Hsp70 protects mitotic cells against heat-induced centrosome damage and division abnormalities
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The effect of heat shock on centrosomes has been mainly studied in interphase cells. Centrosomes play a key role in proper segregation of DNA during mitosis. However, the direct effect and consequences of heat shock on mitotic cells and a possible cellular defense system against proteotoxic stress during mitosis have not been described in detail. Here we show that mild heat shock, applied during mitosis, causes loss of dynamitin/p50 antibody staining from centrosomes and kinetochores, induces division errors in most cells and in the remaining cells progression through mitosis is delayed. Expression of Hsp70 protects against most of the heat-induced division abnormalities. Upon heat shock, Hsp70 is rapidly recruited to mitotic centrosomes and normal progression through mitosis is observed immediately after release of Hsp70 from centrosomes. In addition, Hsp70 expression coincides with restoration of dynamitin/p50 antibody staining at centrosomes but not at kinetochores.

Our data show that during mitosis centrosomes are particularly affected resulting in abnormal mitosis. Hsp70 is sufficient to protect against most of the division abnormalities demonstrating the involvement of Hsp70 in a repair mechanism of heat-damaged mitotic centrosomes.

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Introduction

Centrosomes, the microtubule-organizing centers of the cell, are involved in proper segregation of duplicated DNA, play a role in completion of cytokinesis (14; 33), and are required for progression through G1 phase (14; 17; 22). It has been demonstrated that centrosomes are target organelles affected by different kinds of cellular stress responses, especially during mitosis. In Drosophila embryos, centrosome inactivation occurs in the presence of genotoxic lesions at the onset of mitosis (39; 40; 43), a process that requires Chk2 kinase (43). In this process, multiple components of the γ-Tubulin ring complex (γ-TuRC) disappear from mitotic centrosomes, anastral spindles are formed, and segregation of duplicated DNA is prevented. Mammalian cells also show a mitosis-specific centrosome response to genotoxic stress. When cells enter mitosis with damaged DNA, centrosomes split into mono-centriolar parts that form multiple spindle poles, resulting in mitotic catastrophe (19).

Centrosomes are not only affected after genotoxic stress but also after a proteotoxic stress such as heat shock. In Drosophila embryos, heat shock leads to severe defects in centriole organization specifically when cells are in mitosis (9). Cellular consequences of these heat shock affected mitotic centrosomes have not been studied in this system. In mammalian cells, it has been described that during interphase heat shock causes alterations of centrosomes such as increases in the electron-dense material comprising the pericentriolar mass, disappearance of centrosomal proteins, and disruption of centriolar walls (2; 6; 27; 47; 48). In addition, abnormalities in the first mitoses post-heating were reported such as the appearance of multipolar mitotic spindles (30; 46).

It is known that preconditioning with a mild heat shock can induce a state of heat resistance, i.e. thermotolerance, which also protects against centrosome alterations after a second heat treatment (6; 47). This has been demonstrated for centrosomes in interphase since thermotolerant cells display a more rapid recovery from heat-induced damage to centrosome structure (assayed by antibody staining) and centrosome function (assayed by regrowth of microtubules) (6; 47). However, centrosome alterations in heat-treated mammalian mitotic cells have not been investigated and the fate of cells with heat-affected centrosomes remains unknown.

Thermotolerance is tightly correlated with the elevated expression of heat shock proteins both in interphase (25) and in mitosis (4). Associations of heat shock proteins with mitotic centrosomes have been observed (1; 5; 24; 32; 34) suggesting a role for heat shock proteins in assisting repair of centrosomes after heat shock. Microinjection of Hsp73 antibodies retarded recovery of interphase centrosome structure and microtubule regrowth abilities after heat shock whereas injection of purified Hsp73 prior to heat shock enhanced these processes (6). Since heat shock proteins function as molecular chaperones to repair protein damage (21; 26), it can be suggested that protein aggregation may underlie the centrosome abnormalities after heat shock. Indeed, electron microscopical studies suggested the presence of protein aggregates at the pericentriolar mass following heat shock (48).

Most of the above-mentioned studies have concentrated on effects of severe heat treatments on interphase centrosome structure and function in fixed cells. Here, by performing time-lapse studies we aimed to address the questions what the effects are of mild and severe heat shock on mitotic centrosomes, what the fate is of mitotic cells with heat-affected centrosomes, and whether heat shock proteins can protect heat shock affected centrosomes in mitotic cells.

We show that a severe heat treatment resulted in loss of γ-tubulin and pericentrin antibody labeling at centrosomes and loss of dynamitin/p50 (a 50-kDa subunit of dynactin) antibody labeling at centrosomes and at kinetochores. Under these conditions CREST antiserum did not show an altered localization pattern. However, the organization of mitotic spindles was altered, mitotic progression was strongly delayed, and division errors occurred. After mild heat shock only dynamitin/p50 antibody labeling disappeared and none of the other protein alterations found after severe heat shock were detected. However, under these mild heat
Hsp70 protects mitotic cells against heat-induced centrosome damage and division abnormalities. In thermotolerant cells, the length of heat shock induced mitotic delays and the frequency of heat shock induced division errors were reduced. The reduction in abnormalities correlated with the association of Hsp70, but not Hsp27 or Hsp40, with centrosomes. Overexpression of Hsp70 restored localization of dynamin/p50 antibody labeling at centrosomes and protected cells against most of the heat-induced mitotic abnormalities. Our data suggest a link between protein damage at centrosomes and centrosome function.

Materials and Methods

Cell Lines and Culturing
OT70 hamster lung fibroblasts, were cultured as previously described (31) and maintained at 5% CO₂ in a humidified 37°C incubator. Transient transfections were performed by using Lipofectamine according to the procedure of the manufacturer (GIBCO/BRL, Paisley, UK). Per transfection experiment, the total amount of plasmid DNA was kept constant at 1 µg pDNA per coverslip. The pBOS-H2BGFP construct was purchased from BD Biosciences Pharmingen (CA, U.S.A.). The pHsp70-EYFP was constructed and the fusion protein Hsp70-EYFP has been shown to have similar chaperone activity as the untagged Hsp70 (Rujano M.A. et al., manuscript in preparation).

Heat Shock Treatment
To induce heat shock, petri dishes containing coverslips were pre-incubated in a water bath at 37°C and transferred to a second water bath at the indicated temperatures ± 0.1°C for the indicated times. Thermotolerance was induced by exposing the cells to a priming heat shock of 43°C for 15 minutes followed by 16-20h recovery at 37°C.

Western Blot Analysis
Proteins were separated in SDS/ 10% polyacrylamide gels and transferred to nitrocellulose membranes. Heat-inducible proteins Hsp70, Hsp40, and Hsp27 were detected on the same membrane with anti-Hsp70 mouse (SPA810, Stressgen, Victoria, BC, Canada), anti-Hsp40 rabbit (SPA400, Stressgen), and anti-Hsp27 rabbit (SPA801, Stressgen) primary antibodies. As secondary antibodies, HRP-conjugated goat-anti-mouse and anti-rabbit were used (Amersham, Piscataway, NJ). Signals were detected by enhanced chemiluminescence (ECL, Amersham).

Immunofluorescence Analysis
For immunofluorescence analysis cells were fixed using methanol/acetone as previously described (19). To visualize the centrosomes, polyclonal anti-γ-tubulin (Sigma T3559, St.Louis MO, U.S.A.) was used in combination with swine-anti-rabbit FITC (DAKO, Glostrup, Denmark), monoclonal anti-γ-tubulin (Sigma T6557) in combination with goat-anti-mouse CY3 (Amersham), or polyclonal anti-pericentrin (Babco, Richmond, CA) in combination with goat-anti-rabbit CY3 (Amersham). To visualize the mitotic spindles, monoclonal anti-α-tubulin (Sigma T5168) was used in combination with goat-anti-mouse CY3 (Amersham). To visualize kinetochore components, cells were formaldehyde-fixed and labeled with CREST (kindly provided by B.R. Brinkley, (45)) in combination with secondary antibody rabbit-anti-human (DAKO, A0190) and tertiary antibody swine-anti-rabbit FITC (DAKO) or cells were labeled with monoclonal anti-dynamin/p50 (BD Biosciences Pharmingen, 611002) in combination with secondary antibody goat-anti-mouse CY3 (Amersham) or goat-anti-mouse FITC (The Jackson Laboratory, Bar Harbor, USA). For visualizing heat shock proteins, anti-Hsp70 mouse (SPA810, Stressgen) was used in combination with anti-mouse CY3 (Amersham), anti-Hsc70 rat (SPA815, Stressgen) in combination with rabbit-anti-rat FITC (DAKO), anti-Hsp40 rabbit (SPA400, Stressgen) in combination with swine-anti-rabbit FITC (DAKO), or anti-Hsp27 rabbit (SPA801, Stressgen) in combination with swine-anti-rabbit FITC (DAKO). To visualize the DNA, cells were stained for 10 minutes with 0.2 µg/ml DAPI. Images were obtained with a confocal laser-scanning microscope (Leica TCS SP2, Leica Microsystems, Heidelberg, Germany) with 351/364-, 488-, and 543-nm lasers.
Time-Lapse Imaging

Images were made with a confocal laser-scanning microscope (Leica, SP2). To keep cells alive during the experiment, cells were cultured on round cover slips in a special chamber with 2 ml of medium. The chamber was put in a 37°C-heated microscope stage. A mixture of air with 5% CO₂ was blown into the heated stage. The 63x oil immersion lens was also heated to 37°C. Cells were imaged in 8-10 optical planes (thickness 1-2 μm). In heat shock experiments, time-lapse recordings were started within 10 minutes after heat shock. The cells were imaged every 2 minutes, for up to 3 hours. Cells expressing GFP-fusion proteins were excited with a 488 laser and emission was measured with a BP 500-550 filter. Cells expressing YFP-fusion proteins were excited with a 514 laser and emission was measured with a BP 500-550 filter. Data were analyzed using the Leica Simulator. Maximum projections were made and progression through mitosis was scored as normal when DNA aligned in the metaphase plate and two identical daughter cells were formed. Progression through mitosis was scored as abnormal when two daughter cells were formed containing micronuclei, when more than two daughter cells were formed, when anaphase onset did not occur within 2 hours after heat shock, or when chromatin prematurely decondensed without preceding division of chromosomes.

Results

Heat shock affects mitosis in a dose dependent manner.

To investigate whether and how heat shock affects mitotic cells, time-lapse confocal microscopy was performed. Cells were transiently transfected with the Histon2B-GFP construct to visualize the organization and dynamics of chromosomes. Under control conditions H2B-GFP expressing cells progress normally through mitosis (Figure 1A, http://coo.med.rug.nl/sscb/video.htm Figure 1) and on average these cells proceed from early metaphase to anaphase within 15 minutes (Table 1). This indicates that the intensity of the laser beam used did not cause cell cycle arrest or any abnormalities in cell cycle progression. To analyze the effects of heat shock, cells were heated and immediately thereafter, mitotic cells were selected and progression through mitosis was followed using time-lapse analysis. A variety of abnormalities was seen including division of cells in two daughter cells containing micronuclei (Figure 1B, http://coo.med.rug.nl/sscb/video.htm Figure 2), formation of multipolar spindles (not shown), lack of progression into anaphase within 2 hours after heat shock (not shown), or decondensation of chromatin without a preceding division of chromosomes (Figure 1C, http://coo.med.rug.nl/sscb/video.htm Figure 3). Quantitative analysis showed a heat-dose dependent decline in normal mitoses (Figure 2A and Table 1). For those cells that did complete cytokinesis normally, i.e. after relatively mild heat treatments such as 15’ 42°C or 15’ 43°C, the time from early metaphase to anaphase was significantly increased (Figure 2B and Table 1), indicating that even after these mild heat shock treatments every mitotic cell was affected leading to a delay in progression through mitosis.

It has already been demonstrated that heat shock induces alterations of centrosome structure (2; 6; 9; 27; 47; 48). Also, heat has profound effects on cytoskeleton elements (23; 27; 46), including effects on mitotic spindles (8; 9). To test whether heat-induced alterations of centrosomes are the cause of abnormal mitoses, cells were fixed within 10 minutes after heat shock and immunolabeling was performed using anti-γ-tubulin antibodies and anti-pericentrin antibodies. Like in interphase cells, γ-tubulin and pericentrin staining decreased in mitotic cells after severe heat shock (Figures 3C and F). But, no alterations in γ-tubulin and pericentrin staining were observed after a mild heat shock (compare Figures 3B and E to Figures 3A and D).

Comparable to the dose-dependent heat-induced modifications of centrosomal proteins, the organization of microtubules was also found to be affected after a severe heat shock (Figure 3I), whereas no structural changes of microtubules were observed after a mild heat shock (Figure 3H). These results demonstrate that division errors after severe heat shock can be
Hsp70 protects mitotic cells against heat-induced centrosome damage and division abnormalities explained by the observed changes in centrosomal content and spindle organization, however to explain the division errors after mild heat shock further investigation is required.

Figure 1. Heat shock during mitosis causes division abnormalities.
Cells expressing H2B-GFP under control conditions (A) or after heat shock for 15’ at 43°C (B, C). Time is given in minutes after heat shock. Under control conditions mitotic progression occurred normally (A). After heat shock, mitotic abnormalities were observed such as the formation of two daughter cells with micronuclei (B) or premature decondensation of chromatin (C). Bar, 10 μm. For movies see http://coo.med.rug.nl/sscb/video.htm Figure 1, 2 and 3.

Figure 2. Heat shock during mitosis leads to a heat-dose dependent decrease in normal mitoses and to an increase in mitotic delay.
Mitotic progression of cells expressing H2B-GFP was recorded by time-lapse confocal microscopy. The percentage of normal mitoses (A) and the time in minutes from early metaphase to anaphase onset (B) is given in control cells (no HS), after 15’ heat shock at 42°C (15’ 42°C), after 15’ heat shock at 43°C (15’ 43°C) and after 15’ heat shock at 44°C (15’ 44°C). For each condition more than 20 mitoses were recorded. Data represent the mean ± standard deviation.
Table 1: Overview of heat shock induced mitotic abnormalities in control cells, in thermotolerant cells and in Hsp70-EYFP expressing cells.

<table>
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<tr>
<th>Cells</th>
<th>Treatment</th>
<th>N</th>
<th>Normal mitoses (%)</th>
<th>Average time to anaphase onset (min)</th>
<th>Formation of micronuclei (%)</th>
<th>Multipolar (%)</th>
<th>No anaphase onset (%)</th>
<th>Premature decondensed DNA (%)</th>
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<td>100</td>
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Quantitative analysis of cells transiently expressing either H2B-GFP (-), Hsp70-EYFP (Hsp70-EYFP) or thermotolerant cells (TT). Time-lapse confocal microscopy analysis was started when a mitotic cell was selected under control conditions (no) or within 10 minutes after heat shock (15' 42°C, 15' 43°C, 15' 44°C, 30' 44°C). For each condition the number of recorded mitoses that were followed up to 3 hours after treatment is indicated (N) and the percentage of normal mitoses is given. A normal mitosis is defined as a mitosis in which no division errors were observed. The mean time from heat shock to anaphase onset is calculated using the cells that progressed through mitosis normally. The four different types of division errors are listed and per condition the percentages in which the different mitotic abnormalities occur are indicated.

Kinetochore localization is functionally important for proper mitosis because they are involved in attachment of chromosomes to spindle microtubule plus ends, contribute to force generation for chromosome movement and play a major role in the mitotic spindle checkpoint (10; 16; 37). To investigate whether mitotic division errors as observed after heat shock are associated with alterations in localization of kinetochore components, immunolabeling was performed using antibodies to detect dynamitin/p50 and using CREST antiserum. Dynamitin/p50 is a 50-kDa subunit of dynactin, a component of the kinetochore outer core that contains binding sites for the plus ends of kinetochore microtubules (35). CREST antiserum recognizes CENP-A, -B and -C, components of the kinetochore inner core (28; 45). CREST labeling was unaffected after both mild and severe heat shock compared to control conditions (Figure 3J-L). Staining of unheated mitotic cells with the anti-dynamitin/p50 antibody revealed staining at centrosomes and kinetochores in prometaphase cells (Figure 3M). No staining was found when chromosomes were aligned at the metaphase plate (Figure 3N), consistent with previously published results (11). After both mild and severe heat shock, dynamitin/p50 localization was absent from centrosomes and kinetochores of non-aligned chromosomes during pro-metaphase (Figure 3O and P), in contrast to CREST antiserum labeling. So, loss of dynamitin/p50 from kinetochores and centrosomes is associated with the mitotic delays and division errors induced by both severe and mild heat shock.
Hsp70 protects mitotic cells against heat-induced centrosome damage and division abnormalities.
**Figure 3.** Severe, but not mild, heat shock affects antibody staining of centrosome proteins and organization of microtubules, whereas both severe and mild heat shock affects antibody staining of dynamitin/p50.

Cells were untreated (no HS), mildly heat shocked (15’ 42°C), or severely heat shocked (15’ 45°C). Cells were fixed and labeled with antibodies to γ-tubulin (red, A-C), to pericentrin (red, D-F), or to α-tubulin (red, G-I), with CREST antiserum (green, J-L), or with antibodies to dynamitin/p50 (green M-P). DNA was stained by DAPI (blue, A-P).

**Thermotolerance protects cells against heat-induced mitotic abnormalities.**

One clear response to protein damage is the induction of heat shock proteins (29) leading to a transient state of thermotolerance during which cellular structures are protected against subsequent heat challenges. Recruitment of (induced) heat shock proteins to a specific site can indicate the presence of damaged proteins at that specific site and a need for protection against subsequent heat challenges (21). To test whether induced expression of heat shock proteins can protect mitotic cells against division errors as observed after heat shock treatments, we generated thermotolerant cells by pretreating cells at 43°C for 15 minutes followed by incubation at 37°C overnight. Typically, Hsp27, Hsp40 and Hsp70 expression is increased in thermotolerant cells (Figure 4A). Thermotolerant cells were treated with a second, mild heat shock after which mitotic cells were selected and followed by time-lapse analysis. The percentage of normal mitoses in thermotolerant cells was higher compared to non-thermotolerant cells after the same heat shock (Figure 4B and Table 1). In addition, thermotolerant cells in which cytokinesis occurred normally showed less delay in progression through mitosis (Figure 4C and Table 1). These data show the presence of a mechanism in thermotolerant cells that protects against most of the heat shock induced mitotic abnormalities. This protective mechanism may involve expression of heat shock proteins and/or recruitment of these heat shock proteins to mitotic structures suffering from heat shock induced protein damage.

To test this idea, recruitment of individual heat shock proteins to mitotic structures was examined in thermotolerant cells. As shown in Figure 4, Hsp70 co-localized with γ-tubulin in heat shocked, thermotolerant cells (Figure 4G, K and O) but not in unheated thermotolerant cells (Figure 4F, J and N) indicating that Hsp70 is dynamically recruited to mitotic centrosomes after heat shock. Hsp70 localization at centrosomes was not observed in unheated or heated non-thermotolerant cells (Figure 4D, E, H, I, L, and M) in which Hsp70 is not expressed, indicating that the labeling is specific. None of the other heat shock proteins tested (Hsp27, Hsp40 and Hsc70) showed this localization pattern to centrosomes after heat shock in thermotolerant cells (data not shown), indicating that reallocation of Hsp70 to centrosomes upon heat shock could be sufficient to protect against heat-induced mitotic division errors.
**Hsp70 protects mitotic cells against heat-induced centrosome damage and division abnormalities**

Figure 4. Thermotolerance protects against heat-induced mitotic abnormalities.

Cells were left untreated (no HS) or preconditioned to induce thermotolerance (TT). Expression of Hsp27, Hsp40 and Hsp70 was analyzed by Western Blotting (A) demonstrating that in TT cells Hsp27, Hsp40 and Hsp70 are induced. Heat shock was given to H2B-GFP expressing cells (15' 42°C) or H2B-GFP expressing TT cells (TT + 15' 42°C) and mitotic progression was recorded by time-lapse confocal microscopy. Per condition more than 20 cells were recorded. The percentage of normal mitoses (B) and the mean time ± standard deviation in minutes from early metaphase to anaphase onset (C) is given. After the indicated treatments, cells were methanol/acetone fixed and labeled with monoclonal antibody to Hsp70 (D-G) and polyclonal antibody to γ-tubulin (H-K). DNA was stained by DAPI (D-O). Overlays of the images are shown in L-O. Bar, 10 μm.

**Hsp70 is sufficient to protect against most of the heat-induced division errors.**

To gain insight in the putative protective role of Hsp70 against mitotic errors after heat shock, cells were transiently transfected with Hsp70-EYFP. First, we confirmed that in these cells, Hsp70-EYFP co-localized to centrosomes after heat shock by staining centrosomes with γ-tubulin antibodies (Figure 5A-D). Next, we studied the dynamics of Hsp70 using time-lapse analysis (Figure 5E and F, [http://cee.med.rug.nl/sscb/video.htm](http://cee.med.rug.nl/sscb/video.htm) Figure 4 and 5).
unheated Hsp70-EYFP expressing cells, Hsp70-EYFP was diffusely distributed in mitotic cells and Hsp70-EYFP expressing cells progressed through mitosis normally (Figure 5E and Table 1, http://coo.med.rug.nl/sscb/video.htm Figure 4). In heated Hsp70-EYFP expressing cells, Hsp70-EYFP was present at mitotic centrosomes immediately after heating and disappeared just prior to the onset of anaphase (Figure 5F, 16 minutes) after which the cells went through mitosis normally (Figure 5F, 20-66 minutes). Note that Hsp70-EYFP also decorated centrosomes in interphase cells and this localization was more prolonged than Hsp70-EYFP localization to mitotic centrosomes (Figure 5F, 8-34 minutes).

![Figure 5. Hsp70 localizes transiently to centrosomes of heat shocked mitotic cells.](image)

Cells expressing Hsp70-EYFP were left untreated (E) or treated with heat shock (15’ 42°C; A-D, F). Immediately after heat shock cells were fixed with methanol/ acetone (A-D) and labeled with monoclonal anti-γ-tubulin (B). DNA was stained with DAPI (C,). Hsp70-EYFP (A) co-localizes with γ-tubulin in heat shocked mitotic cells (D). Mitotic cells expressing Hsp70-EYFP were selected for time-lapse analysis under control conditions (E) or after heat shock (F). Time is given in minutes after heat shock. Note that Hsp70-EYFP only localizes to centrosomes of heat-shocked cells (F, 8’) and disappears from centrosomes just before anaphase onset in mitotic cells (F, 16’) and later in interphase cells (F, 66’). Bar, 10 μm. For movies of the time-lapse data in E and F see http://coo.med.rug.nl/sscb/video.htm Figure 4 and 5.

These time-lapse data suggested that, like in thermotolerant cells, expression of Hsp70 might protect against heat-induced mitotic abnormalities. Quantitative analysis indeed confirmed that expression of Hsp70-EYFP resulted in a decrease of mitotic abnormalities (Figure 6A and Table 1) and that, at least after mild heating at 42°C, no mitotic delay was observed (Figure 6B and Table 1). Interestingly, irrespective of the heat treatment used, in more than 75% of the observed normal mitoses after heat shock, loss of Hsp70-EYFP localization at centrosomes preceded or coincided with the onset of anaphase (as seen in Figure 5F).

Because dynamitin/p50 localization at kinetochores and at centrosomes was not detectable in mitotic cells as a result of mild heat shock (Figure 3O and P) we investigated whether Hsp70-EYFP expression would restore the localization pattern of dynamitin/p50 after heat shock. In Hsp70-EYFP expressing cells, centromal localization of dynamitin/p50 was indeed restored (compare Figure 7B,F and Figure 3O), but a pronounced kinetochore
Hsp70 protects mitotic cells against heat-induced centrosome damage and division abnormalities. Labeling as observed under control conditions (Figure 3M) was not observed in mitotic cells regardless whether the DNA was congressed (Figure 7E-H) or not congressed (Figure 7A-D) at the metaphase plate. Our data strongly suggest that Hsp70 plays a role in the repair of centrosome damage induced by heat shock and thereby facilitates normal progression through mitosis.

![Graph](image)

**Figure 6. Hsp70 protects against heat-induced mitotic abnormalities and reduces heat-induced delay in mitotic progression.** Mitotic progression of cells expressing H2B-GFP (control) or Hsp70-EYFP (Hsp70-EYFP) was recorded by time-lapse confocal microscopy. The percentage of normal mitoses (A) and the time in minutes from early metaphase to anaphase onset (B) is given in control cells (no HS), after 15’ heat shock at 42°C (15’ 42°C), and after 15’ heat shock at 43°C (15’ 43°C). For each condition more than 20 mitoses were recorded. Data represent the mean ± standard deviation.

![Images](image)

**Figure 7. Hsp70-expressing cells show dynamin/p50 antibody labeling at centrosomes but not at kinetochores after mild heat shock.** Cells expressing Hsp70-EYFP were mildly heat shocked (15’ 42°C). Cells were fixed and labeled with antibodies to dynamin/p50 (B and F) and DNA was stained by DAPI (C and G). Overlays of the images are shown in D and H.


Discussion

In this study, we show that severe heat treatment during mitosis induces loss of γ-tubulin and pericentrin from centrosomes and loss of dynamitin/p50 antibody labeling from centrosomes and kinetochores. After mild heat shock, only dynamitin/p50 antibody labeling disappeared and none of the other protein alterations found after severe heat shock were detected. Severe and mild heat shock cause delays in normal mitotic progression and result in a variety of mitotic abnormalities. Hsp70 expression is sufficient to protect against most of the heat-induced division errors since in Hsp70-expressing cells the percentage of division errors is decreased compared to non-Hsp70-expressing cells after heat shock. After heat shock Hsp70 is transiently recruited to centrosomes and in most cells normal mitotic progression occurs immediately after exit of Hsp70 from the centrosomes. Expression of Hsp70 and its protection against division errors coincide with restored staining of the dynamitin/p50 antibody at centrosomes but not at kinetochores, suggesting that dynamitin/p50 localization at centrosomes improves the rate of normal mitoses and dynamitin/p50 localization at kinetochores is not essential for mitotic progression. Our data imply that centrosomes are affected by heat shock and the observed division errors are consequences of heat-damaged mitotic centrosomes.

What are the cellular responses to heat shock?

The main molecular event after heat shock is denaturation and subsequent aggregation of proteins. Expression of heat shock proteins can protect the cells from these effects (12). The recruitment of Hsp70 after heat shock to centrosomes, but not to microtubules or kinetochores, suggests that such heat-induced protein damage mainly affects centrosomes and no other mitotic structures. Indeed, ultrastructural analysis of interphase centrosomes after severe heat shock revealed the presence of high electron dense material indicative of protein aggregates (2; 48). Also, in the studies by the group of Watanabe (30), mitotic abnormalities were linked to abnormalities in centrosome structure. Our findings that overexpression of Hsp70 restores dynamitin/p50 antibody labeling at centrosomes and protects against most of the heat-induced division errors support the view that Hsp70 repairs protein damage at the centrosomes, most likely via its well-known chaperone activity (21; 26).

Why are centrosomes sensitive to heat shock?

Since our data indicate that damage to centrosomes is crucial for the heat-induced mitotic defects, the question arises why centrosomes are so extremely sensitive to heat shock. First, it is possible that centrosomal proteins are labile and readily unfold during heat shock and can rapidly form aggregates with other proteins, soluble or insoluble. The decreased staining of several centrosome specific antibodies after heat shock ((Figure 3C, F, O and P) (6; 27; 47; 48)) may be explained by the presence of these aggregates at centrosomes. A second explanation could be that centrosomes are specific sites for storage of heat-induced misfolded proteins (20). Indeed, misfolded proteins were shown to accumulate at the centrosomes in interphase cells (48). These so-called aggresomes (20) may enable cells to coordinateely regulate and dispose damaged proteins at specific cellular sites hereby preventing random protein aggregation and cellular dysfunction.

What is causing the mitotic delay in heat-shocked cells?

Our data suggest that cells contain a mechanism which senses heat damage at centrosomes and induces mitotic delay. We have shown that Hsp70 is recruited to centrosomes in response to heat shock and that progression through mitosis hardly ever occurs as long as Hsp70 is present at the centrosomes. Mitotic progression occurs in most cases immediately after the disappearance of Hsp70 from centrosomes. Severe heat shock induces a longer mitotic delay compared to mild heat shock implying that the more heat-induced damage to centrosomes the longer the mitotic delay. Directly after heat shock,
Hsp70 moves to the centrosomes most likely to repair protein damage and to restore centrosome function. Restoration of centrosome function or disappearance of Hsp70 from centrosomes may trigger the progression through anaphase. However the mitotic delay is also induced in the absence of Hsp70, this indicates that Hsp70 is not required for the delay and additional factors may regulate heat-induced mitotic delay.

Previously it has been reported that hypothermia induces mitotic delay and this delay is Mad2-dependent (38). It remains to be investigated whether the observed heat shock induced mitotic delay is also Mad2 dependent.

**What explains the formation of multipolar spindles and division errors in heat shocked mitotic cells?**

We show that heat shock during mitosis no only causes mitotic delays but can also result in the formation of multipolar spindles. There is an extensive list of publications in which depletion or mutations in proteins from the centrosome, kinetochore or microtubule-associated proteins induce the formation of multipolar spindles (3; 7; 13; 15; 18). Therefore, it can be speculated that multipolar spindles arise during heat shock due to heat inactivation of one of these proteins. Hsp70 expression restored localization of dynamitin/p50 at centrosomes but not at kinetochores and this restored localization at centrosomes coincides with protection against most of the heat-induced mitotic division errors. These data imply that centrosomal dynamitin/p50, but not kinetochore dynamitin/p50, is involved in improving the fidelity of mitoses. After mild heat shock, still 17% of the mitoses in Hsp70 expressing cells results in the formation of micronuclei. It may be possible that this missegregation of DNA is due to incorrect localization of dynamitin/p50 (and perhaps other proteins) at kinetochores, since previously it has been demonstrated that other kinetochore proteins (such as CENP-E, ZW10 and Rod) are required for accurate chromosome segregation (36; 42; 44).

Another abnormality observed in mitotic heat shocked cells is decondensation of chromatin without a preceding division of chromosomes. This premature mitotic exit was also observed in Xenopus tissue culture cells injected with function-blocking antibodies against two proteins (xNdc80 and xNuf2) that localize to kinetochores from prometaphase through anaphase McCleland et al., G&D, 2003). It may be possible that the heat shock conditions used in our experiments inactivate Ndc80, Nuf2 or proteins with a related function and thereby causing premature mitotic exit.

The formation of multipolar spindles and micronuclei indicate that although heat shock itself does not cause DNA damage and hereby is not considered to be a genotoxic stress, genome instability may arise as a secondary consequence of heat-induced centrosomal protein damage ((30), this study). It is likely that most daughter cells that arise after these heat-induced division errors will not be viable and will be eliminated. However, it may be possible that aneuploid cells sporadically are able to divide and survive and finally give rise to cells with a transformed phenotype. Previously, an increased tumor incidence was observed after irradiation in combination with heat treatment as compared to irradiation alone (41). The effect of Hsp70 on viability of heat shock induced aneuploid cells remains to be investigated.

In summary, our data show that heat shock affects mitotic cells predominantly at centrosomes and this heat damage results in division errors. An Hsp70-dependent cellular defense system protects heat-damaged mitotic cells by facilitating restoration of centrosomes and, after a mitotic delay, allowing normal progression through mitosis.

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Chapter 5

References


Hsp70 protects mitotic cells against heat-induced centrosome damage and division abnormalities


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