Hikeshi modulates the proteotoxic stress response in human cells: Implication for the importance of the nuclear function of HSP70s

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Hikeshi mediates the heat stress-induced nuclear import of heat-shock protein 70 (HSP70s: HSP70/HSC70). Dysfunction of Hikeshi causes some serious effects in humans; however, the cellular function of Hikeshi is largely unknown. Here, we investigated the effects of Hikeshi depletion on the survival of human cells after proteotoxic stress and found opposite effects in HeLa and hTERT-RPE1 (RPE) cells; depletion of Hikeshi reduced the survival of HeLa cells, but increased the survival of RPE cells in response to proteotoxic stress. Hikeshi depletion sustained heat-shock transcription factor 1 (HSF1) activation in HeLa cells after recovery from stress, but introduction of a nuclear localization signal-tagged HSC70 in Hikeshi-depleted HeLa cells down-regulated HSF1 activity. In RPE cells, the HSF1 was efficiently activated, but the activated HSF1 was not sustained after recovery from stress, as in HeLa cells. Additionally, we found that p53 and subsequent up-regulation of p21 were higher in the Hikeshi-depleted RPE cells than in the wild-type cells. Our results indicate that depletion of Hikeshi renders HeLa cells proteotoxic stress-sensitive through the abrogation of the nuclear function of HSP70s required for HSF1 regulation. Moreover, Hikeshi depletion up-regulates p21 in RPE cells, which could be a cause of its proteotoxic stress resistant.

1 | INTRODUCTION

Many molecular chaperones address aberrant proteins expressed in response to cellular stress (Stürner & Behl, 2017) to maintain and restore a balanced protein homeostasis. HSP70s comprise the major group of ATP-dependent and ubiquitously expressed molecular chaperones found in all organisms (Kampinga & Craig, 2010; Lindquist & Craig, 1988). HSP70 acts as a protein unfolding machinery, which binds and releases stretches of hydrophobic amino acids, through an ATP-hydrolysis-driven cycle. Furthermore, they also protect the cells against stress-induced apoptosis (Gabai, Mabuchi, Mosser, & Sherman, 2002; Guo et al., 2005; Mosser, Caron, Bourget, Denis-Larose, & Massie, 1997).

HSP70s rapidly and efficiently translocate into the nucleus from the cytoplasm in response to heat stress (Pelham, 1984; Velazquez & Lindquist, 1984; Welch & Feramisco, 1984). We previously identified a nuclear import carrier named Hikeshi, a protein encoded by human C11orf73, that mediates the heat stress-induced nuclear import of HSP70s (Kose, Furuta, & Imamoto, 2012). Crystal structure analysis showed that Hikeshi forms an asymmetric homodimer that is responsible for the interaction with HSP70s (Song et al., 2015). Hikeshi is an evolutionarily conserved protein that exists in most of eukaryotes. Schizosaccharomyces pombe SpHikeshi/Opi10 interacts with Hsp70 homologue Ssa2, and mediates its nuclear import in a HeLa cell-reconstituted transport system (Oda et al., 2014). Hikeshi homologue Hikeshi-like (HKL) protein
in *Arabidopsis thaliana* also interacts with two *Arabidopsis* HSP70 isoforms and regulates its localization (Koizumi, Ohama, Mizoi, Shinozaki, & Yamaguchi-Shinozaki, 2014). These results suggest that the biochemical property of the Hikeshi homologue is conserved among yeast to mammal.

In HeLa cells, depletion of Hikeshi abrogates the heat-stress-induced nuclear import of HSP70 and decreases cell viability after heat stress, probably due to a delay in the attenuation and reversion of the heat stress response (Kose et al., 2012). Recently, we reported that a point mutation of Hikeshi, Val54Leu, induces human genetic white matter disorders called leukoencephalopathies and early death of patients in the Ashkenazi Jewish population (Edvardson et al., 2016). More recently, absence of Hikeshi has been reported to cause infantile hypomyelinating leukoencephalopathy in Finnish patients (Vasilescu et al., 2017).

In spite of the serious effects that dysfunction of Hikeshi has on humans, its cellular function remains largely unknown. Although we previously reported that Hikeshi depletion induces death of HeLa cells after heat stress, we wondered whether such a function of Hikeshi in the protection of cells is universal among human cells, because in the course of studying mouse embryonic fibroblast (MEF) cells prepared by Hikeshi knockout mice, we noticed that the depletion of Hikeshi increases cell viability after heat stress (unpublished results). A previous report also showed that the expression of conventional nuclear localization signal (NLS)-tagged HSC70 partially reversed the cell death caused by Hikeshi depletion after heat stress in HeLa cells (Kose et al., 2012), but its mechanism is not known. In this study, we examined the effects of Hikeshi depletion on cell survival after proteotoxic stress in two human cell lines, HeLa cells and RPE cells, which showed the opposite effect of Hikeshi depletion. In HeLa cells, depletion of Hikeshi rendered cells stress-sensitive, whereas in RPE cells, depletion of Hikeshi rendered them stress-resistant. We found that Hikeshi depletion sustained HSF1 activation in HeLa cells due to lack of nuclear Hsp70, which may partially explain the stress sensitivity of Hikeshi knockout (KO) HeLa cells. However, we found expression of p21 is up-regulated after proteotoxic stress in RPE cells, and Hikeshi depletion further enhanced the up-regulation. This p21 expression seems to rely on activity of p53 because the expression was not seen in HeLa cells that lack transactivation activity of p53. Up-regulation of p21 may explain why Hikeshi KO RPE cells were rendered stress-resistant after proteotoxic stress.

## RESULTS AND DISCUSSION

### 2 | Hikeshi knockout cells require a longer time to recover from stress compared to wild-type cells

Activation of heat-shock factor 1 (HSF1) is crucial for cell survival in a stressful environment including the heat-shock (HS) conditions (Chou, Prince, Gong, & Calderwood, 2012; Pirkkala, Nykänen, & Sistonen, 2001), and HSF1 phosphorylation, including phosphorylation of serine 326, is an established marker of HSF1 activation (Holmberg, Tran, Eriksson, & Sistonen, 2002). To investigate whether the function of Hikeshi is conserved among human cells, we generated Hikeshi knockout (KO) HeLa and human telomerase immortalized retinal pigment epithelial cells (hTERT-RPE1; RPE cells), using a CRISPR-Cas9-mediated genome editing technique (Fig. S1A and B). We confirmed that HS-induced nuclear import of HSP70s was blocked in both Hikeshi KO HeLa and RPE cells (Fig. S1C). Under normal condition, Hikeshi KO did not affect cell growth of both HeLa and RPE cells (Fig. S1D). Tumor cells are well known to be more sensitive to temperature than normal cells (Fukao et al., 2000; Urano, Rice, Epstein, Suit, & Chu, 1983). After examining several different temperatures to heat-shock HeLa and RPE cells, we identified 43 and 45°C, respectively, as the necessary temperatures that affect cell viability upon Hikeshi KO (the viability was affected at 43°C in HeLa cells and at 45°C in RPE cells; see Figure 2a,b and also Fig. S2A,c), and temperature that induced formation of subnuclear structures called nuclear stress granule (nSG) (Fig. S2Aab). nSGs are known to be induced by activation of HSF1 (Cotto, Fox, & Morimoto, 1997; Shi, Mosser, & Morimoto, 1998). Wild-type (WT) and Hikeshi KO HeLa and RPE cells were exposed to HS for 1 h at 43 and 45°C, respectively, and were returned to 37°C for recovery at different time points.

In both WT and Hikeshi KO HeLa cells, an increase in the expression of the phosphorylated form of HSF1 was observed after HS compared to the basal level of the phosphorylated form of HSF1 in control cells grown at 37°C, although the phosphorylation of HSF1 delayed in Hikeshi KO HeLa cells. The marked difference we noticed in Hikeshi KO HeLa cells was sustained phosphorylation of HSF1 after recovery of the stress. Four and a half hours after recovery from HS, the phosphorylated HSF1 disappeared in WT HeLa cells, but was sustained in Hikeshi KO HeLa cells (Figure 1a,c). These results are consistent with our previous studies (Kose et al., 2012) where we reported nSGs are rapidly formed in response to HS in HeLa cells, but are sustained for a longer period after recovery from HS in Hikeshi-depleted cells than in WT cells.

In RPE cells, phosphorylation of HSF1 rather enhanced immediately after heat shock in Hikeshi KO cells compared to WT cells, but sustained phosphorylation was weaker in Hikeshi KO cells compared to HeLa cells. At 4.5 h after recovery from HS, the phosphorylated form of HSF1 almost disappeared in both WT and Hikeshi KO cells (Figure 1b,c). As HSF1 becomes active when the cells challenge the stress conditions (Cotto, Kline, & Morimoto, 1996; Kline &
Morimoto, 1997; Pirkkala et al., 2001; Shamovsky & Nudler, 2008; Voellmy, 1996), the more rapid disappearance of activated HSF1 indicated a faster recovery from stress damage. Therefore, we speculate that RPE cells recovered from HS faster than HeLa cells under conditions in which Hikeshi was depleted.

**FIGURE 1** Hikeshi-depleted HeLa cells require a longer time to recover from heat shock than Hikeshi-depleted RPE cells. WT and Hikeshi KO HeLa (a) and RPE (b) cells were untreated (control) or exposed to heat shock (HS) at 43 and 45°C, respectively, for 1 h and returned to 37°C for 1.5, 3, and 4.5 h after recovery from HS. Cell lysates were prepared using RIPA buffer at the indicated time points and subjected to immunoblotting to detect phosphorylated HSF1 (HSF1/pS326) and HSF1. β-Actin was used as the loading control. (c) Graphs representing the quantification of phosphorylated HSF1 (pS326) protein bands normalized to HSF1.
2.2 | Hikeshi depletion makes HeLa cells sensitive but RPE cells resistant to proteotoxic stress

Then, to investigate the role of Hikeshi in regulating the sensitivity to heat stress, HeLa and RPE cells underwent HS as described in Figure 2a.b. Hikeshi KO HeLa cells showed reduced viability compared to that of WT HeLa cells at 24 and 48 h after recovery from HS (Figure 2a,c). These findings are consistent with our previous results showing that depletion of Hikeshi reduces the viability of HeLa cells upon HS (Kose et al., 2012). In contrast, in RPE cells, the viability of Hikeshi KO cells was higher than in WT cells at 24 and 48 h after recovery from HS (Figure 2b,c). These results show the opposite effects of Hikeshi depletion on stress sensitivity in HeLa and RPE cells; HeLa cells become stress-sensitive, whereas RPE cells become stress-resistant.

We also used the proteasome inhibitor MG132, a small drug that affects the cellular proteome and induces potent proteotoxic stress similar to HS on cells. When the cells were treated with MG132, Hikeshi KO HeLa cells showed reduced viability compared to that of WT HeLa cells. In contrast, Hikeshi KO RPE cells showed a higher viability than WT RPE cells upon MG132 treatment (Figure S2B). These results show that the effect of Hikeshi depletion was not restricted to HS but also to other proteotoxic stressors.

Next, we examined whether or not Hikeshi KO induces apoptosis in HeLa cells and RPE cells upon exposure to proteotoxic stress. For this, we treated HeLa and RPE cells with MG132 for increasing duration (0, 6, 12, 18, and 24 h) and examined apoptosis activity. Tumor cells are well established to be more sensitive to proteasomal inhibition than normal cells (Orlowski et al., 1998). Hence, we initially treated HeLa and RPE cells with different doses of MG132, and doses that affect cell viability most effectively by Hikeshi depletion were determined. We accepted 5 and 10 μM for HeLa cells, and 10 and 15 μM for RPE cells. As shown in Figure 2d, Hikeshi KO HeLa cells showed significantly higher levels of apoptosis than WT cells, whereas Hikeshi KO RPE cells showed significantly less apoptosis than WT cells after MG132 treatment.

In addition to MG132, we used two other proteotoxic stressors, staurosporine, a kinase inhibitor, and heat shock, to induce apoptosis. Upon treatment with staurosporine, we observed higher levels of apoptosis in Hikeshi KO HeLa cells than in WT HeLa cells, but Hikeshi KO RPE cells showed less apoptosis than WT cells (Figure S3A). In response to HS, Hikeshi KO HeLa cells showed higher levels of apoptosis than WT cells, but Hikeshi KO RPE cells showed less apoptosis than WT cells (Figure S3B). Taken together, these results show that after depletion of Hikeshi, HeLa cells become sensitive to proteotoxic stress, whereas RPE cells become resistant to the same proteotoxic stress, which are consistent with the results of our viability assay.
glutamate-induced apoptosis by down-regulating prolonged ERK activity.

We next tried to know why RPE cells become resistant to proteotoxic stress after depletion of Hikeshi, whereas HeLa cells become proteotoxic stress-sensitive. As shown in Figure 1, Hikeshi KO causes delay of HSF1 activation (HSF1 phosphorylation) in HeLa cells, whereas in RPE cells, such delay was not seen. The extent of delay of HSF1 activation
in Hikeshi KO HeLa cells varied among experiments (compare Figures 1 and 3). Although differences of HSF1 activation just after HS might affect viability of HeLa and RPE cells differently after proteotoxic stress, HSF1 was ultimately activated in both Hikeshi KO HeLa and RPE cells, suggesting HSF1 may not be the only cause of the phenomenon we found. Therefore, we searched for alternative possible causes that affect viability of HeLa and RPE cells differently. For this, we examined the expressions of various proteins in HeLa and RPE cells in response to proteotoxic stress. Among the examined proteins, we found the expression level of p21, a cyclin-dependent kinase inhibitor, altered among HeLa cells and RPE cells in response to proteotoxic stress and to Hikeshi depletion.

We observed up-regulation of p53 in both WT and Hikeshi KO HeLa cells treated with MG132 for 12 h. Expression of p53 was diminished after 24 h of MG132 treatment, probably due to death of cells. Under these conditions, the expression of p21 remained unaffected (Figure 3d, upper lane). Treatment with HS at 43°C for 1 h and subsequent recovery at 37°C for up to 24 h resulted in up-regulation of p53 in both WT and Hikeshi KO HeLa cells, but the expression of p21 remained unaffected (Figure S4Ba). Expression of p21 has been reported to depend on activity of p53 tumor suppressor (Di Leonardo, Linke, Clarkin, & Wahl, 1994; Dulic et al., 1994; El-Deiry et al., 1993; Xiong et al., 1993), indicating that the p53–p21 pathway in HeLa cells is not functional. The p53 protein has been reported to be degraded rapidly in HeLa cells because of its association with the E6 protein produced by papillomavirus (Moll, Laquaglia, Benard, & Riou, 1995). Moreover, the function of wild-type p53 has been reported to be disrupted in malignant cells by association with over-expressed mdm2 protein (Barak, Juven, Haffner, & Oren, 1993). This might explain why the p53–p21 pathway did not work in HeLa cells in our study.

In RPE cells, we observed up-regulation of p53 and p21 in both WT and Hikeshi KO cells after 12- and 24-h treatment with MG132. However, the extent of the up-regulation of p21 was higher in Hikeshi KO RPE cells than in WT RPE cells (Figure 3d). We further examined the p53 and p21 expression level in RPE cells in response to different doses of MG132 treatment, which again confirmed higher up-regulation of p21 in Hikeshi KO RPE cells than in WT RPE cells (Figure 3e, f). When we treated RPE cells with HS at 45°C for 1 h and returned the cells to 37°C for up to 24 h, we observed higher up-regulation of p53 and p21 in Hikeshi KO RPE cells than in WT RPE cells (Fig. S4Bbc). A previous study showed that the stabilization and activation of p53 in response to genotoxic stress requires activation of HSF1 and subsequent up-regulation of p21 (Logan et al., 2009). p53-dependent p21 could be up-regulated in Hikeshi KO RPE cells after stress because Hikeshi depletion strongly induced HSF1 after heat stress (Figure 1b).

In this study, we found HeLa cells become proteotoxic stress-sensitive after depletion of Hikeshi due to the abrogation of the nuclear function of HSP70. In RPE cells, we found that the depletion of Hikeshi enhanced the up-regulation of p21 after proteotoxic stress. It is known that p21 is functional in RPE cells (Lambrus et al., 2016; Wang et al., 2014). p21 binds to CDK complexes and prevents cell growth (El-Deiry et al., 1994). p21 is also well known for its ability to inhibit apoptosis (Chang et al., 2000; Moon, Choi, & Kim, 2011). Anti-apoptotic effect of p21 can be induced as a consequence of its growth inhibitory effect, and by several different mechanisms that involve its transcription activity and interaction with molecules involved in the regulation of apoptosis (Coqueret, 2003; Jung, Qian, & Chen, 2010). We presume up-regulation of p21 is one cause of RPE cells for rendering it proteotoxic stress-resistant upon depletion of Hikeshi. However, we must carefully examine its molecular mechanisms. Hikeshi depletion also affects activation of HSF1, and this might also contribute to RPE cells rendering stress-resistant. Hikeshi depletion affects multiple molecules. It is also important to understand the mechanism of p21 up-regulation in Hikeshi-depleted cells. In addition, it is important to determine which types of cancer cells become stress-sensitive upon Hikeshi depletion. With this information, Hikeshi could become a potential therapeutic target.

3 EXPERIMENTAL PROCEDURES

Experimental procedures of this study are described in the Supporting information.
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FIGURE 3  Depletion of Hikeshi abrogates nuclear function of HSP70 for HSF1 regulation and up-regulates the p53–p21 pathway in RPE cells. (a) Schema representing experiments. (b) Cell lysates prepared before heat shock (normal condition), immediately after heat shock, and 3, 6, and 12 h after recovery from heat shock were subjected to immunoblotting. β-Actin was used as the loading control. Lanes are indicated by numbers. (c) Graphs representing the quantification of phosphorylated HSF1 (pS326) protein bands normalized to β-actin bands carried out by ImageJ software. (d) WT and Hikeshi knockout (KO) HeLa (upper lane) and RPE cell (lower lane) lysates from untreated control (0 h) or MG132 (10 μM)-treated cells for the indicated duration were subjected to immunoblotting. (e) WT and Hikeshi KO RPE cell lysates prepared from untreated control (0 h) or treated cells with the indicated doses of MG132 for 24 h were subjected to immunoblotting. β-Actin was used as the loading control. (f) The p21 band intensity was quantified using ImageJ software and normalized to the intensity of β-actin.

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