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CELL DEATH AS A BARRIER AGAINST CIN AND ANEUPLOIDY

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List of Abbrevations:

53BP1	p53 binding protein1
APC/C	anaphase promoting complex/ cyclosome
BCL2	B-cell lymphoma 2
BID	BH3-interacting domain death agonist
cGAS	cyclic GMP-AMP synthase
LC-3	microtubule associated light chain 3
MCL1	myeloid cell leukaemia sequence 1
MOMP	mitochondrial outer membrane permeabilization
mTORC1	mammalian TOR complex 1
NOXA/PMAIP	Phorbol-12-myristate-13-acetate-induced protein 1
P62	sequestosome1; ubiquitin-binding protein p62
RAPTOR	regulatory associated protein of mTOR
STING	stimulator of interferon genes
TOR	target of rapamycin
UPR	unfolded protein response

USP28	ubiquitin specific protease 28

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Abstract

Aneuploidy describes the gain or loss of entire chromosomes or chromosome arms. Already more than 100 years ago, aneuploidy was described to be featured in cancer and it is known today to be present in 68-90 % of cancers, depending on tumour entity. Aneuploidy is implicated to affect cancer growth, therapy response and frequently affects prognosis. Chromosomal instability (CIN) is recognized as the main cause of aneuploidy and is characterised as the fluent process of gaining or losing chromosomes. Chromosomally instable cells need to be kept in check, or cleared, to prohibit the sampling of aneuploid karyotypes able to drive tumorigenesis. Of note, even aneuploid cancer cells often show CIN, a feature that promotes therapy related drug-resistance. Here, we review how CIN can be prevented or limited to spread by the induction of cell death and the relevance of different p53 responses triggered in response to mitotic perturbations to prohibit the formation of cancer driving aneuploidies.

Introduction

The faithful duplication of genomic information during each cell division cycle and physical its separation into newly emerging daughter cells during mitosis pose several challenges in order to maintain genome integrity. Multiple highly complex but error-susceptible processes are involved to fulfil this task. Starting from correct DNA-synthesis by DNA-polymerase in S-phase timed entry from G2 into M-phase upon completion of whole genome duplication, chromosome condensation, mitotic spindle formation and attachment of chromosomes to microtubules, as well as their proper alignment in a metaphase plate prior separation into two daughter cells completed by cytokinesis, many things can go wrong that ultimately affect cell fate and genome integrity. Cell cycle progression hence involves passage through multiple checkpoints, some of them key to prevent chromosomal segregation errors and CIN. When theses checkpoints cannot be satisfied, different fail safe mechanisms kick in that are geared to prevent perturbation of genome integrity at various levels. As a final consequence, cells that enter mitosis with under-replicated DNA or unresolved DNA damage, inheriting mis-segregated chromosomes or chromosome arms, or unable to complete cytokinesis, frequently resort to trigger the activation of cell death to prevent the spread of potentially pathogenic karyotypes. Here, we aim to review the established links between the cell cycle and apoptotic cell death machinery in response to delayed mitosis, unfaithful chromosome segregation and defective cytokinesis, that acts as a barrier of malignant transformation as well as therapy-resistance.

Mitotic Surveillance by the Spindle Assembly Checkpoint

The major cellular signaling complex guarding against CIN during cell division is the so-called mitotic spindle assembly checkpoint (SAC). The SAC prevents cell cycle progression into anaphase by inhibiting the anaphase promoting complex/cyclosome (APC/C) E3 ubiquitin ligase complex from engaging its critical coactivator, CDC20. Induction of metaphase to anaphase transition is mediated by APC-dependent ubiquitination and degradation of securin and Cyclin B1, leading to a loss of CDK1 activity and separase activation allowing separation of sister chromatids and mitotic exit. The SAC involves at least 15 proteins, first and foremost those that build up the mitotic checkpoint complex (MCC). The MCC includes MAD2 (mitotic arrest deficient 2) and the mitotic checkpoint proteins BUB3 and BUBR1 that sequester CDC20 away from the APC, thereby preventing the degradation of its mitotic substrates (1). Importantly, for proper SAC function all proteins have to work in conjunction to induce mitotic arrest when even a single kinetochore remains unattached (1). When the SAC has become activated, cell have three possibilities to respond. Cells may either die in mitosis or eventually complete cell division with severe delays, which may lead to chromosomal mis-segregation and CIN, or slippage from mitosis into the next interphase without completing cytokinesis. Thereby, cellular or nuclear ploidy as well as centrosome number increase (2-4). Consequences of polyploidization are discussed towards the end. For a more detailed description of the spindle assembly checkpoint, we politely refer the reader to excellent reviews on this topic (1, 5).

Preventing CIN by the Induction of Apoptosis

Mitotic arrest and apoptosis

In general, cell death during extended mitotic arrest is executed along the intrinsic apoptosis pathway (Figure 1). Intrinsic apoptosis is a highly regulated largely non-inflammatory cellular suicide program that is executed by the BCL2 protein family, regulating organogenesis, tissue homeostasis and self-tolerance (6, 7). The BCL2 protein family consists of pro survival proteins (BCLX, BCL2, MCL1, A1/BFL1, BCLB), pro-apoptotic or BH3-only proteins (BIM, PUMA, NOXA, BID, BAD, BIK, BMF, HRK) and pro-apoptotic effector proteins (BAX, BAK1, BOK) (7, 8). In steady-state, these proteins balance each other in their activity but upon stress apoptotic trigger translate into the activation of pro-apoptotic BAX (BCL2-associated X protein) and BAK1 (BCL2 antagonist 1). This can be achieved by transcriptional activation of BH3-only proteins, their post-transcriptional accumulation or different post-translational modification, allowing them to effectively bind and neutralize pro-survival proteins, or activate BAX/BAK directly (9). BAX and BAK dimerization is considered the nucleating event, driving the assembly of mixed higher oligomeric structures and pore formation in the MOM (mitochondrial outer membrane). Upon mitochondrial outer membrane permeabilization (MOMP) cytochrome c release triggers activation of a proteolytic cascade involving members of the caspase family, cysteine-dependent aspartate-specific proteases executing this form of cell death (10, 11).

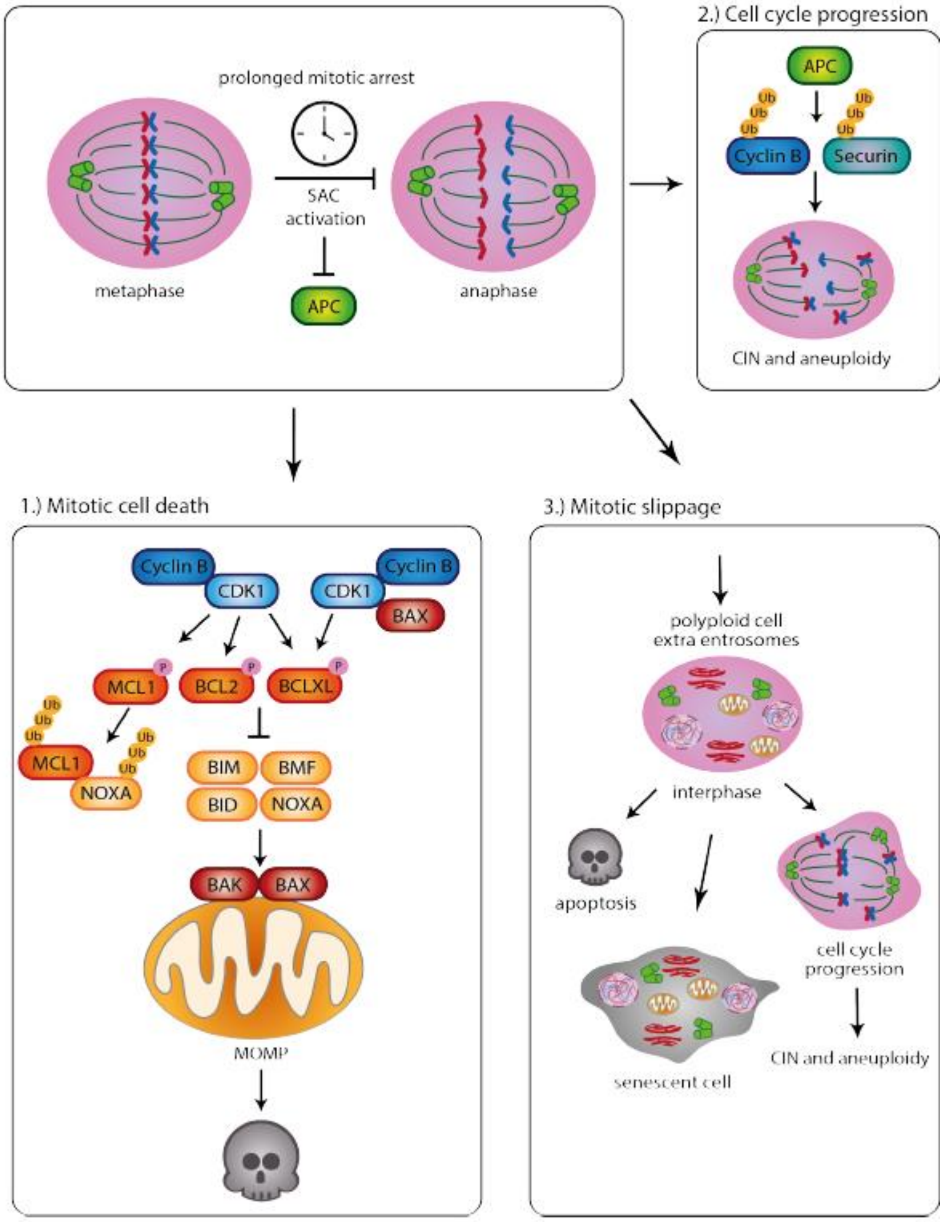

Figure 1: Proper distribution of chromosomes during mitosis is guarded by the spindle assembly checkpoint (SAC), which upon misguided microtubule attachment inhibits the anaphase promoting complex/cyclosome (APC/C), the E3 ubiquitin ligase for Cyclin B1 and Securin. Upon mitotic arrest cells may either 1.) undergo cell death in mitosis executed by the BCL2 protein family interaction, 2.) complete cell division, resulting in chromosomal mis-segregation and improper DNA content distribution within the next cell-cycle or 3.) fail mitosis and slip into the next interphase resulting in duplication of DNA-content and centrosomal imbalance.

As mentioned, mitotic arrest upon lack or faulty sister-chromatid to kinetochore attachment depends on the inhibition of Cyclin B1 degradation (1). Nevertheless, Cyclin B1 is still degraded slowly by non-canonical APC activity in the absence of CDC20 (12). When falling below a critical level, cells may eventually slip out of mitotic arrest, disregarding the SAC still being active (12). To prevent chromosome mis-segregation during slippage, apoptosis is induced when caspase activity exceeds a certain threshold within this timeframe. However, the kinetics of caspase activation appears quite heterogeneous when comparing different cell types and different cell lines (2). These observations have been the basis of the competing network hypothesis (13), where both pathways, BCL2-regulated caspase activation vs. APC-mediated Cyclin B degradation are kick-started in parallel, deciding on cell fate, being either death in mitosis or mitotic slippage (2, 13, 14). While the APC appears to display a constitutive low level activity in mitosis, the net balance of the BCL2 network needs to be tilted towards MOMP to trigger caspase-activation so that apoptosis can be induced (9). Towards this end, CDK1 (Cyclin dependent kinase 1), which forms a complex with Cyclin B1 during mitosis, is able to phosphorylate pro-survival proteins BCL2 (B-cell lymphoma 2) and BCLX (B-cell lymphoma extra-large) (15). Similarly, MCL1 (myeloid cell leukaemia sequence 1) is also targeted by CDK1 and this PTM affects speed of degraded by the proteasome (16-19). As such MCL1 levels influence the time cells arrest in mitosis and can be considered as a “molecular timer” (20). Multiple studies suggest that MCL1 degradation is the critical driving force in mitotic cell death, aided by NOXA/PMAIP (Phorbol-12-myristate-13-acetate-induced protein 1), the BH3-only protein with a high binding preference for MCL1 (7). Physical MCL1/NOXA interaction is driving MCL1 degradation, lowering the threshold to mitotic cell death by, e.g., by unshackling pro-apoptotic protein BIM, that can be neutralized by interaction with MCL1, giving way to MOMP (21, 22). Multiple E3 ligases, including the APC/C itself, SCF$^{\beta\text{-TrCP}}$, MULE/HUWE1 or SCF$^{\text{FBW7}}$ have been implicated in MCL1 turnover, also during mitotic arrest (16, 18, 23). In addition, we noted that degradation of MCL1/NOXA is co-regulated by the mitochondria-resident E3-ligase MARCH5 (membrane-associated ring finger 5) and that lack of MARCH5 sensitizes cancer cells to microtubule targeting agents (24). Similarly, when interfering with mitosis by depletion of CHAMP1 (chromosome alignment maintaining phosphoprotein), a protein participating in microtubule attachment to kinetochores (25), cells showed an increase in CIN, and cell death. Increased cell death was accompanied by reduced levels of MCL1, although CHAMP1 dependent MCL1 stabilization was not specific for mitosis (26), similar to findings made with MARCH5 (24).

While the MCL1/NOXA/BIM axis appears to be most critical to delete mitotically arrested cells as a barrier against CIN in multiple epithelial cancer cell lines (21, 22), the BH3-only protein BMF (BCL2 modifying factor), originally described to be a regulator of anoikis (27), was suggested to contribute to death in mitosis of human dermal fibroblasts. When entering mitosis, BMF expression is limited by binding of FOXM1 (forkhead box protein M1) transcription factor (28). Additionally FOXM1 is upregulating *Cyclin B* transcription to aid mitotic entry (29). Upon reduction of FOXM1 expression, cells tend to die in mitosis as BMF repression is alleviated (28). Furthermore, FOXM1 is repressed by p53 upon DNA damage (30, 31). As FOXM1 is an important mitotic regulator (29), and deficiency can lead to chromosomal mis-segregation, observed effects may be lingering on cell death due to impaired mitosis and subsequent p53 responses.

Moreover, the BH3-only protein BID (BH3-interacting domain death agonist) and BAD (BCL2-associated agonist of cell death) (32, 33) have also been implicated in mitotic cell death. While the contribution of BAD was only noted upon overexpression, questioning physiological relevance, phosphorylation of BID on serine 66 early upon mitotic entry appears to facilitate its mitochondrial translocation and priming in colon cancer cell lines, rendering these cells more prone to cell death upon paclitaxel treatment (32). The kinase responsible for this phosphorylation event remains to be identified.

Activation of BAX and BAK1 are actually thought to be the last BCL2 family members involved in the events promoting apoptosis execution in mitosis. However, recently it has been suggested that they are not simply effectors waiting for upstream signals to be integrated, but that they play an active role in mitotic cell death by facilitating CDK1 mediated phosphorylation of pro-survival proteins (34). BAX, and to a lesser degree BAK1, appear to be able to interact with CDK1, targeting it to the outer mitochondrial membrane, where it can phosphorylate BCL2 and BCLX to facilitate apoptosis (34). Phosphorylation of both BCL2 and BCLX was found to be reduced in BAX/BAK double mutant cells. The increased potential of BAX in shuttling CDK1 to the OMM, compared to BAK, can be explained by the fact that BAX is mainly cytoplasmatic, while upon insertion into the OMM it is retro-translocated back into the cytosol upon interaction with BCLX (35). It remains to be seen if phosphorylation of BCLX by CDK1 may reduce its capacity to shunt BAX back into the cytosol, leading to its accumulation at the OMM and pore-formation.

Certainly, it remains difficult to predict if cells slip or undergo apoptosis *in situ* when they experience mitotic delays. Moreover, although MOMP is frequently seen as a point of no return in apoptosis signalling, it does not need to be quantitative and not all cells need to die after caspase activation. In fact, limited MOMP, referred to as minorityMOMP, has been shown to lead to genomic instability by the activation of caspase-activated DNase (CAD) (36). It is worth mentioning that limited caspase activation-induced CAD activity can cause DNA damage specifically at telomeres in mitosis. Protection of telomeres by TRF2 (telomeric repeat binding factor 2) is lost within this process, leading to a DNA-damage response and p53 activation (37-39). Loss of TRF2 sensitises cells to mitotic cell death (40). However, some cells may still slip into the next interphase, which may be aided by TRF2 overexpression. The consequences of a subsequent G1 arrest after slippage will be discussed further below.

Mitotic arrest, metabolism and mitophagy

One feature frequently linked to mitochondrial quality control, also implicated in tuning mitochondrial cell death is mitophagy, which is the process of mitochondrial clearance after damage by autophagy (41). In mitosis mitochondria are divided and distributed equally within the daughter cells, a process that is regulated by DRP1 (dynamin-related protein 1), a GTPase that is phosphorylated and activated by CDK1 during mitosis (42). In HeLa cells, loss of DRP1 led to vast increase of cell death during mitotic arrest, accompanied by an increase of mitophagy (43). Additionally in a lung adenocarcinoma xenograft model, tumour proliferation was decreased upon inhibition of DRP1 (44). In both cases, the effects noted did not seem to be linked to the fission-promoting function of DRP1, but may be a consequence of corrupted mitochondrial metabolism, as mitosis is highly energy consuming. When entering mitosis, CDK1 tunes cellular metabolism that becomes highly dependent on mitochondrial respiration. A fraction of Cyclin B / CDK1 can be found in the matrix and phosphorylate components of complex I of the respiratory chain (45). During extended mitotic arrest, however, mitochondria get cleared by mitophagy and glycolysis becomes the main energy source for ATP production. This response depends on AMPK (AMP-activated protein kinase) of PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3) ramping up glycolysis for survival (46, 47). Of note, glucose-deprivation may again promote cell death by engaging the BH3-only protein NOXA (48). As such, it can be hypothesised, that loss of mitochondria via mitophagy as well as inhibition of glycolysis should render cells more susceptible to mitotic inhibitors during cancer therapy.

Another metabolism-regulating target of CDK1 and AMPK during mitosis is RAPTOR (regulatory associated protein of mTOR), which is phosphorylated during mitosis, supressing its activity (49, 50). RAPTOR is forming a complex with mTOR in mTORC1 (mammalian TOR complex 1), ensuring its functionality. The TOR (target of rapamycin) kinase pathway plays an essential role in nutrient response, cell growth and proliferation (51). Although mitosis is a highly nutrient dependant process, mTORC1 levels are generally low, as mTOR *per se* does not seem to be necessary for mitotic progression or during prolonged mitotic arrest (52). mTORC1 inhibition is achieved by RAPTOR phosphorylation on multiple sides (49). Nevertheless, expression of a RAPTOR phosphorylation mutant in HeLa cells could prevent the loss of mTORC1 and cells were less prone to cell death during prolonged mitosis. Cell survival was secured by an increase in anti-apoptotic BCLX levels and a decrease in PDCD4 (programmed cell death protein 4). This suggests that cells able to sustain mTORC1 function in M-phase may be more likely to slip after mitotic arrest and that mTORC1 downregulation during mitosis may be essential to impede CIN (52). Dual mTORC1/2 inhibition is currently tested together with paclitaxel in high-grade serous ovarian carcinoma carrying MYC amplifications (53).

Cell Death or Survival in Response to CIN – All about Bribing p53?

Clearly, p53 is one of the most studied proteins and the plethora of its effector functions apparently needed for active tumour suppression have made it difficult to understand its precise mode of action. Identification of relevant transcriptional outputs is challenging and the existence of multiple p53 isoforms as well as interaction partners make therapeutic exploitation of our vast knowledge difficult (54, 55). As the modalities and consequences of p53 activation differ substantially between cell types and tissues , many studies limit themselves to use p53 activation as a surrogate read out for tumour suppression, senescence or cell death initiation (56). The picture is clearer upstream of p53 and multiple pathways leading to its activation have been deciphered that are critical to prevent the CIN and aneuploidy (Figure 2).

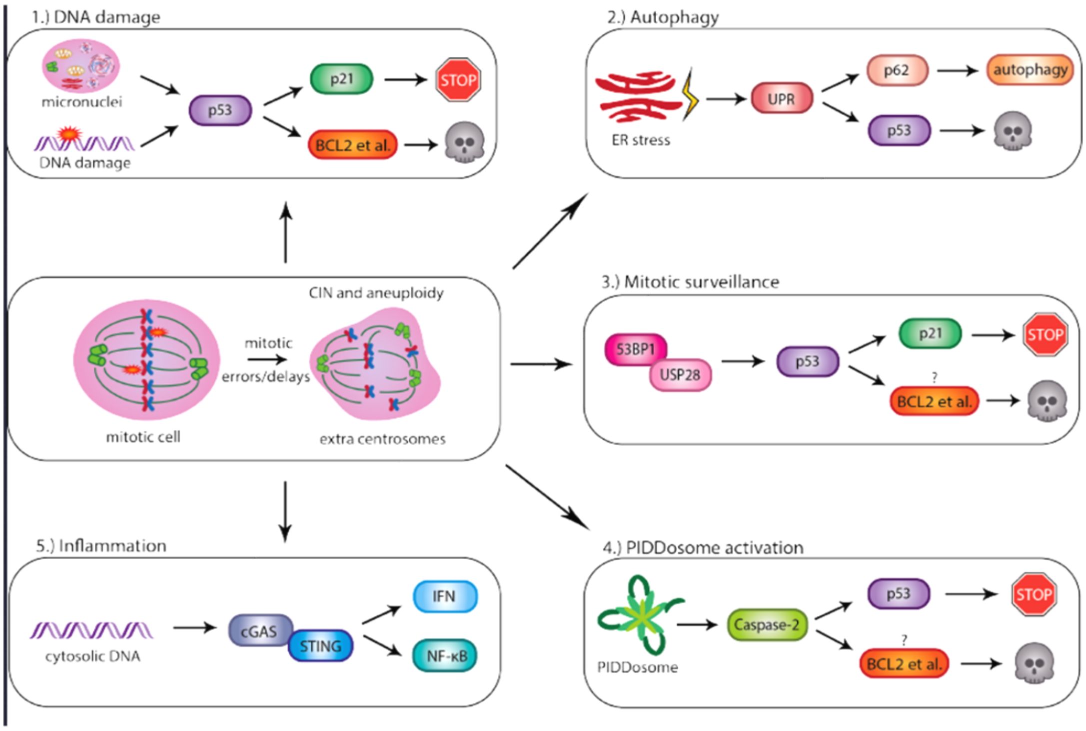

Figure 2: Consequences of chromosomal mis-segragation or mitotic slippage are divers. 1.) p53 activation due to DNA damage often resulting from micronuclei formation and can trigger p21 dependant cell cycle arrest or BCL2 protein family dependant cell death. 2.) ER-stress and subsequent unfolded protein response (UPR,) as a result of aneuploidy induced protein imbalances and aggregation, can trigger autophagy regulated by p62 and result in p53 activation leading to cell death. 3.) Loss of centrosomes can trigger a DNA-damage independent p53 response, resulting from p53 binding protein 1 (53BP1) and ubiquitin specific protease 28 (USP28) limiting MDM2-dependent p53 degradation. Cells may either arrest or die. 4.) Similarly, independent of DNA-damage, amplification of centrosomes triggers PIDDosome activation, MDM2-cleavage, p53 stabilization and p21 dependent cell cycle arrest. Whether extra centrosomes trigger cell death remains uncertain 5.) Aggregation of cytosolic DNA, e.g., as a result of micronuclei formation, leads to cGAS-STING dependant sterile inflammation, resulting in interferon (IFN) production or induction NF-κB signalling.

p53 activation due to chromosomal mis-segregation

The tumour suppressor p53 plays a major role in the DNA damage response, able to promote cell death. There is a clear correlation between aneuploidy and *Tp53* mutations across cancers (57, 58) and it has been shown that deletion of p53 promotes aneupoloidy induction (47, 59). Therefore, p53 seems to play a major role in restraining or eliminating cells with a high rate of CIN. Hereby, activation of p53 is likely not due to aneuploidy *per se*. Instead, DNA damage as a consequence of chromosomal mis-segregation, either by DNA-double-stranded breaks or micronuclei formation and chromothripsis, can lead to a p53 response and p21 dependent cell cycle arrest in the next G1 phase (60, 61). Interestingly, Histone H3.3^{Ser31} phosphorylation on lagging chromosomes during anaphase can also trigger p53 dependent G1 arrest upon chromosomal mis-segregation in the absence of DNA damage (62). How aneuploid G1 cells that carry serine phosphorylated H3.3 activate p53 remains to be established and it will be interesting to test if the somatic mutations in H3.3. found in pediatric glioblastoma patients functionally interfere with this type of aneuploidy checkpoint (63, 64). The consequences of p53 activation beyond p21 induction in such cells also need to be established, but defective cell death pathway engagement may contribute to the rise of MYCN amplified disease.

P53 activation in the light of CIN induced protein imbalance

Protein aggregation leads to an overflow of proteins at the endoplasmic reticulum (ER) and consequently insufficient protein folding. “ER-stress” initiates a number of signalling events summarized in the unfolded protein response (UPR). The UPR adjusts protein load within the cell by inhibiting ribosomal translation, giving way to more sufficient protein folding (65, 66).

In budding yeast, aneuploidy induced protein aggregation, either due to protein complex stoichiometry imbalance or due to poor lysosomal aggregate clearance, is leading to proteotoxic stress and proliferation defects. This phenotype was random and not dependent on aneuploidy of specific chromosomes or the number of aneuploid chromosomes (67-69). In mammalian cells the UPR is strongly interconnected with autophagy. Global transcriptome and proteome analysis in human HCT116 and RPE1 cell lines harbouring extra chromosomes have shown that autophagy is induced in a p62 (sequestosome1; ubiquitin-binding protein p62) specific manner (70). Additionally, upon CIN induced by SAC inhibition, HeLa cells showed high levels of ER-stress and consecutive autophagy induction depending on p62 (71). P62 acts as a cargo receptor for non-functional ubiquitinated proteins and further associates itself with LC3 (microtubule associated light chain 3) to ensure formation of the autophagosome, its conjugation with lysosomes and autophagolysosomal degradation

(72, 73). It was also shown that p53 induction was accompanying the UPR and p62 enrichment in those cells. The modalities leading to p53 stabilization in this setting were not explored but may involve PERK mediated alternative start site activation in the *p53* gene locus or sequestration of MDM2 by ribosomal proteins (74). As a result, cells were dying by apoptosis upon protein accumulation in a dose dependent manner (71), establishing another link between aneuploidy, autophagy and the apoptotic machinery. Apoptosis-induction in the context of ER-stress has been linked to the BCL2 family proteins BIM and PUMA (75).

Interestingly, although drugs causing proteotoxic stress lead to p53 activation and apoptosis in trisomic MEFs (mouse embryonic fibroblasts), the same drugs showed antiproliferative effects on aneuploid cell lines independent of p53 mutation status (76). Nevertheless, DNA damage induced by doxorubicin increased autophagy and cell death in trisomic ES cells (embryonic stem cells) and cell death was alleviated by inhibition of autophagy (77), suggesting pathway interconnection. Recently Singla et. al. described that in mouse embryos, aneuploidy induced by SAC inhibition leads to apoptotic cell death of aneuploid cells in the epiblast during early embryogenesis and that removal of aneuploid cells triggered compensatory proliferation of diploid cells (78). Both p53 and LC3 were found upregulated in aneuploid embryos while *Bcl2* levels were reduced, limiting proliferation of those cells. Inhibition of p53 resulted in decreased activation of autophagy, whereas inhibition of autophagy had no influence on *p53* mRNA levels. This suggests an interconnection between p53 and autophagy rather than two events occurring in parallel, as here p53 activation due to aneuploidy triggers autophagy and this drives elimination of aneuploid cells by apoptosis (78). While the induction of autophagy in aneuploidy cells appears a more general response, the requirement for p53 in this process may differ, as it was shown that upon chromosomal mis-segregation in RPE1 cells, lysosomal activation was comparable between p53 proficient and deficient cells (79). Although certain facts point to a possible p53 dependant activation of autophagy in response to CIN and aneuploidy, more work has to be done proofing a direct interaction and dissecting the effectors of cell death involved in the elimination of aneuploid cells that engage autophagy prior apoptosis.

P53 activation due to delayed mitotic progression

Of note, cells experiencing delays to pass through mitosis, e.g., caused by delayed satisfaction of the SAC or due to defects in centriole biogenesis, also induce a p53 response when entering the next G1 phase. While in cell line studies p21-induced cell cycle arrest appears the primary response to centriole loss, acentriolar mitoses caused by SAS-4 deletion promote massive apoptosis in the developing mouse embryo (80, 81). Importantly, p53 activation appeared to be independent of DNA damage, suggesting the existence of a “centrosome surveillance” pathway to limit the growth or survival of cells that experience errors in the centrosome duplication (80, 81). Loss of centrioles interferes with mitotic timing (81, 82) and an early study also reported the phenomenon that a prolonged prometaphase blocks proliferation, suggesting the existence of a “mitotic stop watch” (83). Three studies eventually provided a mechanistic explanation how p53 becomes activated in cells that experience mitotic delays, independent of the type of perturbation (84-86). In one of those, a CRISPR-based genetic screen identified the p53 binding protein 53BP1 and the deubiquitinase USP28 along with the E3 ligase TRIM37 as key regulators of p53 stabilisation upon centriole depletion and acentriolar proliferative capacity, respectively (85). Here, 53BP1 appears to act independently of DNA damage and exerts a scaffold function allowing interaction of p53 with USP28 that antagonizes MDM2-regulated ubiquitination (86). How 53BP1 is activated in order to exert its function in the mitotic surveillance pathway remains to be fully established but a role for p38MAPK kinase signalling has been proposed (83, 87). The apoptosis-inducing potential of p53 in this response has been best documented in early embryogenesis where loss of SAS-4, abrogating centriole biogenesis, causes premature lethality in utero. Co-deletion of USP28, however, allows embryogenesis to proceed significantly further (88). Similarly, loss of USP28 can rescue neuronal progenitor cells from p53-induced apoptosis in mice that carry mutations found in patients developing microcephaly due to defects in centriole biogenesis that also cause mitotic delays (89). Upon DNA damage, p53 is able to directly target pro-apoptotic BCL2 proteins, mostly PUMA (p53 upregulated modulator of apoptosis), NOXA and BAX, leading to MOMP, cytochrome c release and the inducing the apoptotic cascade (90). How p53 actually triggers cell death in the context of the mitotic surveillance pathway has not been addressed, but hematopoietic cells appear to prefer cell death initiation over cell cycle arrest upon PLK4 inhibition (personal observations). As such targeting USP28 in cancer, with the idea to block its role in DNA-repair function may become a boomerang, as it may allow survival of cells prone for CIN after experiencing delays in mitosis. Moreover, loss of p53 cannot fully restore embryonic development in SAS-4 mutant mice (88) , suggesting alternative players to be involved in removing cells facing CIN and at risk for aneuploidy in response to mitotic delays. Finally, while loss of the 53BP1-USP28-p53-p21 axis allowed for the outgrowth of cells facing centrosome loss (86), it did not seem to be involved in limiting the growth of cells that experience cytokinesis failure or centrosome amplification after slippage (85).

p53 activation by extra centrosomes

Accumulating evidence suggest that mitotic slippage, discussed in the context of prolonged SAC activation above, as well as failed cytokinesis, e.g. due to lagging chromosomes or persisting DNA bridges, are the main routes of polyploidization and centrosome duplication that both greatly contribute to the development of CIN and aneuploidy. Both events generate polyploid cells with a concomitant increase in centrosome number, a prominent feature of many cancer cells. Supernumerary centrosomes in turn can promote multipolar cell division and chromosome alignment errors leading to CIN and aneuploidy, clearly highlighting the importance of centrosome number surveillance as an onco-suppressive mechanism (91-93).

Previous research has shown, that in the presence of supernumerary centrosomes cells activate a multi-protein complex called the PIDDosome, which prevents these cells from re-entering the cell cycle (94). Fava et.al. demonstrated that the PIDDosome acts as a sensor for centrosome number, thus controlling ploidy levels in mammalian cells (95). The PIDDosome comprises the death-domain (DD) containing proteins PIDD1 (P53-Induced Death Domain Protein 1), RAIDD (RIP-Associated ICH1/CED3-Homologous Protein with Death Domain) as well as Caspase-2, an aspartate-specific endopeptidase (96). Upon assembly, the PIDDosome facilitates proximity-induced dimerization and autoproteolytic activation of Caspase-2. Several lines of evidence suggest activated Caspase-2 can contribute to apoptosis and cell cycle arrest in the context of different mitotic perturbations (97-99), but its link to centrosome surveillance appears most robust, thus establishing also a link to its reported role as a tumour suppressor wherein it may act by removing aneuploid cells (100-102).

Interaction of PIDD1 with extra mature centrosomes, mediated by the distal appendage protein, ANKRD26, promotes PIDDosome assembly in polyploid cells after cytokinesis failure (103). As a result, Caspase-2 starts cleaving its substrates, most notably MDM2, an E3 ubiquitin ligase that acts as a negative regulator of p53. This leads to p53 stabilization and to the transcription of p53 target genes, including *p21* thus facilitating cell cycle arrest and preventing the outgrowth of polyploid cells and the onset of CIN (95, 104). As with loss of *p21*, cells deficient for any of the PIDDosome components display impaired cell cycle arrest after cytokinesis failure, establishing a clear link between the acquisition of multiple centrosomes and a p53 dependent cell cycle arrest in cancer cells forced to fail cytokinesis and in primary hepatocytes, undergoing scheduled cytokinesis failure (95, 105).

Beyond its role to facilitate cell cycle arrest in a PIDDosome dependent manner, Caspase-2 has frequently been reported to engage MOMP and thus induce apoptosis, e.g., in response to DNA damage or spindle poisons (97-99). Whether Caspase-2 can delete cells with extra centrosomes has not been addressed. How Caspase-2 promotes MOMP has also been a matter of debate (99, 106). Notably, it has been previously reported that Caspase-2 is able to cleave the BCL2 family protein BID to its truncated, pro-apoptotic form tBID (98, 107). However, the substrate specificity of Caspase-2 for BID is low and primary cells from

Caspase-2 deficient mice do not display severe apoptotic defects after mitotic perturbations (108). While evidence accumulates that Caspase-2 might act as a barrier against CIN in cells with extra centrosomes, the mechanistic details remain to be elucidated.

Preventing the spread of CIN by sterile inflammation

Chromosome mis-segregation associates with micronuclei formation. Due to reduced stability of lamin B in the nuclear envelop wrapping chromosomes that fail to integrate in the main nucleus, dsDNA becomes detectable in the cytoplasm (109, 110). cGAS (cyclic GMP-AMP synthase) binds cytosolic DNA, leading to the generation of a second messenger cyclic dinucleotide, cGAMP (cyclic guanosine monophosphate – adenosine monophosphate), which binds to STING (stimulator of interferon genes). Although it is an evolutionary conserved pathway against viral or bacterial invasion, DNA-binding of cGAS is not sequence specific and hence not limited to dsDNA from pathogens (111, 112). For cGAS-STING activation size matters, as more cytosolic DNA leads to a stronger inflammatory response (113). Hence, CIN is a very potent trigger of innate immunity and RPE1 cells did show a different immune response gene pattern after chromosomal mis-segregation, when compared to cells arrested due to DNA damage (114). Of note, aneuploid cells arrested in G1-phase show an upregulation of cell surface ligands, increasing immunological visibility, rendering these cells more susceptible to NK-cell (natural killer cell) attack. In co-culture systems, aneuploid cells were rapidly eliminated. Contrary to that, euploid cells did not show increased cell death (114).

Spinning the ball back to an earlier topic, there also seems to be a connection between cGAS-STING activation and autophagy. It would be easy to conclude both pathways being independently active in response to aneuploidy, but autophagy appears required to dispose cGAS-activating dsDNA (115). Additionally, Krivega et. al. showed that in constitutively trisomic cell lines, upon deletion of cGAS and STING, the expression of TFEB (transcription factor EB), which is the master regulator of lysosomal biogenesis, and its target genes (LC3, p62, LAMP2) was reduced, when compared with diploid cells. The same results could be shown in trisomic human embryonic fibroblasts, suggesting that cGAS-STING is not only responsible for the induction of inflammation after CIN, but also transcriptionally upregulates autophagy in response to aneuploidy, as shown in cells from humans with down syndrome (116).

Since this inflammatory response to aneuploidy and CIN seems to be genoprotective, e.g. by fostering NK cell recognition, it is puzzling that cGAS and STING are rarely mutated (<1%) in cancer (117). This is consistent with recently published data documenting that patientderived triple negative breast cancers exhibiting high CIN showed higher cGAS activity and were more likely to form metastasis than those with low CIN. Interestingly, STING dependant NF-κB signalling seemed to positively affect tumour metastasis. Depletion of STING or NF-κB inhibtion limited metastatic capacity while in cancers with low CIN the addition of cGAMP improved metastasis capacity (118). Upon deletion of cGAS using CRISPR-Cas9 or upon chemical inhibition of cGAS, several human and mouse breast cancer cell lines (BT594, 4T1) showed higher rates of apoptosis upon chromosomal mis-segregation. The same was true for STING inhibition, in line with NF-κB signalling inducing the expression of several pro-survival proteins, e.g. within the BCL2 family. In vivo tumour growth of the chromosomally instable 4T1 cells was clearly reduced when lacking cGAS or STING (119), indicating that STING activation in the context of CIN is not onco-protective *per se.* This notion points out a *caveat* for the application of STING agonists in the context of cancer therapy.

Conclusions & outlook

The role of CIN and aneuploidy in cancer formation and evolution is firmly established. The relevance of apoptosis or other cell death modalities as a barrier against CIN or the removal of aneuploid cells, as compared to the induction of cell cycle arrest and senescence, is less clear. Moreover, while multiple pathways have been delineated that lead to p53 activation in response to CIN and aneuploidy, its outcome has mostly been investigated in model cell lines and rather superficially. Of note, the ability of p53 to induce cell cycle arrest or cell death for tumour suppression *in vivo*, however, has been challenged in its importance (55), begging the question, are we looking in the right direction. Clearly, it remains to be seen if all routes of p53 activation subsequent of CIN lead to the same transcriptional core signature or inflammatory outcome in different cell types and tissues. The long lasting conundrum why different cell types elicit different cell fates in response to p53 activation still lurks around in the attic. Future studies addressing this question with new sequencing technologies and live-cell imaging approaches will shed light about the plasticity of the response to limit CIN and aneuploidy at the cellular and organism-wide level.

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