Heat shock induces rapid resorption of primary cilia

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Summary

Primary cilia are involved in important developmental and disease pathways, such as the regulation of neurogenesis and tumorigenesis. They function as sensory antennae and are essential in the regulation of key extracellular signalling systems. We have investigated the effects of cell stress on primary cilia. Exposure of mammalian cells in vitro, and zebrafish cells in vivo, to elevated temperature resulted in the rapid loss of cilia by resorption. In mammalian cells loss of cilia correlated with a reduction in hedgehog signalling. Heat-shock-dependent loss of cilia was decreased in cells where histone deacetylases (HDACs) were inhibited, suggesting resorption is mediated by the axoneme-localised tubulin deacetylase HDAC6. In thermotolerant cells the rate of ciliary resorption was reduced. This implies a role for molecular chaperones in the maintenance of primary cilia. The cytosolic chaperone Hsp90 localises to the ciliary axoneme and its inhibition resulted in cilia loss. In the cytoplasm of unstressed cells, Hsp90 is known to exist in a complex with HDAC6. Moreover, immediately after heat shock Hsp90 levels were reduced in the remaining cilia. We hypothesise that ciliary resorption serves to attenuate cilia-mediated signalling pathways in response to extracellular stress, and that this mechanism is regulated in part by HDAC6 and Hsp90.

Key words: Heat shock, Molecular chaperone, Primary cilia

Introduction

The cellular response to environmental stress is complex and encompasses mechanisms to regulate cell cycle checkpoints, modulate energy metabolism and maintain macromolecular integrity (Kültz, 2005). Key to this response is the synthesis of cytoprotective heat shock proteins (Hsps) and a decrease in the translation of components of the normal proteome. Hsp expression is primarily regulated by heat shock transcription factors and is required for the development of thermotolerance (Cotto and Morimoto, 1999; Anckar and Sistonen, 2007).

Although elevated temperature affects multiple cellular compartments, the consequences for the primary cilium have not been well analysed. The majority of mammalian cells have a single primary cilium protruding from their surface. Primary cilia function as sensory antennae in the signal transduction of extracellular stimuli from the environment and other cells (Davenport and Yoder, 2005; Singla and Reiter, 2006; Berbari et al., 2009). They play an important role in the normal function of key intercellular signalling pathways. In particular they are essential for ligand-dependent sonic hedgehog (Shh) signalling (Corbit et al., 2005; Haycraft et al., 2005; Rohatgi et al., 2009) and platelet-derived growth factor-α signalling (Schneider et al., 2005). Primary cilia also have cell-type-specific roles, dependent on the receptors localised to them. This includes acting as mechanosensors in kidney epithelial cells where polycystin-2, a member of the transient receptor potential family of ion channels, monitors urine flow (Nauli et al., 2003). Unsurprisingly, abnormal primary cilium function is linked to both monogenic disorders and complex diseases (Fliegauf et al., 2007; Baker and Beales, 2009; Nigg and Raff, 2009).

The structure of the primary cilium is closely related to that of motile cilia and flagella. These are all microtubule-based organelles that consist of a basal body and an axoneme of nine microtubule doublets covered by a ciliary membrane. Microtubules are dynamic and maintenance of a constant cilium length is dependent on the balance between rates of assembly and disassembly at the axoneme tip. The elongation of the axoneme from the basal body is dependent on intraflagellar transport (IFT), a bidirectional process that facilitates axonemal turnover (Rosenbaum and Witman, 2002; Pedersen et al., 2008). Kinesin-2 motor complexes mediate the anterograde transport of axonemal precursors and other cargos to the site of assembly at the distal tip of the cilium. Concurrently, IFT particles are returned to the cell body by cytoplasmic dynein-2 motors. In addition to IFT proteins a number of other proteins have been identified as necessary for normal ciliogenesis and maintenance.

Assembly and disassembly of the primary cilium is a dynamic process that is regulated through the cell cycle. Although there is evidence that primary cilia are present on some proliferating cells, resorption generally occurs at mitotic entry followed by re-emergence after cell division (Seeley and Nachury, 2010). In

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addition to the loss of primary cilia by resorption, observed in mammalian cells as part of their normal function, unicellular flagellates (e.g. *Clamydomonas*) also exhibit deflagellation. Flagellar excision occurs at the transition zone between the axoneme and the basal body and is dependent on microtubule severing. Deflagellation occurs in response to environmental stresses, including heat shock. It is a conserved cellular process, with deciliation of motile cilia from epithelia, such as the upper respiratory tract, reported to occur by this mechanism (Quarmby, 2004).

In mammalian cells, primary cilia disassemble in response to addition of serum. This is dependent on an Aurora-A kinase pathway (Pugacheva et al., 2007). In this pathway the pro-metastatic scaffolding protein HEF-1 (NEDD9) activates Aurora-A kinase. This is sufficient to induce rapid ciliary resorption through phosphorylation of histone deacetylase 6 (HDAC6), which then deacetylates ciliary tubulin and destabilises the axonemal microtubules (Pugacheva et al., 2007).

Intriguingly, heat shock factor-1 (HSF-1), the key mammalian HSF, has been shown to be necessary for the maintenance of beating of motile (9+2) cilia in murine respiratory epithelium. This is most likely because HSF-1 regulates the expression of constitutively expressed Hsp90, a molecular chaperone protein that plays a role in tubulin polymerisation (Takaki et al., 2007). Moreover, HDAC6 is an Hsp90 deacetylase and has been reported to trigger the disassociation of an HDAC6/Hsp90/HSF-1 complex in response to the accumulation of ubiquitinated protein (Boyault et al., 2007). This allows activation of HSF-1 and subsequent expression of stress inducible molecular chaperones.

Recent evidence demonstrates that primary cilia are lost from mammalian cells in response to chemical agents such as chloral hydrate by deciliation (Overgaard et al., 2009). Cilia have also been observed to exhibit altered frequency and length in response to mechanical stimuli (McGlashan et al., 2010). However, little is known of how these sensory organelles respond to extracellular stress. We report that primary cilia are highly sensitive to sub-lethal elevated temperature and are quickly lost from many cells by resorption. Importantly, heat-shock-induced loss of primary cilia reduced cilium-mediated signalling. This heat-shock-induced cilium resorption is dependent on HDAC activation. Hsp90 function was found to be required for normal ciliary maintenance. Moreover heat shock reduced levels of Hsp90 in cilia. HDAC6 and Hsp90 are known to form a complex in the cytosol (Boyault et al., 2007), and we demonstrate partial colocalisation of these proteins within ciliary axonemes. We hypothesise that this complex may exist in the axoneme and that it potentially disassembles upon heat-shock-promoting HDAC6-mediated ciliary resorption.

Results

Heat shock causes a loss of primary cilia and decreases hedgehog signalling in mammalian cells

We hypothesised that primary cilia would be responsive to extracellular stress. To test this we exposed ciliated NIH3T3 (mouse embryonic fibroblast) cells to a sub-lethal heat shock. NIH3T3 cells were cultured for 24 hours after seeding, until 70–80% confluent, and then transferred to serum-free conditions for 20 hours to promote cilia formation. Cells were then maintained at 37℃ or exposed to a 42℃ heat shock for up to 30 minutes. After fixation, cells were then processed for immunofluorescent detection of acetylated tubulin and pericentrin (Fig. 1A). The frequency of primary cilia was quantified in cells exposed to control and heat shock conditions (Fig. 1B). Primary cilia were detected on 85±9% of the cells maintained at 37℃. Remarkably, exposure to a 42℃ heat shock resulted in a significant reduction in the number of cells that exhibited primary cilia after only 5 minutes ($P<0.01$). Furthermore, after 30 minutes at the elevated temperature just 34±5% cells had a detectable primary cilium. Western analyses revealed that levels of acetylated tubulin and pericentrin were not altered by the heat shock (Fig. 1C). To confirm the heat shock conditions used were sufficient to rapidly activate stress response associated signalling cascades, we immunoblotted for the phosphorylated forms of extracellular signal-regulated kinases (Erk1/2) (Ng and Bogoyevitch, 2000) (Fig. 1C). Like the ciliary axoneme, the cytoplasmic bridges connecting daughter cells in cytokinesis are highly enriched in acetylated tubulin in many cell types. The number of these structures was not altered in populations of cells exposed to a 42℃ heat shock for 30 minutes (data not shown).

To confirm that the observed loss of primary cilia was due to their absence and not a lack of acetylated tubulin staining in the ciliary axoneme we stained cells with an additional marker (supplementary material Fig. S1). ADP-ribosylation factor-like protein 13B (Arl13b) is a small GTPase that has previously been reported to localise to primary cilia (Caspary et al., 2007). NIH3T3 cells maintained at 37℃ or exposed to 42℃ were processed for dual-immunofluorescence. We observed colocalisation of acetylated tubulin and Arl13b in the primary cilia of control cells and cells exposed to elevated temperature (supplementary material Fig. S1). In all cilia examined acetylated tubulin and Arl13b staining overlapped along the entire axoneme. Moreover, in control and heat shocked cells we were unable to identify any putative ciliary structures that did not label with both markers. These data further support a loss of primary cilia.

To test if the loss of primary cilia in response to heat shock was a general phenomenon we investigated their frequency in IMCD3 (murine inner medullary collecting duct) and ARPE19 (human retinal pigment epithelial) cells maintained at 37℃ or exposed to elevated temperature. For these cell types we also observed a rapid loss of primary cilia in response to heat shock (supplementary material Fig. S2).

The functional consequences of heat-induced resorption on primary-cilium-mediated signal transduction, were tested by analysing Shh signalling. NIH3T3 cells, which contain all of the components required to activate target gene transcription when exposed to ligand (Taipale et al., 2000; Rohatgi et al., 2009), were transfected with a Gli-dependent luciferase reporter and a constitutively active Renilla reporter. 16 hours post-transfection cells were heat shocked at 42℃ for up to 30 minutes, allowed to recover for 4 hours, and then treated with a pathway agonist for 12 hours before measurement of luciferase activity. Luciferase activity was significantly reduced in cells exposed to a 30 minute heat shock (Fig. 1D).

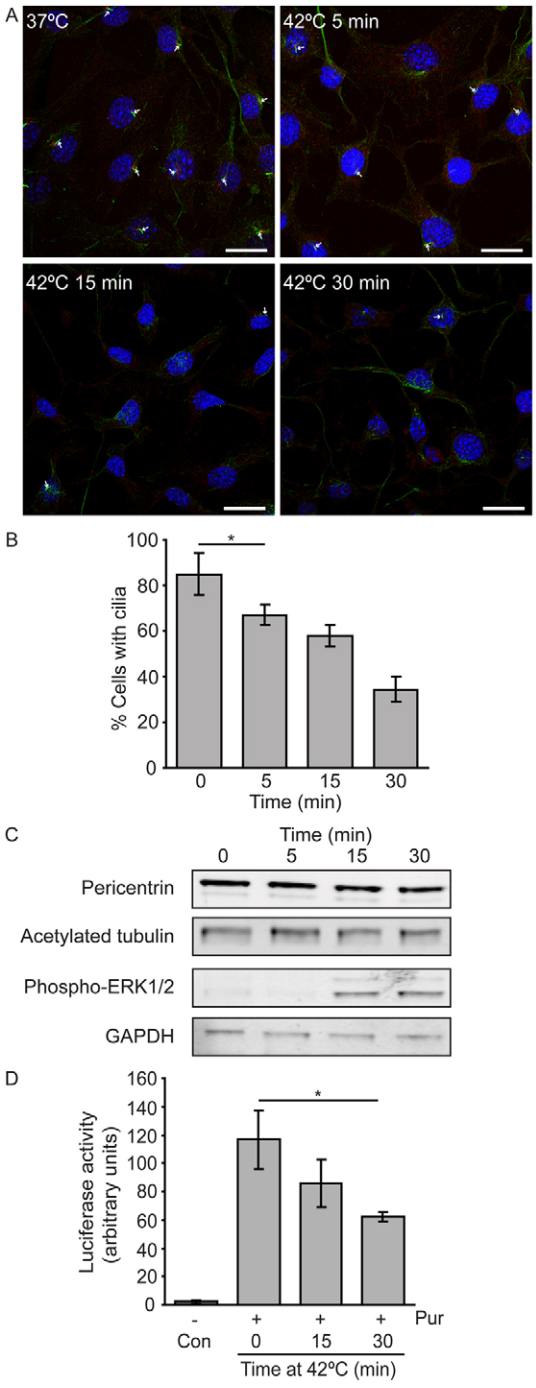
Heat shock causes loss of cilia by resorption

Having established that the number of primary cilia are reduced in populations of mammalian cells in response to heat shock, we next investigated the mechanism of their disappearance. Measurement of mean axonemal length revealed that on average cilia were becoming progressively shorter with increased exposure to elevated temperature (Fig. 2A,B). This was suggestive of ciliary resorption rather than excision at the

transition zone. In support of this, ultracentrifugation and subsequent western analyses did not detect elevated levels of acetylated tubulin in cell culture supernatants from cells exposed to heat shock (not shown).

Cells reassemble primary cilia following heat shock

The reduction in Shh signalling observed after heat shock suggested that primary cilia were not reassembled immediately. In NIH3T3 cells exposed to a 30 minute heat shock we observed no significant increase in cilia incidence or length up to 4 hours after heat shock (Fig. 2C,D). Although the percentage of NIH3T3 cells possessing primary cilia recovered to pre-heat-shock levels by 16 hours, they were significantly shorter up to 24 hours after exposure to elevated temperature ($P<0.05$). After the 24 hour time point, the serum-free conditions required for cilium formation began to impact on NIH3T3 cell survival. Therefore to investigate reassembly of primary cilia over a longer period we utilised IMCD3 cells. A high incidence of cilia occur on confluent IMCD3 cells in the presence of serum, making them more suitable than NIH3T3 cells for longer time course experiments. Exposure to 42℃ for 30 minutes reduced the frequency of primary cilia in populations of IMCD3 cells from 77±2% to 33±2% (Fig. 2E). 6 hours after heat shock the percentage of cells possessing primary cilia had not significantly increased. 24 hours after heat shock there was evidence of ciliary reassembly as the organelle was present on 56±2% of cells. The frequency of cilia remained slightly lower than before the heat shock for up to 4 days later ($P<0.05$). 24 hours after heat shock the average axoneme length was also less than in control cells (Fig. 2F). Together these data suggest that cells that have lost primary cilia in response to elevated temperature do not reassemble them immediately.



Primary cilia are sensitive to heat shock in vivo

Primary cilia are essential for the normal function of signalling pathways in vertebrates, with zebrafish (*Danio rerio*) widely used as a model system to study their function in vivo. To investigate the effect of elevated temperature on cilia in vivo we established a 5 minute 42℃ heat shock was non-lethal for zebrafish embryos at the 24 hours post-fertilisation (hpf) stage. In 42℃ exposed fish and control fish, maintained at 28.5℃, we detected ciliary axonemes by staining for acetylated tubulin. Confocal analyses of the caudal region of embryos, using the tip of the tail as a reference for orientation, was performed (Fig. 3A,B) and the number of cilia within a defined region quantified from a Z-series of images. Both the number of ciliated cells (Fig. 3C) and the length of cilia (Fig. 3D) were reduced in the tails of heat-shocked fish. We also observed a reduction in acetylated tubulin staining in the pronephric duct of heat-shocked fish, which predominantly contains motile cilia, along a dense brush border (Fig. 3E). Gaps in the acetylated tubulin staining of the pronephric duct were not observed in fish maintained at 28.5℃ but were seen in fish exposed to 42℃, indicating a reduction in the frequency of motile cilia in these embryos. Thus heat shock triggered the rapid onset of cilia loss in zebrafish embryos analogous to the disassembly of primary cilia seen in cultured mammalian cells. Although cilium

Fig. 1. Primary cilia are lost and hedgehog signalling reduced in mammalian cells exposed to elevated temperature. (**A**) NIH3T3 cells stained to detect the ciliary axoneme with anti-acetylated tubulin (green), basal body with anti-pericentrin (red) and nuclei with DAPI (blue). Cells were maintained at 37℃ or exposed to 42℃ for 5, 15 or 30 minutes. Scale bars: 20 μm. (**B**) The percentage of cells with primary cilia was quantified. The number of cilia, defined by both acetylated tubulin and pericentrin labelling, and the number of nuclei (to give the total number of cells) were counted in 10 randomly selected fields from three replicates for each experimental condition. (**C**) Western blot analyses of levels of pericentrin, acetylated tubulin, phospho-Erk1/2 and GAPDH in NIH3T3 cells exposed to 42℃ for the indicated times. (**D**) Luciferase activity was measured in NIH3T3 cells transfected with a Gli-dependent reporter and normalised to the activity of a constitutively active renilla reporter. 16 hours post-transfection cells were exposed to 42℃ for the indicated times, cells were then allowed to recover for 4 hours prior to the addition of medium containing purmorphamine or vehicle (DMSO) for 12 hours. Error bars indicate 2× s.e.m. *$P<0.05$.

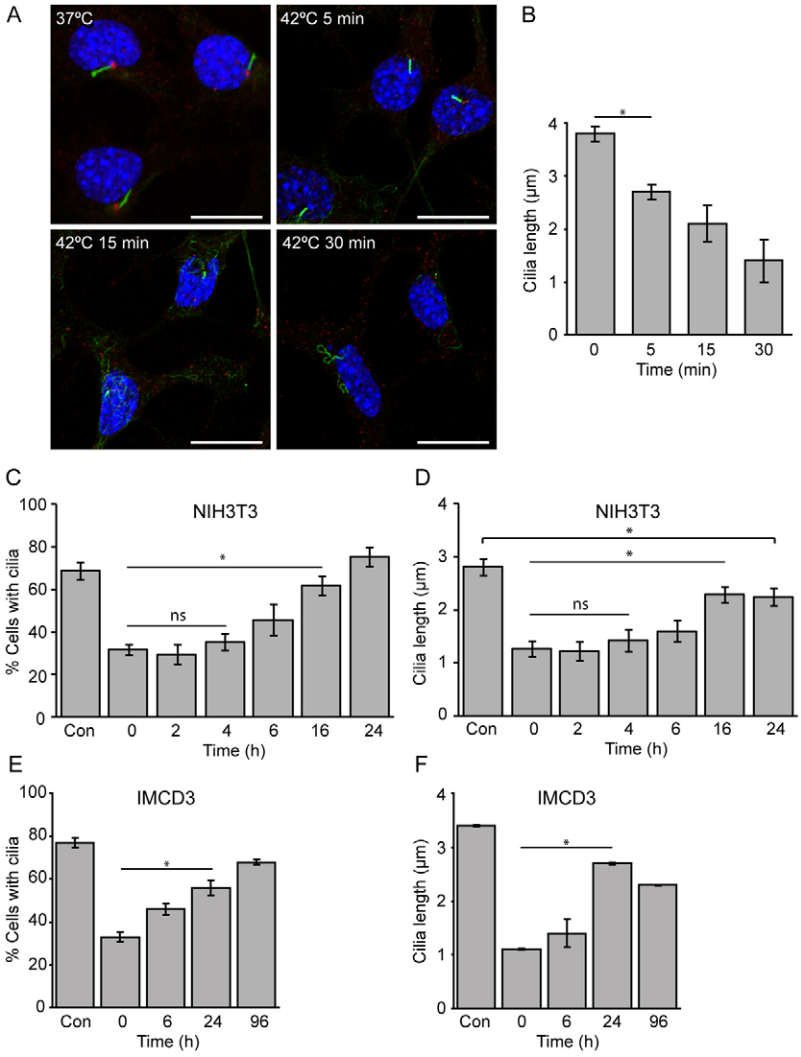


Fig. 2. Primary cilia rapidly shorten upon heat shock and do not immediately reassemble. (**A**) Cilium shortening in NIH3T3 cells, revealed by staining with anti-acetylated tubulin (green) and anti-pericentrin (red). Nuclei were labelled with DAPI (blue). Cells were maintained at 37℃ or exposed to 42℃ for 5, 15 or 30 minutes. Scale bars: 20 μm. (**B**) Mean axoneme length was quantified from confocal images of at least 50 randomly selected ciliated cells for each experimental condition. (**C–F**) To investigate the dynamics of cilium reassembly after heat shock NIH3T3 (C,D) cells were exposed to 42℃ for 30 minutes and then returned to 37℃ for up to 24 hours . (E,F) Cilium recovery was also quantified in IMCD3 cells cultured for up to 96 hours. Controls (Con) were not heat shocked. (C,E) The percentage of cells with primary cilia was quantified. The numbers of cilia and nuclei were counted in 10 randomly selected fields for each experimental condition in three biological replicates. (D,F) Mean axoneme length was quantified from confocal images of at least 50 randomly selected ciliated cells for each experimental condition. Error bars indicate 2× s.e.m. *$P<0.05$.

loss was observed, the heat shock conditions were sub-lethal, as assessed by examining the gross development and head/trunk angle up to 8 hours after the 42℃ heat shock (supplementary material Fig. S3).

Heat-shock-induced cilium loss is dependent on a HDAC-mediated pathway

The resorption of primary cilia, in mammalian cells, in response to serum has previously been shown to be dependent on an Aurora-A/HDAC6 pathway (Pugacheva et al., 2007) (Fig. 4A). We used inhibitors to test if this pathway was implicated in heat-shock-induced cilium resorption. Inhibition with 0.5 μM PHA-680632, which specifically targets Aurora-A (Pugacheva et al., 2007), did not inhibit thermally induced cilium resorption (Fig. 4B,C). The effectiveness of this inhibitor in NIH3T3 cells was confirmed by testing its ability to prevent serum-induced cilium resorption (supplementary material Fig. S4). In contrast, after 30 minutes heat shock ciliary resorption was significantly reduced in cells treated with the mammalian class I and II HDAC inhibitor trichostatin A (TSA; $P<0.01$; Fig. 4D). In TSA-treated heat-shocked cells, remaining primary cilia were also longer than in untreated cells (Fig. 4E). HDAC6 has previously been localized to the ciliary axoneme (Pugacheva et al., 2007). HDAC6 staining was often punctuate along the length of the cilium and commonly localised to the distal end of the axoneme (supplementary material Fig. S5). Immunofluorescent staining identified that HDAC6 still localised to remaining primary cilia in cells exposed to heat shock. No significant differences were observed in the number of HDAC6-positive cilia or the intensity of HDAC6 staining in axonemes (Fig. 4F; quantification not shown). These data are consistent with heat-shock-induced primary cilium resorption involving HDAC6. They also further confirm that primary cilia are lost from heat-shocked cells by resorption.

Primary cilium resorption in response to heat shock is reduced in thermotolerant cells

The cellular response to sub-lethal heat shock involves the synthesis of Hsps and the development of thermotolerance. We hypothesised that primary cilia would be less sensitive to elevated temperature in thermotolerant cells. To make NIH3T3 cells thermotolerant we exposed them to a 30 min priming heat shock (42℃) 4 hours prior to passage. After seeding, these cells were cultured for 24 hours and then transferred to serum-free medium for 16 hours to promote cilium formation. Western analyses confirmed these cells expressed Hsp70, indicating the cellular stress response had been induced (Fig. 5A). Resorption of primary cilia in response to heat shock, was significantly

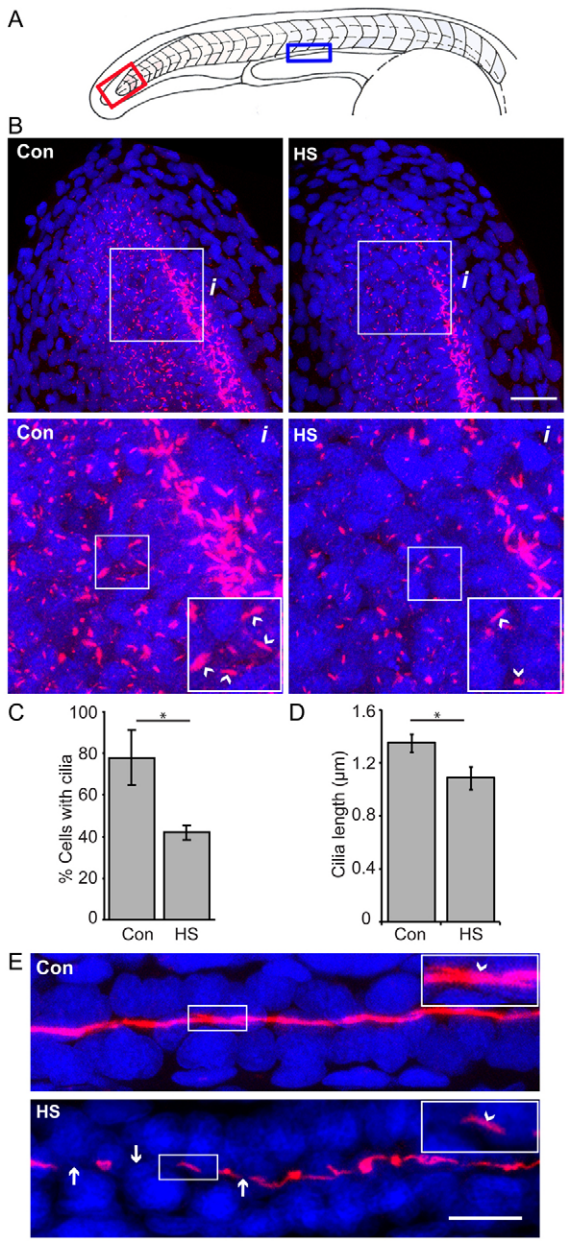


Fig. 3. Heat-shock-induced resorption of cilia occurs in vivo. (**A**) In zebrafish embryos we investigated the effects of exposure to elevated temperature on primary cilia in the area of the tip of the tail (red box) and motile cilia in the pronephric duct (blue box). 24 hour post-fertilisation embryos were either maintained at the normal growth temperature (28.5℃) or transferred to 42℃ for 5 minutes. Immediately after this non-lethal heat shock fish were fixed and ciliary axonemes stained with anti-acetylated tubulin (red) and nuclei with DAPI (blue). (**B**) Imaging of fish tails was performed to generate confocal z-stacks. Maximum intensity projections of these z-stacks are presented. Scale bars: 20 μm. Boxed regions indicated by *i* in the top panel are shown enlarged in the lower panel with a further enlarged insert, where individual cilia are indicated by arrowheads. (**C**) From this confocal data the percentage of cells with primary cilia was quantified. The numbers of primary cilia and nuclei were counted in a defined region at the tip of the tail of five fish per treatment. (**D**) Mean axoneme length was quantified from confocal images of at least 30 randomly selected ciliated cells from five fish for each experimental condition. Error bars indicate 2× s.e.m. *P<0.05. (**E**) A representative confocal imaging of the pronephric ducts. Arrows indicate areas of motile cilia loss. In the enlarged insert arrowheads indicate individual cilia.

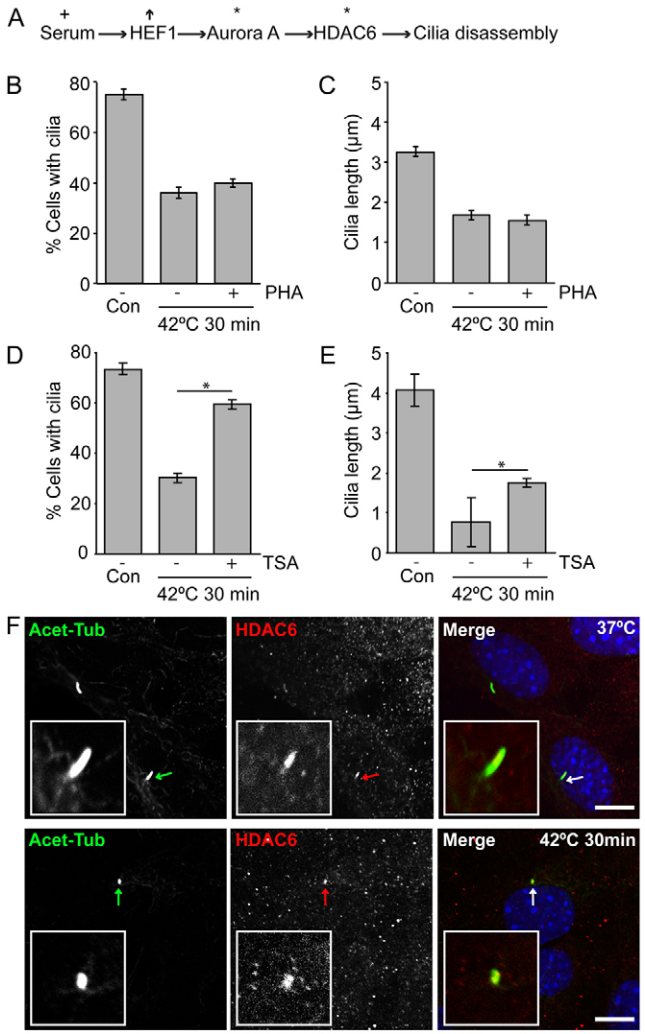


Fig. 4. Heat-shock-induced cilium resorption is HDAC6 dependent. (**A**) Schematic of the Aurora-A–HDAC6 cilium resorption pathway (Pugacheva et al., 2007). Upward arrow indicates increased expression and asterisks indicate activation. To test whether Aurora-A (**B**,**C**) and HDAC6 (**D**,**E**) activities are required for heat-induced cilium resorption, NIH3T3 cells were cultured in medium containing the Aurora-A inhibitor PHA-680632 (PHA), the HDAC inhibitor trichostatin A (TSA), or DMSO vehicle for 4 hours, prior to exposure to 42℃ for 30 minutes or continued maintenance at 37℃ (Con). (B,D) The percentage of cells with primary cilia was quantified. The numbers of cilia and nuclei were counted in 10 randomly selected fields for each experimental condition. (C,E) Mean axoneme length was quantified from confocal images of at least 50 randomly selected ciliated cells for each experimental condition. (**F**) HDAC6 localises to primary cilia in NIH3T3 cells maintained at 37℃ or exposed to 42℃ for 30 minutes. Cells were stained to detect the ciliary axoneme with anti-acetylated tubulin (green) and HDAC6 (red). Nuclei were detected with DAPI (blue). Enlarged images of the cilia indicated by arrows are shown in the insets. Scale bars: 10 μm.

reduced in these thermotolerant cells (P<0.01; Fig. 5B), furthermore remaining cilia were longer than in non-thermotolerant cells (P<0.01; Fig. 5C).

Hsp90 plays a role in primary cilium maintenance

The observation that the primary cilia of thermotolerant cells are more resistant to heat shock suggested a possible role for Hsps in ciliary maintenance. One candidate protein for such a role is

Hsp90. This molecular chaperone has been reported to be associated with ciliary microtubules in a protozoan (Williams and Nelsen, 1997) and is implicated in the normal function of motile cilia in mice (Takaki et al., 2007). Moreover, the constitutive (HSP90AB) and inducible (HSP90AA) cytosolic Hsp90 proteins, as well as the ER resident Grp94 (HSP90B) have been identified as being in the ciliary proteome (Gherman et al., 2006). Inhibition of Hsp90s with the benzoquinone ansamycin geldanamycin (GA) resulted in a reduction in the frequency (Fig. 5D) and average length (Fig. 5E) of primary cilia in a

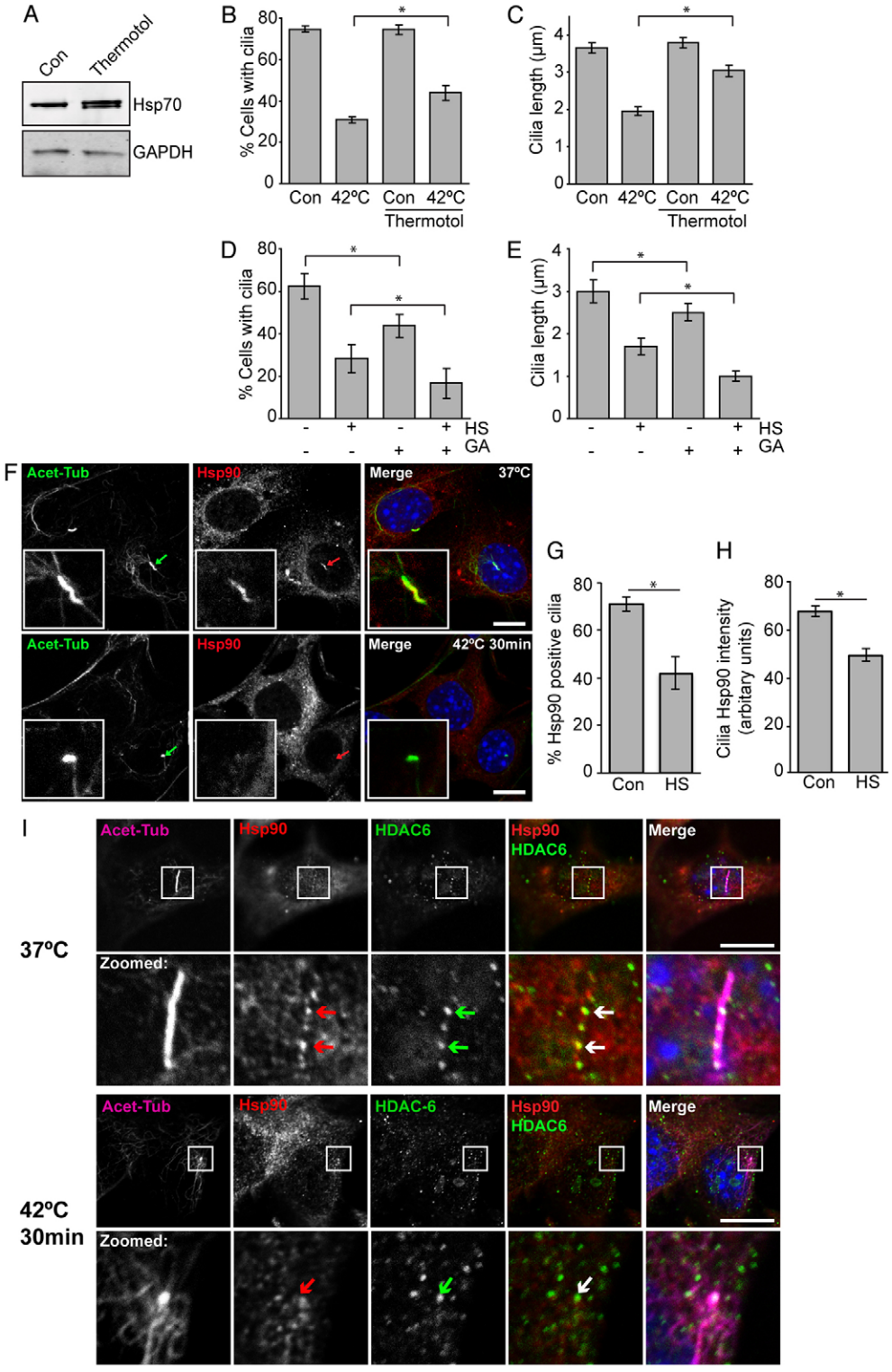


Fig. 5. See next page for legend.

population of NIH3T3 cells maintained at 37℃. In cells exposed to 42℃, GA treatment further reduced cilia frequency and length relative to the heat shock alone. These data indicate a possible role for Hsp90 in the maintenance of primary cilia.

Localisation of Hsp90 to primary cilia is reduced by heat shock

To further investigate the association between primary cilia and Hsp90 we performed immunofluorescent staining, using an antibody that detects both the constitutively expressed and inducible cytosolic Hsp90s (Fig. 5F; supplementary material Fig. S5). Hsp90 has previously been reported to be present in the nucleus and cytoplasm with some enrichment at centrosomes (Lange et al., 2000). In 71±3% of ciliated NIH3T3s Hsp90 staining was detected in axonemes (Fig. 5F,G). For cells that retained primary cilia after 30 minutes of heat shock, the incidence of cilia with an Hsp90-positive axoneme was reduced to 42±7%. Therefore, heat shock resulted in a significant decrease ($P<0.01$) in the percentage of primary cilia that were positive for Hsp90. Cells that retained primary cilia also had significantly less intense Hsp90 staining in the organelle ($P<0.01$; Fig. 5H). This was quantified by measuring the Hsp90 immunofluorescent signal that colocalised with acetylated tubulin in individual primary cilium. Together these data suggest that Hsp90 levels are decreased in cilia upon heat shock. They could also indicate that cilia that are positive for Hsp90 are more readily lost upon heat shock, however given that GA treatment reduces cilia frequency this seems unlikely.

Fig. 5. Hsp90 is required for maintenance of primary cilia and is lost from the axoneme upon heat shock. (**A–C**) Thermotolerant cells are resistant to heat-induced resorption of primary cilia. NIH3T3 cells, which had received a 20 minute priming heat shock 4 hours before passage (Thermortol), and control cells where seeded and cultured for 24 hours prior to a further 16 hours culture in serum-free conditions, to promote cilium formation. Cells were then maintained at 37℃ (Con) or exposed to 42℃ for 30 minutes. Induction of a heat-shock response in thermotolerant cells was confirmed by immunoblot of Hsp70 (A). The prevalence (B) and length (C) of cilia were quantified in control and thermotolerant cells. (**D–E**) To examine whether Hsp90 was involved in maintenance of cilia under normal and heat-shock conditions NIH3T3 cells were treated for 3 hours with the inhibitor geldanamycin (GA) or vehicle (DMSO) alone. Cells were then either maintained at 37℃ or exposed to 42℃ for 30 minutes. The prevalence (D) and length (E) of cilia were then quantified. The numbers of cilia and nuclei were counted in 10 randomly selected fields for each experimental condition (B,D). Mean axoneme length was quantified from confocal images of at least 50 randomly selected ciliated cells for each experimental condition (C,E). (**F–H**) Hsp90 is lost from the axoneme upon heat shock. NIH3T3 cells were maintained at 37℃ or exposed to 42℃ for 30 minutes. (F) NIH3T3 cells were stained to detect the ciliary axoneme with anti-acetylated tubulin (green) and Hsp90 (red). Nuclei were detected with DAPI (blue). Enlarged images of the cilia indicated by arrows are shown in the insets. Scale bars: 10 μm. The incidence of primary cilia with detectable levels of Hsp90 (G) and the intensity of Hsp90 staining in axonemes, relative to acetylated tubulin levels (H), were quantified. 100 primary cilia were assessed for Hsp90 staining in three separate experiments and fluorescence intensities quantified in 50 randomly selected axonemes. Error bars indicate s.e.m. *$P<0.05$. (**I**) NIH3T3 cells maintained at 37℃ or exposed to 42℃ for 30 minutes were stained with anti-acetylated tubulin (magenta) to detect the ciliary axoneme. They were co-stained for HDAC (green), Hsp90 (red) and with DAPI. Boxed regions in the top panel are shown enlarged in the lower panel (zoom). Arrows indicate areas where HDAC6 and Hsp90 stainings overlap. Scale bars: 10 μm.

As HDAC6 and Hsp90 have previously been reported to interact we investigated if they colocalised in the ciliary axoneme by triple immunofluorescent staining (Fig. 5I; supplementary material Fig. S6). Cells were stained for acetylated tubulin to reveal cilia as well as HDAC6 and Hsp90. This triple staining required the use of a different HDAC6 antibody that needed an additional methanol fixation step. This fixation gave a more punctate cilia staining for both Hsp90 and HDAC6 (alone and in combination). We observed partial colocalisation of Hsp90 and HDAC6 in cilia of cells maintained at 37℃. This was particularly evident in puncta along longer cilia. In cells exposed to 42℃ for 30 minutes it was difficult to identify Hsp90 staining in the remaining shortened ciliary axonemes. This data further supports that, in contrast to HDAC6, Hsp90 levels are reduced in cilia upon heat shock.

Discussion

Understanding the assembly and disassembly of primary cilia is important because of the role of this organelle in human development and disease. For example, primary cilia are critical in processes such as neurogenesis (Breunig et al., 2008) and Shh driven oncogenesis (Han et al., 2009).

We demonstrate here that primary cilia undergo rapid resorption in response to heat shock. After heat-induced loss, primary cilia eventually reappear in mammalian cells, however this takes significantly longer than their resorption. This is consistent with attenuation of the cilium-mediated Shh signalling pathway in cells exposed to heat shock. Data from zebrafish experiments indicated that cilia are also sensitive to non-lethal exposure to elevated temperature in vivo. This was the case for primary cilia in the fish tail and motile cilia in the pronephric duct. It has been suggested that the regulated disassembly or shortening of cilia may serve as a rheostat to limit the cellular response to overly persistent or abnormal growth cues in the extracellular environment (Pugacheva et al., 2007). Similarly, we speculate that ciliary resorption may also play a role in regulating gene expression under conditions of extracellular stress. The cellular response to heat shock includes downregulating expression of non-essential proteins and it is possible that resorption of primary cilia, leading to signalling downregulation, would represent another component of this cellular survival mechanism. That both mammalian and zebrafish primary cilia are reduced in response to elevated temperature indicates that rapid resorption is likely to be an evolutionarily conserved phenomenon amongst vertebrates. Interestingly not all cells in the population lost primary cilia after a 30 minute heat shock, indicating variability in this response. Multiple factors are known to regulate biogenesis and resorption of primary cilia and it seems possible that in some cells ciliogenesis pathways could counteract heat-induced loss. Alternately, some cells may be more stress tolerant and thus resistant to the effects of elevated temperature on cilia.

Previously it has been demonstrated that resorption of cilia is dependent on an Aurora-A/HDAC6 pathway that regulates deacetylation of microtubules (Pugacheva et al., 2007). Our data is consistent with resorption under heat shock conditions being, at least in part, HDAC modulated yet downstream of Aurora-A. Importantly, the finding that HDAC inhibition reduced heat-shock-induced resorption, in addition to the rapidity of cilia loss, also implies that cilium resorption is a signalling response, as opposed to the result of an inability of the cell to maintain the

organelle. We localised Hsp90 and HDAC6 to the ciliary axoneme. These proteins have previously been reported to exist in a complex that disassociates as part of the cellular stress response, activated by cytotoxic protein aggregate formation. We propose a model where cilium resorption in response to heat shock is mediated by the deactylase activity of HDAC6 and loss of axoneme Hsp90 function, possibly after disassembly of an HDAC6/Hsp90 complex.

Although our data supports resorption as part of a regulated response to heat shock, it is also likely that heat shock may reduce the pool of dimeric soluble tubulin available for IFT and subsequent incorporation into ciliary microtubules. Indeed, it has recently been demonstrated in vitro that dimeric soluble tubulin is prone to aggregation even at physiological temperatures and furthermore that Hsp90 has been shown to protect tubulin against thermal denaturation (Weis et al., 2010). Consistent with this, we observed that inhibition of Hsp90 reduced the frequency of ciliated cells and cilium length under both control and heat shock conditions. We also observed that in thermotolerant cells where relative levels of Hsp90 and Hsp70 proteins are elevated, primary cilia are more resistant to heat-shock-induced resorption.

In conclusion, the cellular stress response is multifaceted and adaptive and we hypothesise the loss of cilia may be a normal cellular mechanism that downregulates non-essential signalling pathways during stress conditions.

Materials and Methods

Cell culture

All cell lines where from the American Type Culture Collection. NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM). IMCD3 and ARPE19 cells were grown in DMEM at a 1:1 ratio with Ham's F12 medium. Cells were maintained in medium supplemented with 10% fetal calf serum (FCS) containing 100 U/ml penicillin and 100 mg/ml streptomycin. To promote the appearance of primary cilia on NIH3T3 cells serum was withdrawn for 20 hours, as previously described (Pugacheva et al., 2007).

Heat shock, recovery, thermotolerance and drug inhibition

Cells were grown in 35 mm cell culture dishes that contained a 10 mm coverslip. For heat shock experiments 37℃ medium was rapidly exchanged for 42℃ medium and dishes immediately transferred to a 42℃ water bath. For experiments with a recovery period after heat shock cells were returned to a 37℃ incubator. To generate thermotolerant cells a 42℃ 30 minute heat shock was administered 4 hours before passaging into cell culture dishes. For Hsp90 inhibition NIH3T3 cells were treated for 3 hours with 2 μM geldanamycin (Sigma) For Aurora-A inhibition cells were treated with PHA-680632 (Selleck Chemicals, Houston) at a concentration of 0.5 μM for 4 hours. For HDAC inhibition cells were treated for 4 hours with trichostatin A (Sigma) at final concentration of 10 nM. Vehicle only (DMSO treated) controls were also performed. These inhibitors have been used previously to establish the Aurora-A cilium resorption pathway (Pugacheva et al., 2007). After treatments coverslips were processed for immunofluorescent staining and the remaining cells in each dish collected for western analyses.

Immunofluorescence detection and quantification of primary cilia

For detection of cilia using immunofluorescence, cells grown on coverslips were fixed in 4% (v/v) formaldehyde followed by detergent permeabilization with 0.05% Triton X-100. For triple staining, a 5 minute additional methanol fix was included immediately after the formaldehyde fixation (Pugacheva et al., 2007). Monoclonal anti-acetylated tubulin, clone 611 B-1, mouse ascites 1:1000 (Sigma), and rabbit polyclonal anti-pericentrin, 1:2000 (Abcam), were used for detection of the ciliary axoneme and basal body respectively. Rabbit polyclonal anti-HDAC6 (phosphor-22; Abcam) and goat polyclonal anti-HDAC6 (Santa Cruz) were used at titers of 1:200 and 1:50 and anti-Hsp90 (Abcam) was used at a titer of 1:500. Appropriate Alexa-Fluor-488- and Alexa-Fluor-568-conjugated secondary antibodies (Molecular probes) were used and nuclei were detected with DAPI (4′,6-diamidino-2-phenylindole). Imaging and quantification of the percentage of ciliated cells and cilium length was performed using a Zeiss LSM510 laser scanning confocal microscope and the Zeiss ZEN software. For quantification both acetylated tubulin and pericentrin labelling were used to define cilia. The proportion of ciliated cells in a given field was determined by counting the number of cilia and the number of nuclei present and is expressed as % of the total cell population. Cilium prevalence was measured for 10 fields (>20 cells/field) in three separate experiments giving a total of approximately 600 cells. Sequential 0.5 μm thick z-stacked sections were imaged through the entirety of the cellular profile using a 63× objective lens and were used to create maximum intensity projections (MIPs). Zen analysis software (Zeiss) was used to trace and measure the length of cilia in MIPs. Due to the differences in the z resolution of the microscope compared with the x and y planes, only cilia that were approximately 90° to the incident light were selected. Z-stacks were carefully examined prior to measurement to ensure vertical cilia were excluded. Axonemal length of at least 30 cilia were measured from five fields for each experimental condition. Experiments were conducted in triplicate. Measurements were made blind to experimental status and statistical significance determined by unpaired *t*-test or one-way ANOVA as appropriate.

Hedgehog signalling assay

NIH3T3 cells were transfected, using Lipofectamine Plus (Invitrogen), with a Gli-binding site luciferase reporter plasmid (pGBS-luc) and a constitutive expressing vector for Renilla (pRL, from Promega). Cells were allowed to recover from transfection and then serum starved for 16 hours to promote cilium formation. Heat shock for the specified times was then performed and cells were allowed to recover for 4 hours prior to addition of 2 μM purmorphamine. 16 hours later a Dual-Luciferase Reporter Assay (Promega) was performed and luciferase activity normalized to renilla values.

Western blot analyses

For western blot analyses of levels of acetylated tubulin and pericentrin in cell lysates the same antibodies as for immunofluorescence were used, Hsp70 proteins and GAPDH were detected using anti-Hsp70 clone BRM-22 and rabbit anti-GAPDH (Santa Cruz Biotechnology). Infrared-dye-conjugated secondary antibodies were used for detection of bands with an Odyssey Infrared Imaging System (LI-COR). Cell lysates were prepared for western analyses as described previously (Meimaridou et al., 2011).

Heat shock and detection of cilia using immunofluorescence in zebrafish

24 hour post-fertilization zebrafish (*Danio rerio*) were heat shocked at 42℃ for 5 minutes. Immediately after heat shock whole fish were fixed in 4% paraformaldehyde overnight prior to processing for immunofluorescent detection of primary cilia, as described above. Cilium frequency and length were quantified from z-series through the full thickness of the tail in a region up to 100 μm from the tip. The number of cells with cilia was determined by counterstaining nuclei with DAPI. Z-stacks were generated from five fish for each treatment. The lengths of at least 30 randomly selected cilia were quantified for each fish using Zen software giving a total of at least 150 cilia. Head–trunk angle measurements were made between a line drawn through the middle of the ear and eye, and a second line parallel to the notochord in the mid-trunk region (myotomes 5–10).

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