected into an eppendorf tube by pippeting up and down. After 5 minutes of incubation at room temperature, 200 µl chloroform was added and thoroughly vortexed for 15 sec and further incubated for 3 minutes at room temperature. Then the tubes were centrifuged at 12000 g and 4ºC for 10 min and the upper aqueous phase was transferred to a new reaction tube. RNA was precipitated with 500 µl isopropanol at -20ºC for 1 h and then centrifuged for 10 min at 12000 g and 4ºC. The pellet was washed with 75 % ethanol (in DEPC water) and resuspended in 25 µl DEPC water.

2.2.2.2 Reverse transcription (RT)

To transcribe RNA into cDNA, 2-2.5 µg RNA was mixed with 0.5 µg random primer (0.5 mg/ ml) and brought to 10 µl with DEPC water. After incubation at 70ºC for 5 minutes, the samples were left for 1 min at 4ºC and then mixed with 5 µl M-MLV 5X reaction buffer, 6.25 µl dNTPs (2 mM), 0.5 µl Ribolock RNase inhibitor (40 U/µl), 0.5 µl M-MLV-RT (200 U /µl) and 2.75 µl H_2O. For cDNA synthesis, the samples were incubated at 37ºC for 60 min and then inactivated for 15 min at 70ºC.

2.2.2.3 Quantitative real-time PCR (qRT-PCR)

To determine the amount of a specific mRNA compared to a housekeeping gene, the following reaction was prepared:

Standard reaction mix:

12.5 µl absolute qRT-PCR SYBER Green Mix

10.5 µl H_2O

1 µl fw / rev primer mix (10 pmol/µl each)

1 µl cDNA

Standard PCR program (40 cycles):

95 °C 15 min

95 °C 15 s

60 °C 1 min

The relative expression of a gene compared to a housekeeping gene was calculated with this formula: $2^{-\Delta\Delta Ct}$

where $\Delta\Delta Ct = \Delta Ct$ (sample) – ΔCt (reference)

and ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

The standard deviation of $\Delta\Delta Ct$ was calculated with:

$s=\sqrt{(s1^2+s2^2)}$

where s1 = standard deviation (gene of interest)

and s2 = standard deviation (housekeeping gene)

The margin of error for $2^{-\Delta\Delta Ct}$ was determined by this formula: $2^{-\Delta\Delta Ct+/-s}$ and the error used for the error bars was calculated with: $2^{-\Delta\Delta Ct+/-s} - 2^{-\Delta\Delta Ct}$

2.2.3 Biochemical methods

2.2.3.1 Whole cell lysates

Cells were scraped with cold PBS and centrifuged for 5 min at 3000 rpm and 4°C. The pellet was resuspended with 10 times its amount of TNN buffer (with freshly added protease inhbitor cocktail in 1:1000 ratio) by vortexing and incubating on ice for 20 minutes. Then the lysates were centrifuged at 14000 rpm for 10 min at 4ºC to remove cell debris. The supernatant was transferred in a new reaction tube and protein was quantified by Bradford method (see section 2.2.3.2). The required amount of protein was immediately used for immunoprecipitation (see section 2.2.3.3) or boiled with 3X ESB and loaded on SDS-polyacrylamide gel for SDS-PAGE (see section 2.2.3.4). Rest of the lysate was stored at -80ºC.

2.2.3.2 Quantification of protein by Bradford method

The protein concentration was determined with the method described by Bradford (Bradford, 1976). 1 µl of whole cell lysate was mixed with 100 µl 0.15 M NaCl and 1 ml of Bradford solution. Extinction at 595 nm was measured and compared to a standard BSA dilution series.

2.2.3.3 Immunoprecipitation

For immunoprecipitation, between 0.5 and 1 mg of whole cell lysate was incubated for 4 hours or overnight, with the desired antibodies on a rotating wheel at 4°C. 40 µl of protein A- (polyclonal antibodies) or protein G-sepharose (monoclonal antibodies) were added and incubated for additional 1 h at 4 °C on the roating wheel. The beads were washed 5 times with TNN and centrifuged between the washing steps for 1 minute at 3000 rpm at 4°C. After the last wash, the supernatant was removed completely with a Hamilton syringe and the beads were resuspended in 40 µl 3X ESB and heated for 5 minutes at 95°C. Samples were stored at -20°C or directly used for electrophoresis.
In parallel, 5-10 % of the protein amount used for immunoprecipitation was heated with 3X ESB at 95 °C for 5 min and used as input.

2.2.3.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed using the discontinuous method (Laemmli, 1970). 8 - 14 % resolving gel was prepared and after polymerization, the stacking gel was poured on the top. The gel compositions were as follows:

Separating gel (10 %)	Stacking gel
6.1 ml H_2O	6.9 ml H_2O
3.7 ml 1.5 M Tris pH 8.8	1.4 ml 0.5 MTris pH 6.8
5 ml Acrylamid/Bisacrylamid	1.6 ml Acrylamid/Bisacrylamid
75 µl 20 % SDS	50µl 20 % SDS
100 µl 10 % APS	50µl 10 % APS
10 µl TEMED	10 µl TEMED

Electrophoresis was carried out in 1X SDS running buffer for about 1.5 h at constant current of 35 mA/gel. The gels were then used for immunoblotting.

2.2.3.5 Immunoblotting

The transfer of proteins onto PVDF membranes was done via electroblotting using a BioRad Wet Blot gadget. The PVDF membrane was preincubated for 1 min with 100 % methanol and rinsed with blotting buffer. The membrane was laid onto a layer of Whatman filter paper and the SDS-polyacrylamide gel was placed on the membrane, followed by a second layer of filter paper. This “sandwich” was clasped on both sides by sponges and placed in a cooled wet blotting tank (Biorad). The transfer was done for 1-2 h (depending on the size of the protein to be detected) at constant voltage of 100 V in 1X Blotting Buffer. Successful and equal transfer of proteins was visualized by staining of the membrane with a Ponceau S solution and destaining with H_2O.
To detect specific proteins with their respective antibodies, the membranes were blocked with 3 % milk powder in TBST (blocking solution) for 1 h, and then incubated overnight at 4°C with the primary antibody diluted in blocking solution. Afterwards, the membrane was washed 3 times for 5 minutes in TBST and incubated with the secondary HRP-conjugated antibody (diluted in blocking solution) for 1 h at room temperature. After 3 wash steps of 5 minutes in TBST, specific bands were detected using a Luminol-substrate-solution. The membrane was wrapped in plastic foil and exposed to an ECL-film.

2.2.3.6 Chromatin immmunoprecipitation (ChIP)

For ChIP, 4.5×10^6 cells were seeded in 15 cm dishes in 20 ml medium and 24 h later treated with various inhibitors. After 24 h, the cellular protein-DNA complexes were cross-linked by adding 540 µl 37 % formaldehyde to the dishes and incubating at room temperature (slowly shaking) for 10 minutes. The reaction was stopped by adding 2.5 ml 1

M glycine and incubated for 5 minutes at room temperature. Afterwards cells were washed two times with ice cold PBS and scraped into 15 ml falcons and then pelleted by centrifugation at 1200 rpm for 5 minutes at 4ºC. The cells were lysed in 10 times lysis buffer (depending on the pellet) for 10 min. After centrifugation for 5 minutes, at 2800 rpm at 4ºC, the nuclei were lysed in 800 µl nuclei lysis buffer for 10 min. Lysed nuclei were sonicated for 10 min using the Bioruptor with 30 sec on/ 30 sec off cycle with high intensity. Thereby chromatin was fragmented into 250-1000 bp fragments. To remove cell debris chromatin was centrifuged at 14000 rpm for 10 minutes at 4ºC and 50 µl of the chromatin was used to check the chromatin size. For this, 2 µl of 5 M NaCl and 1 µl RNase A was added to the chromatin and incubated O/N at 65ºC. After 2 h treatment with 2 µl Proteinase K (10 mg/ml) the chromatin size was analyzed on a 1.2 % agarose gel.
The remaining chromatin was diluted 1:10 with dilution buffer and 2 ml chromatin was use per IP. 20 µl of the diluted chromatin was removed as input and stored overnight at -20ºC. After addition of the antibodies the samples were incubated at 4ºC overnight on a rotating wheel. The following day immunoprecipitations were collected by adding 50 µl magnetic Protein-G Dynabeads beads to the chromatin for 1-2 hours at 4ºC. The beads were washed 7 times with 1 ml LiCl-washing buffer and eluted with 100 µl elution buffer for 15 min. A second elution step was performed with additional 150 µl elution buffer for 15 min and both supernatants were combined. To reverse the crosslinking, 10 µl 5 M NaCl and 5 µl RNase A was added to the eluted chromatin as well to the input samples and incubated overnight at 65ºC. Proteins were degraded by incubation with 2 µl proteinase K (10 mg/ml) for 2 h at 55ºC. Thereafter the DNA was purified using Qiagen PCR purification kit according to the manufacturers manual. The chromatin was eluted in 50 µl elution buffer (Qiagen).
1 µl of the purified chromatin was used for quantitative PCR analysis and precipitated samples were compared to input chromatin.

2.2.4 Molecular biology

2.2.4.1 Isolation of plasmid DNA from bacteria

2.2.4.1.1 Mini preparation

Single colonies were picked from an LB agar plate after transformation and incubated in 3 ml LB medium containing ampicillin over night in a shaker at 37°C. 1.5 ml bacterial culture was pelleted and resuspended in 200 µl S1. The bacteria were lysed by adding 200 µl S2 for 5 minutes. This reaction was neutralized with 200 µl S3. The bacterial debris was pelleted for 10 minutes at full speed at 4ºC and plasmid DNA in the supernatant was

precipitated with 500 µl isopropanol. After centrifugation for 20 minutes at 4ºC, the pellet was washed with 1 ml 70 % ethanol at 4ºC. The pellet was air dried and resuspended in 50 µl H_2O. Positive bacterial clones were identified by restriction digestion (see section 2.2.4.4.4).

2.2.4.1.2 Midi and Maxi preparation

A single colony was picked from a LB agar plate after transformation and cultured in 3 ml LB medium containing ampicillin at 37 °C for 6-8 h. 500 µl of this culture was transferred into either 100 ml (Midi preparation) or 200 ml (Maxi preparation) LB medium containing ampicillin and left growing over night in a shaker at 37°C. Plasmids were purified with Midi- or Maxi-Kits from Invitrogen according to the manufacturer's instructions.

2.2.4.2 Isolation of plasmid DNA fragments from agarose gels

Plasmid DNA was digested with the desired restriction enzymes and incubated at 37°C for 1-2 h. The restriction digest was loaded on a 0.8 – 1.4 % agarose gel and fragments were separated by electrophoresis (see section 2.2.4.4.2) at 110 V for 1-2 h. The desired bands were cut out and isolated with the JetStar gel extraction kit (Genomed) according to the manufacturer's instructions.

2.2.4.3 Isolation of PCR products after restriction

To purify PCR products after restriction digestion, the QIAquick PCR purification kit from Qiagen was used according to the manufacturer's protocol.

2.2.4.4 Standard cloning methods

2.2.4.4.1 PCR for cloning of DNA fragments

To amplify DNA fragments for cloning, the Phusion™ High Fidelity polymerase (Finnzymes) was used, which is a special proof reading polymerase and has a 3'- 5' proof reading / exonuclease activity.

Standard reaction mix:

Template DNA	100 ng
Polymerase buffer	10 µl
Phusion polymerase	0.5 µl
dNTPs (2mM)	5µl

Forward primer (10µM)	3µl
Reverse primer (10µM)	3µl
ddH_2O	add to 50µl

Standard PCR conditions:

Initial denaturation	30 sec	98ºC
Denaturation	10 sec	98ºC
Annealing	30 sec	45-59ºC (primer dependent)
Elongation	30 sec/kb	72°C → back to step 2, 30 cycles 72°C
Final elongation	10 min	72ºC

For further cloning, PCR products were separated on a 0.8-1.4 % agarose gel by electrophoresis (see section 2.2.4.4.2), excised and then eluted from the gel. Afterwards they were digested with the appropriate restriction enzymes (see section 2.2.4.4.3).

2.2.4.4.2 Agarose gel electrophoresis

DNA fragments from restriction digests (see section 2.2.4.4.3) or cloning PCRs (see section 2.2.4.4.1) were separated for analytical or preparative purpose via agarose gel electrophoresis. The desired amount of agarose (0.8 – 1.4 %) was added to 1X TAE buffer and then heated in a microwave until it was completely dissolved. Ethidium bromide was added in the concentration of 1 µg/100 ml to TAE to enable the visibility of the DNA fragments under UV light. DNA samples were mixed with 6X DNA-Loading buffer and loaded into the pockets of the gel. As a marker, 1 kb DNA ladder (Fermentas) was used. Electrophoresis was performed at 110 V for about 1-2 h. DNA bands were visualized under UV light and then photographed and/or excised.

2.2.4.4.3 Restriction digestion

Restriction digestion of plasmid DNA and PCR fragments was performed with an adequate restriction endonuclease and the recommended buffer for approximately 3 h at 37°C.

Standard reaction mix:

DNA	0.5-5 µg

10X buffer	5 µl
Enzyme	0.5 µl
ddH_2O	add to 50µl

Digested DNA fragments from a vector were separated and analyzed by agarose gel electrophoresis. Digested PCR products were purified with the QIAquick PCR purification kit.

2.2.4.4.4 Ligation

Ligation was performed with T4-DNA-ligase (NEB) in a molar ratio of 1:3 and 1:5 (vector to insert). The mixture was set up in a 15 µl volume, with 1 unit T4-DNA-ligase and ~50 ng vector DNA and incubated at room temperature for 1 h. 5 µl of ligation mix was used for transformation (see section 2.2.4.4.5).

2.2.4.4.5 Transformation of DH5α by heat shock

For transformation, chemical competent bacteria cells (DH5α) were used. These cells (stored at -80°C) were first thawed on ice for 10 min. Then 100 ng of plasmid DNA or the 5 µl ligation reaction were mixed with 60 µl of the bacteria in a reaction tube and the mixture was set on ice for 10 min. Next, the bacteria were given heat shock for 90 seconds at 42°C and then cooled on ice for 3 minutes. After addition of 400 µl warm LB media (without antibiotics), the sample was incubated for 15-30 min at 37°C. The bacterial cells were centrifuged for 2 minutes at 1000 g and the supernatant was discarded. The pellet was resuspended in 50 µl LB medium and plated on LB agar plates (with required antibiotic) and incubated over night at 37°C. The colonies were picked for plasmid isolation.

2.2.4.4.6 Sequencing

All sequencing was done by LGC genomics

3 Results

3.1 Inhibition of Aurora B results in polyploidy and induction of the cell cycle inhibitor p21

To investigate the effect of Aurora B inhibition on cell cycle progression, two chemical inhibitors of Aurora B kinase, ZM447439 (Ditchfield et al. 2003) and AZD1152-HQPA (Yang et al. 2007; Wilkinson et al. 2007) were used. In addition, Aurora B was also depleted by a specific siRNA. Further, two cell lines U2OS and HCT116 were employed for the study.

3.1.1 Inhibition of Aurora B in U2OS cells results in polyploidy and induction of p21

Given the critical requirement of Aurora B during cell cycle, impairment of its function is associated with polyploidy (Hauf 2003; Wilkinson et al. 2007; Ditchfield et al. 2003) and cell cycle arrest due to induction of the CDK inhibitor p21(Cip1) (Gizatullin et al. 2006; Trakala et al. 2013). Indeed these phenotypes were observed when Aurora B was inhibited in U2OS cells by ZM447439 (Fig. 10A and B). U2OS cells were treated with ZM447439 for 24 h and then processed for immunostaining to visualize nuclei and p21 (Fig. 10A), which indicated that a large fraction of cells become multinucleated (indicating failure of cytokinesis) and show very strong p21 induction upon Aurora B inhibition. DNA content analysis of ZM447439 treated U2OS cells by flow cytometry (PI FACS) further confirmed the polyploidy status of these cells as compared to normal cell cycle profile of DMSO treated control cells (Fig. 10B). Absence of sub-G1 population in the PI FACS profile of ZM447439 treated cells (Fig. 10B) indicated that there was no apoptosis in U2OS cells after Aurora B inhibition. Also treatment of U2OS cells with a more specific Aurora B inhibitor AZD1152-HQPA (Mortlock et al. 2007) resulted in induction of p21 in time and concentration dependent manner, as shown by immunoblotting for p21 (Fig 10C). To rule out the possibility that the observed cellular response is an artifact of pharmacological inhibition, Aurora B was depleted by a specific siRNA, which also resulted in strong induction of p21 protein (Fig. 10D).

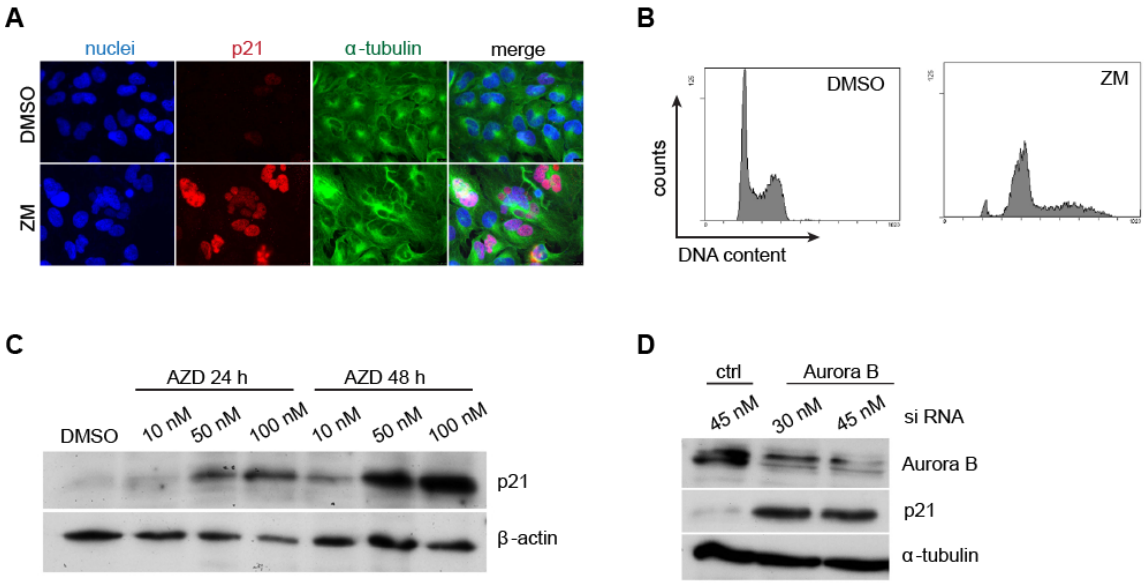

Figure 10: Inhibition of Aurora B in U2OS cells results in polyploidy and induction of p21. (A) U2OS cells were treated with DMSO or 1 µM ZM447439 for 24 h. p21 (in red) and α-tubulin (in green) were detected by immunostaining. Nuclei (in blue) were stained with Hoechst 33528. (B) U2OS cells were treated as in (A) and analyzed by PI FACS. (C) U2OS cells were treated with DMSO or indicated concentrations of AZD1152-HQPA for 24 h or 48 h and p21 levels were determined by immunoblotting. β-actin served as loading control. (D) U2OS cells were transfected with indicated concentrations of control siRNA or Aurora B specific siRNA for 48 h. p21 and Aurora B levels were determined by immunoblotting. α-tubulin served as loading control.
Parts of this figure were published in similar form in Kumari et al. 2013.

3.1.2 Inhibition of Aurora B in HCT116 cells results in polyploidy and induction of p21

To further confirm that the effects of Aurora B inhibition are not specific for one cell line, another cell line HCT116 was used. Treatment of HCT116 cells with ZM447439 or AZD1152-HQPA also resulted in induction of p21 protein in a time and concentration dependent manner (Fig. 11A). The flow cytometry profiles of HCT116 cells treated with two different Aurora B inhibitors also indicated generation of multinucleated cells (increase in proportion of cells with DNA content of 4N or more) (Fig 11B). The fraction of cells with 4N DNA content increased after prolonged treatment for 48 h (as compared to 24 h) (Fig 11B), indicating that some cells escaped arrest at 4N and continued through additional cell cycles, a phenotype of Aurora B inhibition shown previously (Ditchfield 2003; Gizatullin et al. 2006). These findings are in line with previous studies (Tao et al. 2008; Kaestner at al. 2009; Nair et al. 2009) and imply that p21 induction and generation of polyploidy is a general response to Aurora B inhibition and is not restricted to only one cancer cell type, confirming Aurora B as a key regulator of cell division.

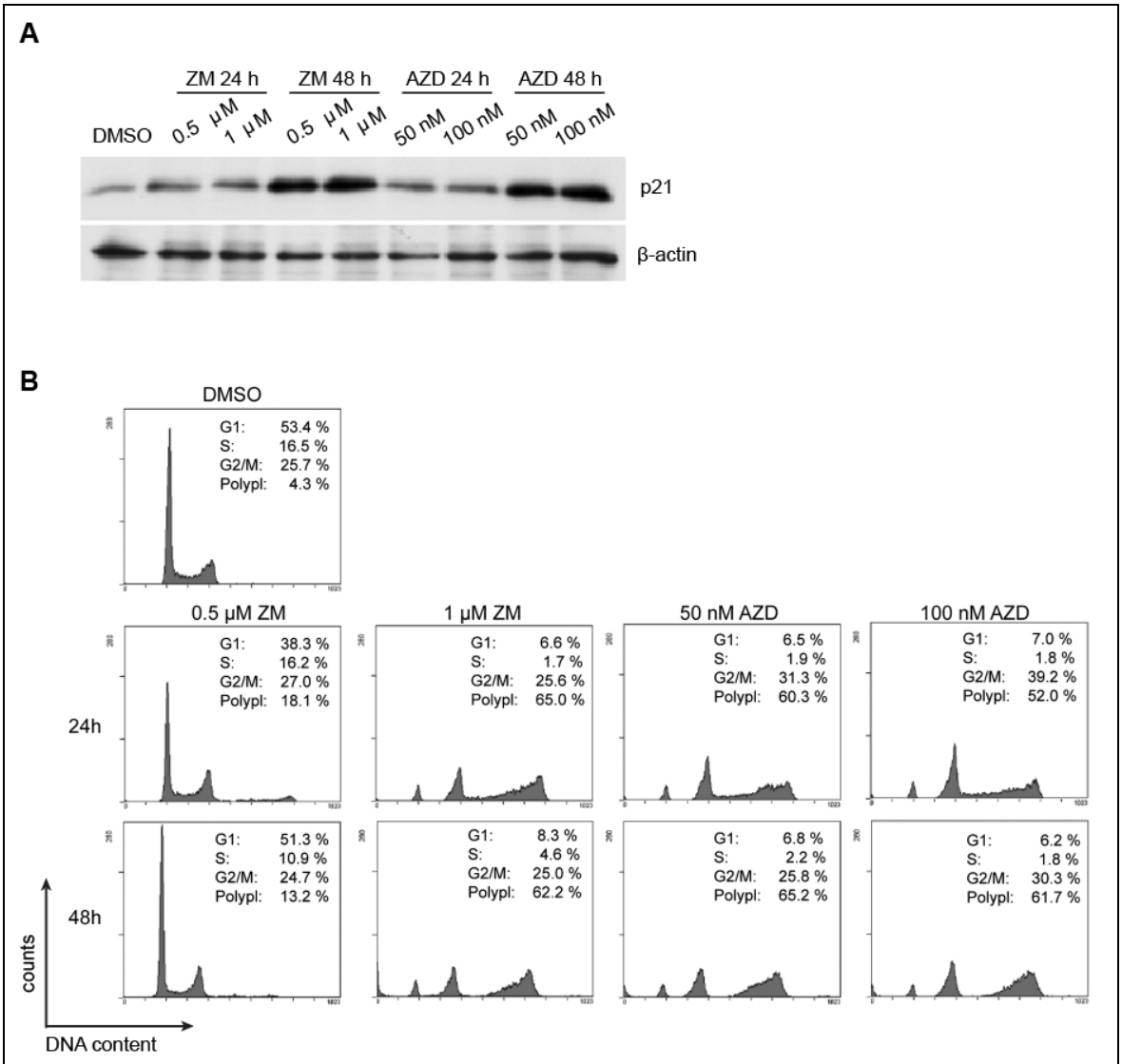

Figure 11: Inhibition of Aurora B in HCT116 cells results in polyploidy and induction of p21.

(A) HCT116 cells were treated with DMSO or with the indicated concentrations of ZM447439 or AZD1152-HQPA for 24 h or 48 h. p21 levels were determined by immunoblotting. β-actin served as loading control. (B) FACS analysis of HCT116 cells treated with DMSO, ZM447439 (0.5 µM or 1 µM) or AZD1152-HQPA (50 nM or 100 nM) for 24 h or 48 h.

This figure was published in similar form in Kumari et al. 2013.

3.2 Induction of p21 in response to Aurora B inhibition depends on p53

Since p21 is a well-known p53 target gene (El-Deiry et al. 1993) and recent studies showed a role of Aurora B in mediating p53 degradation (Wu et al. 2011; Gully et al. 2012), it was of considerable importance to test the requirement of p53 for p21 induction after Aurora B inhibition. Indeed strong induction of p53 was observed upon treatment of U2OS cells with Aurora B inhibitor ZM447439 (Fig. 12A). To test whether induction of p21 is p53 dependent, HCT116 p53 null cells (Bunz, 1998) were used. Treatment of HCT116

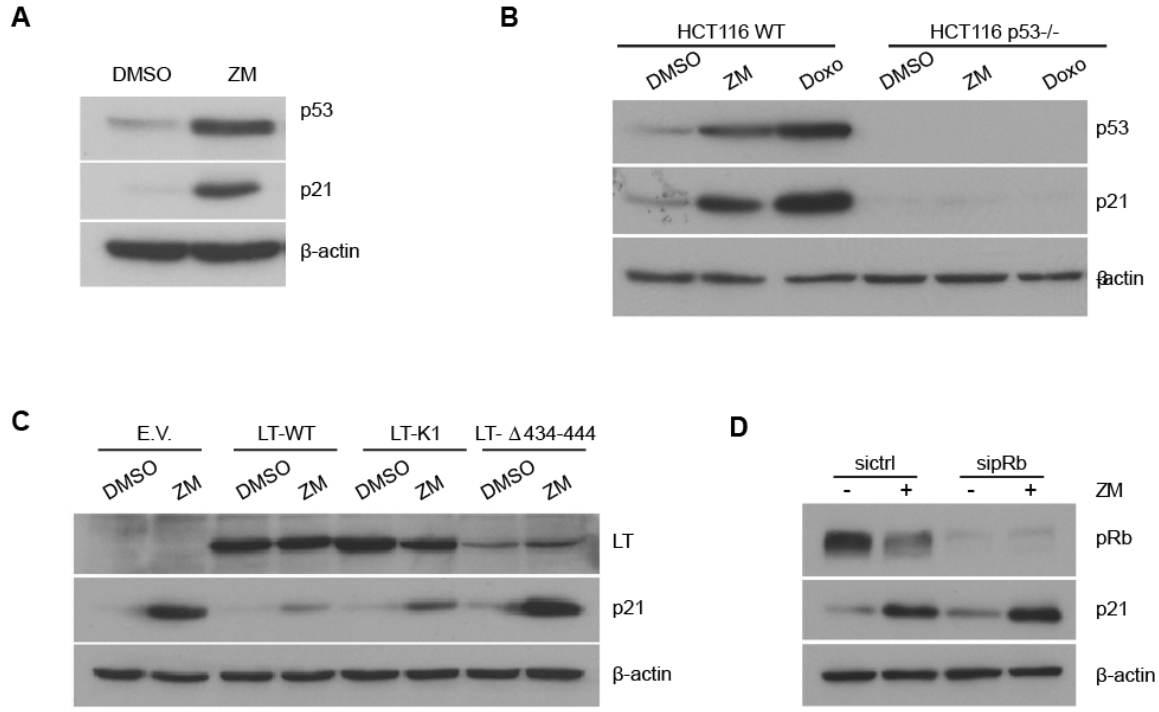

Figure 12: Induction of p21 in response to Aurora B inhibition depends on p53.
(A) U2OS cells were treated with DMSO or 1 µM ZM447439 for 24 h. p53 and p21 levels were determined by immunoblotting. β-actin served as loading control. (B) HCT116 wild type (WT) and p53 negative cells were treated with DMSO, 1 µM ZM447439 for 48 h or with 1 µM doxorubicin (Doxo) for 24 h. p53 and p21 levels were determined by immunoblotting. β-actin served as loading control. (C) U2OS-EcoR cells stably expressing empty vector (E.V.) or SV40-Large T antigen (wild type, K1 or Δ434-444) were treated with DMSO or 1 µM ZM447439 for 24 h. Large T and p21 levels were analyzed by immunoblotting. β-actin served as loading control. (D) U2OS cells were transfected with 12 nM control siRNA or pRb specific siRNA for 48 h and then treated with DMSO or 1 µM ZM447439 for 24 h. pRb and p21 protein levels were determined by immunoblotting. β-actin was used as loading control.
Parts of this figure were published in similar form in Kumari et al. 2014.

wild-type (WT) cells with ZM447439 or doxorubicin (a DNA damaging agent, used as positive control) activated p53 and induced p21, whereas ZM447439 and doxorubicin failed to induce p21 in isogenic HCT116 p53 null cells (Fig. 12B), indicating that p53 is

required for p21 induction in response to Aurora B inhibition. To further verify the requirement of p53 for p21 induction, U2OS-EcoR cells stably expressing the SV40 large T (LT) antigen that binds and inhibits pRb family members and p53 (Ali and DeCaprio 2001), were generated. Two mutants of LT, LT-K1 and LT-Δ434-444, were also used to test which pathway (pRb or p53) is involved in p21 induction. The LT-K1 mutant can no longer bind to and inactivate pRb and thus only targets p53 (Stubdal et al. 1997). In contrast, LT-Δ434-444 cannot bind to p53 and only targets pRb and the related 'pocket proteins' p107 and p130 (Kierstead and Tevethia 1993). Expression of LT wild-type and its mutants was verified by immunoblotting (Fig. 12C). Expression of all three LT antigens was detectable and not changed after Aurora B inhibition, although expression of LT-Δ434-444 was weaker as compared to LT-wild-type and LT-K1, as has been demonstrated previously (Ye et al. 2007) (Fig. 12C). Induction of p21 after inhibition of Aurora B was blocked by wild-type LT (Fig. 12C). The K1 mutant was also able to block p21 induction, while LT-Δ434-444 did not prevent induction of p21 after Aurora B inhibition by ZM447439 treatment. However, since the LT-Δ434-444 mutant was expressed at low levels as compared to wild-type and K1 mutant, to directly test the requirement of pRb for p21 induction after Aurora B inhibition, pRb was knocked down by pRb specific siRNA (Fig. 12D). Knockdown of pRb had no effect on p21 induction after Aurora B inhibition (Fig. 12D). Taken together, these findings indicate that p53 but not pRb-proteins are required for induction of p21 in response to Aurora B inhibition.

3.3 p38 MAPK is required for induction of p21 in response to Aurora B inhibition

Cell cycle arrest by p53-p21 activation due to impairment of cytokinesis and resulting tetraploidy has been reported to involve the stress-activated kinase, p38 MAPK (Ganem and Pellman 2007; Mikule et al. 2007; Thompson and Compton 2010). Hence, it was of interest to investigate if p38 MAPK is involved in p21 activation in response to Aurora B inhibition. To test this, an antibody against the active phosphorylated form of p38 MAPK was used. Strikingly, p38 MAPK was phosphorylated and hence activated in a time dependent manner after Aurora B inhibition (Fig. 13A). The total p38 protein levels remained unchanged after treatment with ZM447439. There was a correlation in activation of p38, induction of p53 and activation of p21 in a time dependent manner after Aurora B inhibition, suggesting that inhibition of Aurora B could result in activation of p38-p53-p21 pathway that blocks the proliferation of cells (Fig. 13A). The next important question to be addressed was whether p38 is required for activation of p53 and p21 in response to

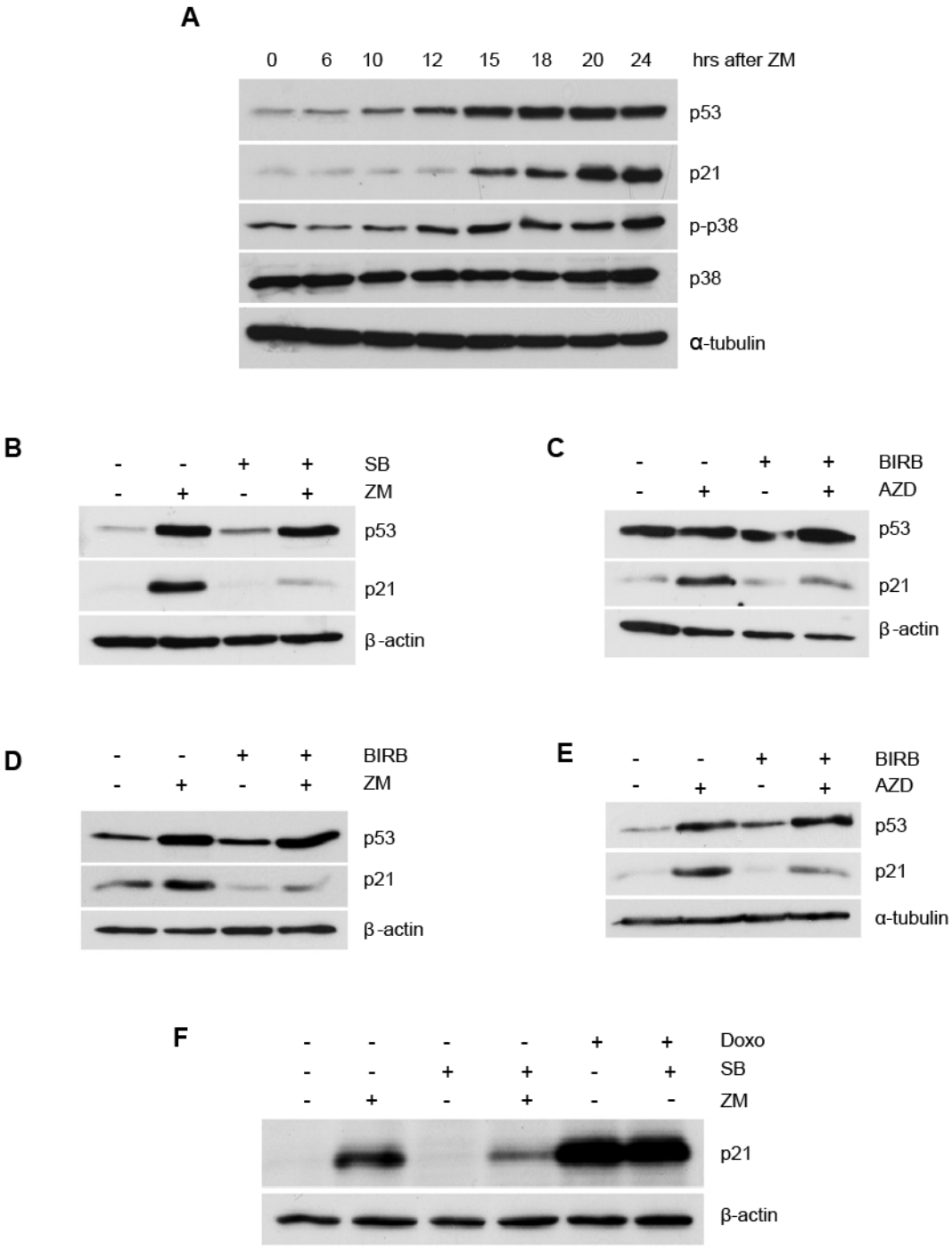

Figure 13: p38 MAPK is required for induction of p21 in response to Aurora B inhibition. (A) U2OS cells were treated for the indicated timepoints with 1 μM ZM447439. Levels of p53, p21, phosphorylated p38 (p-p38), total p38 and α-tubulin (control) were determined by immunoblotting. (B) U2OS cells were treated with DMSO, 1 μM ZM447439 alone, 10 μM SB202190 alone or pretreated with 10 μM SB202190 for 2 h and then treated with 1 μM ZM447439 for 24 h. Levels of p21 and p53 were determined by immunoblotting. β-actin served as loading control. (C) U2OS cells were treated with DMSO, 50 nM AZD1152-HQPA alone, 1 μM BIRB796 alone or pretreated with 1 μM BIRB796 for 2h and then treated with 50 nM AZD1152-HQPA for 24 h. Levels of p21 and p53 were determined by immunoblotting. β-actin served as loading control. (D) HCT116 cells were treated with DMSO, 1 μM ZM447439 alone, 1 μM

BIRB79 alone or pretreated with 1 μM BIRB796 for 2 h and then treated with ZM447439 (1 μM) for 24 h. p21 and p53 protein levels were determined by immunoblotting. β-actin served as loading control. (E) HCT116 cells were treated as in (D), but with a different Aurora B inhibitor, AZD1152-HQPA (50 nM). α-tubulin served as loading control. (F) U2OS cells were treated with DMSO, 1 μM ZM447439 alone, 10 μM SB202190 alone, 1 μM doxorubicin (Doxo) alone or pre-treated with 10 μM SB202190 for 2 h, and then treated either with 1 μM ZM447439 or 1 μM doxorubicin (Doxo) for 24 h. Levels of p21 were determined by immunoblotting. β-actin served as loading control.
This figure was published in similar form in Kumari et al. 2013.

Aurora B inhibition. For this, two chemical inhibitors of p38 MAPK, SB202190 (inhibits all the four isoforms p38α, p38β, p38 γ and p38δ) (Lee et al. 1994) and BIRB796 (inhibits specifically p38α and p38β isoforms) (Kuma et al. 2005) were used. Co-treatment with SB202190 and ZM447439 blocked the p21 induction mediated by ZM447439 alone, indicating that indeed p38 is required for p21 induction after inhibition of Aurora B (Fig. 13B). The other more specific p38 inhibitor BIRB796 also blocked the p21 induction mediated by AZD1152-HQPA (Fig. 13C) as well as ZM447439 (Fig. 13D and Fig 13E) in both U2OS and HCT116 cells, confirming that p38 signaling contributes to p21 induction. Interestingly inhibition of p38 (by SB202190 or BIRB796) did not affect p53 activation after Aurora B inhibition, suggesting that p53 induction does not require activation of p38 (Fig. 13B, C, D, E). To test whether requirement of p38 MAPK was specific for the p21 induction observed after Aurora B inhibition, cells were treated with the chemotherapeutic drug doxorubicin (a DNA damaging agent), which also induces p21 (Fig. 13E, lane 5). However, this p21 induction was not blocked by SB202190 co-treatment, indicating that p38 is not required for p21 activation following a different type of cellular stress, such as DNA damage. These results emphasize that p38 MAPK is specifically required for activation of p21 after impairment of Aurora B function.

3.4 Co-inhibition of Aurora B and p38 inhibits cell proliferation in p53 dependent manner

Previous data from our group indicated that long-term co-treatment of cells with ZM447439 and SB202190 resulted in reduced colony formation due to apoptosis (data from Tanja Ulrich, published in Kumari et al. 2013). To investigate whether this cooperation requires p53, U2OS-EcoR cells stably expressing shRNA against p53 were generated. The knock down of p53 was confirmed by RT-qPCR of the RNA isolated from cells stably expressing shp53 (Fig. 14A). As a control, cells expressing empty vector were used. These cells were seeded at very low density (6000 cells/10 cm dish) and treated with DMSO, ZM447439 alone, SB202190 alone or a combination of ZM447439 and SB202190 for 14 days and then the colonies were visualized by crystal violet staining.

While a strong reduction in colony formation was observed in cells expressing empty vector upon combination of ZM447439 and SB202190 treatment, this co-operation was weaker in cells in which p53 was knocked down (Fig. 14B). In light of the previous finding from our group that SB2020190 co-operates with ZM447439 to inhibit colony formation by inducing apoptosis, this result supports that functional p53 is required for the induction of apoptosis when p38 is inhibited along with Aurora B.

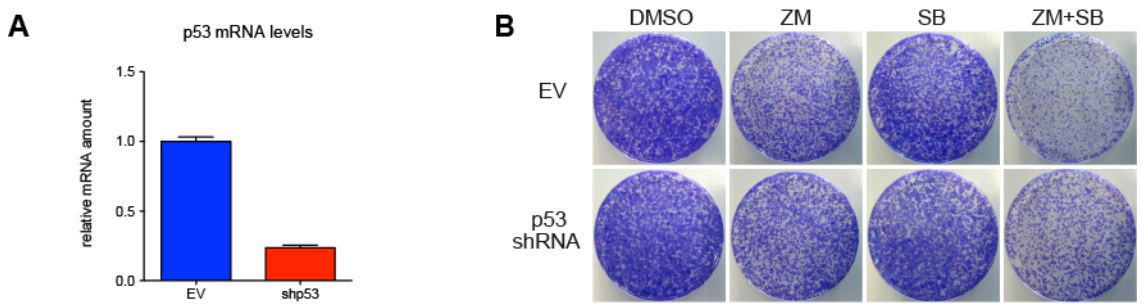

Figure 14: Co-inhibition of Aurora B and p38 inhibits cell proliferation in p53 dependent manner.
(A) Quantification of p53 mRNA levels in U2OS-EcoR cells stably expressing empty vector (EV) or shRNA against p53 (shp53). (B) U2OS-EcoR cells stably expressing empty vector (EV) or shRNA against p53 (shp53) were treated with DMSO, 0.5 µM ZM447439 alone, 10 µM SB202190 alone or a combination of 0.5 µM ZM447439 and 10 µM SB202190 for 14 days. The colonies were fixed and stained with crystal violet.

3.5 Cell cycle arrest after Aurora B inhibition requires p21 and is mediated by inhibition of E2F-dependent transcription

To directly test the requirement of p21 in cell cycle arrest and hence preventing endoreduplication following Aurora B inhibition, HCT116 p21 null cells (Waldman et al. 1995) were used. Upon treatment with ZM447439, the proportion of cells with 4N DNA content was significantly increased in HCT116 p21 null cells as compared to isogenic HCT116 wild-type (WT) cells, indicating that p21 prevents polyploidization after inhibition of Aurora B (Fig. 15A).

To investigate if impairment of Aurora B function also regulates other genes besides p21, a genome-wide microarray analysis was performed in our group (performed by Tanja Ulrich and published in Kumari et al. 2014). For this, U2OS cells were treated with DMSO, ZM447439 alone, SB202190 alone or pretreated with SB202190 for 2 h and then treated with ZM447439 for 24 h. The results of this analysis indicated that in addition to a large number of genes, which were up regulated by 2-fold on ZM447439 treatment (730 genes) some genes were also down regulated (430 genes) by 2-fold. The expression of 53 %

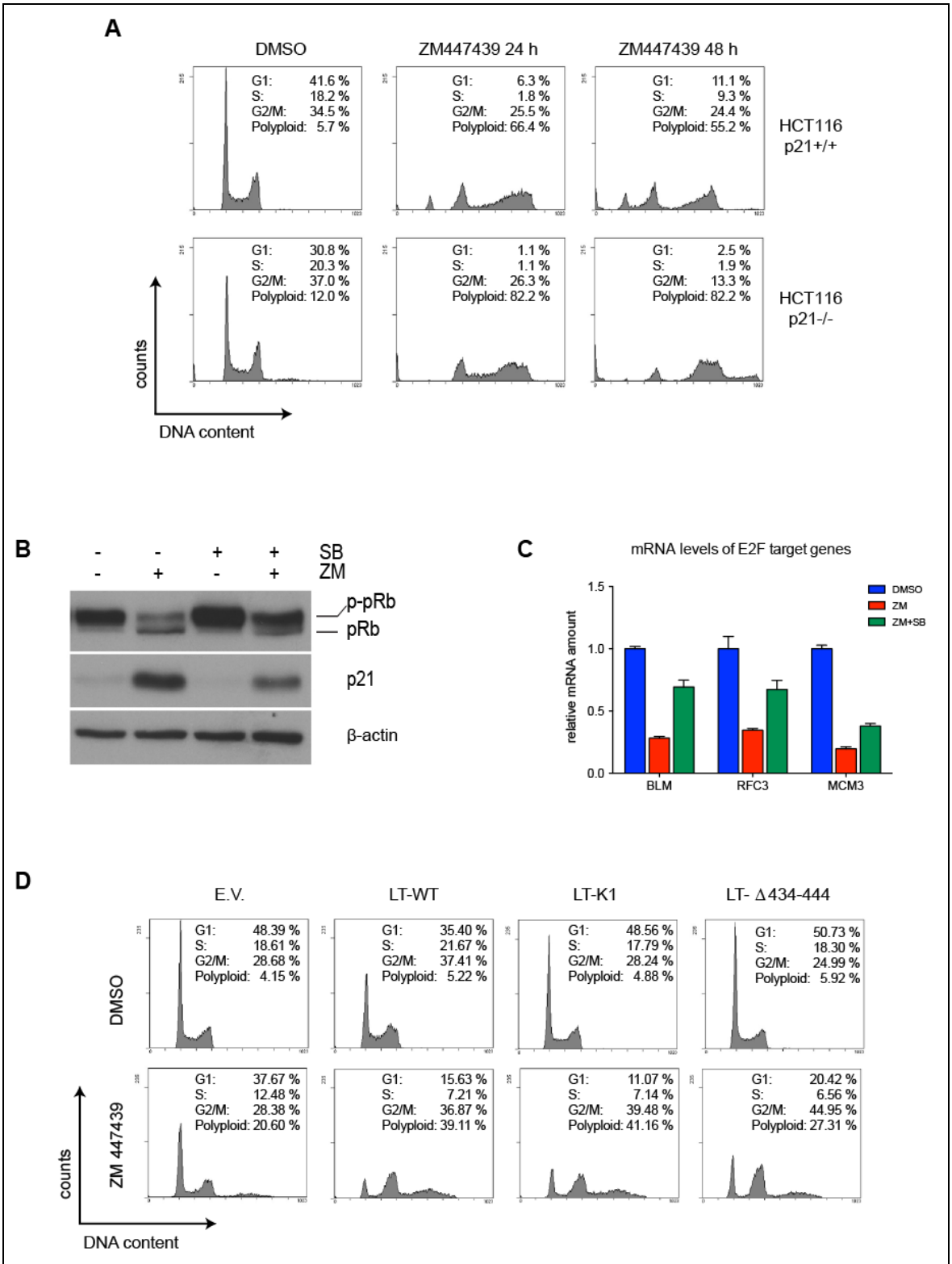

Figure 15: Cell cycle arrest after Aurora B inhibition requires p21 and is mediated by inhibition of E2F-dependent transcription.

(A) HCT116 wild type (WT) and HCT116 p21 null cells were treated with 1 µM ZM447439 for 24 h and 48 h. Cell cycle distribution was analyzed by FACS. (B) U2OS cells were treated with DMSO, 1 µM ZM447439 alone, 10 µM SB202190 alone or pretreated with 10 µM SB202190 for 2 h and then treated with 1 µM ZM447439 for 24 h. Levels of p21, hyperphosphorylated Rb (p-

pRb) and hyphophosphorylated Rb (pRb) were determined by immunoblotting. β-actin served as loading control. (C) U2OS cells were treated as described in (B) and mRNA levels of the indicated E2F-target genes were determined by RT-qPCR. (D) The indicated U2OS cell lines were treated with either DMSO or 1 μM ZM447439 for 24 h and flow cytometry was performed to determine the fraction of cell in the different phases of the cell cycle.
Parts of this figure were published in similar form in Kumari et al. 2013 and Kumari et al. 2014.

(220 genes) of these down-regulated genes was restored upon p38 co-inhibition by SB202190 treatment. Hence these genes required p38 for their expression and might be involved in cell cycle arrest upon Aurora B inhibition. Gene Ontology (GO) analysis of these down-regulated genes whose expression was restored upon p38 co-inhibition demonstrated that they play roles in DNA-replication and repair, cell cycle regulation and mitosis and that many of them are known targets of the pRb/E2F pathway. Owing to the fact that p21 is a CDK inhibitor (Harper et al. 1993; Sherr and Roberts 1995) and therefore indirectly inhibits phosphorylation of the retinoblastoma (pRb) proteins by CDKs (Heuvel and Dyson 2008; Dimova and Dyson 2005), we hypothesized that p21 induced as a result of Aurora B inhibition resulted in cell cycle arrest by indirect dephosphorylation of pRb, as phosphorylation of pRb is required for cell cycle progession. Expression of p21 thus results in repression of E2F-regulated genes through the formation of repressive pocket-protein/ E2F complexes. To investigate whether phosphorylation of pRb is indeed inhibited after inhibition of Aurora B, the phosphorylation status of pRb was analyzed before and after treatment with ZM447439. Inhibition of Aurora B resulted in dephosphorylation of pRb (Fig. 15B) and this dephosphorylation of pRb was prevented by SB202190 co-treatment, indicating that p38 signaling is indeed required for formation of the active, repressive state of pocket-proteins via activation of p21, which in turn results in cell cycle arrest. This effect of p21 on pRb and hence the targets of pRb/E2F pathway, was further verified for some of the genes from microarray analysis. mRNA levels of E2F-target genes BLM, RFC3 and MCM3 were down-regulated by ZM447439 and restored upon SB202190 co-treatment (Fig. 15C), suggesting that the cell cycle arrest after Aurora B inhibition requires E2F target genes. Also, when pRb proteins were inhibited by expression of Large T antigen (wild type or its Δ434-444 mutant), the proportion of polyploid cells increased as compared to the control cells (expressing empty vector) upon ZM447439 treatment (Fig. 15D). Collectively these results suggest that inhibition of Aurora B leads to cell cycle arrest through p53 and p38-dependent p21 induction, pocket-protein dephosphorylation and inhibition of E2F-dependent transcription.

3.6 p38 MAPK is required for transcriptional induction of p21 but not for its protein stability

To investigate if p38 has a role in transcription of the p21 gene in response to Aurora B inhibition, mRNA levels of p21 were analyzed by real time RT-qPCR. Treatment of cells with ZM447439 resulted in induction of p21 mRNA levels and this induction was blocked by SB202190 co-treatment (Fig. 16A), indicating that p21 transcriptional induction after Aurora B inhibition depends on p38 signaling. p38 has been reported to stabilize p21 by

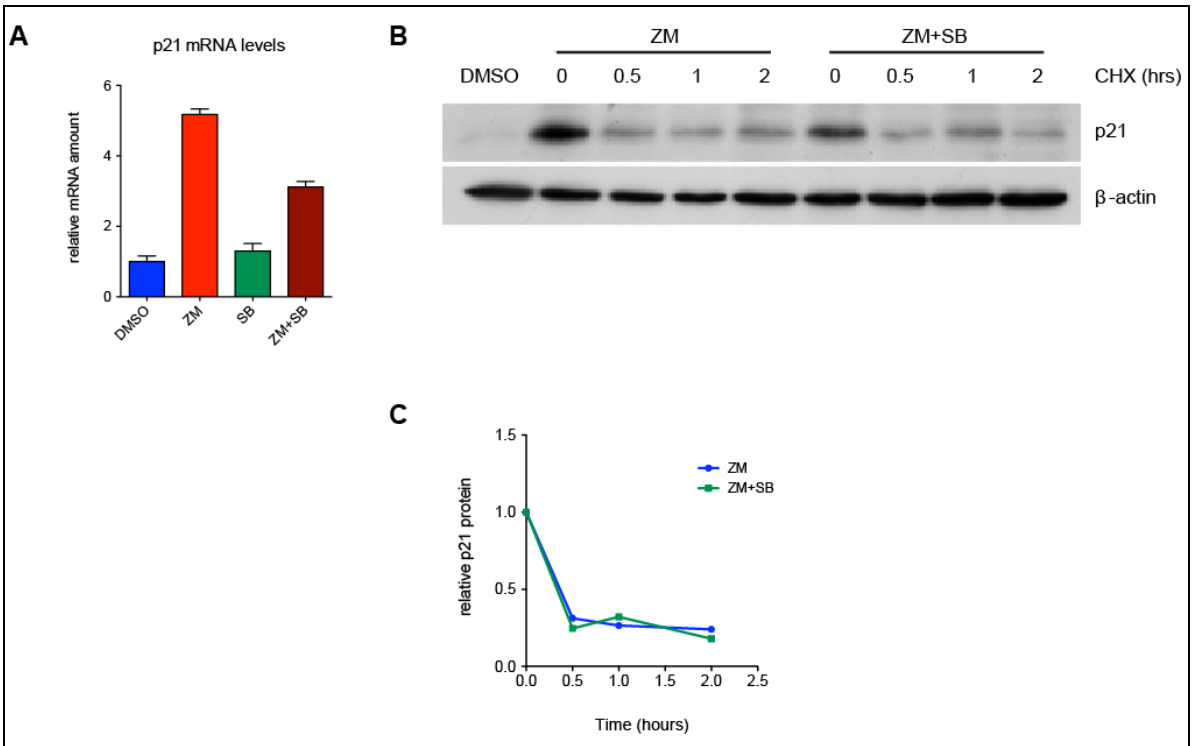

Figure 16: p38 is required for transcriptional induction of p21 but not for its protein stability.
(A) U2OS cells were treated with DMSO, 1 μM ZM447439 alone, 10 μM SB202190 alone or pretreated with 10 μM SB202190 for 2 h and then treated with 1 μM ZM447439 for 24 h. mRNA level of p21 was determined by RT-qPCR. (B) U2OS cells were treated with DMSO, 1 μM ZM447439 or pretreated with 10 μM SB202190 for 2 h followed by treatment with 1 μM ZM447439 for 22 h and then with 20 μg/ml cycloheximide (CHX) for the indicated time points to block protein synthesis. p21 levels were determined by immunoblotting. β-actin served as loading control. (C) The protein bands of p21 and β-actin of the blot shown in (B) were quantified by ImageJ. The amount of p21 protein (relative to β-actin) was plotted against time.
Parts of this figure were published in similar form in Kumari et al. 2013.

phosphorylating it at Ser-130 in response to stress signals induced by TGFβ (Kim et al. 2002). To examine if p38 activated as a consequence of Aurora B inhibition has an effect on p21 protein stability, the half-life of p21 protein induced after Aurora B inhibition (in

absence and presence of p38 co-inhibition) was determined by using cycloheximide (a protein synthesis inhibitor). U2OS cells were treated with ZM447439 or pretreated with SB202190 for 2 hours followed by ZM447439 treatment for 22 hours and then either left untreated or were treated with cycloheximide for 0.5, 1 and 2 hours. The stability of p21 protein induced after Aurora B inhibition was not affected by co-inhibition of p38, indicating that p38 does not influence p21 protein stability under these conditions (Fig. 3.6B).

3.7 p38 MAPK is not required for p53 binding to p21 promoter in response to Aurora B inhibition

Having shown that p38 is required for transcriptional induction of p21 without affecting its protein stability and total p53 protein levels; it was tempting to study the transcriptional regulation of p21 gene in more detail. For this, first the binding of p53 to the p21 promoter was studied by chromatin immunoprecipitation (ChIP) assay at p21 gene locus using an antibody specific for p53. Cells were treated with DMSO, ZM447439 or with a combination of SB202190 (pretreatment for 2 h) and ZM447439. Chromatin was isolated and immunoprecipitated with a p53 specific antibody. As a control, nonspecific IgG was used. Promoter regions were detected by quantitative real time PCR. p53 binding was robustly induced at the high affinity p53 binding site 1 (p53BS1) upon treatment with ZM447439 (Fig. 17B) or AZD1152-HQPA (Fig. 17D). No significant binding of p53 was observed at the distal regions of the p21 gene, as expected (Fig. 18B). In addition, no significant binding was observed at the GAPDH2 promoter used as a control. Interestingly, p53 binding was not affected by p38 inhibition with SB202190 (Fig. 17B) or BIRB796 (Fig. 17D). Thus, p38 is not required for binding of p53 to its binding site on p21 promoter, implicating that p38 acts at a step subsequent to p53 binding at the p21 gene locus.

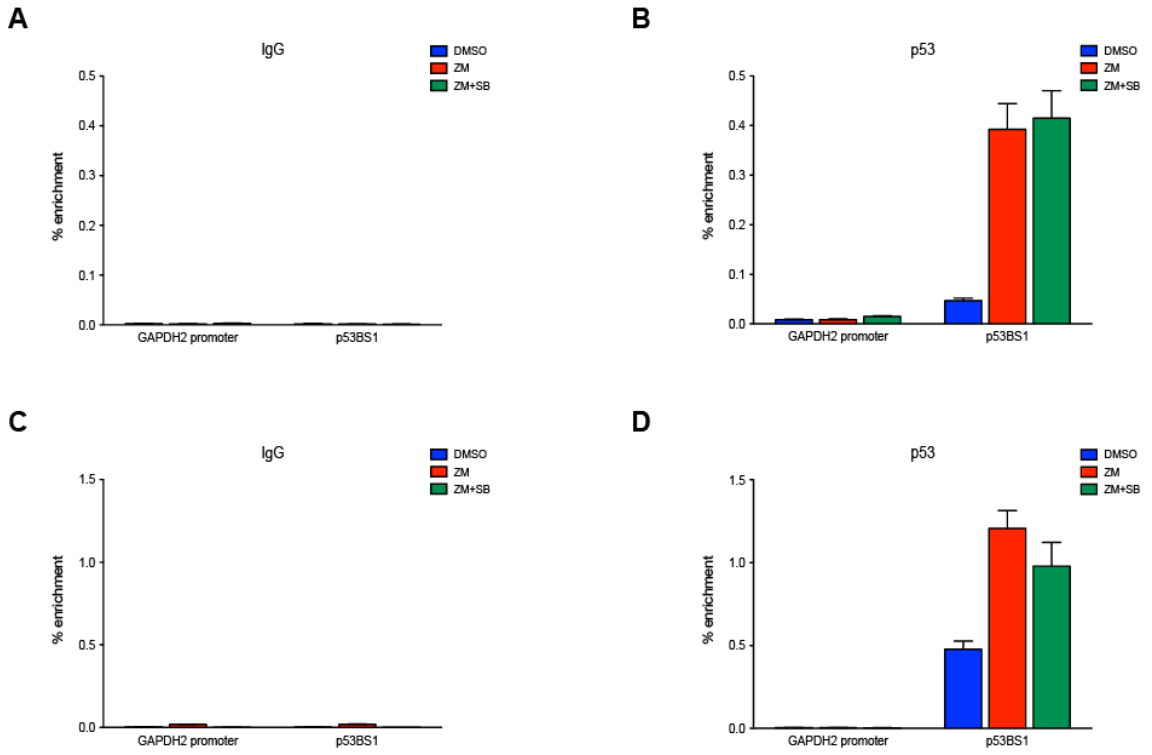

Figure 17: p38 MAPK is not required for p53 binding to p21 promoter in response to Aurora B inhibition.
(A) and (B) U2OS cells were treated with DMSO, 1 μM ZM447439 alone, or pretreated with 10 μM SB202190 for 2 h and then treated with 1 μM ZM447439 for 24 h. Chromatin was isolated and precipitated with antibody specific for p53 or nonspecific IgG. ChIP enriched DNA was amplified by qPCR using the indicated amplicons. GAPDH2 promoter was analyzed as a control. (C) and (D) HCT116 cells were treated with DMSO, 50 nM AZD1152-HQPA alone, or pretreated with 1 μM BIRB796 for 2h and then treated with 50 nM AZD1152-HQPA for 24 h. Chromatin was isolated and precipitated with antibody specific for p53 or nonspecific IgG. ChIP enriched DNA was amplified by qPCR using the indicated amplicons. GAPDH2 promoter was analyzed as a control.

3.8 p38 MAPK is required for transcriptional elongation of p21 in response to Aurora B inhibition

Regulation of p21 expression has been shown to occur at the level of transcriptional elongation (Gomes et al. 2006; Valin et al. 2013). To explore this possibility, ChIP assay with antibody against RNA Polymerase II (RNA Pol II) was performed at the p21 gene locus. Significant amount of RNA Pol II was observed at the core promoter (-20 region) of p21 gene even before stimulation by inhibition of Aurora B (Fig. 18D), consistent with previous reports showing that RNA Polymerase II is paused at the promoters of stress responsive genes (Adelman and Lis 2012). Very low binding of RNA Pol II was observed in the transcribed region of the p21 gene, indicating that the polymerase is paused at the p21 promoter. Upon inhibition of Aurora B by ZM447439 treatment, loading of RNA Pol II

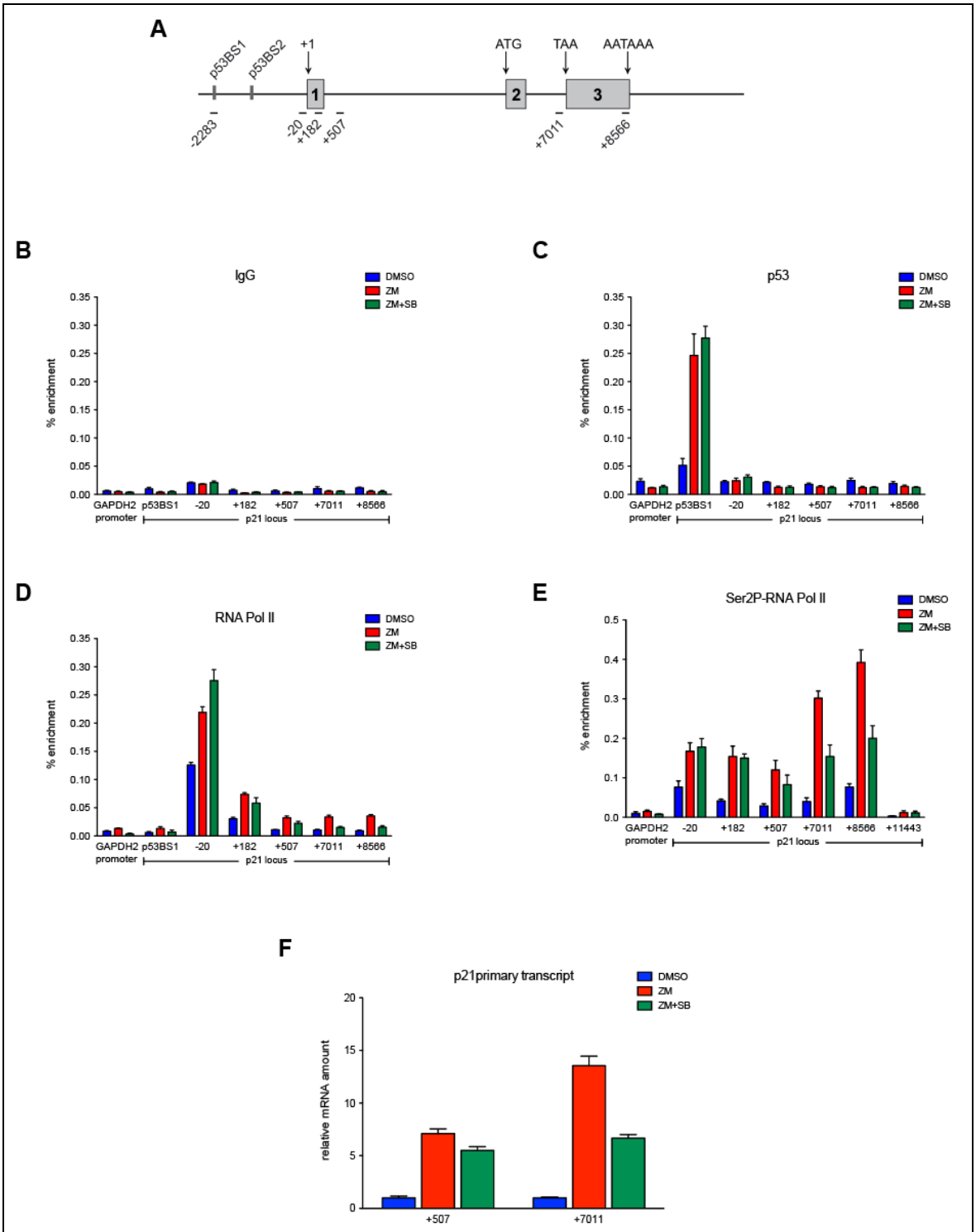

Figure 18: p38 MAPK is required for transcriptional elongation of p21 in response to Aurora B inhibition.

(A) Schematic diagram of the p21 gene locus and position of the amplicons used for ChIP analysis and primary transcript analysis. U2OS cells were treated with DMSO, 1 µM ZM447439 alone, or pretreated with 10 µM SB202190 for 2 h and then treated with 1 µM ZM447439 for 24 h. Chromatin was isolated and precipitated with nonspecific IgG (B), antibody specific for p53 (C), total RNA polymerase II (D) and phospho-ser2 RNA Pol II (E). ChIP enriched DNA was

amplified by qPCR using the indicated amplicons. The GAPDH2 promoter was analyzed as a control. (F) U2OS cells were treated with DMSO, 1 μM ZM447439 alone, or pretreated with 10 μM SB202190 for 2 h and then treated with 1 μM ZM447439 for 24 h. Total RNA was isolated and subjected to RT-qPCR using primers specific for shorter (+507) and longer region (+7011) primary p21 transcripts.
This figure was published in similar form in Kumari et al. 2013.

moderately increased at the core promoter (less than 2-fold), whereas it's loading in the distal regions of the p21 gene was strongly induced (3-4 fold) (Fig. 18D). The loading of RNA Pol II at the core promoter (-20 region) was not affected by p38 inhibition by SB202190 co-treatment (instead there was a slight increase binding), whereas its recruitment to the distal regions of the p21 gene was strongly blocked upon p38 inhibition (Fig. 18D). These findings indicate a role for p38 MAPK in mediating transcriptional elongation of p21 gene after recruitment of RNA Pol II, in response to Aurora B inhibition. To confirm the requirement of p38 in elongation stage of the transcriptional induction of p21, ChIP assay was performed with antibody against RNA polymerase II phosphorylated at serine 2 (Ser 2P RNA Pol II), which represents the elongating form of RNA Polymerase II (Phatnani and Greenleaf 2006; Egloff et al. 2012). Interestingly the elongating form of RNA polymerase II was strongly enriched towards the 3' region of the p21 gene after Aurora B inhibition by ZM447439 and this enrichment was impaired by p38 inhibition by SB202190 co-treatment (Fig. 18E), emphasizing the notion that p38 is required for transcriptional elongation of p21 gene. To corroborate the finding that p38 is required for the elongation of the p21 gene and hence is required to generate the full-length p21 mRNA, nascent p21 transcripts were analyzed by RT-qPCR. Short p21 transcripts were analyzed by with primers specific for an amplicon in the first intron of the gene (+507) whereas long p21 transcripts were analyzed with primers specific for the second intron just before the third exon (+7011). Inhibition of Aurora B by ZM447439 treatment induced short transcripts, but their induction was not prevented by co-inhibition of p38. Longer p21 transcripts were also induced after Aurora B inhibition. Importantly, accumulation of the longer transcript was significantly reduced when p38 was inhibited by SB202190 co-treatment (Fig. 18F). Together these data indicate that p38 signaling is required for transcriptional elongation of p21 after Aurora B inhibition.

3.9 Transcriptional elongation of p21 in response to replication stress is dependent on p38 MAPK

Several studies have investigated the transcriptional elongation control of p21 in response to stress (Espinosa et al. 2003; Mattia et al. 2007; Beckerman et al. 2009; Gomes 2006; Valin et al. 2013). Beckerman et al. (2009) demonstrated that blocking DNA replication by hydroyxurea (HU, induces S-phase arrest by replication block) induced p53 but did not activate a subset of p53 targets including p21 due to marked reduction in its transcriptional elongation. The authors further showed that, Chk1 is employed in sensing the replication block caused due to stalled replication forks in presence of HU and inhibiting Chk1 by caffeine co-treatment relieved the elongation block of p21 gene leading to significant accumulation of p21 mRNA and protein. In order to test the requirement of p38 MAPK in this elongation pathway, levels of phosphorylated p38 were analyzed under conditions where elongation block was relieved (i.e. under caffeine and HU co-treatment conditions). Indeed p38 was phosphorylated and hence activated under these conditions when p21 was induced, whereas total p38 levels remained unchanged (Fig. 19A). As reported previously (Gottifredi et al. 2001; Beckerman et al. 2009) HU treatment induced p53 and co-treatment with caffeine and HU induced p21, without affecting p53 protein levels induced by HU alone (Fig. 19A). To further confirm the requirement of p38 in this pathway, the p38 MAPK specific inhibitor BIRB796 was used to inhibit p38. Inhibition of p38 partially blocked the p21 induction upon caffeine and HU co-treatment, without affecting the p53 protein levels (Fig. 19B). Interestingly, the enrichment of elongating form of RNA polymerase II in the distal regions of the p21 gene observed upon caffeine and HU co-treatment conditions was also partially blocked by p38 co-inhibition by BIRB796 (Fig. 19C). Taken together these findings strongly support the importance of p38 signaling in mediating transcriptional elongation p21 gene under stress conditions.

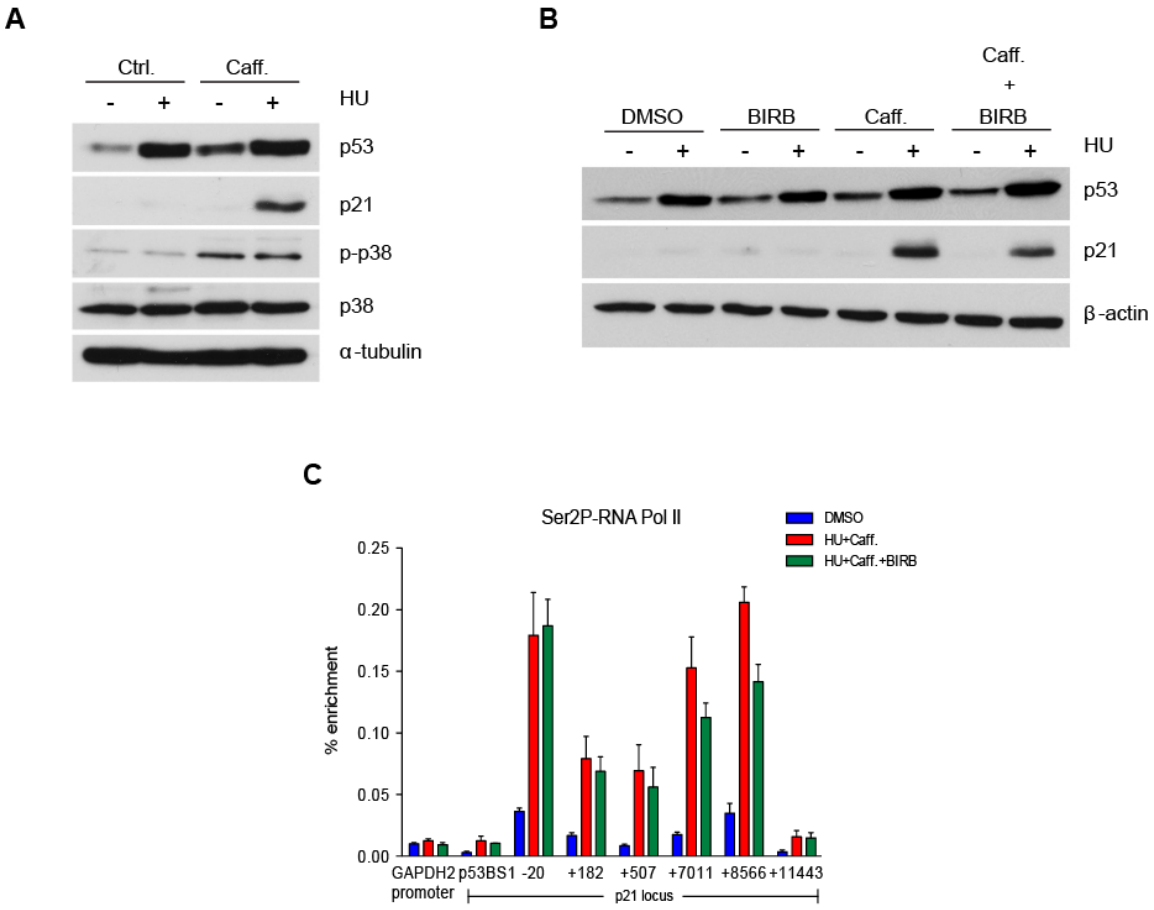

Figure 19: Transcriptional elongation of p21 in response to replication stress is dependent on p38 MAPK.
(A) U2OS cells were untreated (unt.) or pretreated with 4 mM caffeine for 1 h and then left untreated or were treated with 1.7 mM HU for 24 h. The levels of respective proteins were determined by immunoblotting. α-tubulin served as loading control. (B) U2OS cells were pretreated with DMSO, 4 mM caffeine, 1 μM BIRB796 or a combination of 4 mM caffeine and 1 μM BIRB796 and then left untreated or were treated with 1.7 mM HU for 24 h. p53 and p21 protein levels were determined by immunoblotting. β-actin served as loading control. (C) U2OS cells were treated as in (B). Chromatin was isolated and precipitated with antibody specific for phospho-ser2 RNA Pol II. ChIP enriched DNA was amplified by RT-qPCR using the indicated amplicons. GAPDH2 promoter was analyzed as a control.

3.10 Inhibition of Aurora B activates both α and β isoforms of p38 MAPK without affecting their subcellular localization

Phosphorylation of p38 triggers a conformational change leading to its translocation into the nucleus (Wood et al. 2009). Subcellular localization is an important mechanism by which substrate specificity of p38 MAPK is regulated (Roux and Blenis 2004; Raman et al. 2007). Hence, I was prompted to study the subcellular localization of p38 after Aurora B inhibition. Since SB202190 inhibits all the four isoforms of p38 (p38α, p38β, p38γ and p38δ), whereas BIRB796 specifically inhibits only the α and β isoforms, the findings so far suggest the preferential activation of p38α and/or p38β (as compared to p38γ and p38δ) due to Aurora B kinase inhibition. So, future experiments designed to explore the mechanism by which p38 was mediating the activation of p21, were concentrated mainly on p38α and p38β isoforms.

To study subcellular localization of p38α and p38β, U2OS-EcoR cells stably expressing haemagglutinin (HA) tagged versions of p38α and p38β (HA-p38α and HA-p38β) were generated. The expression of HA-p38α and HA-p38β was verified by immunoblotting (Fig. 20A). Further, these cells showed stabilization of p53 and induction of p21 upon Aurora B inhibition by ZM4447439 treatment, indicating that overexpression of these constructs did not had any off target effects and did not alter the outcome of Aurora B inhibition. Aurora B was inhibited by ZM447439 treatment and the lysates were immunoprecipitated with a HA-tag specific antibody and then immunoblotted with an antibody against phosphorylated p38. Both α and β isoforms of p38 were phosphorylated upon Aurora B inhibition (Fig. 20A). To test whether inhibition of Aurora B or co-inhibition of Aurora B and p38 affects the subcellular localization of these isoforms of p38, cells expressing either HA-p38α or HA-p38β were treated with ZM447439 alone, SB202190 alone or pretreated with SB202190 for 2 h and then treated with ZM447439 for 24 h, fixed and analyzed by immunostaining using HA-tag specific antibody. Both HA-p38α and HA-p38β were localized in cytoplasm as well in nucleus under control conditions (DMSO panel in Fig. 20B and C) in accordance with published data (Raingeaud et al. 1995). The subcellular localizations of both the isoforms remained unchanged on ZM447439 treatment or ZM447439 and SB202190 co-treatment (Fig. 20B and C). From these data it is clear that the subcellular localization of p38α and p38β is not influenced by Aurora B inhibition.

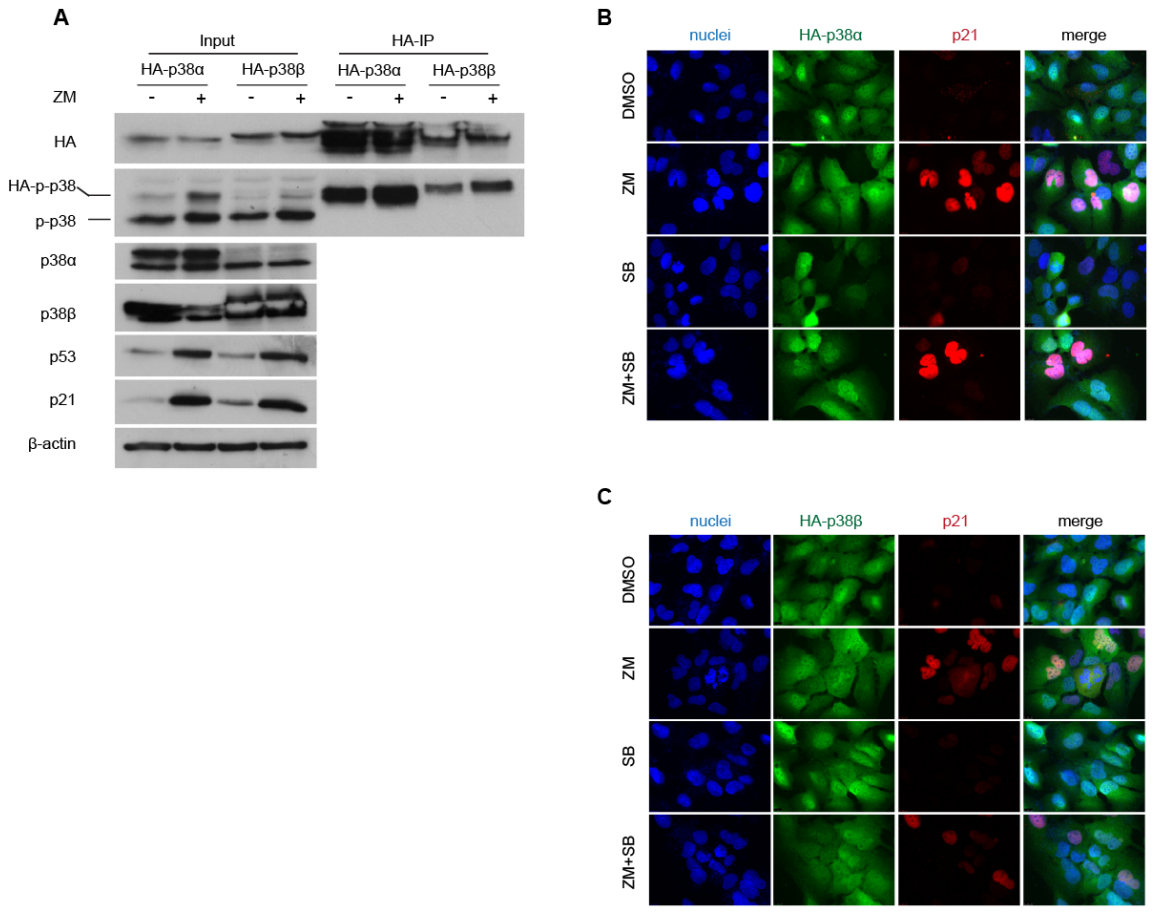

Figure 20: Inhibition of Aurora B activates both α and β isoforms of p38 MAPK without affecting their subcellular localization.
(A) U2OS-EcoR cells stably expressing either HA-p38α or HA-p38β were treated with 1 μM ZM447439 for 24 h and lysates were immunoprecipitated with HA-tag specific antibody. Immunoprecipitated proteins were analyzed by blotting with antibodies against HA and phosphorylated p38 (p-p38). The input sample was analyzed for with antibodies against HA, p-p38, p38, p53 and p21. β-actin served as loading control. (B) and (C) U2OS-EcoR cells stably expressing either HA-p38α or HA-p38β were treated with DMSO, 1 μM ZM447439, 10 μM SB202190 or first pretreated with 10 μM SB202190 for 2 h and then treated with 1 μM ZM447439 for 24 h. HA-tagged proteins (in green) and p21 (in red) were detected by immunostaining. Nuclei (in blue) were stained with Hoechst 33528.

3.11 Neither p38α nor p38β bind to the p21 gene upon Aurora B inhibition

It is known that the yeast p38-related MAP kinase, Hog1 associates with the transcribed region of osmoresponsive genes and promotes their transcriptional elongation in response to osmotic stress (Proft et al. 2006). To investigate, if this is true for human p38 MAPK as well, the binding of HA-p38α and HA-p38β to the p21 gene locus was studied by ChIP. U2OS-EcoR cells stably expressing either HA-p38α or HA-p38β were treated with

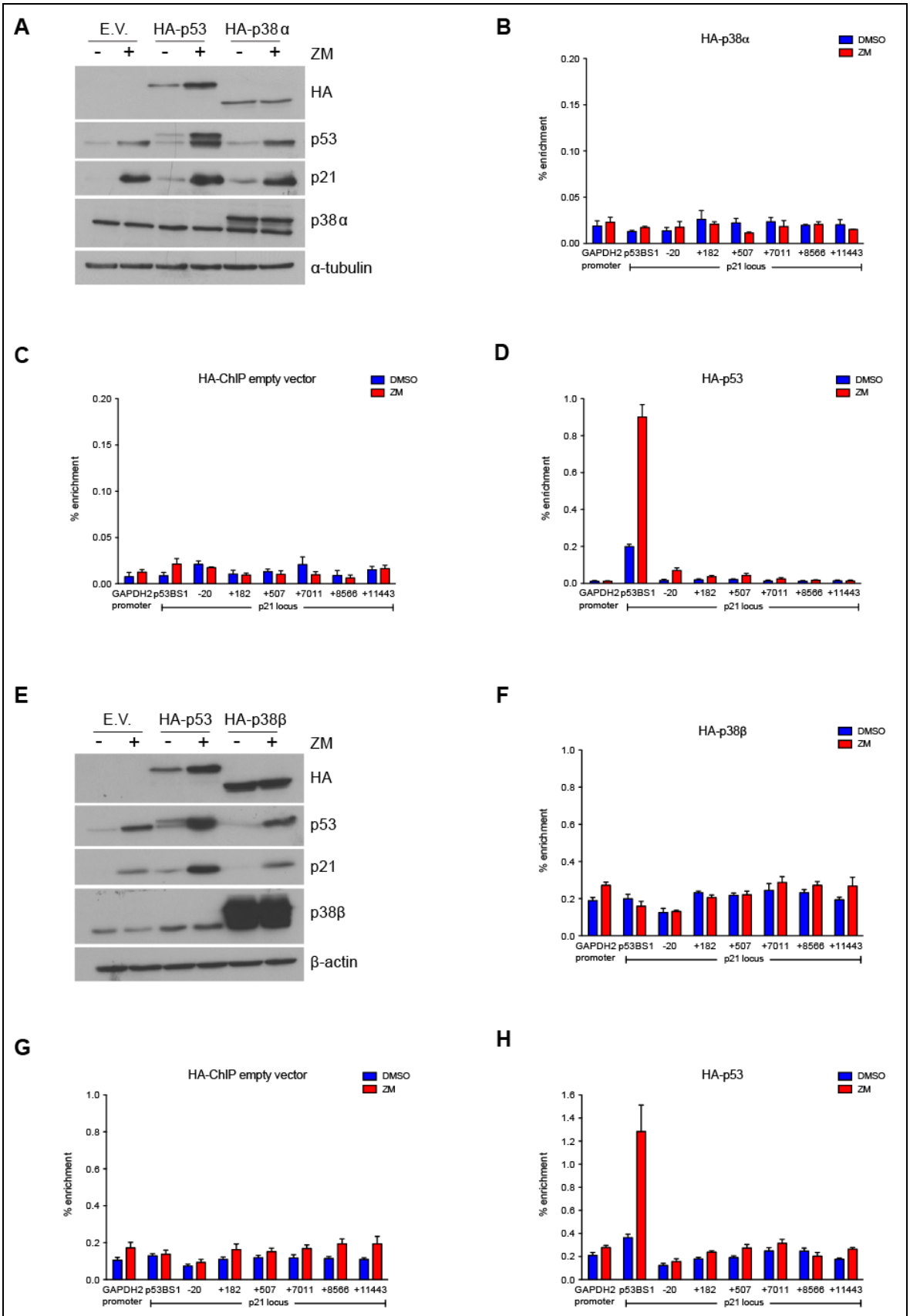

Figure 21: Neither p38α nor p38β bind to the p21 gene upon Aurora B inhibition.
(A) U2OS-EcoR cells stably expressing empty vector (EV), HA-p53 or HA-p38α were treated with DMSO or 1 μM ZM447439 for 24 h and lysates were subjected to analysis for respective

proteins indicated. (B) U2OS-EcoR cells stably expressing HA-p38α, empty vector (C) or HA-p53 (D) were treated with DMSO or 1 μM ZM447439 for 24 h and chromatin was immunoprecipitated with a HA-tag specific antibody. ChIP enriched DNA was amplified by qPCR using the indicated amplicons. The GAPDH2 promoter was analyzed as a control. (E) U2OS-EcoR cells stably expressing empty vector (EV), HA-p53 or HA-p38 β were treated with DMSO or 1 μM ZM447439 for 24 h and lysates were subjected to analysis for respective proteins indicated. (F) U2OS-EcoR cells stably expressing HA-p38β, empty vector (G) or HA-p53 (H) were treated with DMSO or 1 μM ZM447439 for 24 h and chromatin was immunoprecipitaed by HA-tag specific antibody. ChIP enriched DNA was amplified by qPCR using the indicated amplicons. GAPDH2 promoter was analyzed as a control.

ZM447439 and chromatin was isolated and immunoprecipitated with an antibody against the HA-tag. U2OS-EcoR cells stably expressing empty vector were used as negative control, whereas for positive control U2OS-EcoR cells stably expressing HA-p53 were used. Expression of HA-p38α and HA-p38β and induction of p53 and p21 upon Aurora B inhibition was verified by immunoblotting (Fig. 21A and E). In ChIP analysis, HA-p38α did not show any significant binding to the complete p21 gene locus (similar to empty vector expressing cells) either in untreated conditions or upon ZM447439 treatment (Fig. 21B and C). Similarly HA-p38β showed the same background binding to the p21 gene locus as empty vector (Fig. 21F and G). On the other hand HA-p53 binding was strongly induced at the p53 binding site 1 (p53BS1) in the p21 promoter upon Aurora B inhibition by ZM447439 treatment as expected (Fig. 21D and H). These results suggest that p38α and p38β are not recruited to p21 gene locus after Aurora B inhibition.

3.12 Elongin A binding to the p21 gene locus is induced upon Aurora B inhibition

Elongin A has been reported to bind p21 gene and mediate its transcriptional elongation in response to various cellular stresses (Kawauchi et al. 2013). To investigate the involvement of Elongin A in p21 transcriptional elongation, U2OS-EcoR cells stably expressing HA-tagged mouse Elongin A (HA-mEloA) were generated. The expression of HA-mEloA was verified by immunobloting (Fig. 22A). Further, treatment of these cells with ZM447439 resulted in p21 induction and this induction was blocked by p38 inhibition by SB202190 co-treatment, indicating that over expression of mouse Elongin A does not interfere with the activation of p21 upon Aurora B inhibition (Fig. 22A). U2OS-EcoR cells stably expressing HA-mEloA were treated with DMSO, ZM447439 or pretreated with SB202190 and then treated with ZM447439. Chromatin was isolated and immunoprecipitated with antibody against HA-tag. The binding of HA-mElongin A was induced at the core promoter as well as in the coding region the p21 gene towards the distal regions on Aurora B inhibition by ZM447439 treatment (Fig. 22B). Interestingly this

enrichment was blocked when p38 was inhibited by SB202190 co-treatment. This suggests that Elongin A recruitment to p21 gene locus might be dependent on signaling from p38. To confirm this possibility the effect of knockdown of Elongin A on p21 induction upon Aurora B inhibition needs to be investigated.

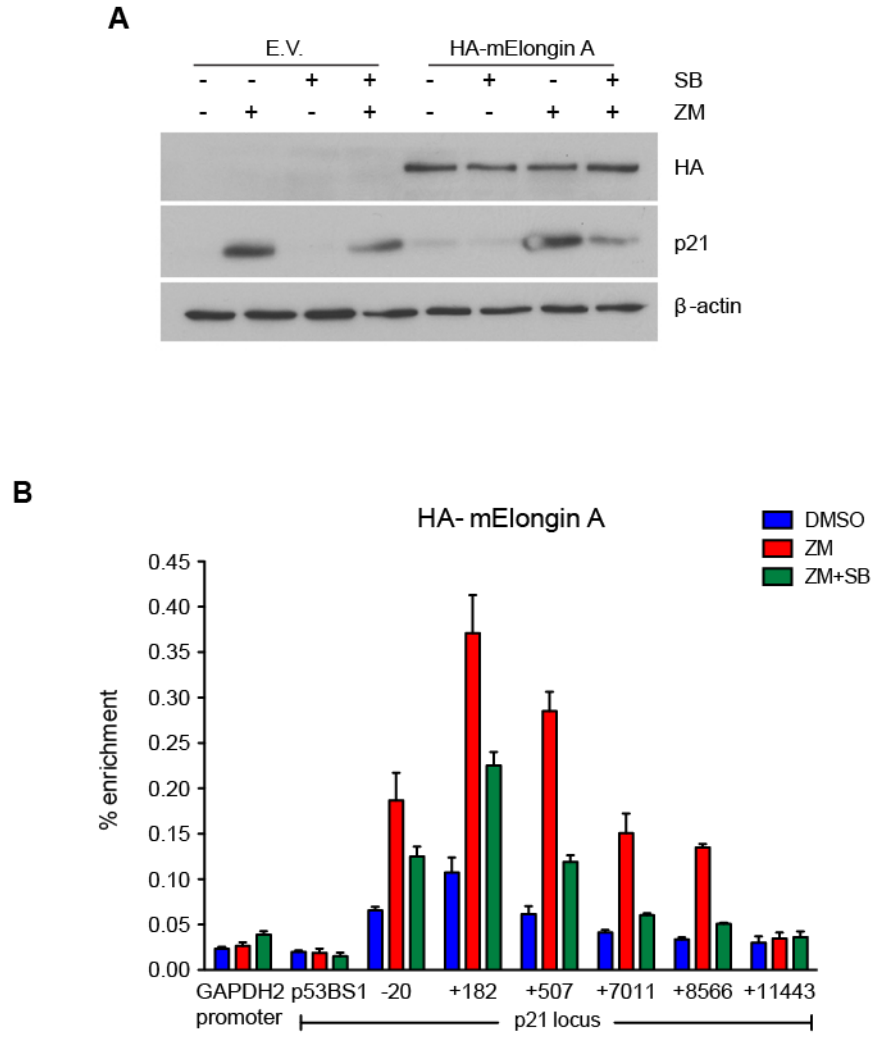

Figure 22: Binding of Elongin A to the p21 gene locus is induced upon Aurora B inhibition.
(A) U2OS-EcoR cells stably expressing empty vector (EV) or HA-mEloA were treated with DMSO, 1 μM ZM447439 alone, 10 μM SB202190 alone or pretreated with 10 μM SB202190 for 2 h and then treated with 1 μM ZM447439 for 24 h. Levels of HA-mEloA and p21 were determined by immunoblotting. β-actin served as loading control. (B) U2OS-EcoR cells stably expressing HA-mEloA, were treated as in (A) and chromatin was immunoprecipitated by HA-tag specific antibody. ChIP enriched DNA was amplified by qPCR using the indicated amplicons. GAPDH2 promoter was analyzed as a control.

3.13 Aurora B inhibition in interphase is not sufficient for induction of p21

Recently it has been proposed that Aurora B has a function outside mitosis, during the interphase where it mediates degradation of p53 by phosphorylation (Gully et al. 2012). In order to test whether the interphase function or the mitotic function of Aurora B is necessary for p21 induction, cells were arrested at the G1/S border by thymidine. The G1/S arrest was confirmed by flow cytometry (Fig. 23B). Inhibition of Aurora B in interphase (in G1/S arrested cells) by ZM447439 treatment did not induce p21 (Fig. 23A, lane 3), whereas p21 was strongly induced (Fig. 23A, lane 5) when the cells were first released from G1/S block and then treated with ZM447439 (inhibition of Aurora B outside interphase). As control, asynchronous cells were treated with ZM447439, which showed p21 induction (Fig. 23A, lane 6). Although G1/S arrested cells already showed p53 stabilization, this stabilization increased when cells were released from G1/S block and then treated with ZM443439. Taken together these findings support the conclusion that the induction of p53 and p21 is not due to inhibition of interphase function of Aurora B, but due to inhibition of its function during mitosis.

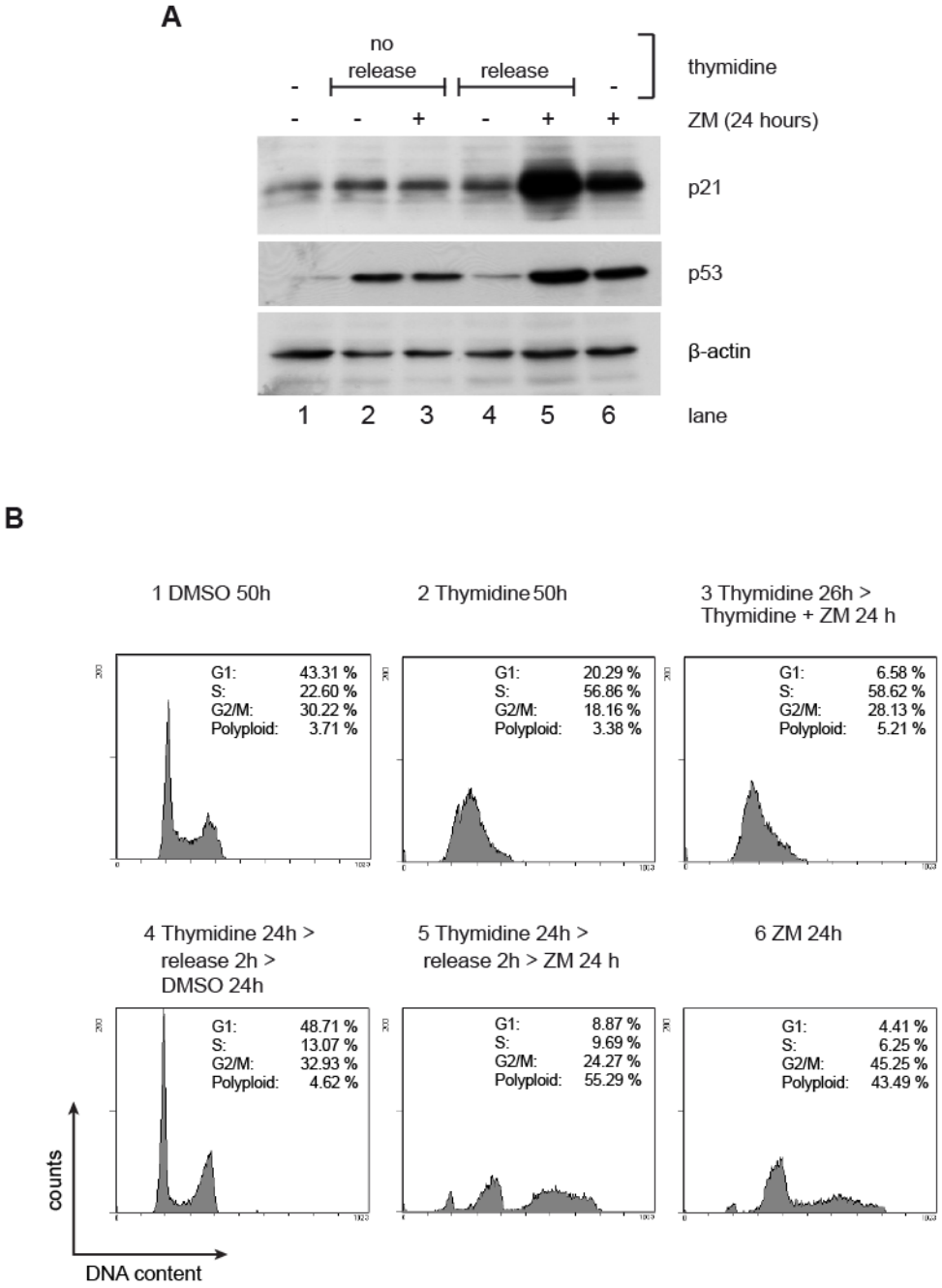

Figure 23: Aurora B inhibition in interphase is not sufficient for induction of p21.
(A) U2OS cells were synchronized in G1/S by treatment with 2.5 mM thymidine for 24 h (lanes 2-5). Thymidine-blocked cells were treated with DMSO or with 1 µM ZM447439 for 24 h either without release (lane 2 and 3) or after a 2 h release from the thymidine block (lane 4 and 5). Asynchronous cells (lane 1) and asynchronous cells treated with 1 µM ZM447439 for 24 h (lane 6) served as controls. Levels of p21 were determined by immunoblotting. β-actin served as a loading control. (B) Cell cycle synchronization of the experiment shown in (A) was confirmed by flow cytometry.
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3.14 Partial inhibition of Aurora B is sufficient to induce p21 without any cytokinesis defects

Given the various important functions of Aurora B during mitosis and cytokinesis like proper chromosome orientation and equal segregation of chromosomes leading to generation of euploid cell progeny (Ruchaud et al. 2007; Vader and Lens 2008; Carmena et al. 2012) and the proposed involvement of a 'tetraploidy checkpoint' in activation of p53 after cytokinesis failure (Andreassen et al. 2001; Margolis et al. 2003), it was of considerable interest to study if binucleation and hence failure of cytokinesis is necessary for activation of p53 and p21 after Aurora B inhibition. For this, U2OS and HCT116 cells were treated with different doses of Aurora B inhibitors and p21 induction as well as cellular defects was studied.

3.14.1 Partial inhibition of Aurora B by low doses of ZM447439 treatment in HCT116 cells results in p21 induction without binucleation

HCT116 cells were treated with increasing concentrations (0.1 μM-1 μM) of ZM447439 for 24 h and the phosphorylation status of histone H3 (pH3) was monitored by immunoblotting, which served as readout for extent of Aurora B inhibition (Fig. 24A) (Crosio et al. 2002). At low doses of ZM447439 (0.3 μM and 0.4 μM), Aurora B was partially inhibited as compared to the high dose (1 μM). Immunobloting and immunofluorescence revealed that p53 and p21 were induced at these low doses (Fig. 24B and C). Interestingly at these low doses of ZM447439, there were no cytokinesis defects as indicated by the presence of mononuclear cells (with p21 induction) and absence of multinucleated cells (Fig. 24C). In contrast, at the high dose of 1 μM ZM447439, all the cells became multinucleated and enlarged due to repeated failed cytokinesis (Fig. 24C). The absence of polyploid cells at low doses of ZM447439 as compared to high dose was also confirmed by flow cytometry (Fig. 24D). These findings suggest that subtle mitotic defects are sufficient to activate p53 and p21 in response to Aurora B inhibition and a complete failure of cytokinesis is not required.

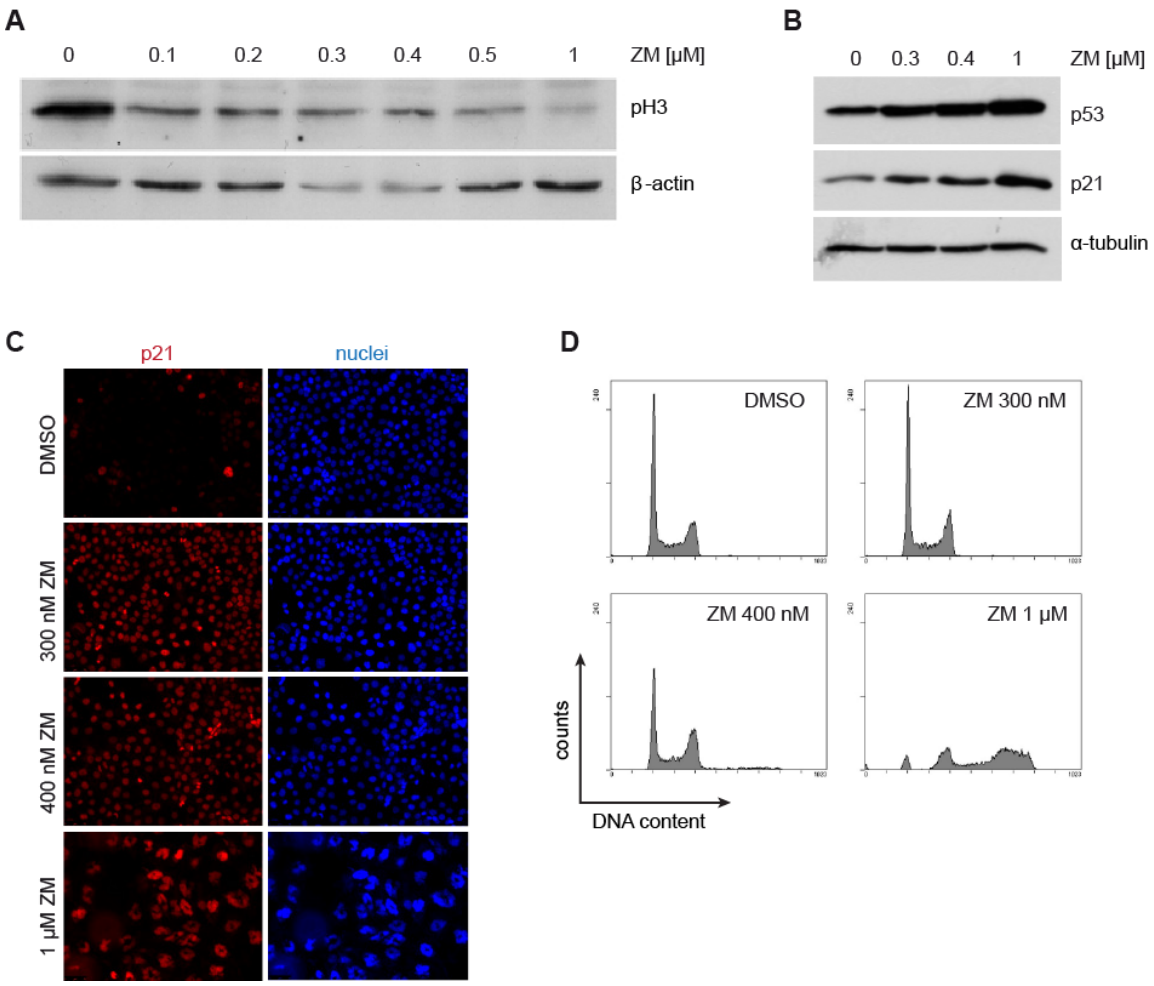

Figure 24: Partial inhibition of Aurora B by low doses of ZM447439 treatment in HCT116 cells results in p21 induction without binucleation.
(A) HCT116 cells were treated with various concentrations of ZM447439 for 24 h. Histone H3 phosphorylated at serine 10 (pH3) was analyzed by immunoblotting. β-actin served as a loading control. (B) HCT116 cells were treated with 0.3 µM, 0.4 µM or 1 µM ZM447439 for 24 h. Levels of p53 and p21 proteins were determined by immunoblotting. α-tubulin served as loading control. (C) HCT116 cells were treated as in (B) and level of p21 was determined by immunostaining. DNA was counterstained with Hoechst 33258. (D) HCT116 cells were treated as in (B) and FACS was done to determine the fraction of cells in different phases of the cell cycle.
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3.14.2 Partial inhibition of Aurora B by low doses of ZM447439 treatment in U2OS cells results in p21 induction without binucleation

To confirm that the effects seen at low doses of ZM447439 are not specific for HCT116 cells, another cell line U2OS was also treated with different doses (0.5 µM and 1 µM) of ZM447439. Treatment of U2OS cells with 0.5 µM ZM447439 did not lead to cytokinesis defects, but induced p21 at comparable levels to the higher dose of 1 µM (Fig. 25A). Also, p53 and p-p38 were activated at these low doses of Aurora B inhibitor (Fig. 25B),

indicating that the p38-p53-p21 pathway activation in response to Aurora B inhibition is sensitive to defects in mitosis and not to inhibition of cytokinesis. The cell cycle profiles of U2OS cells treated with 0.5 µM and 1 µM ZM447439, further confirmed that there was no cell division failure at the low doses (Fig. 25C).

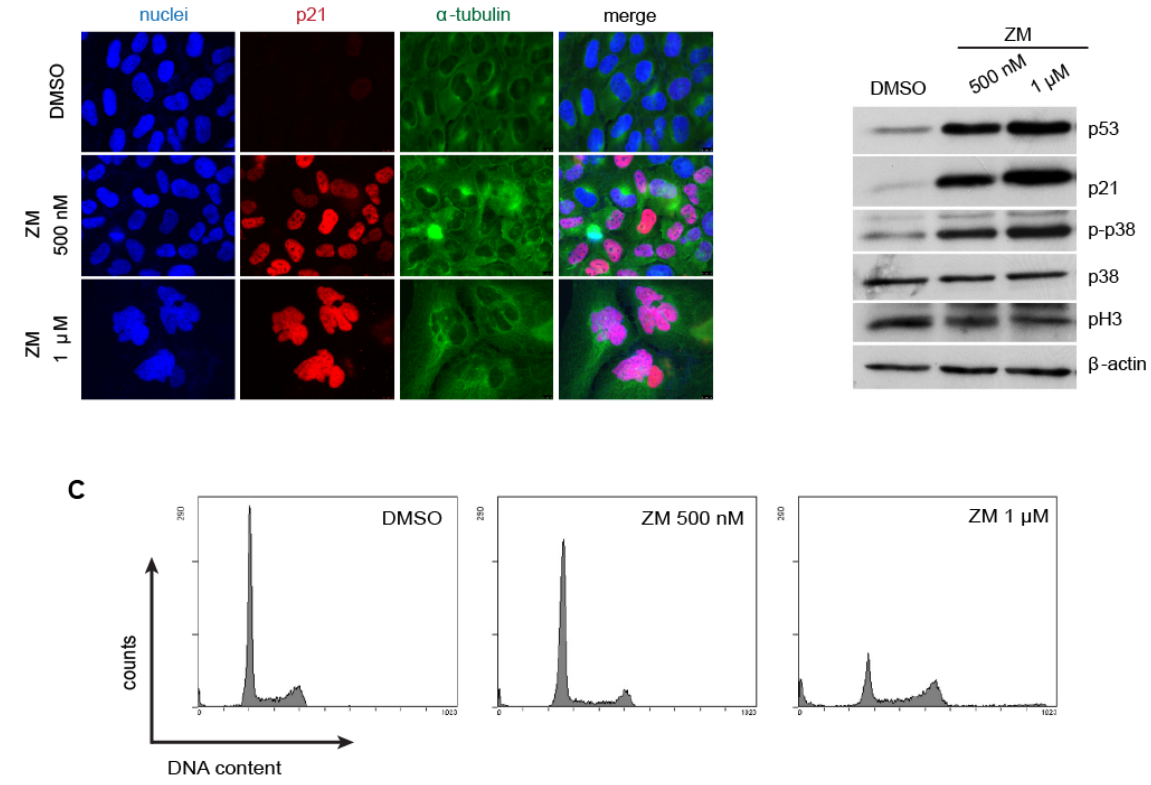

Figure 25: Partial inhibition of Aurora B by low doses of ZM447439 treatment in U2OS cells results in p21 induction without binucleation.
(A) U2OS cells were treated with DMSO, 0.5 µM or 1 µM ZM447439 for 24 h. p21 (in red) and α-tubulin (in green) was detected by immunostaining. Nuclei (in blue) were stained with Hoechst 33528. (B) U2OS cells were treated as in (A) and respective protein levels were determined by immunoblotting. (C) U2OS cells were treated as in (A) and analyzed by FACS to determine cells in different phases of cell cycle.
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3.14.3 Partial inhibition of Aurora B by low doses of AZD1152-HQPA treatment in U2OS cells results in p21 induction without binucleation

To exclude the possibility that the phenotypes observed in U2OS and HCT116 cells by low doses of ZM447439 treatment are artifact, another inhibitor of Aurora B, AZD1152-HQPA was used. U2OS cells were treated with 50 nM and 100 nM AZD1152-HQPA. Although both concentrations of AZD1152-HQPA induced comparable levels of p53, p-p38 and p21 (Fig. 26A), there were no binucleated or multinucleated cells at the lower

dose of 50 nM as compared to higher dose of 100 nM AZD1152-HQPA. Taken together, all these data with low doses of two different Aurora B inhibitors in two different cell lines indicate that tetraploidy and hence cytokinesis failure is not required for p38-p53-p21 activation in response to Aurora B inhibition.

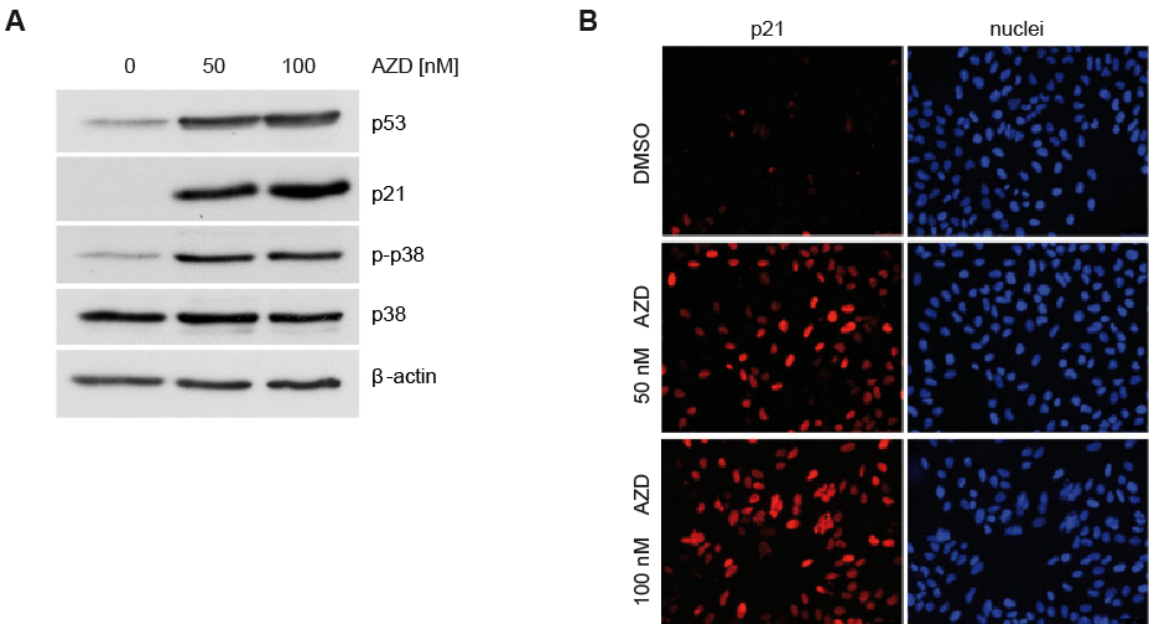

Figure 26: Partial inhibition of Aurora B by low doses of AZD1152-HQPA treatment in U2OS cells results in p21 induction without binucleation.
(A) U2OS cells were treated with DMSO, 50 nM or 100 nM AZD1152-HQPA for 24 h. Respective protein levels were determined by immunoblotting. (B) U2OS cells were treated as in (A) and p21 levels (in red) were determined by immunostaining. Nuclei (in blue) were stained with Hoechst 33528.
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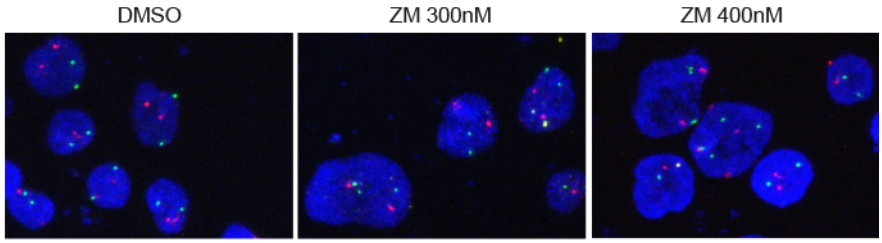
3.15 Partial Aurora B inhibition results in increased aneuploidy

The activation of p38-p53-p21 pathway in absence of any cytokinesis defects upon partial Aurora B inhibition suggests that chromosomal segregation errors due to Aurora B inhibition might be responsible for activation of this pathway. To answer this possibility the frequency of aneuploidy was determined by carrying out interphase FISH assays using fluorescent probes specific for chromosome 7 and 8. Partial inhibition of Aurora B by low doses of (0.3 μM or 0.4 μM) ZM447439 treatment (for 48 h) in HCT116 cells indeed caused increased aneuploidy as indicated by an increase in the deviation from mode for chromosome 7 from 5.01 % to 6.47 % (with 0.3 μM ZM447439) and to 11.84 % (with 0.4 μM ZM447439) and for chromosome 8 an increase from 3.34 % to 6.47 % (with 0.3 μM ZM447439) and to 14.11 % (with 0.4 μM ZM447439) (Fig. 27A and B). Hence p53 and

p21 activation on partial Aurora B inhibition correlates with increased chromosome segregation errors in absence of tetraploidy.

A



B

	chromosome analyzed	number of FISH signals per cell 1	2	3	4	>4	cells off the mode (%)
DMSO	7	18	625	7	6	2	5.01
	8	8	636	5	7	2	3.34
ZM 300 nM	7	26	622	9	7	1	6.47
	8	16	622	15	11	1	6.47
ZM 400 nM	7	31	506	18	17	1	11.84
	8	19	493	32	24	6	14.11

Figure 27: Partial Aurora B inhibition results in increased aneuploidy.
(A) Example microphotographs of interphase FISH assays of HCT116 cells treated with DMSO, 0.3 µM or 0.4 µM ZM447439 for 48 h. Chromosome 7: red; chromosome 8: green (B) Quantification of interphase FISH signals for chromosomes 7 and 8 in DMSO and ZM447349 treated HCT116 cells shown in (A). The difference in the percentage of cell off the mode after treatment with 400 nM ZM447439 was statistically significant (chromosome 7 or 8 p-values <0.001). With 300 nM ZM447439 the difference in percentage of cells of the mode was not significant for chromosome 7 but was significant for chromosome 8 (p=0.009). Chi-squared test was used to analyze the data.
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3.16 p21 induction after partial Aurora B inhibition does not involves DNA damage

A plausible cause of p21 activation due to partial Aurora B inhibition might be chromosomal segregation errors causing DNA damage, which has been reported to activate p53 (Thompson and Compton 2010b; Janssen et al. 2011). Presence of DNA damage can be detected by classical markers like presence of phosphorylated H2A.X (a widely used marker for DNA damage response as a result of double strand breaks) or presence of activated ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) kinases (Zhou and Elledge 2000; Falck et al. 2005). All these possibilities were

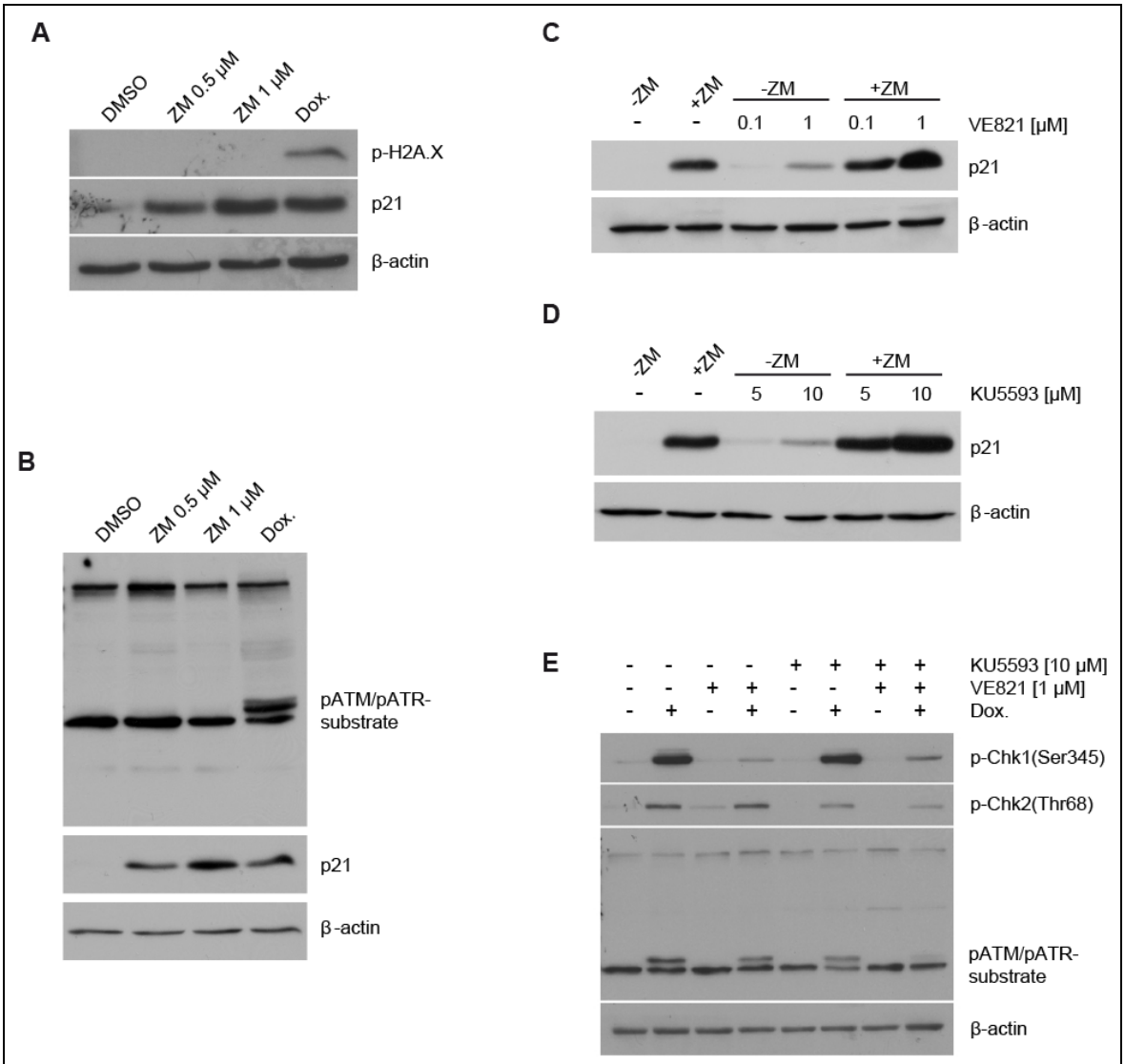

Figure 28: p21 induction after partial Aurora B inhibition does not involve DNA damage.
(A) U2OS cells were treated with DMSO, 0.5 µM ZM447439, 1 µM ZM447439 for 24 h or with 1 µM doxorubicin (Dox.) for 6 h. p21, p-H2A.X and β-actin protein levels were determined by immunoblotting. (B) U2OS cells were treated as in (A) and protein levels of phosphorylated ATM/ATR substrates and p21 were detected by immunoblotting. β-actin served as a control. (C) U2OS cells were pretreated with the indicated concentration of VE821 for 2 h and then treated with 1 µM ZM447439 for 24 h. p21 levels were determined by immunoblotting. β-actin served as a control. (D) U2OS cells were pretreated with the indicated concentration of KU5593 for 2 h and then treated with 1 µM ZM447439 for 24 h. p21 levels were determined by immunoblotting. β-actin served as a control. (E) U2OS cells were pretreated with DMSO, 1 µM VE821, 10 µM KU5593 or a combination of 1 µM VE821 and 10 µM KU5593 for 2 h and then treated with 1 µM doxorubicin or left untreated for 6 h. p-Chk1(Ser345), p-Chk2(Thr68) and p-ATM/p-ATR substrate protein levels were determined by immunoblotting. β-actin served as a control.
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explored. However there was no evidence of DNA damage upon partial Aurora B inhibition as evident by absence of p-H2A.X in U2OS cells upon ZM443439 treatment,

whereas p-H2A.X was detected by treatment with the DNA damaging agent, doxorubicin (used as positive control) (Fig. 28A). Also, phosphorylation of ATM/ATR kinase substrates was not detected in response to ZM447439 treatment (at both low and high doses) (Fig. 28B) while a positive result was obtained with doxorubicin, which induced the phosphorylation of ATM/ATR substrates (Fig. 28B). To further confirm that ATM and ATR kinases are not involved in p21 induction in response to Aurora B inhibition, the effects of ATM inhibitor KU5593 (Hickson et al. 2004) and ATR inhibitor VE821 (Reaper et al. 2011) on p21 induction mediated by ZM447439 treatment was investigated. However, none of these inhibitors were able to prevent the p21 induction upon ZM447439 treatment, indicating that they are not involved in the activation of the p53-p21 pathway after Aurora B inhibition (Fig. 28B and C). In order to test if the inhibitors were working, the effect of ATM inhibitor KU5593 on p-Chk2(Thr68) and of ATR inhibitor VE821 on p-Chk1(Ser345) induction upon doxorubicin treatment was studied. As expected, the phosphorylation of Chk1 at Ser-345 was efficiently blocked by VE821 and the phosphorylation of Chk2 at Thr-68 was efficiently blocked by KU5593, while the combined inhibitor treatment efficiently blocked the phosphorylation of ATM/ATR substrates (Fig. 28E). Overall, these results indicate that the DNA damage does not contributes to p21 activation after impairment of Aurora B function.

3.17 Partial inhibition of Aurora B results in proteotoxic stress but no autophagy

Aneuploidy has been linked with increased proteotoxic stress due to protein imbalance, in humans as well yeast (Stingele et al. 2012; Oromendia et al. 2012). Proteotoxic stress leads to formation of ubiquitinated protein aggregates, which can be detected by immunoblotting. Indeed increased poly-ubiquitin aggregates were detected in both U2OS and HCT116 cells upon partial Aurora B inhibition by ZM447439 treatment (Fig. 29A). Partial Aurora B inhibition by AZD1152-HQPA treatment in U2OS cells also resulted in generation of poly-ubiquitin aggregates (Fig. 29B). Since aneuploidy has also been linked to increased autophagy (Stingele et al. 2013), the effect of partial Aurora B inhibition on two autophagy markers (p62 and LC3) was investigated. However the protein levels of these markers (p62 and LC3) remained unchanged upon partial Aurora B inhibition by low dose ZM447439 treatment in both U2OS and HCT116 cells (Fig. 29C). These findings demonstrate that aneuploidy caused due to partial Aurora B inhibition generates proteotoxic stress.

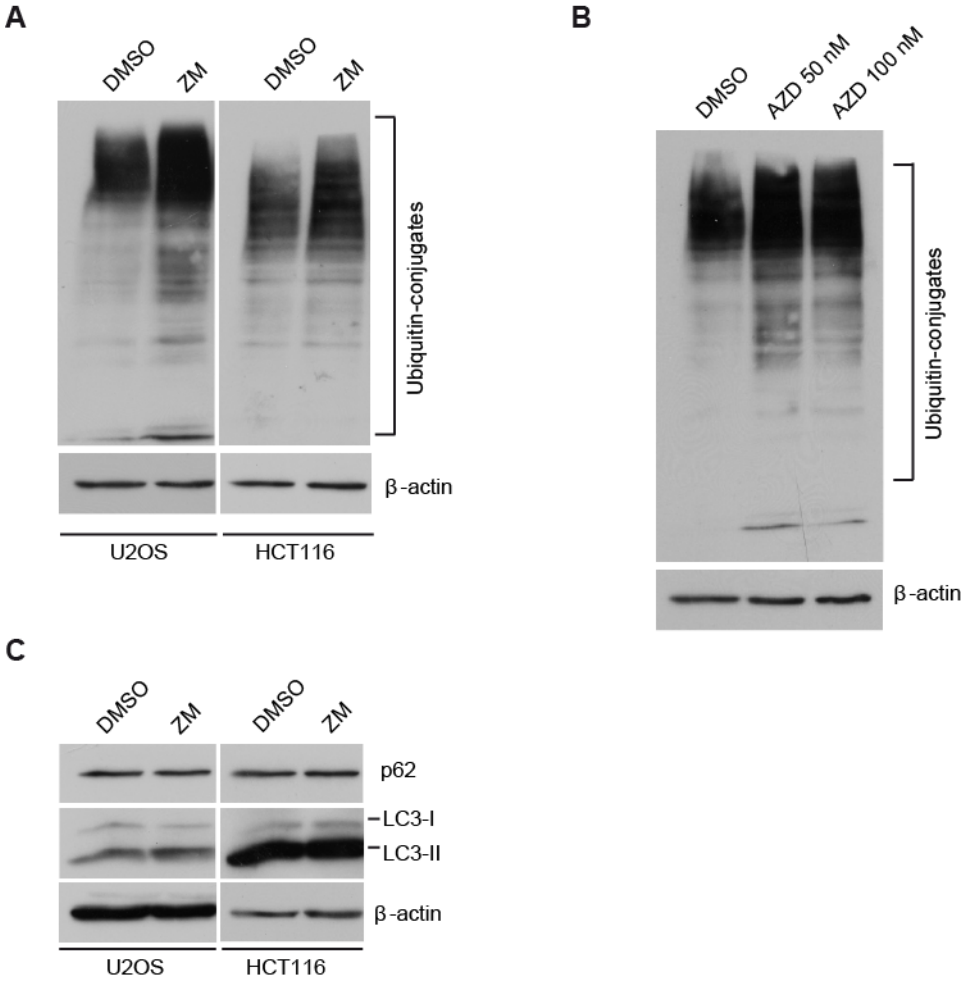

Figure 29: Partial inhibition of Aurora B results in proteotoxic stress but no autophagy. (A) U2OS and HCT116 cells were treated with 0.5 µM (for 24 h) or 0.3 µM (for 48 h) ZM447439, respectively. As a control, cells were treated with DMSO. Ubiquitin conjugates were detected by immunoblotting. β-actin served as a control. (B) U2OS cells were treated with DMSO, 50 nM or 100 nM AZD1152-HQPA for 24 h. Ubiquitin conjugates were detected by immunoblotting. β-actin served as a control. (C) U2OS and HCT116 cells were treated as in (A) and protein levels of p62 and LC3 were determined by immunoblotting. β-actin served as a control.

3.18 Partial inhibition of Aurora B correlates with increased generation of reactive oxygen species (ROS)

The metabolic imbalance resulting due to aneuploidy results in generation of reactive oxygen species (ROS) (Li et al. 2010). Hence it was speculated that ROS might be generated due to aneuploidy after partial Aurora B inhibition. To test the production of ROS, the dye H2DCF-DA was used, which is a general oxidative stress indicator and gives green fluorescence in presence of ROS. Interestingly partial Aurora B inhibition by ZM447439 treatment resulted in generation of significant amount of ROS (Fig. 30A).

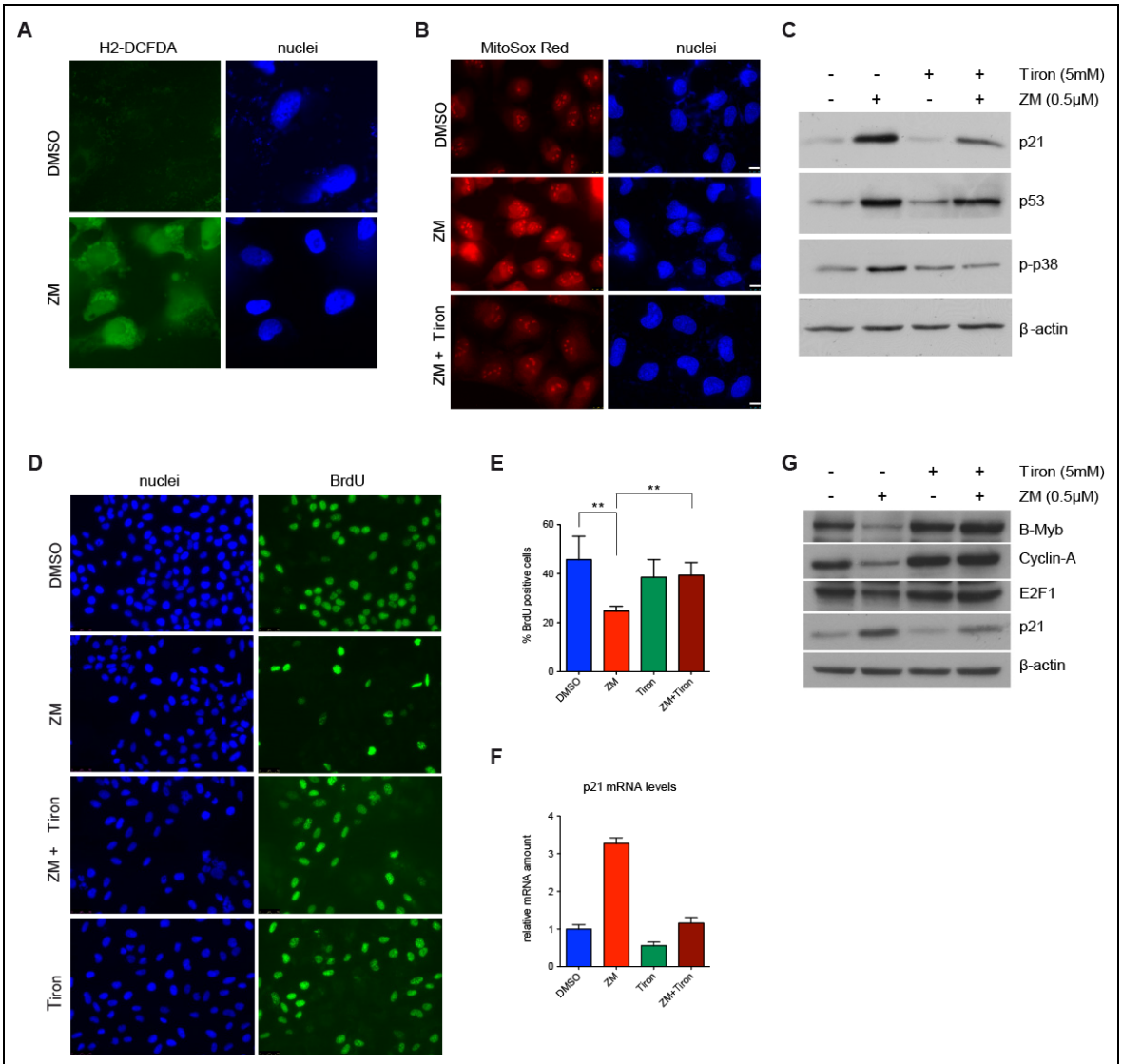

Figure 30: Partial inhibition of Aurora B correlates with increased generation of reactive oxygen species (ROS).

(A) Detection of ROS with the fluorescent dye H2DFC-DA in U2OS cells treated with DMSO or 0.5 μM ZM447439 for 24 h. (B) ROS generation was analyzed with MitoSox Red in U2OS cells treated with DMSO, 0.5 μM ZM447439 or co-treated with 0.5 μM ZM447439 and 5 mM Tiron for 24 h. Bar 10 μm. (C) U2OS cells were treated as in (B). Levels of p53, p21, phosphorylated p38 (p-p38) and β-actin were determined by immunoblotting. (D) U2OS cells were treated for 48 h with DMSO, 0.5 μM ZM447439, 5 mM Tiron or 0.5 μM ZM447439 and 5 mM Tiron. Cells were pulse labeled with 15 μg/ml BrdU for 2 h. The percentage of BrdU-positive cells was determined by immunofluorescence. (E) Quantification of the BrdU positive cells shown in (D). Error bars represent standard deviation of four independent experiments. The differences between DMSO and ZM447439 (p=0.005) and between ZM447439 and ZM447439+Tiron (p=0.0019) were statistically significant (student's t-test). (F) U2OS cells were treated as in (B) and p21 mRNA levels were determined by qRT-PCR. (G) U2OS cells were treated with DMSO, 0.5 μM ZM447439 or co-treated with 0.5 μM ZM447439 and 5 mM Tiron for 72 h. Protein levels of p21 and the E2F target genes (E2F1, B-Myb, Cyclin A) were determined by immunoblotting. β-actin served as loading control.

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To specifically test the type of ROS generated, MitoSox Red (which detects mitochondrial superoxide free radicals) was used. Staining by MitoSox Red gave a strong red fluorescence in presence of partial Aurora B inhibition (Fig. 30B), suggesting that mitochondrial superoxide anions are generated under conditions of aneuploidy generated due to partial Aurora B inhibition.

The next question was whether superoxide anions are required for activation of the p38-p53-p21 pathway. To answer this, an antioxidant 4,5-dihydroxybenzene-1,3-disulfonate (Tiron, a Vitamin E analog), was used which is a superoxide anion scavenger. Indeed, co-treatment with Tiron and ZM447439 rescued the induction of p-p38 and p21 observed upon ZM447439 treatment alone (Fig. 30C). Tiron co-treatment also partially prevented the activation of p53 by ZM447439 (Fig. 30C). Hence these findings implicate that ROS is involved upstream of p53 and p38 MAPK in response to partial Aurora B inhibition.

Further, it was important to know if ROS is also required for the cell cycle arrest observed on partial Aurora B inhibition. For this, DNA synthesis was measured by BrdU incorporation. Treatment with ZM447439 induced cell cycle arrest and hence resulted in a decrease in the percentage of BrdU positive cells. Interestingly, co-treatment with Tiron rescued the BrdU incorporation to a level similar to the control treated cells (Fig. 30D and E). Collectively these data indicate that partial Aurora B inhibition leads to generation of ROS, which in turn is required for cell arrest by mediating p53 and p38 dependent activation of p21.

3.19 Drugs that target aneuploid cells synergize with inhibition of Aurora B

In order to explore the functional and clinical significance of these studies, the ability of Aurora B inhibitors to synergize with drugs to which aneuploid cells are sensitive, for example the heat shock protein 90 (Hsp90) inhibitor 17-AAG and AICAR an activator of the AMP-activated protein kinase (AMPK) (Tang et al. 2011) was analyzed. To do this, HCT116 cells were seeded at very low density (700 cells/well in a 6-well plate) and treated with DMSO, AICAR alone, 17AAG alone, combination of low dose of ZM447439 (400 nM) and AICAR or combination of low dose of ZM447439 (400 nM) and 17AAG. The number of colonies was determined after 14 days, by fixing the cells and staining with crystal violet. Treatment with AICAR alone moderately inhibited proliferation of cells whereas with 17AAG alone and low dose of ZM447439 had no effect (Fig. 31A). Interestingly the combination of low dose of ZM447439 with either AICAR or 17AAG markedly reduced the colony formation (Fig. 31A). This effect of the combination of ZM447439 with these drugs was further confirmed in U2OS cells (Fig. 31C) and validated by quantification of the dye extracted from these assays (Fig. 31B).

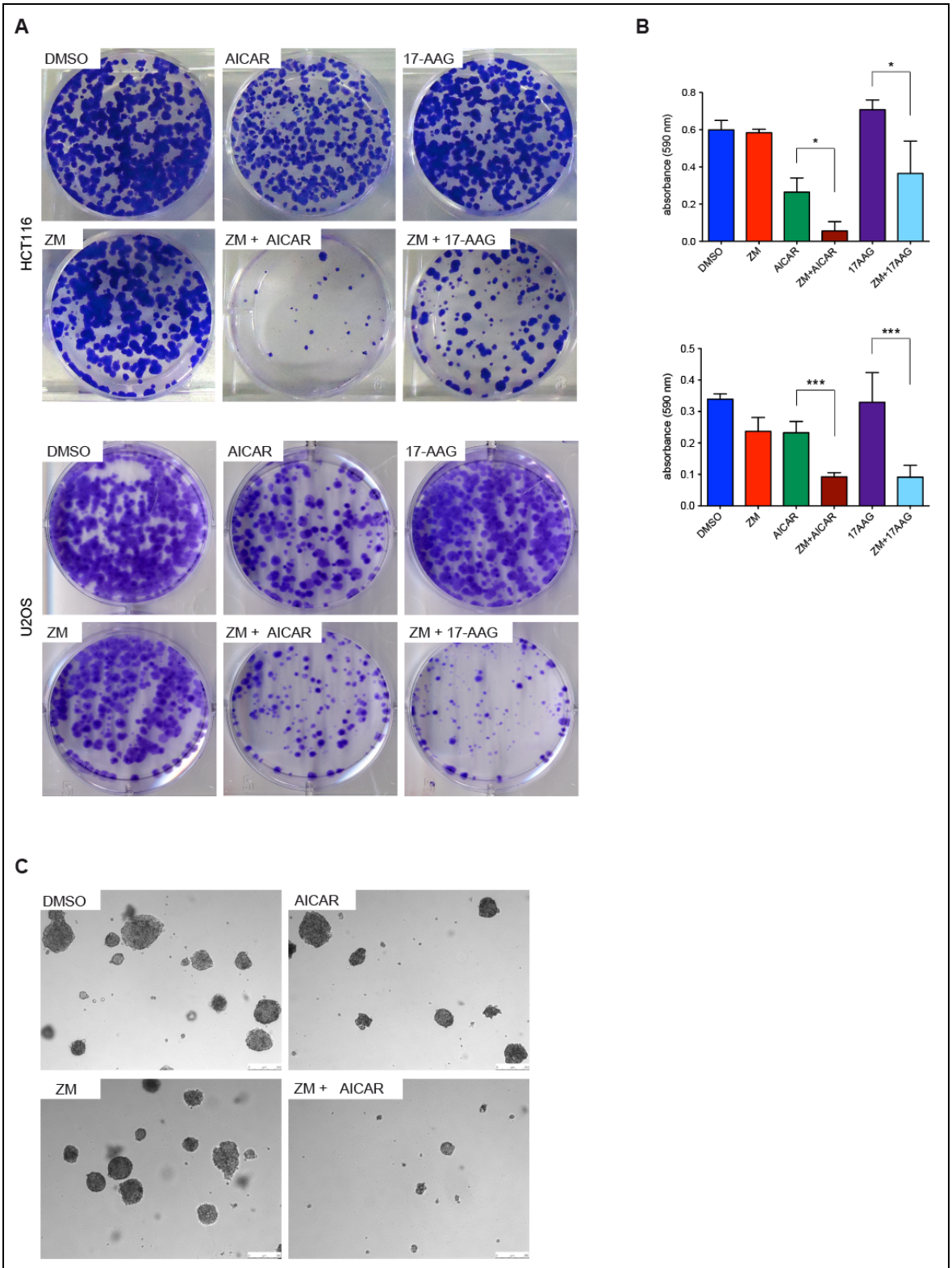

Figure 31: Drugs that target aneuploid cells synergize with inhibition of Aurora B.
(A) HCT116 and U2OS cells were treated with DMSO, AICAR (200 µM), 17-AAG (8nM), ZM447439 (0.4 µM for HCT116, 0.5 µM for U2OS), a combination of ZM447439 and AICAR or a combination of ZM447439 and 17AAG. 14 days later, colonies were fixed and stained with crystal violet. (B) Quantification of the colonies shown in (A). The dye was extracted and absorbance was determined at 590 nm. The experiment was performed in triplicates. Error bars

represent standard deviation. (*P<0.05, ***P<0.01, student's t-test). (C) Soft-agar assays were performed to analyze anchorage independent growth of HCT116 cells treated with DMSO, AICAR (200 μM), ZM447439 (0.4 μM) or a combination of 0.4 μM ZM447439 and 200 μM AICAR.
This figure was published in similar form in Kumari et al. 2014.

Next, the ability of the combination of ZM447439 and AICAR to inhibit oncogenic transformation was explored by determining the ability of HCT116 cells to grow independently of anchorage in presence of these drugs. Low dose ZM447439 or AICAR treatment had no effect on colony formation, whereas the combined treatment strongly prevented the ability of HCT116 cells to grow independent of anchorage (Fig. 31C). Together these results support the notion that low doses of Aurora B inhibitor ZM447439 synergize with drugs (AICAR and 17AAG), to which aneuploid cells are sensitive, to inhibit cell proliferation and transformation.

3.20 The synergism of Aurora B inhibitor with AICAR/17AAG in decreasing cell proliferation is due to a cooperative effect on induction of cell cycle inhibitor proteins

The decrease in proliferation of cells upon co-treatment with ZM447439 and AICAR (and 17AAG) might be due to an additive effect on activation of cell cycle inhibitor p21 (Chae et al. 2012; Vaseva et al. 2011). In order to explore this possibility U2OS cells were treated with DMSO, ZM447439 (0.5 μM), AICAR (200 μM), 17AAG (8 nM), a combination of ZM447439 and AICAR or a combination of ZM447439 and 17AAG for 12 h or 24 h. Interestingly, AICAR cooperated with ZM447439 to induce p21 and p53 at 12 h and also to induce p27 after 24 h (Fig. 32A and B). 17AAG also augmented p21 induction after 12 h co-treatment with ZM447439 (Fig. 32C). These results indicate that AICAR and 17AAG act together with ZM447439 at an early time-point to cause growth inhibition by increasing the expression of well-known cell cycle inhibitors p53, p21 and p27.

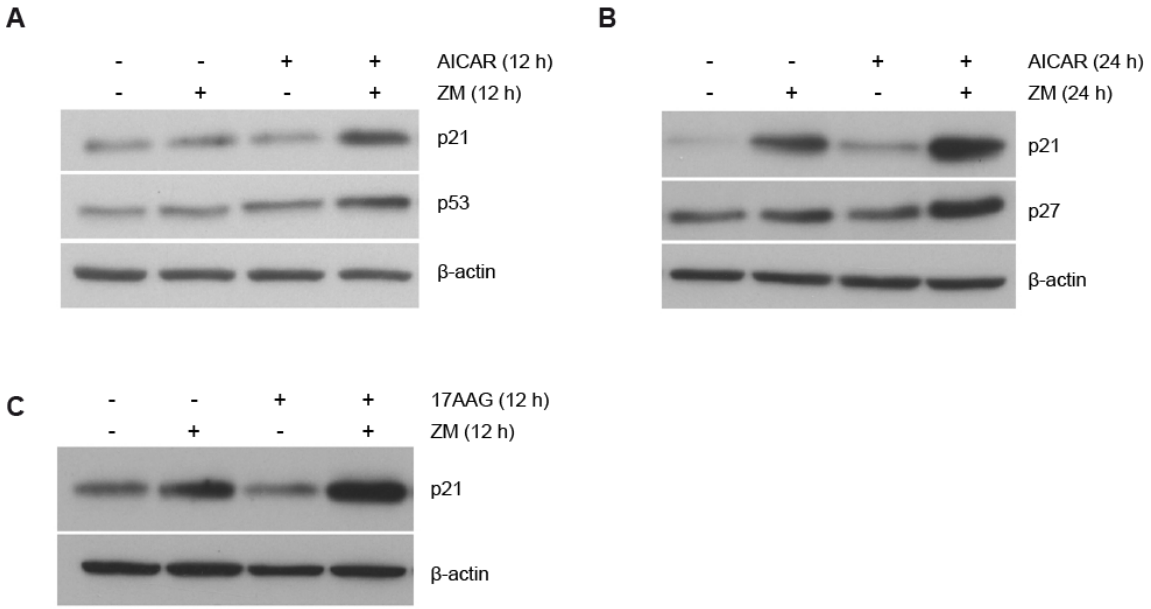

Figure 32: AICAR and 17AAG cooperate with ZM447439 to induce p21, p53 and p27.
(A) U2OS cells were treated with DMSO, 0.5 µM ZM447439, 200 µM AICAR or a combination of ZM447439 (0.5 µM) and AICAR (200 µM) for 12 h and protein levels of p53, p21, and β-actin were determined by immunoblotting. (B) U2OS cells were treated with DMSO, 0.5 µM ZM447439, 200 µM AICAR or a combination of ZM447439 (0.5 µM) and AICAR (200 µM) for 24 h and protein levels of p21 and p27 were determined by immunoblotting. β-actin served as loading control. (C) U2OS cells were treated with DMSO, 0.5 µM ZM447439, 8 nM 17AAG or a combination of ZM447439 (0.5 µM) and 17AAG (8 nM) for 12 h and protein levels of p21 and β-actin were determined by immunoblotting.
Parts of this figure were published in similar form in Kumari et al. 2014.

4 Discussion

It has been reported that the tumor suppressor p53 and its target gene p21 (a cell cycle inhibitor) are activated as a consequence of Aurora B inhibition. However the detailed molecular requirements for p21 induction have not been established. In this study I investigated the pathways leading to p21 activation due to Aurora B inhibition in detail. Using chemical inhibitors against Aurora B (ZM447439 and AZD1152-HQPA) and against p38 MAPK (SB202190 and BIRB796), I demonstrate here that, induction of p21 in response to Aurora B inhibition requires activation of p38 MAPK, while p53 induction is independent of p38 MAPK activity. p38 mediates the activation of p21 by stimulating the transcriptional elongation of p21 gene by RNA Polymerase II. Further I show that partial inhibition of Aurora B by low doses of Aurora B inhibitors is sufficient for activation of p38, p53 and p21 in the absence of any cytokinesis defects. Instead, aneuploidy and subsequent generation of oxidative stress accomplish the activation of p38-p53-p21 pathway under partial Aurora B inhibition conditions. Finally I provide evidence that cancer cells treated with low doses of Aurora B inhibitor are sensitive to drugs that specifically target aneuploid cells such as AICAR and 17AAG.

4.1 p38 MAPK is necessary for p21 induction and is required for transcriptional elongation stage of p21 gene regulation in response to Aurora B inhibition

Inhibition of Aurora B activates p38, a stress activated mitogen kinase by inducing phosphorylation of p38 (active form of p38 MAPK) without changing the total p38 protein levels (Fig. 13A). Both isoforms of p38, p38α and p38β are phosphorylated upon Aurora B inhibition (Fig. 20A). Inhibition of p38 MAPK (by two chemical inhibitors SB202190 and BIRB796) prevents the p21 induction mediated by two different Aurora B inhibitors (ZM447439 and AZD1152-HQPA) in two different cell lines (Fig. 13B-E). Conversely, p38 inhibition does not block the p21 activation in response to DNA damage induced by doxorubicin (Fig. 13F). These data strongly point towards the specific requirement of p38 in p21 induction in context of Aurora B inhibition. In fact a number of other studies have reported the involvement of p38 MAPK in cell cycle arrest and activation of p53 and p21 due to mitotic stress such as disruption of centriole function (Mikule et al. 2007; Uetake et al. 2007; Thompson and Compton 2010).

Inhibition of p38 does not influence p53 induction, indicating that it is regulating p21 without affecting the protein levels of p53 (Fig.13B-E). Also p38 does not modulate the stability of the p21 mRNA (as observed by Tanja Ulrich and published in Kumari et al.

2013) or protein (Fig. 16B and C). Chromatin immunoprecipitation analysis at the p21 gene locus revealed that p53 binding is strongly induced at its binding site (p53BS1) in the p21 promoter upon Aurora B inhibition but this binding remains unaltered upon p38 co-inhibition (Fig. 17B and D, Fig. 18C). This indicates that p53 binding to the p21 gene locus does not require p38 signaling. The loading of RNA Polymerase II to the promoter of p21 gene also occurs independently of p38 activity (Fig. 18D). Interestingly, the enrichment of the elongating form of RNA Polymerase II in the p21 coding region and formation of full-length p21 primary transcripts requires p38 signaling (Fig. 18E and F). Collectively, these data identify p38 MAPK as a downstream mediator of Aurora B in regulating p21 gene expression through its transcriptional elongation. Regulation of p21 gene expression by modulation of transcriptional elongation by RNA Polymerase II has been demonstrated by several other studies (Gomes et al. 2006; Kawauchi et al. 2013; Valin et al. 2013). Regulation at the transcriptional elongation stage occurs at the promoters of stress responsive genes, which are characterized by the presence of paused RNA Polymerase II at the promoter in the uninduced state. This ensures rapid induction of gene transcription once the cell receives a stress signal (Muse et al. 2007; Price 2008; Adelman and Lis 2012). In fact I also found that the RNA Polymerase II was paused at the p21 gene promoter in absence of any stimulation (Fig. 18D). Collectively these findings demonstrate a yet unknown role of p38 MAPK in mediating transcriptional elongation of p21 gene and hence its importance in fine-tuning the stress signaling activated due to Aurora B inhibition.

4.2 p38 is not recruited to p21 gene locus after Aurora B inhibition

In light of the published findings that the yeast counterpart of human p38, Hog1 binds to the coding regions of genes and has a role in transcriptional elongation (Proft et al. 2006), it was of interest to study if the mammalian counterpart also has the same function. As the commercially available antibodies for p38 and p-p38 do not work in ChIP, I performed HA-ChIP assays with cells stably expressing HA-p38α and HA-p38β. However, none of the p38 isoforms bound to p21 gene locus upon Aurora B inhibition (Fig. 21B and F). Nevertheless, the possibility that the endogenous p38 is recruited to the p21 gene locus after Aurora B inhibition cannot be excluded. It is possible that p38 is binding to the p21 gene locus through interaction with the RNA Polymerase II transcription machinery via transient interactions, and that these interactions are too weak to be detected by the crosslinking conditions used in the ChIP assay. There is evidence that mammalian p38 MAPK is recruited to the promoter as well as to the coding regions of stress responsive genes such as c-Fos, IL8, Cox2 via the respective transcription factors Elk1, NFκB and

AP1, in response to osmostress (Ferreiro et al. 2010). Besides the possibility of direct binding of p38 to the p21 gene locus, another possibility is that p38 is recruiting (or phosphorylating resulting in differential activity) other mediators of the transcriptional elongation machinery (transcription factors and other DNA-binding proteins such as chromatin modifiers) to the p21 gene locus to activate its elongation. For instance, p38 has an essential role in muscle differentiation by phosphorylating BAF60c, a subunit of SWI/SNF complex, which promotes the recruitment of the chromatin remodeling complex SWI/SNF along with the muscle transcription factor MyoD (Simone et al. 2004; Forcales et al. 2012). p38 also phosphorylates several transcription factors including myocyte enhancer factors 2C and 2A (MEF2C and 2A) (Han et al. 1997; Zhao et al. 1999), p53 (Bulavin et al. 1999) and C/EBP (c-AMP response element binding protein) family of transcription factors (Wang and Ron 1996).

Regarding transcriptional elongation control by p38, one interesting candidate is Elongin A. Kawauchi et al. (2013) recently demonstrated that Elongin A is necessary for transcriptional elongation of stress responsive genes including p21 and ATF3 under stress conditions. In fact I could also demonstrate that recruitment of Elongin A to the p21 gene locus is induced by Aurora B inhibition and also that this recruitment is blocked upon p38 co-inhibition (Fig. 22B). Requirement of Elongin A for p21 induction requires further validation by studying the effect of Elongin A knockdown (by siRNA/shRNA) on p21 activation after Aurora B inhibition. Another relevant mediator in transcriptional elongation of p21 after Aurora B inhibition is CDK8, a component of the Mediator complex. Recently it was shown that under hypoxia conditions, HIF1A recruits CDK8 to stimulate transcriptional elongation by RNA Polymerase II (Galbraith et al. 2013). It will be interesting to study if CDK8 is also recruited to p21 gene locus upon impairment of Aurora B function, and if yes, then whether its recruitment is p38 dependent. Exploring the mechanism by which p38 is mediating transcriptional elongation of p21 gene upon disruption of Aurora B function will be of foremost importance in future experiments.

4.3 Disruption of mitotic function of Aurora B is necessary for p21 induction, but tetraploidy is not required

Chemical inhibition of Aurora B by ZM447439 and AZD1152-HQPA generates polyploidy and induces p21 at both mRNA and protein levels (Fig. 10, Fig. 11 and Fig. 16A). Knockdown of Aurora B also induces p21 (Fig. 10D). This induction of p21 is p53 dependent as absence of p53 or disruption of its function (by SV40 large T antigen) failed to induce p21 upon Aurora B inhibition (Fig. 12B and C). These findings are in line with a number of reported studies (Gizatullin et al. 2006; Tao et al. 2008; Kaestner et al. 2009).

p53 is stabilized due to prevention of its degradation by Aurora B when Aurora B is inhibited as reported by Gully et al. (2012). p53 is a tumor suppressor and guardian of genome, which prevents cell cycle progression under stress conditions by regulating the induction of cell cycle inhibitor protein p21. After defects in mitosis, p53 causes a pseudo-G1 arrest (cells arrested in G2/M phase with 4N DNA content) (Lanni and Jacks 1998). This is evident by the accumulation of cells with 4N DNA content (Fig. 10B and 11B), p21 induction (Fig. 10C) and presence of dephosphorylated pRb upon ZM447439 treatment (Fig. 15B). This ensures that the cells with abnormal DNA content and altered ploidy status are eliminated by apoptosis after prolonged cell cycle arrest or undergo senescence. An interesting question to be answered is that, when during the cell cycle, is p53 activated after Aurora B inhibition. For example, in mitosis, as proposed by (Blagosklonny 2006) or in the subsequent G1 phase. In support of the second possibility, a recently published study reported that, Aurora B also plays a role during interphase in mediating p53 degradation by phosphorylating it, in addition to it's functions during mitosis (Gully et al. 2012). Hence, it might be possible that p53 stabilization resulting from Aurora B inhibition during interphase is responsible for p21 induction. Alternatively, it is also possible that p21 induction is due to signaling pathways activated due to failed mitosis. In order to tease apart these alternate hypotheses, Aurora B was inhibited in interphase or during mitosis. When cells are treated with ZM447439 during interphase, p21 is not induced, whereas a strong p21 induction occurs when cells progress through mitosis after treatment with ZM447439 (Fig. 23). Hence, in order for p21 to be induced, inhibition of Aurora B during mitosis is necessary. The requirement of mitotic entry for Aurora B inhibition mediated p53 induction has been shown by a previous study which demonstrated that inhibition of mitotic entry by CDK1 inhibitor prevented the p53 induction by Aurora B inhibitor (Dreier et al. 2009). Hence, these findings imply that defects during mitosis are responsible for activation of p53-p21 pathway. This result is not in agreement with the recently published findings, where it was shown that knocking out Aurora B (using conditional MEFs) during quiescence (G0) phase induces p21 (Trakala et al. 2013). The observed disparity between my study and by Trakala et al. might be due to the different cell systems used (human cells vs. primary mouse cells respectively) and hence point towards different requirements for p21 induction after Aurora B inhibition in these two systems, which certainly requires further investigation.

Treatment of U2OS and HCT116 cells with doses of Aurora B inhibitors, which are half (or below) of the dose at which severe cytokinesis defects are observed, still induces p53, p38 and p21 (Fig. 24B and C, Fig. 25A and B, Fig. 26A). Interestingly, under conditions of partial Aurora B inhibition there is no cytokinesis failure as indicated by the presence of

mononuclear cells with low doses of Aurora B inhibitors as compared to a large number of multinucleated cells observed with higher doses (Fig. 24C, Fig. 25A, Fig. 26B). Also the cell cycle profiles of cells with partial Aurora B inhibition resemble with that of control cells, showing no obvious changes in their profile as compared to cells with complete Aurora B inhibition, which have a highly distorted profile with a large polyploid fraction (Fig. 24D and Fig. 25C). The protein levels of phosphorylated histone H3 (pH3, a Aurora B substrate) decreased with increasing concentrations of ZM447439, indicating the extent of Aurora B inhibition (Fig. 24A). Since the effect of partial Aurora B inhibition on cell division, cell cycle profile and the p38-p53-p21 pathway was confirmed using two chemical inhibitors against Aurora B and in two cell lines, the possibility that these effects were artifact is highly unlikely. This finding implies that complete inhibition of cytokinesis and subsequent generation of a tetraploid state is not necessary for induction of the p38-p53-p21 pathway, hence supporting the notion that the cell cycle arrest does not requires a 'tetraploidy checkpoint'. It has been proposed that the tetraploidy resulting due to cytokinesis failure activates a 'tetraploidy checkpoint' which counts the number of chromosomes and centrosomes resulting in cell-cycle arrest in subsequent G1 phase by activation of p53 and p21 (Andreassen et al. 2001; Margolis et al. 2003). In addition to the evidence presented here, other studies (Uetake 2004; Wong and Stearns 2005) also support that a tetraploidy checkpoint does not exists.

4.4 Partial Aurora B inhibition generates aneuploidy and subsequently proteotoxic stress and oxidative stress

When Aurora B is inhibited partially, although no tetraploidy was observed, an increase in chromosome misseggregation (as quantified by FISH assay) and hence aneuploidy is observed (Fig. 27). This could be due to inhibition of the ability of Aurora B to resolve merotelic attachments (where one kinetochore is attached to microtubules emanating from opposite spindle poles). Indeed it has been reported previously that at low doses of Aurora B inhibitor ZM447439, the frequency of merotelic attachments increased from 8 to 50% in Ptk1 cells (Cimini et al. 2006). Cimini et al. (2006) proposed that this is due to increased stabilization of microtubule-kinetochore interactions upon partial Aurora B inhibition resulting in anaphase lagging chromosomes. The same study by Cimini et al. shows that at low doses of ZM447439, there is only a partial inhibition of histone H3 phosphorylation, consistent with my findings (Fig. 24A). Induction of p53 and p21 due to aneuploidy is also consistent with the findings of Thompson and Compton (2010), where they show that chromosomal missegregations caused either by monastrol washout or by siRNA mediated knockdown of the mitotic kinesin MCAK (mitotic centromere-associated

kinesin) induces p53 and p21. Aneuploidy generated by other means such as due to compromised SAC components also induces p21 (Li et al. 2010).

The findings of the present study suggest that when Aurora B activity is partially blocked, its functions in regulating kinetochore-microtubule turnover (required for faithful chromosome segregation) are preferentially affected, while its functions at the midbody (required for proper execution of cytokinesis) remain unperturbed resulting in only chromosome segregation defects (causing aneuploidy) in absence of any cytokinesis defects. This may be due to abundance of PP1 and PP2A phosphatases near the centromeres at the kinetochores as compared to other regions in the cell such as chromosome arms and midbody, due to preferential targeting of these phosphatases to the centromere (Kitajima et al. 2006; Posch et al. 2010). In future experiments it will be interesting to study the inhibition of kinetochore substrates (Hec1, MCAK) vs. midbody substrates (MgcRacGAP, MKLP1) of Aurora B under conditions of partial and complete Aurora B inhibition.

In a state of aneuploidy, the chromosome complement is not an exact multiple of the haploid karyotype, as a result of which there is a disturbance in the stoichiometric ratio of the proteins due to absence/presence of extra chromosomes in the cell. This in turn causes burden on the mechanisms for protein quality control (chaperone system) and protein clearance/degradation (proteasome, autophagy), causing proteotoxic stress (Fig. 33).

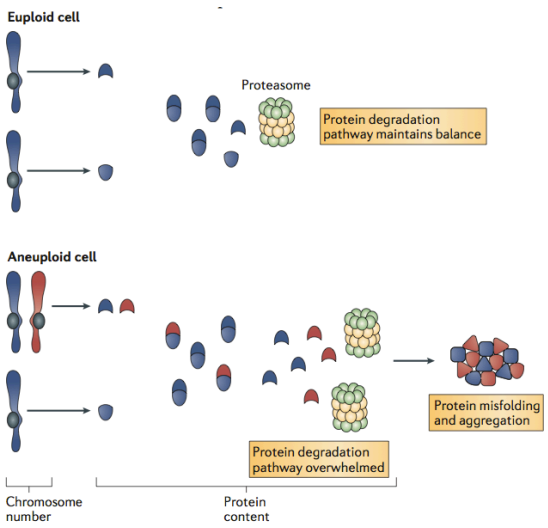

Figure 33: Proteotoxic stress in response to aneuploidy.
Figure taken from (Gordon et al. 2012).

Indeed ubiquitin aggregates are accumulated in the cell as a result of proteotoxic stress caused by partial Aurora B inhibition (Fig. 29A and B). A similar correlation between

aneuploidy and proteotoxic stress has been demonstrated in yeast (Oromendia et al. 2012) as well human cell lines (Stingele et al. 2012). Although proteotoxic stress causes activation of autophagy (Stingele et al. 2013), there was no evidence of autophagy under conditions of partial Aurora B inhibition (Fig. 29C). However involvement of proteasomal pathway for degradation of the accumulated ubiquitinated protein aggregates remains to be explored. This could be addressed by using proteasomal inhibitors.

Partial Aurora B inhibition also causes generation of oxidative stress as indicated by production of reactive oxygen species (ROS). Additionally ROS is required induction of p-p38 and p21 as well as for cell-cycle arrest under partial Aurora B conditions (Fig. 30). Specifically superoxide anions ($O2^{\bullet -}$) are generated by partial Aurora B function impairment as indicated by positive MitoSox Red staining upon low dose ZM447439 treatment (Fig. 30B). Scavanging of superoxide anions by antioxidant Tiron, prevents the activation of p-p38 and p21, as well rescues the cell cycle arrest and expression of E2F target genes caused by partial Aurora B inhibition (Fig. 30C-G). These data demonstrate that ROS is required for activation of the p38-p53-p21 signaling and for mounting subsequent cell cycle arrest after Aurora B inhibition. ROS might be generated due to the disturbed metabolic profile of the cell caused by the protein imbalance as a result of an altered chromosome complement (Li et al. 2010; Fang and Zhang 2011). Aneuploidy has been shown to activate p53 through ATM and ROS (Li et al. 2010). In summary, aneuploidy and resulting proteotoxic stress caused by Aurora B inhibition generates ROS which acts upstream of p38 to activate p21 and this in turn cause cell cycle arrest. One mechanism by which ROS could activate p38 is by activating the upstream kinase of p38, ASK1 by causing its dissociation from glutathione-S-transferase Mu and Thoiredoxin (Trx) (Fig. 34) (Dorion 2002; Liu 2002). Alternatively, ROS may act by preventing the activation of p38 MAPK phosphatases (PPM1D, Wip1, DUSP26), implicated in inactivation of p38 by oxidation of the active site cysteine residue (Fig. 34) (Bulavin et al. 2002; Bulavin et al. 2004; Yu et al. 2007). Unveiling the requirement of the above-mentioned MAPK phosphatases and kinases upstream of p38 in future will provide more details for the p38-p53-p21 signaling axis activated due to Aurora B inhibition.

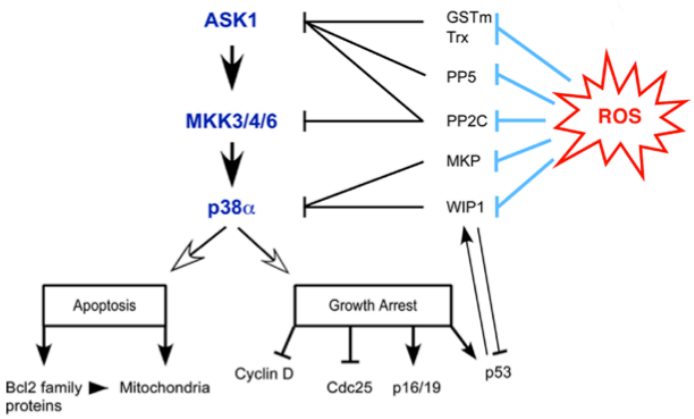

Figure 34: Regulation of p38 MAPK pathway by ROS.
ROS affects the p38 MAPK cascade by regulating its upstream activators (ASK1 and MKK3/4/6) through regulating Trx, GSTm, PP5 and PP2C, or by having an effect on phosphatases (MKP, Wip1), which in turn regulate p38. Figure taken from (Kennedy et al. 2007).

4.5 DNA damage pathway is not implicated in p21 induction after Aurora B inhibition

Aurora B inhibition did not induce any of the well known DNA damage markers such as p-H2A.X and phosphorylation of ATM/ATR substrates (Fig. 28A and B). Further, the finding that neither the ATM (KU5593) inhibitor nor the ATR inhibitor (VE821) have any effect on p21 induction by Aurora B inhibition, confirms that DNA damage response is dispensable for p21 activation (Fig. 28C and D). However it remains to be tested whether ROS generated due to aneuploidy is causing oxidative damage to DNA. This could be done by for example, measuring the 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels in genomic DNA. DNA damage is a well-known contributor towards activation of p53 through activation of ATM pathway (Shiloh and Ziv 2013) and opposing forces at a merotelicaly attached centromere from the spindle poles or randomly segregating chromosomes stuck at the cleavage furrow might cause DNA damage (Janssen et al. 2011). However similar to findings in this study, Thompson and Compton (2010) also did not observe generation of p-H2A.X foci after chromosome missegregations (after monastrol washout). Although Li et al. (2010) demonstrate that ATM is required for activation of p53 and p21 in aneuploid cells, they also failed to depict activation of p-Chk1/Chk2 or presence of p-H2A.X in aneuploid HCT116 cells and propose a noncanonical pathway for activation of ATM via oxidative stress. Thus interfering with Aurora B function does not activate the classical DNA damage response (which activates ATM/ATR kinases resulting in generation of p-H2A.X foci).

4.6 Cell cycle arrest due to Aurora B inhibition depends on p21 and E2F target genes

One aim of this study was to investigate the requirement of CDK inhibitor p21 and genes regulated by E2F/pRb pathway in mediating cell cycle arrest after Aurora B inhibition. Absence of p21 (in case of HCT116 p21 null cells) or inhibition of the retinoblastoma protein (pRb) by SV40-Large T antigen increases the percentage of polyploid cells upon Aurora B inhibition (Fig. 15A and D), due to failure of cells to undergo cell cycle arrest. This finding demonstrates that p21 and pRb proteins are required for mediating cell cycle arrest after Aurora B inhibition. Aurora B inhibition by ZM447439 de-phosphorylates the pRb protein (Fig. 15B, lane 2). Since Aurora B is known to phosphorylate pRb at serine 780 (Nair et al. 2009), the cell cycle arrest after Aurora B inhibition could be due to its direct effect on pRb. Alternatively, it might be an indirect effect by p21, which is induced due to Aurora B inhibition and blocks the cyclin/CDK complexes, required to keep pRb in hyper-phosphorylated (p-pRb) form, resulting in formation of the hypo-phosphorylated form of Rb (pRb), which causes a decrease in E2F gene transcription leading to cell cycle arrest. The finding that inhibition of p21 induction by SB202190 co-treatment restores the hyper-phosphorylated form of pRb (Fig. 15B, lane 4) supports the second hypothesis. Hence, pRb protein is not required for p21 induction (conclusion from Fig. 12), but it is required for cells to undergo arrest after Aurora B inhibition. This finding supports the use of Aurora B inhibitors for pRb null/non-functional tumors, as in such cases the tumors will continue dividing because of their inability to mount cell cycle arrest and finally die by apoptosis, although this has to be demonstrated in suitable experimental systems.

4.7 Drugs to which aneuploid cells are sensitive synergize with Aurora B inhibitors, to inhibit cell proliferation via a cooperative effect on induction of cell cycle inhibitor proteins

Long-term treatment of U2OS and HCT116 cells (seeded at very low density) with low doses of ZM447439 does not affect (or slightly reduces in case of U2OS cells) their ability to proliferate and form colonies (Fig. 31A and B). However combining low doses of ZM447439 with AICAR (a chemical compound that activates AMPK and induces energy stress) (Corton et al. 1995) or with 17AAG (a chemical compound that inhibits proper protein folding by inhibiting the chaperone Hsp90) (Young et al. 2001) significantly prohibits the ability of these cancer cells to grow and form colonies (Fig. 31A and B). On the other hand treatment with 17AAG alone has no impact and AICAR alone slightly restrains the colony formation ability of both the cell lines. HCT116 cells have the ability to grow independent of anchorage when seeded in media with soft agar, which is drastically

reduced in the combined presence of AICAR and low doses of ZM447439 (Fig. 31C). AICAR and 17AAG selectively kill aneuploid cells without having any impact on euploid cells due the disturbed energy balance and proteotoxic stress in aneuploid cells (Tang et al. 2011). Requirement of extra energy for transcription, translation and degradation of proteins produced from the extra copies of genome imposes energy stress, metabolic stress and proteotoxic stress on aneuploid cells (Stingele et al. 2012; Oromendia and Amon 2014). One possible molecular explanation for this co-operative ability of Aurora B inhibitor ZM447439 and AICAR/17AAG in impeding cell-growth is augmentation of induction of cell cycle inhibitors p21, p53 and p27 (Fig. 32A, B and C). The combined treatment induces more p21, p53 and p27 at very early time points as compared to treatment with AICAR, 17AAG or ZM447439 alone. Co-treatment of ZM447439 with AICAR was more effective in colorectal carcinoma cell line (HCT116) and with 17AAG was more effective in osteosarcoma cell line (U2OS). These different sensitivities might be due to different set of mutations in these two types of cancers and detailed understanding of genetic background of these cell lines will provide information about the mechanism of action of these drugs as well as help develop targeted therapies for subgroups of tumors with common markers/mutations. AICAR and 17AAG had no cooperative affect on phosphorylation of p38 MAPK caused by ZM447439 alone (data not shown), indicating that they induce p21 and p53 through an independent pathway that does not requires p38. Also the combination of ZM447439 and AICAR/17AAG did not alter the apoptotic pathway (as determined with apoptotic markers such as cleaved-PARP and cleaved-caspase) and the activation of autophagy markers (LC3, p62 and Hsp70) (data not shown).

In support of these findings are data from yeast, where presence of one extra chromosome sensitizes them towards proteasome inhibitors, due increased burden on proteasome system to clear poly-ubiquitinated proteins for degradation (Torres et al. 2007). Primary MEFs which become spontaneously aneuploid due to a compromised spindle assembly checkpoint (SAC) (as a result of impaired BubR1 allele or by checkpoint resistant Cdc20 allele) are also sensitive to AICAR and 17AAG (Tang et al. 2011). In the future it will be important to validate the effects of combined Aurora B inhibitor and AICAR/17AAG in in-vivo xenograft mouse models. These results suggest that combinations of low doses of Aurora B inhibitor with certain drugs to which aneuploid cell are sensitive might be effective for cancer therapy. This will not only increase the efficacy of Aurora B inhibitors, but also lead to fewer side effects due to usage of low doses of Aurora B inhibitors. Currently both AICAR and 17AAG are in clinical trials (Gordon et al. 2012). The combination of Aurora B inhibitors with the classes of drugs that interfere with

the metabolic balance and proteostasis of cells certainly needs to be tested in trials going on clinic.

4.8 Hypothesis and working model

Taken together, the findings of this study provide evidence for a yet unknown role of the p38 MAPK in transcriptional elongation of the p21 gene and present sufficient details of the signaling pathways downstream of Aurora B which support the use of combination Aurora B inhibitors with drugs to which aneuploid cells are sensitive, for treatment of cancer. We propose the following working model (Fig. 35): Impairment of Aurora B kinase function by chemical inhibitors (ZM447439/AZD1152-HQPA) or by siRNA mediated knockdown results in stabilization of p53 as proposed by (Gully et al. 2012) and induces its binding to the p53 binding site (p53BS1) in the p21 gene promoter. However this binding is not sufficient for p21 activation because p38 MAPK, which is also activated by Aurora B inhibition is required for mediating the transcriptional elongation of p21 gene by inducing the recruitment of the elongating form of RNA Polymerase II (P-Ser2-RNA Polymerase II) to the p21 coding region, resulting in transcriptional induction of p21 gene. Activation of p38 MAPK in turn correlates with generation of aneuploidy and depends on generation of reactive oxygen species (ROS). Further, the induced p21 leads to dephosphorylation of retinoblastoma protein (pRb) owing to the ability of p21 to inhibit cyclin-CDK complexes, which are required to maintain pRb in phosphorylated state (inactive state). The resulting inactive pRb-E2F complexes prevent expression of E2F target genes and hence block G1-to-S transition causing G1 arrest.

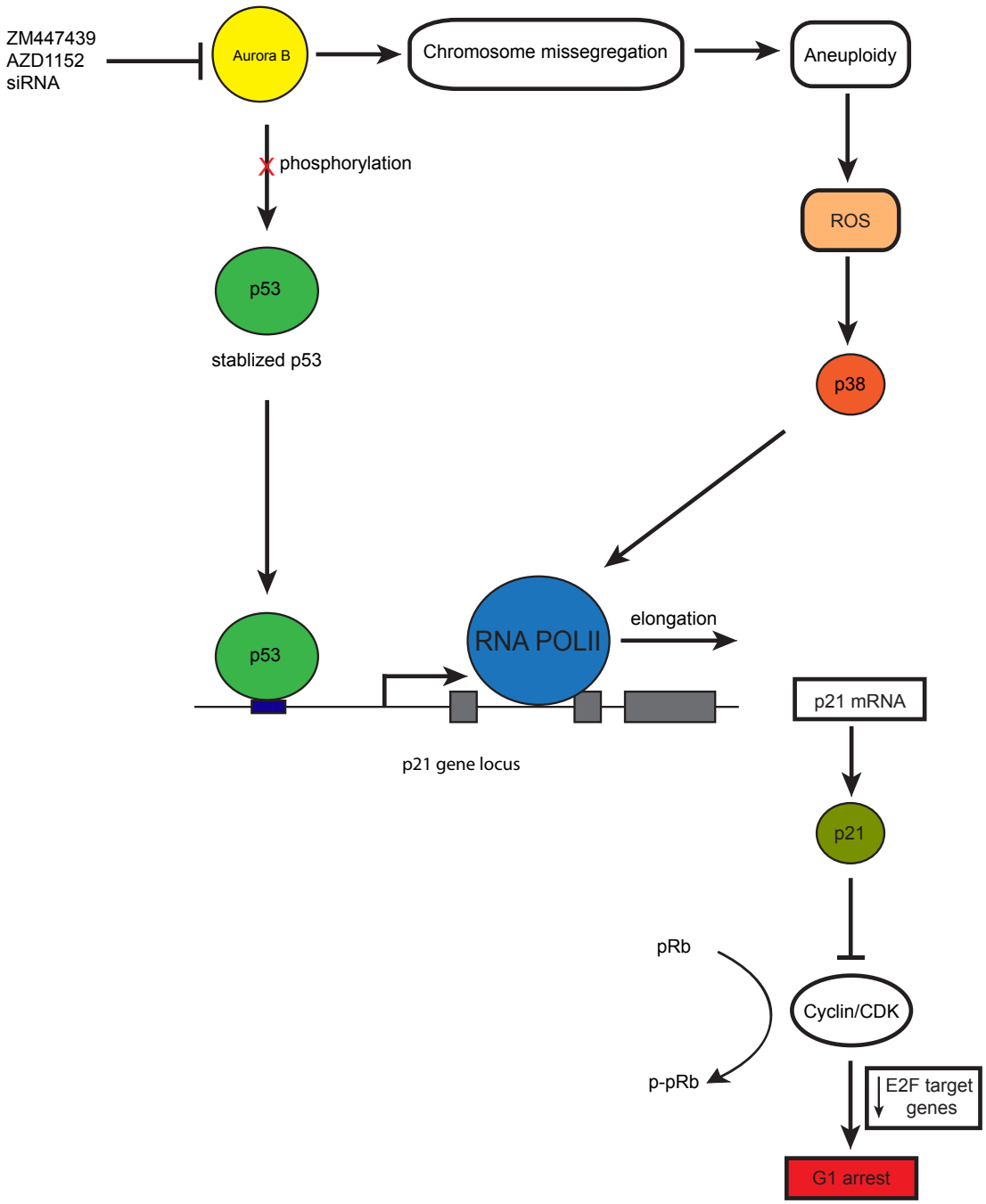

Figure 35: Model for p21 induction and cell cycle arrest due to Aurora B inhibition.
See text for details.
Parts of this figure were published in similar form in Kumari et al. 2013 and Kumari et al. 2014.

5 Summary

Aurora B is a mitotic kinase that is essential for cell division. Because it is mutated or overexpressed in a range of cancer types, it has been suggested as a novel therapeutic target. Currently chemical inhibitors against Aurora B are in various phases of clinical trials for treatment of solid tumors and leukemia. Information regarding the molecular requirements for the reported phenotypes of Aurora B inhibition such as cell cycle arrest, activation of the tumor suppressor p53 and its target p21 are not well understood.

In this study, I investigated the requirements for p21 induction after Aurora B inhibition. I found that p38 is phosphorylated and activated when Aurora B is inhibited. Experiments with chemical inhibitors against p38 indicate that p38 is required for p21 induction and cell cycle arrest in response to Aurora B inhibition. p53 induction after impairment of Aurora B function and the recruitment of p53 to its binding site in the p21 gene promoter occur independently of p38 signaling. Instead, I found that p38 is required for the enrichment of the elongating RNA Polymerase II in the coding region of the p21 gene. Furthermore, p38 is required for formation of the full-length p21 mRNA transcript. These data indicate that p38 promotes the transcriptional elongation of p21 gene in response to Aurora B inhibition. In further experiments I could show that the p21 causes cell cycle arrest due to a decrease in E2F-dependent transcription by promoting the dephosphorylation of the retinoblastoma protein.

Using synchronized cells I could show that the induction of p21 in response to Aurora B inhibition requires transition through an aberrant mitosis and does not occur in cells that are arrested in interphase. Interestingly, p38, p53 and p21 are already induced by partial inhibition of Aurora B, which results in aneuploidy but not in cytokinesis failure and in tetraploidy. This supports the notion that activation of p38-p53-p21 signaling correlates with aneuploidy but not with tetraploidy or binucleation. Partial inhibition of Aurora B also leads to increased generation of reactive oxygen species (ROS), which are required for the activation of p38, p21 and cell cycle arrest. Based on these observations I propose the following model: Inhibition of Aurora B leads to chromosome missegregation resulting in aneuploidy. This results in increased generation of ROS (reactive oxygen species) possibly through proteotoxic stress caused by an imbalance of protein synthesis in aneuploid cells. ROS triggers the activation of p38, which then stimulates the transcriptional elongation of p21 resulting in cell cycle arrest.

Aneuploidy, proteotoxic stress and oxidative stress are hallmarks of cancer cells. Based on my results reported in this study, I suggest that the combination of Aurora B inhibitors with drugs that specifically target aneuploid cells might be a novel strategy for cancer therapy, as this is a lethal combination for proliferation of cancer cells.

6 Zusammenfassung

Aurora B ist eine mitotische Kinase, die entscheidende Funktionen in der Zellteilung ausübt. Aurora B ist außerdem in einer Vielzahl von Krebsarten mutiert oder überexprimiert. Daher ist die Aurora B Kinase ein attraktives Ziel für die Tumortherapie. Gegenwärtig werden Aurora B-Inhibitoren zur Behandlung von soliden Tumoren und Leukämien in verschiedenen klinischen Studien getestet. Es fehlen jedoch Informationen, welche molekularen Mechanismen den beschriebenen Phänotypen wie Zellzyklusarrest, Aktivierung des Tumorsuppressors p53 und seines Zielgens p21 nach Aurora B-Hemmung zugrunde liegen.

Hauptziel dieser Arbeit war es die Mechanismen der p21-Induktion nach Hemmung von Aurora B zu untersuchen. Es konnte gezeigt werden, dass nach Hemmung von Aurora B die p38 MAPK phosphoryliert und somit aktiviert wird. Experimente mit p38-Inhbitoren belegen, dass p38 für die Induktion von p21 und den Zellzyklusarrest benötigt wird. Die Stabilisierung von p53 nach Aurora B-Inhibition und die Rekrutierung von p53 an den p21-Genpromotor erfolgen jedoch unabhängig vom p38-Signalweg. Stattdessen ist p38 für die Anreicherung der elongierenden RNA-Polymerase II in der kodierenden Region des p21-Gens und für die Bildung des p21 mRNA Transkripts notwendig. Diese Daten zeigen, dass p38 transkriptionelle Elongation des p21-Gens nach Aurora B Hemmung fördert. In weiteren Untersuchungen konnte ich zeigen, dass die Aurora B-Hemmung zu einer Dephosphorylierung des Retinoblastoma-Proteins führt und dadurch eine Abnahme der E2F-abhängigen Transkription bewirkt. Dies löst indirekt einen Zellzyklusarrest aus.

Weiterhin konnte mit Hilfe von synchronisierten Zellen gezeigt werden, dass p21 nach Durchlaufen einer abnormalen Mitose induziert wird, jedoch nicht nach Aurora B-Hemmung in der Interphase. Interessanterweise werden p38, p53 und p21 schon bei partieller Inhibition von Aurora B aktiviert. Die partielle Inhibition von Aurora B führt zu chromosomaler Instabilität aber nicht zum Versagen der Zytokinese und zur Bildung polyploider Zellen. Damit korreliert die Aktivierung des p38-p53-p21-Signalweges nicht mit Tetraploidie sondern mit vermehrter Aneuploidie. Die partielle Hemmung von Aurora B führt außerdem zur vermehrten Entstehung von reaktive Sauerstoffspezies (ROS), welche für die Aktivierung von p38, p21 und für den Zellzyklusarrest benötigt werden. Basierend auf diesen Beobachtungen kann folgendes Modell postuliert werden: Die Hemmung von Aurora B führt zu Fehlern in der Chromosomenverteilung in der Mitose und damit zu Aneuploidie. Dies führt zu vermehrter Produktion von ROS, möglicherweise durch proteotoxischer Stress, hervorgerufen durch die Imbalanz der Proteinbiosynthese in

aneuploiden Zellen. ROS bewirkt eine Aktivierung der p38 MAPK und trägt damit zur Induktion von p21 und dem resultierenden Zellzyklusarrest bei.

Aneuploidie, proteotoxischer und oxidativer Stress stellen Schlüsselmerkmale von Tumorkrankungen dar. Anhand der Ergebnisse dieser Arbeit könnte die Kombination von Aurora B-Hemmstoffen mit Medikamenten, die gezielt aneuploide Zellen angreifen, in Tumorerkrankungen therapeutisch wirksam sein.

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8 Appendix

8.1 List of figures

8.2 Abbreviations

AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AMPK	5' adenosine monophosphate-activated protein kinase
APC/C	Anaphase promoting complex/Cyclosome
APS	Ammonium persulfate
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CDK	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
CHX	Cycloheximide
Co-IP	Co-immunoprecipitation
Ctrl.	Control
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC	Deoxycholate
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
e.g.	Example
ESB	Electrophoresis sample buffer
FACS	Flourescence activated cell sorting
FCS	Fetal calf seum
Fig.	Figure
G0, G1, G2	Gap phases
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	Hours
HBS	Hepes buffered saline
HDAC	Histone deacetylase
HRP	Horseradish peroxidase

Hsp90	Heat shock protein 90
HU	Hydroxyurea
IF	Immunofluorescence
IP	Immunoprecipitation
kDa	kilodalton
LB	Luria broth
LiCl	Lithium chloride
MAPK	Mitogen-activated protein kinase
$MgSO_4$	Magnesium sulphate
M phase	Mitosis and cytokinesis
NP-40	Nonylphenoxylpolyethoxyl-ethanol
O/N	Over night
PBS	Phosphate buffer saline
PCR	Ploymerase chain reaction
PFA	Paraformaldehyde
pH3	phospho histone H3 (Ser10)
PI	Propidium iodode
PMSF	Phenylmethanesulfonylflouride
pRb	Retinoblastoma protein
qPCR	Quantitative real time PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
shRNA	short hairpin RNA
siRNA	small interfering RNA
S-phase	Synthesis phase
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBST	Tris buffered saline-Tween
TEMED	N'N'N'N'-tetramethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultra violet light
WT	Wild type
17AAG	17-*N*-allylamino-17-demethoxygeldanamycin

8.3 Own publications and conference contributions

2014 Geeta Kumari, Tanja Ulrich, Michael Krause, Florian Finkernagel and Stefan Gaubatz. “Induction of p21CIP1 and cell cycle arrest after inhibition of Aurora B kinase is attributed to aneuploidy and reactive oxygen species.” J Biol Chem. 2014 April 29; doi: 10.1074/jbc.M114.555060.

2013 Geeta Kumari*, Tanja Ulrich*, and Stefan Gaubatz. "A role for p38 in transcriptional elongation of p21CIP1 in response to Aurora B inhibition". Cell Cycle. 2013 Jul 1;12(13):2051-60. doi: 10.4161/cc.25100. Epub 2013 Jun 6. *These authors contributed equally to this work.

Conference contributions (Talks and Posters)

04-09.02.2014 Cancer Epigenetics and Transcriptional Regulation joint meeting. Keystone Symposia held at Santa Fe Community and Convention Center, New Mexico, USA.
Poster: A role for p38 in transcriptional elongation of p21CIP1 in response to Aurora B inhibition. Geeta Kumari, Tanja Ulrich and Stefan Gaubatz.

09-10.10.2013 8th International GSLS Symposium. “Scientific Crosstalk” held at RVZ, Wuerzburg, Germany.
Poster: Aneuploidy due to inhibition of Aurora B induces p21 through activation of p38 by proteotoxic stress and ROS. Geeta Kumari, Tanja Ulrich and Stefan Gaubatz.

25-27.09.2013 Retreat “Schloss Pommersfelden 2013” by the Integrated Graduate College (IGC), “Ras-Dependent pathways in human Cancer” held at Pommersfelden, Germany.
Talk: A role for p38 in transcriptional elongation of p21CIP1 in response to Aurora B inhibition.

13-15.02.2012 Retreat “Kloster Schöntal 2012” by the Integrated Graduate College (IGC), “Ras-Dependent pathways in human Cancer” held at Schöntal, Germany.

Talk: Role of human DREAM complex in tumor suppression.

19-20.10.2011 6th International GSLS Symposium. “Bio Bang” held at RVZ, Wuerzburg, Germany.

Poster: Role of human DREAM complex in tumor suppression. Geeta Kumari, Nina Reichert, Stefanie Hauser and Stefan Gaubatz.

29-31.07.2011 GSLS Annual Retreat, 2011 held at Nürnberg, Germany.

Talk: Role of DREAM complex in Quiescence and Differentiation.

8.4 Curriculum vitae

8.5 Acknowledgements

First and foremost, I convey my gratitude and sincere thanks to my supervisor Prof. Dr. Stefan Gaubatz for his supervision of my PhD thesis, advice and guidance during my PhD project. His positive attitude and keen interest in research is highly motivating and always inspired me to advance during my PhD work. It has been an invaluable experience so far and I learned a lot since the time I arrived here in Wuerzburg.

Besides my supervisor, I extend my gratitude and thanks to other members of my thesis committee: Prof. Dr. Peter Gallant and Prof. Dr. Manfred Alsheimer for their encouragement and insightful comments during our meetings.

I thank the Graduate School of Life Sciences (GSLS), University of Wuerzburg for funding my PhD project and providing this great opportunity to pursue research here in Germany. The opportunity to attend an international conference and various courses with GSLS funding was a great experience and is an additional asset for my scientific career.

I thank all the former lab members, Steffi, Tanja, Jasmina and Piero for their advice and support. I am extremely thankful to Steffi for her help both in the lab as well outside the lab, during the initial days when I started my PhD project. A special thanks to Andrea our former lab secretory, for her support and help. I thank all the present lab colleagues Sabine, Patrick, Fabian, Marc and Katja for all their help and support as well for the various fun moments in the lab. Special thanks to Sabine, for translating the summary of this work from English to German. Thanks to Adelgunde and Susi for their technical assistance and for the fun chats we had despite their difficulties in speaking English.

I thank all my friends here in Wuerzburg, specially Priya, Aruna and Prema for making my life easy and full of fun and joy when I was outside the lab.

Most importantly I am grateful my family members, specially my father and mother for their continuous unconditional love and support throughout my life and career.

8.6 Affidavit

I hereby confirm that my thesis entitled "Molecular Characterization of the Induction of Cell Cycle Inhibitor p21 in Response to Inhibition of the Mitotic Kinase Aurora B" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Wuerzburg, Date

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation “Untersuchungen zur Induktion des Zellzyklusinhibitors p21 nach Inhibition der mitotischen Kinase Aurora B” eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, Date