Stress responses

Mammalian cells exhibit complex cellular responses to genotoxic stress, such as cell cycle checkpoints, DNA repair and apoptosis. Inactivation of these important biological events may result in genomic instability and cell transformation (147). Genotoxic stress can be the result of intrinsic processes (e.g. replication), and can be induced by radicals formed by cellular metabolic processes. Genotoxic stress can also be induced by extrinsic sources such as oxidative stress, irradiation or chemical agents. Another form of cellular stress, a non-genotoxic form, is proteotoxic stress induced by heat shock. The best characterized heat shock response is the induction of the heat shock proteins (HSP) which function as molecular chaperones and that exert cell cycle regulatory and anti-cell death activities (126). Thus, proteotoxic stress induced by heat shock in addition to genotoxic stress can affect progression through the cell cycle (71).

Centrosomes, the microtubule-organizing centers (MTOC) of the cell, can be affected by genotoxic stress (150; 159) as well as by proteotoxic stress (6; 12; 26; 96; 163; 164). The aim of this thesis was to investigate in detail centrosome responses to different forms of stress and to investigate what the consequences are for cells with affected centrosomes.

In the sections below an overview is given of cellular responses to genotoxic stress and to proteotoxic stress. Subsequently a summary is given about centrosomes and the relation between abnormal centrosomes and various kinds of stress. Finally the scope of this thesis will be described. Unless otherwise mentioned, all the responses and characteristics we describe here have been studied in mammalian cells.

Genotoxic stress

Checkpoints

Cells can respond to DNA damage by blocking cell cycle progression and therefore provide the cell with sufficient time to repair the damage before DNA replication and cell division. Such a block in cell cycle progression, called a cell cycle checkpoint, is a regulatory mechanism that induces cell cycle arrest at characteristic time points in response to DNA damage caused by internal or natural processes or by external agents (50; 63; 107; 130; 155; 165; 168; 169). Three major cell cycle checkpoints that respond to DNA damage are the G1/S, the G2/M, and the intra-S-phase checkpoint. The last checkpoint temporarily inhibits further DNA synthesis if the cells are in the S phase at the time of the DNA-damaging insult. Once the DNA damage is repaired, the regulating cellular machinery will trigger the cell to continue the cell cycle (83; 130).

DNA damage checkpoints can be activated by various kinds of DNA damage, including UV-lesions, replication errors, DNA cross-links, base damage and DNA strand breaks (50). These checkpoints respond to different types of primary damage at different stages of the cell cycle. Only lesions that cause the most serious damage, if not repaired before the next stage of the cell cycle, will provide signals or the recognition of signals to activate the DNA damage checkpoint. For example, DNA gaps or single strand breaks (SSB) will cause an arrest at G1/S because failure to arrest would permit their conversion to double strand breaks (DSB). In contrast, this checkpoint does not respond to unexcised dimers (yeast and mammalian cells: (114; 151)) or a DSB (yeast: (140)), while the arrest at G2/M is dramatically sensitive to even one DSB (yeast: (81)). Failure to arrest at G2/M while a DSB is present would lead to a chromosome break and thus to the irreversible loss of a chromosome fragment. Below, the three different checkpoints and a selection of players involved in the checkpoints will be discussed.
Arrest of the cell cycle at the G$_1$/S checkpoint caused by DNA damage generally induces increased cellular levels of the tumor suppressor p53. The presence of DNA strand breaks leads to p53 induction, which requires the high-molecular weight phosphoinositide 3 (PI3)-kinase ATM (Ataxia-Telangiectasia Mutated) (152). Through its transcriptional activity, p53 increases the expression of p21, a Cyclin-dependent kinase (Cdk) inhibitor that, in turn, inhibits the Cdk2-CyclinE complex required for progression of the cell cycle from the G$_1$ to S phase. The intra-S-phase checkpoint also requires ATM activation, but is distinct from the G$_1$/S checkpoint. For example, the intra-S-phase checkpoint is not dependent on p53 activity, but it requires several other proteins in a complex such as BRCA1, Mre11, Nbs1 and Rad50 (167; 178). The G$_2$/M checkpoint regulates the inhibition of the dephosphorylation of Cdk1. Cdk1-CyclinB is required for the progression from G$_2$ into M phase. Key players in the G$_2$/M checkpoint response are the kinases ATM and ATM-related protein ATR (1), as well as checkpoint kinases Chk2 and Chk1 (2; 62). Cells that contain diminished ATR (22; 172) or Chk1 (90) activity show a clear defect in the triggering of the G$_2$/M checkpoint in response to IR. However, cells deficient in ATM show no gross defects in the triggering of G$_2$/M arrest in response to IR (129; 174; 177). Likewise, cells deficient in Chk2 are able to trigger the G$_2$/M checkpoint in response to IR (53). Thus, conditions that require the ATM/Chk2 pathway to elicit G$_2$/M arrest have yet to be described. In Figure 1 an overview is given of the major players involved in cell cycle checkpoints as mentioned in this section.

![Diagram of cell cycle checkpoints in mammalian cells.](image)

**Figure 1. Cell cycle checkpoints in mammalian cells.**

An overview of the G$_1$/S checkpoint, S-phase checkpoint, G$_2$/M checkpoint and the spindle checkpoint in mammalian cells. Solid arrows illustrate direct interactions and dotted arrows indicate that there are multiple steps in between.

In addition to cell cycle checkpoints that respond to DNA damage, cells contain another checkpoint: the spindle checkpoint. This spindle checkpoint does not respond to DNA damage. The spindle checkpoint monitors the attachment of microtubules to kinetochores and the tension at the kinetochores. In case chromosomes are not attached, or altered tension is monitored, anaphase progression is inhibited and chromosomal instability due to unequal segregation of chromosomes during cell division is prevented (82; 179). At the heart of the spindle checkpoint is the kinetochore, a multi-layered proteinaceous complex that assembles on the centromeric DNA of each chromosome. During mitosis, the kinetochore mediates the interaction between the chromosome and spindle microtubules. At the very beginning of prometaphase, immediately after the nuclear envelope breakdown (NEB), kinetochores are not attached to microtubules. Subsequently, one kinetochore on a
chromosome captures microtubules from one spindle pole. When its sister kinetochore captures microtubules from the other pole, the now bi-oriented chromosome moves to the equatorial plane, the metaphase plate; this process is known as chromosome-congression (145). Finally, all the chromosomes are attached through both kinetochores to microtubules from two opposite spindle poles and aligned at the metaphase. The spindle checkpoint ensures that, only when all the chromosomes are properly attached and aligned at the equatorial plane, anaphase onset is triggered, allowing the splitting of sister chromatids and their movement to each spindle pole (179). The spindle checkpoint regulates the APC ubiquitin ligase (anaphase promoting complex, or cyclosome) that ubiquitinates M phase cyclins (for cyclins: section ‘centrosomes and the cell cycle’) thereby regulating the exit from M phase.

The spindle checkpoint is also defined as metaphase-anaphase checkpoint, mitotic checkpoint, or as kinetochore checkpoint. However, although in contrast to what the name ‘spindle checkpoint’ would suggest, this checkpoint does not monitor defects in spindle architecture itself like multipolar spindles (154). In our opinion, this checkpoint is better called the kinetochore checkpoint. It first monitors the attachment of microtubules to kinetochores and after all kinetochores are attached to microtubules it monitors the tension at the kinetochores generated by the attached microtubules. Only when tension is generated at all kinetochores, anaphase onset is triggered. However, the term spindle checkpoint is the most widely used and therefore we will use spindle checkpoint in this thesis.

**DNA repair**

Checkpoint proteins arrest the cell cycle in the presence of DNA damage to allow the cell time for repair. Moreover, the checkpoints are proposed to act not simply as a switch for arrest, but also to directly recruit the DNA repair machinery or perhaps even play direct roles in repairing DNA. For example, ATM, the main G1/S checkpoint regulator, is also involved in regulating the repair of DNA damage by homologous recombination (HR) (117).

There are five main DNA damage repair pathways in mammalian cells (147; 158; 173). These are the direct reversal (DR), mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), and the recombinational repair pathways. DR is the simplest repair mechanism in which one single enzyme removes the DNA damage in a one step reaction. MMR, NER and BER rely on an intact complementary strand and can repair various kinds of damage. Recombinational repair can be divided into HR and non-homologous end joining (NHEJ) and is responsible for the repair of DSB and interstrand DNA crosslinks. DSB, as well as SSB, can be caused by spontaneous hydrolysis of the phophodiester bonds in the sugar backbone of DNA, by errors in completing DNA replication, or by ionizing radiation (15).

In this thesis, only the recombinational repair pathways will be discussed in more detail, as these repair pathways are most relevant to the experimental work performed. In HR, the repair of one DSB in one chromatid uses the undamaged sister chromatid as a template. HR almost exclusively takes place in the S and G2 phase of the cell cycle and is a multi step process catalyzed by many gene products (HR reviewed in (121; 149)). Before genetic exchange of the two homologous sequences, two DNA duplexes need to be partially unwound in order to recognize each other by base pairing. These events are coordinated to ensure that the event only occurs between large homologous DNA stretches. In this way, the exact sequence of the genome is maintained (121; 149). However, during G1, homology can solely be found in the other copy of the chromosome or in repetitive sequences. The alignment is problematic and it is more important for the cell to immediately rejoin two broken strands and maintain the configuration of the DNA than to spend time on finding the exact homologous sequence. Therefore, in most cases DSB are repaired by NHEJ when cells are in G1/G0. This pathway involves the direct rejoining of the broken, non-complementary DNA ends of a DSB without the exchange of homologous sequences (NHEJ reviewed in: (52; 55; 56; 131)). As a consequence, deletions and basepair substitutions will arise, which makes this pathway error-prone. Thus, NHEJ does not faithfully restore the original DNA sequence but does certify rejoining of DSB. Nevertheless, due to the high
content of non-coding DNA in diploid somatic cells of multicellular organisms, these mutations are expected to be tolerable in most cases (132).

**Cell death expression / execution**

When the above-mentioned DNA repair pathways fail, one possibility is that the cell executes programmed cell death, referred to as apoptosis. This is a tightly regulated phenomenon ensuring that cells that accumulate irreversible DNA damage do not replicate and divide. Apoptosis is morphologically defined by cytoplasmic shrinkage, chromatin condensation and nuclear fragmentation with chromatinolysis. Apoptosis is characterized by biochemical events including the activation of caspases, DNA fragmentation, and the permeabilization of the outer mitochondrial membrane (MMP), with the consequent release of multiple death effectors, such as the caspase activator cytochrome C, from the mitochondrial intermembrane space (9; 36; 143; 166).

Cells that do not initiate apoptosis may die by a necrotic form of cell death. Also, in case of proliferating cells such as fibroblasts, DNA damaged cells may still remain biochemically active, but they undergo permanent G1 arrest or terminal differentiation (24) that also prohibits cells from generating progeny with a damaged genome.

It also has been demonstrated that after failure of the G1/M checkpoint, cells can undergo mitotic catastrophe in M phase (16; 17). Whereas this was long considered as an uncontrolled process, due to chromosomal damage, there are now (at least in *Drosophila*) indications that this also may be a regulated process to prevent cytokinesis and hence transfer of genetic errors to its progeny (159). Finally, cells that do undergo cytokinesis with damaged DNA mostly will yield daughter cells with unbalanced numbers of chromosomes and may undergo secondary apoptosis or necrosis. However, certain daughter cells may stay alive and will carry mutations that eventually may lead to cancer (in somatic cells) or that may cause inheritable diseases (in germ cells).

**Proteotoxic stress**

A variety of intrinsic and extrinsic stress conditions can be classified as proteotoxic stresses. These can be defined as stresses that increase the fraction of proteins that are in an unfolded state, thereby enhancing the probability of formation of potentially toxic intracellular protein aggregates. A typical example of a proteotoxic, non-genotoxic stress is heat shock. When cells are exposed to high temperatures, energy is absorbed throughout the cell, damaging nearly all cellular structures and functions. Under normal physiological conditions, an equilibrium of folded and unfolded proteins exists within the cell. Upon administration of heat shock, this equilibrium is shifted, resulting in an increase in the fraction of unfolded, denatured proteins that have lost part of their quaternary and tertiary structure. Hydrophobic domains that are normally buried inside native proteins are now exposed. These hydrophobic domains have a tendency to stick to other hydrophobic domains that will lead to the formation of aggregates of denatured proteins within the cell and this formation of aggregates is only partially reversible. Heat stress can lead to cell cycle arrest and cell death, depending on the temperature and the duration of the exposure.

Heat causes a variety of morphological, biochemical, and molecular changes in the cell (Table 1) (13; 60; 79; 85; 115; 127). Virtually all classes of macromolecules are affected by heat shock. RNA and DNA at temperatures up to 48°C are reversibly damaged and this minor damage does not lead to a dysfunction of these macromolecules (58). Similarly, although membrane lipids may be fluidized during hyperthermia, which depends on the transition temperature of the lipids and the severity of the heat treatment, this process itself is not the cause of heat-induced cell death (70). Numerous lines of evidence have led to the conclusion that protein damage is the major molecular event leading to the biological effects of heat stress (60). As elevated temperatures affect nearly all structures and organelles, critical cellular targets are difficult to define and it is also not known which effect leads to the induction of cell cycle arrest or cell death.
Cell cycle arrest

Cells accumulate in specific phases of the cell cycle after they have been exposed to heat treatments. The major blocks are at the $G_1$/S and the $G_2$/M transitions. The accumulation at specific phases depends on cell type and the phase in which the treatment was given. For example, Chinese Hamster Ovary (CHO) cells heated in $G_1$ exhibit a $G_1$/S block, while HeLa cells progress through the $G_1$/S border after the same heat treatment, accumulate in $S$ phase and then undergo spontaneous premature chromosome condensation (PCC), leading to cell death (94).

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<th>Table 1. Heat causes a variety of morphological, biochemical, and molecular changes</th>
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These data were based on references (13; 60; 79; 85; 128).

The absence or presence of the $G_1$/S block may not be caused by the ability or inability to activate the $G_1$/S checkpoint. Rather, the response may simply be a passive process because of protein damage that inhibits replication initiation. HeLa cells will still enter $S$ phase and initiate replication after heat shock, unlike CHO cells. If HeLa cells are further heat stressed, $S$ phase arrest will occur due to protein damage which inhibits replication elongation and subsequently triggers PCC formation (93). Therefore, cell cycle progression after heat shock may be purely the result of obstruction of essential processes, rather than the elegant signaling found after DNA damage. Similarly, the accumulation of cells at the
Chapter 1

G2/M border (120) might be less dependent on signaling events, and merely caused by the disturbance of processes needed to initiate mitosis, such as damage to centrosomes and microtubules.

**Thermotolerance**

Thermotolerance is defined as a state of transient heat-resistance caused by exposure to an initial heat shock followed by recovery at 37°C (so-called acute thermotolerance). Thermotolerance can also occur during continuous mild heating, whereby initial cell death gradually levels off and cells become resistant to further heating (so-called chronic thermotolerance). Thermotolerance is strongly linked to the expression of HSP that accumulate after the first heat shock or under chronic mild heating. Transcription of heat shock genes is specifically induced under such conditions, while transcription of other genes is inhibited by heat (108).

Although thermotolerance can develop in the absence of new HSP synthesis (60), several observations have indicated that HSP play an important role. First, there is good agreement between the kinetics of the transient heat-induced increase in HSP expression and the kinetics of induction and decay of thermotolerance (21; 61; 86). Also, agents that induce HSP, such as heavy metals and ethanol, induce thermotolerance (47; 171). Furthermore, heat-resistant cell lines have elevated expression of one or more HSP (3; 80) and over-expression of some of the single members of the various HSP families has been shown to result in a permanent thermoresistant state (75; 84; 116).

In mammalian cells, there are four major classes of HSP, namely the group of Hsp90, Hsp70, Hsp60 and the group of small HSP such as Hsp27 (25). They all belong to the family of ‘molecular chaperones’. Molecular chaperones are defined as proteins that bind to and stabilize an otherwise unstable conformer of another protein. By controlled binding and release, molecular chaperones facilitate the correct fate in vivo of the unstable protein. HSP have the ability to bind specifically to exposed hydrophobic protein domains. Binding to these denatured peptides prevents their sticking to other hydrophobic domains and as such prevents excessive protein aggregation. The Hsp70 machine (i.e. Hsp70 plus a number of regulators of its activity: (35)) has been thought to be especially crucial in the development of thermotolerance (85).

**Cell death**

If checkpoints are not functional, cells may attempt to progress through the S phase, but nuclear protein damage may obstruct DNA synthesis, leading to replication errors, lethal chromatin aberrations and cell death (28). When cells enter mitosis with unresolved protein damage (or when heated in mitosis), cells may undergo mitotic catastrophe or aberrant divisions (112) that will mostly result in secondary apoptosis or necrosis of the daughter cells. There are clear indications that accumulation of heat-unfolded proteins at centrosomes are linked to these aberrant mitoses (112).

**Centrosomes**

The central question in this thesis is how do the centrosomes respond to various stresses during the cell cycle. First, an overview is given of centrosome structure, duplication and function. Then, the responses of centrosomes to various kinds of stress during the cell cycle are discussed.

**Centrosomes and the cell cycle**

The duplication of centrosomes is linked in time to the DNA replication cycle and should happen only once per cell cycle. Both processes involve cyclins, the regulatory proteins that are present at high levels at specific phases in the cell cycle. Cyclins are needed for the kinase activity of serine/threonine kinases (Cdk’s) and each combination of Cdk-Cyclin is specific for a certain cell cycle phase. For example, Cdk2-CyclinE regulates the progression into the S phase as well as the initiation of the centrosome duplication (section ‘centrosome duplication’).
Figure 2. A schematic structure of a centrosome.

The centrosome is composed of two centrioles, connected by interconnecting fibers and surrounded by the PCM. Microtubules are nucleated from the PCM and the appendages of the mother centriole. Over 80 proteins have been localized to this complex. Reproduced with permission from Nature Reviews Molecular Cell Biology (30) copyright (2001) Macmillan Magazines Ltd. (www.nature.com/reviews).

Structure and biochemical composition

Centrosomes consist of two centrioles facing perpendicular to each other, surrounded by a fibrous meshwork termed pericentriolar material (PCM). The two centrioles are barrel shaped and each consist of nine triplet microtubules of ~400 nm in length (97). These stable microtubules are composed of large numbers of polypeptides, including α-, β-, and δ-tubulin (19), and centrin (5). The two centrioles differ both structurally (123) and biochemically (78) and one is called the mother centriole and the other the daughter centriole. The mother centriole is the oldest centriole and contains distal appendages. Some proteins, such as cenexin (77) and Cep170 (44), only associate with the mother centriole, by localizing to the distal appendages.

There are four classes of centrosomal proteins each of which are associated with a particular function, including structural, microtubule-nucleating, anchoring, and regulating. Examples of proteins from these four classes of centrosomal proteins are given in Table 2. The structural proteins are required for maintenance of the morphology of centrosomes. Microtubule-nucleating proteins assist the nucleation of microtubules with the minus ends to the centrosome and are required for aster formation or microtubule growth from the centrosome. Because of this function the centrosome is also referred to as the MTOC of the cell. The microtubule-nucleating capacity is the classic function of centrosomes and this capacity differs between the mother and daughter centriole. While both centrioles nucleate microtubules, only the mother centriole anchors them, probably through its sub-distal appendages containing ninein (133). Anchoring proteins are required to anchor microtubules and regulating proteins to the centrosome and have at least two domains, namely an enzyme-binding motif and a cellular localization motif. Anchoring proteins form the interface.
between microtubule-nucleating proteins and regulating proteins. Regulating proteins are enzymatic proteins that regulate processes at the centrosomes. Regulating proteins include kinases, phosphatases, signaling molecules and proteasomal components and can direct the microtubule nucleating proteins (42). Cyclins and Cep135 are examples of regulating proteins (118). In Figure 2 a schematic structure of a centosome is shown and includes the localization of some of the centrosomal proteins mentioned in Table 2.

**Figure 3. The centrosome duplication cycle.**

Centrioles are indicated as cylinders, and are numbered. Distal appendages are shown on centrioles 1 and 2, indicating that these are mother centrioles. The line encircling the centrioles indicates the PCM. The dotted line on the S phase centrosome B indicates that this centrosome is immature and that S phase centrosome A has inherited the PCM. Reprinted from Cell, Vol. 105, Stearns, T., Centrosome duplication. a centriolar pas de deux, 417 - 420, Copyright (2001), with permission from Elsevier.

**Centrosome duplication**

The duplication of the centrosome is highly coordinated and can be divided into several steps that will be discussed in the next sections. In short, duplication of centrosomes occurs as follows. During G1 only one centrosome is present containing two centrioles: one mother and one daughter centriole. At G1/S the two centrioles lose their orthogonal orientation and each centriole forms a template for a newly synthesized procentriole. These procentrioles elongate during S and so the centrosome contains four centrioles in this phase: one old mother centriole, one old daughter centriole and two new daughter centrioles. In G2 phase the link between the two old centrioles, also called parental centrioles, is broken. Each of the two centrosomes matures, so that in the end both centrosomes will contain a mother centriole, a daughter centriole, and PCM. At the onset of mitosis both centrosomes migrate to opposite ends of the nucleus and each centrosome forms a mitotic pole of the bipolar spindle. After completion of cytokinesis the two daughter cells each have inherited one centrosome and the cycle starts over again. All phases of the duplication are controlled by kinases and phosphatases as described below. A schematic overview is given in Figure 3.

**Centriole duplication and elongation (G1/S phase)**

It has been demonstrated in Xenopus that the initial round of centrosome duplication is triggered by calcium/calmodulin-dependent protein kinase II (CaMKII), which is activated by
sudden increases in intracellular free calcium ions. This suggests that calcium oscillations in the cell cycle are linked to centrosome duplication (99). In mammalian cells, the calcium-binding protein centrin is a member of this calmodulin super family (170).

Other factors have been identified that play a role in centrosome duplication and in Xenopus oocytes and early embryos Cdk2-CyclinE activity is required for centrosome duplication. Cdk2-CyclinE is also involved in centrosome duplication in mammals as outlined below. In G1 phase, each cell has one centrosome and the association of phosphoprotein nucleophosmin prevents its premature duplication. In early S phase, nucleophosmin is phosphorylated by Cdk2-CyclinE and dissociates from the centrosome (74; 119). After disappearance of nucleophosmin from the centrosome, the two centrioles lose their orthogonal orientation by Skp1-Cullin-F-box (SCF) mediated proteolysis (Figure 3, G1/S phase). The SCF ubiquitin ligase also ubiquitinates G1 cyclins and thereby regulates the progression from the G1 into S phase. Each of the two centrioles forms a template for the procentriole that is formed adjacent to the proximal wall and that elongates gradually during the next phases (73) (Figure 3, S phase). Directly after nucleophosmin has disappeared from the centrosome, Cdk2 activates mMps1p kinase to drive the centrosome duplication process (37). The mMps1p kinase is localized to centrosomes during the entire cell cycle. Cdk2 activity is not required for the centrosomal localization of mMps1p, but is required for stabilization of mMps1p protein levels during S phase resulting in centrosomal accumulation of mMps1p. This accumulation restricts the mMps1p kinase-dependent centrosome duplication to the S phase (37).

Factors regulating parental centriole separation (G2 phase)
Just before G2 phase, the procentrioles elongate and the centrosome now consists of four centrioles (Figure 3, S phase), namely two parental centrioles that are the old mother (Figure 3, centriole 1) and old daughter centriole (Figure 3, centriole 2) and two procentrioles (Figure 3, centrioles 3 and 4) forming the new daughter centrioles. The proximal ends of the two parental centrioles are still connected by a proteinaceous link that consists of C-Nap1. A constant delivery of microtubule subunits towards the centrosome-associated microtubule minus ends determines the concentration of PCM components at the centrosome. In addition, the microtubule network may play a role in the balance of kinases (NIMA family member kinase Nek2) and phosphatases (PP1α, Cdc14A) that control regulatory processes at the proteinaceous link (103). The dynamic interaction of Cdc14A with the centrosome may help to generate this balance (95). Cdc14A interacts with interphase centrosomes and this interaction is independent of microtubules or Cdc14A phosphatase activity. The amount of Cdc14A is reduced at the centrosomes after entry into mitosis although it is not entirely absent. Parental centriole separation is inhibited by the phosphatase PP1α, which is believed to be a physiological antagonist of Nek2. At the onset of mitosis PP1α is inhibited (138) and then Nek2 kinase is able to phosphorylate C-Nap1 (101). The regulation of C-Nap1 is cooperatively regulated by Nek2 and Aurora-A, a member of the Aik (Aurora/Ipl1-related kinase) family (98). After phosphorylation of C-Nap1, the duplicated centrosome is not connected by a proteinaceous link anymore but still the two, immature, centrosomes localize close together.

Centrosome maturation (G2 phase)
Directly after breakage of the link between the two parental centrioles (Figure 3, centrioles 1 and 2), a difference is observed in protein content between the immature centrosomes. The oldest centrosome (Figure 3, A), which contains the old mother centriole, has inherited the PCM and is most mature. The newest centrosome (Figure 3, B), which contains the old daughter centriole, has to acquire newly synthesized PCM as well as distal appendages at the old daughter centriole to become the new mother centriole (Figure 3, G2 phase). ε-Tubulin has been shown to localize only to the PCM of the oldest centrosome and later on to the PCM of both centrosomes. At the same time ε-tubulin is acquired, centexin is acquired at the new mother centriole (19). In addition to the gain of new PCM, maturation of centrosomes involves the phosphorylation of centrosomal proteins, and the increase of microtubule-nucleating capacity (124).
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Migration to poles (M phase)
At the end of G2 phase, the two mature centrosomes localize just outside the nucleus. At this moment the amount of γ-tubulin associated with the centrosome suddenly increases and this γ-tubulin recruitment to centrosomes coincides with an increase of the centrosomal microtubule-nucleation capacity during prometaphase (67). Withdrawal of the nuclear membrane by the molecular motor dynein creates membrane tension, causing tearing and resulting in NEB and subsequently in the release of several proteins such as nucleophosmin and Ran-GTP. Nucleophosmin associates with the two centrosomes preventing them from premature duplication. Ran-GTP, recruited and/or activated by Polo-like kinase Plk1 (54), causes increased microtubule nucleation from the centrosomes. Each centrosome moves to one side of the nucleus. The formation of the spindle by interaction of microtubules and chromosomes and the trigger to anaphase onset is described in the section ‘checkpoints’. At the end of telophase, the two daughter cells are still connected by the midbody, a cytoplasmic bridge consisting of microtubules.

Cytokinesis completion
Each of the two daughter cells inherits one centrosome and while the mother and daughter centriole of one centrosome are always close together during the rest of the cell cycle, they separate into individual units just after telophase. The mother centriole remains almost stationary near the geometrical center of the forming daughter cell and bears the microtubule aster, while the daughter centriole is more mobile and only nucleates a few, if any, microtubules (133). The mother centriole then suddenly moves towards the midbody (133). The cytoplasmic bridge narrows, the microtubules within the bridge depolymerize, and cytokinesis is completed when the mother centriole moves back to the daughter centriole (134) after which the movements of the daughter centriole reduce (133). Remarkably, the movement of the monocentriolar unit described here is required for the fidelity of the completion of cytokinesis.

Centrosome function in mitosis
Although centrosomes play an important role in the M phase, the centrioles of the centrosome are not essential for assembling spindles (49), segregating chromosomes (49), directing cell division, or forming centrosomes. In the absence of centrioles, these processes depend on self-organization of microtubules (48). In the absence of centrioles, centrosomal components, organelles, and microtubules interact with each other to produce polarized microtubule arrays that resemble centrosomes. In addition, chromosomes and microtubules can influence each other’s behavior to form spindles. However, the absence of centrioles often results in cytokinesis failure (49) because chromosomes persist in the midbody (66) and because cytokinesis does not complete if the mother centrioles do not move towards the midbody (134; 146).

Although the centriole of the centrosome is dispensable for mitosis, its presence increases the fidelity of mitosis (144; 146). The centriole induces the formation of centrosomes at specific times and in specific places thereby increasing the accuracy and speed of the processes the centrosome directs.

Centrosome function in interphase
As described in the section ‘centrosomes and the cell cycle’, the classic function of centrosomes is their role as the MTOC in the cell. Through its nucleating and anchoring capabilities, the centrosome organizes the cytoplasmic microtubule complex. However, a typical interphase microtubule array can be formed in the absence of a centrosome. Under these conditions, microtubules are nucleated randomly within the cytoplasm and organized into an aster by the action of multivalent microtubule molecular motors such as dynein. These a-centrosomal microtubule arrays support all normal intracellular trafficking, but it is not known whether they support all microtubule-mediated functions (144).

While the centrosome is dispensable to the cytoskeleton, this organelle is essential for the progression from G1 into S phase (31; 49). It is not known whether this is because the
centrosome is part of a checkpoint pathway (110), or because the centrosome is required simply to catalyze reactions needed for progression into the S phase (144).

**Centrosomes and genotoxic stress**

Under normal conditions, centrosome duplication and DNA replication are coupled. Centrosomes are essential for progression into S phase, the DNA synthesis phase, and play a role in increasing the fidelity of cytokinesis. Remarkably, in the presence of damaged DNA during mitosis, centrosomes appear to be important target organelles for regulating cell division. For example in *Drosophila* embryos, the presence of damaged or incompletely replicated DNA during mitosis triggers the dissociation of several components of the γ-tubulin ring complex (γ-TuRC) from the centrosome, leading to loss of microtubule nucleation and to defects in spindle assembly. Anastral spindles are formed that are unable to segregate the DNA. These features occur in wild-type embryos treated with replication inhibitors or DNA-damaging agents (150; 159). Various types of agents that can cause DNA lesions trigger the mitosis-specific centrosome inactivation, e.g. DNA replication inhibitors aphidicolin and hydroxyurea, DNA-damaging agents such as UV radiation and X-rays, the radiomimetic drug bleomycin, Topoisomerase I or II inhibitors camptothecin, etoposide VM-26 and ICRF-193, and also injection of phage DNA that was restriction-digested, single-stranded or supercoiled (150; 159).

A null mutation in the *Drosophila* Chk2 tumor suppressor homologue (DmChk2/Dmnk) blocks the mitosis-specific centrosome inactivation response to DNA lesions and also prevents loss of defective nuclei from the cortex. In addition, DNA damage leads to increased DmChk2/Dmnk localization to the centrosome and spindle microtubules. DmChk2/Dmnk is therefore essential for a ‘mitotic catastrophe’ signal that disrupts centrosome function in response to genotoxic stress and ensures that aneuploid nuclei are eliminated from the embryonic precursor pool (159).

Whether a mitosis-specific centrosomal inactivation pathway also exists in mammalian cells was not known at the start of this project and one part of this thesis is dedicated to investigate this.

**Centrosomes and proteotoxic stress**

Proteotoxic stress also leads to centrosomal abnormalities, although this might be an indirect response. Misfolded proteins that arise after heat shock are able to aggregate with each other or with other proteins in the cell. Misfolded proteins were shown to accumulate at the centrosomes in interphase cells (164). These so-called aggresomes (57) may enable cells to coordinate regulation and dispose of protein damage at specific cellular sites and thereby preventing random protein aggregation and cellular dysfunction. However, it is not unlikely, although not proven yet, that protein aggregates at the centrosome may also lead to dysfunction of the centrosomes in cell cycle progression.

In *Drosophila* embryos, heat shock leads to severe defects in centriole organization specifically when cells are heated in mitosis (26). Cellular consequences of these heat shock affected mitotic centrosomes have not been studied in this system.

At the start of this project, centrosomal effects of heat shock specifically given during mitosis had not been studied in mammalian cells. However, during interphase, centrosomal abnormalities caused by heat treatments were observed. These centrosomal abnormalities include increases in the electron-dense material comprising the PCM, disappearance of centrosomal proteins such as γ-tubulin and pericentrin, and disruption of centriole walls (6; 12; 96; 163; 164). In addition, abnormalities in the first mitoses post-heating were reported including the appearance of multipolar mitotic spindles (112; 162).

Protection of centrosomes from protein damage during heat treatments has been demonstrated for centrosomes in interphase because thermotolerant interphase cells display a more rapid recovery from heat-induced damage to the centrosome structure and centrosome function (12; 163). However, a protective role of HSP to protein damage at the
centrosomes of mitotic cells was not investigated and remained elusive at the start of this thesis.

Nevertheless, in untreated conditions a role for HSP in centrosome function has been suggested. For example, Hsp90 is localized at the centrosome throughout the cell cycle, at different stages of development in Drosophila and vertebrates. Disruption of Hsp90 function by mutations in the Drosophila hsp90 gene or treatment of mammalian cells with an Hsp90 inhibitor, results in abnormal centrosome separation and maturation, aberrant spindles and impaired chromosome segregation (76). Another example is that Hsp73, the constitutive form of Hsp70, is intimately associated with the centrosome during all stages of the cell cycle in untreated conditions, suggesting that Hsp73 is involved in centrosome function (11; 142). It was speculated that Hsp73 participates in the process of centrosome assembly. Following the duplication of centrioles during S phase, Hsp73 mediates the assembly of the proteins that comprise the PCM. This is consistent with the finding that Hsp73 has a role in the return of centrosomal structure and microtubule-nucleating function following heat shock. Furthermore, these authors suggested that the presence of Hsp73 at the centrosome during other stages of the cell cycle might facilitate the movement of proteins into and out of the organelle (12).

**Centrosomes and cancer**

One characteristic phenotype of many cancer cell lines is the presence of multiple centrosomes (10; 23; 87; 139; 148). Centrosome amplification and abnormalities occur exclusively in aneuploid, but not diploid cancer cell lines, and correlates with numerical chromosomal alterations (43). In addition to abnormal centrosome numbers, centrosomes in human tumors display also significant alterations compared to centrosomes in normal tissue. These alterations include supernumerary centrioles, excess PCM, disrupted centriole barrel structure, unincorporated microtubule complexes, centrioles of unusual length, accumulation of inappropriate phosphorylation of centrosomal proteins, and increased numbers of MTOC nucleating unusually large arrays of microtubules (88; 89; 135; 136). However, it remains unclear whether multiple centrosomes are merely a consequence of the genomic instability in transformed cells, or whether they also play a driving force in the process of carcinogenesis.

In particular it has been suggested that important tumor suppressors like p53 or BRCA1 are associated with centrosomes. It can therefore be speculated that their loss of function may amongst others result in a defective regulation of centrosome duplication, either in the absence or in the presence of (induced) DNA damage. However, both proteins are also otherwise linked to genomic instability, namely via checkpoint control or DNA repair. Hence it needs to be tested whether the abnormal number of centrosomes in cells lacking BRCA1 or p53 is due to a direct loss of control on centrosomes or rather due to an indirect result of the involvement of these tumor suppressors in cell cycle regulation and DNA repair.
Table 2. Centrosomal proteins, categorized by function.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Localization in centrosome</th>
<th>Remarks</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-tubulin</td>
<td>Centriole</td>
<td>αβ-dimers stack head-to-tail, aggregate laterally and form cylinder.</td>
<td>(51)</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>Centriole</td>
<td>αβ-dimers stack head-to-tail, aggregate laterally and form cylinder.</td>
<td>(51)</td>
</tr>
<tr>
<td>Cenexin</td>
<td>Mother centriole</td>
<td>Marker for maturation: it is acquired by immature centriole at G2/M transition.</td>
<td>(77)</td>
</tr>
<tr>
<td>Centrin</td>
<td>Distal lumen of centrioles</td>
<td>Probably involved in centrosome reproduction cycle. Calcium-binding protein.</td>
<td>(125)</td>
</tr>
<tr>
<td>C-Nap1</td>
<td>Proximal (closed) centriole end; proteinaceous link between parental centrioles</td>
<td>Key component of dynamic, cell cycle-regulated structure that mediates centriole-centriole cohesion. (C-Nap1 = Cep250)</td>
<td>(41; 102)</td>
</tr>
<tr>
<td>δ-tubulin</td>
<td>Between centrioles and between centrosomes</td>
<td></td>
<td>(19)</td>
</tr>
<tr>
<td>Dyn2</td>
<td>Centrioles and PCM</td>
<td>Directly interacts with γ-tubulin. Dyn2 (Dynamin 2) is dynamic exchangeable component of centrosome and is participant in centrosome cohesion. Dyn2 is a GTPase.</td>
<td>(160)</td>
</tr>
<tr>
<td>ε-tubulin</td>
<td>PCM</td>
<td>Marker for maturation: it associates with oldest centrosome.</td>
<td>(19)</td>
</tr>
<tr>
<td>Odf2</td>
<td>G0/G1 mother centriole, G1/S mother and daughter centriole</td>
<td>May be involved in maturation of daughter centrioles. (Outer dense fiber 2)</td>
<td>(111)</td>
</tr>
</tbody>
</table>
### Table: Localization in centrosome

<table>
<thead>
<tr>
<th>Protein</th>
<th>Location</th>
<th>Remarks</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKAP450</td>
<td>PCM</td>
<td>Anchors CK1δ and probably also CK1ε. The association between centrioles and AKAP450 is critical for centrosome integrity and reproduction. (A-Kinase Anchoring Protein; = AKAP350 = CG-NAP)</td>
<td>(65; 153)</td>
</tr>
<tr>
<td>Cep110</td>
<td>Mother centriole</td>
<td>Marker for maturation: it appears at daughter centrosome during telophase-G1 transition.</td>
<td>(122)</td>
</tr>
<tr>
<td>EB1</td>
<td>Mother centriole</td>
<td>Anchors cytoplasmic MT minus ends to subdistal appendages of mother centriole. (End-binding protein 1)</td>
<td>(92)</td>
</tr>
<tr>
<td>Kendrin</td>
<td>Centrosome</td>
<td>Anchors γ-tubulin. Kendrin binds DISC1 (Disrupted-In-Schizophrenia 1). N-terminal regions of Kendrin share significant sequence homology with pericentrin.</td>
<td>(38; 105)</td>
</tr>
<tr>
<td>Ninein</td>
<td>Mother centriole and PCM</td>
<td>Marker for maturation: it appears at open end of daughter centrosome during telophase-G1 transition; Proposed to have dual role as MT minus-end capping and anchoring protein; Promotes MT nucleation by docking γ-TuRC at centrosome; Can be phosphorylated by Aik and PKA.</td>
<td>(8; 20; 27; 106; 122)</td>
</tr>
<tr>
<td>Nlp</td>
<td>Mother centriole</td>
<td>Is coordinately regulated at G2/M by Plk1 and Nek2 and is displaced from centrosome upon mitotic entry. It binds γ-tubulin.</td>
<td>(141)</td>
</tr>
<tr>
<td>Pericentrin</td>
<td>PCM</td>
<td>Anchors γ-TuRC at spindle poles, thereby playing a role in MT nucleation.</td>
<td>(180)</td>
</tr>
<tr>
<td><strong>MT nucleation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-tubulin</td>
<td>PCM</td>
<td>Recruits αβ dimers.</td>
<td>(51)</td>
</tr>
<tr>
<td>Ran</td>
<td>Centrosome</td>
<td>Centrosomal interaction mediated by AKAP450. When AKAP450 is delocalized from centrosome, Ran is also delocalized, and MT regrowth or anchoring is altered. Ran is a GTPase.</td>
<td>(64)</td>
</tr>
<tr>
<td>RanBP1</td>
<td>Centrosome</td>
<td>RanBP1 (Ran-binding protein 1) is major effector of Ran. Overexpression of RanBP1 induces centrosome splitting during mitosis, which requires microtubule integrity and Eg5 activity.</td>
<td>(29; 113)</td>
</tr>
<tr>
<td>TCP-1</td>
<td>Centrosome</td>
<td>Microtubule regrowth.</td>
<td>(11)</td>
</tr>
</tbody>
</table>
### Regulating

<table>
<thead>
<tr>
<th>Localization in centrosome</th>
<th>Remarks</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aik</td>
<td>Spindle pole (prophase to anaphase)</td>
<td>Possibly involved in centrosome function such as chromosome segregation or spindle formation.</td>
</tr>
<tr>
<td>Aik3</td>
<td>Mitotic centrosome (anaphase to cytokinesis)</td>
<td>Suggested to play role in centrosome function at later stages of mitosis.</td>
</tr>
<tr>
<td>APC10/ Doc1</td>
<td>Mitotic centrosomes</td>
<td>APC10/Doc1 (APC subunit 10) may be one of core subunits of APC, the ubiquitin ligase which specifically targets mitotic regulatory factors such as Pds1/Cut2 and cyclinB.</td>
</tr>
<tr>
<td>Aurora-A</td>
<td>Centrosomes (from S phase after centriole duplication till early G1)</td>
<td>Involved in centrosome separation, duplication, maturation, bipolar spindle assembly and stability. Aurora-A is potential oncogene. (Aurora-A = Stk15); Phosphorylates Cdc25B at centrosome during mitosis and might locally participate in control of mitotic onset. NB: Cdc25B phosphatase is activator of Cdk’s at mitosis (e.g. Cdk1-cyclin B1).</td>
</tr>
<tr>
<td>hCdc14A</td>
<td>Centrosomes</td>
<td>hCdc14A is phosphatase with dual specificity: it dephosphorylates hCdh1 and activates APC(Cdh1), but it does not affect APC(Cdc20) activity; NB: APC(Cdh1) ubiquitinates mitotic cyclins, allowing mitotic exit.</td>
</tr>
<tr>
<td>Cdc20</td>
<td>Centrosomes</td>
<td>Cdc20 is activator of APC/C and has rapid turnover at spindle poles.</td>
</tr>
<tr>
<td>Cdk1</td>
<td>Centrosomes (elevated levels G2/M)</td>
<td>Modification of centrosome bringing about formation of mitotic spindle. (Cdk1 = p34cdc2)</td>
</tr>
<tr>
<td>Cep135</td>
<td>PCM</td>
<td>Role in centrosomal function of organizing microtubules.</td>
</tr>
<tr>
<td>Cep170</td>
<td>Interphase mother centriole</td>
<td>Marker for maturation: localizes to subdistal appendages, typical of the mature mother centriole. Thus, one centriole during G1, S, and early G2, but two centrioles during late G2 phase.</td>
</tr>
<tr>
<td>CK1δ</td>
<td>Interphase centrosome</td>
<td>Contribution to cell cycle progression (vesicular trafficking, spindle formation). (Casein kinase 1)</td>
</tr>
<tr>
<td>CK1ε</td>
<td>Interphase centrosome</td>
<td>Contribution to cell cycle progression (vesicular trafficking, spindle formation).</td>
</tr>
<tr>
<td>Localized in Centrosome</td>
<td>Remarks</td>
<td>Ref</td>
</tr>
<tr>
<td>------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Cul1</td>
<td>Centrosomes. Member of SCF complex.</td>
<td>(39)</td>
</tr>
<tr>
<td>CyclinE</td>
<td>Centrosomes. Has modular centrosomal-targeting domain essential for promoting S phase entry in Cdk2-independent manner.</td>
<td>(100)</td>
</tr>
<tr>
<td>Erk</td>
<td>Centrosomes. Links extracellular signaling to centrosome dynamics by Nek2A. Interacts with Nek2A via conserved Erk2 docking site.</td>
<td>(91)</td>
</tr>
<tr>
<td>Katanin</td>
<td>PCM. Regulates number of microtubule ends in spindle (it is 'MT-severing'). Katanin's activities are segregated into subunit (p60) that possesses enzymatic activity and subunit (p80) that targets the enzyme to centrosome.</td>
<td>(14; 46)</td>
</tr>
<tr>
<td>NM23</td>
<td>Centrosomes. NM23-H1 is putative tumor suppressor. It associates with Aurora-A/STK15.</td>
<td>(32)</td>
</tr>
<tr>
<td>Nek2</td>
<td>Centrosomes. Centrosome separation at the G2/M transition. (NIMA (never in mitosis A)-related kinase)</td>
<td>(40)</td>
</tr>
<tr>
<td>Nucleophosmin</td>
<td>Mitotic centrosome (till anaphase) involved in nucleolar assembly, centrosome duplication, ribosome assembly, and transport. Centrosomal localization and function regulated by Nek2A. (=NPM/B23 = NO38 = Numatrin)</td>
<td>(18; 176)</td>
</tr>
<tr>
<td>p53</td>
<td>Mitotic centrosome. ATM is essential for p53 centrosomal localization (required for activation of post mitotic checkpoint after spindle disruption, thus for inhibiting DNA replication). P53 is tumor suppressor.</td>
<td>(109; 161)</td>
</tr>
<tr>
<td>Plk1</td>
<td>Centrosome. Regulator of chromosome segregation, mitotic entry, and mitotic exit; Plk1 (= Polo; = Plx1) may play centrosomal roles through recruitment and/or activation of Ran/RanBPM (Ran-binding protein in MTOC).</td>
<td>(54)</td>
</tr>
<tr>
<td>PML</td>
<td>Centrosomes. Direct role in control of centrosome duplication through suppression of Aurora-A activation to prevent centrosome reduplication. (promyelocytic leukemia gene)</td>
<td>(175)</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>Mitotic centrosomes. Interacts with Cdc20 resulting in inhibition of APC activity and thereby regulates mitotic progression. RABP1 (RASSF1A-binding protein 1 = RAS association domain family protein 1A = C19orf5) is required for recruitment of RASSF1A to spindle poles and for inhibition of APC-Cdc20 activity during mitosis. RASSF1A is tumor suppressor.</td>
<td>(156; 157)</td>
</tr>
<tr>
<td>Skp1</td>
<td>PCM. Role in proper chromosomal segregation. Member of SCF complex.</td>
<td>(39; 137)</td>
</tr>
</tbody>
</table>
Aim of the thesis

A well-established role for centrosomes is to increase the fidelity of mitosis, although centrosomes themselves are not required for progression through mitosis. Only recently it has been demonstrated that centrosomes also play a novel role in responses of cells to various kinds of stress. *Drosophila* embryos respond to genotoxic stress by a mitosis-specific centrosomal inactivation pathway. This pathway is dependent on DmChk2. The aim of this thesis was to investigate whether there exists a comparable centrosomal inactivation pathway in mammalian cells. Therefore we investigated in detail whether and how various stress events affect mitotic centrosomes in mammalian cells and what the consequences are for dividing cells with altered centrosomes.
Chapter 1

References


