PRKCSH contributes to tumorigenesis by selective boosting of IRE1 signaling pathway

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Unfolded protein response (UPR) is an adaptive mechanism that aims at restoring ER homeostasis under severe environmental stress. Malignant cells are resistant to environmental stress, which is largely due to an activated UPR. However, the molecular mechanisms by which different UPR branches are selectively controlled in tumor cells are not clearly understood. Here, we provide evidence that PRKCSH, previously known as glucosidase II beta subunit, functions as a regulator for selective activation of the IRE1α branch of UPR. PRKCSH boosts ER stress-mediated autophosphorylation and oligomerization of IRE1α through mutual interaction. PRKCSH contributes to the induction of tumor-promoting factors and to tumor resistance to ER stress. Increased levels of PRKCSH in various tumor tissues are positively correlated with the expression of XBP1-target genes. Taken together, our data provide a molecular rationale for selective activation of the IRE1α branch in tumors and adaptation of tumor cells to severe environmental stress.
Cancer development is often associated with cytotoxic conditions such as nutrient deprivation, oxidative stress, and metabolic changes. These conditions trigger the unfolded protein response (UPR) that helps the cell to cope with the stress and to reestablish normal endoplasmic reticulum (ER) function by attenuating translation and promoting protein folding, secretion, and degradation. Although prolonged ER stress can result in apoptotic cell death, chronic ER stress responses have been documented in most major types of human tumors and play a crucial role in tumor growth and survival. UPR is also involved in tumor development and progression by promoting the expression of tumor growth factors such as TNF-α, IL-8, and VEGF. UPR signaling is independently mediated by three distinct components: RNA-dependent protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1α (IRE1α/ERN1). Various tumors display altered activation of different UPR branches according to cell types and tissue origins; UPR activation is crucial for tumor cell adaption to ER stress. However, the molecular mechanisms by which different UPR branches in tumors are selectively activated remain poorly understood.

IRE1α is the ancestral branch of the ER stress response and is an ER-resident transmembrane protein acting as a proximal sensor of the UPR. Among various protein kinases, IRE1α has been proposed as a major contributor to tumor progression. IRE1α is the ancestral branch of the ER stress response and is linked to cell survival and tumor progression under stress. Indeed, the IRE1α pathway is linked to the expression of several tumor-promoting factors. IRE1α is activated by autophosphorylation and oligomerization, resulting in activation of its endoribonuclease (RNase) to cleavage and initiation of the splicing of the X-box binding protein 1 (XBP1) mRNA. XBP1 is a unique transcription factor that regulates genes responsible for ER-associated degradation (ERAD) and protein folding. Aside from RNase activity of IRE1α, phosphorylation of its kinase domain recruits TRAF2 to facilitate JNK and p38 MAPK activation under ER stress. Activated IRE1α also induces ERK MAPK activation through dissociation from the SH2/SH3 domain-containing adaptor Nck upon ER stress. The activation of IRE1α is regulated by the UProsome, a complex protein platform at the ER membrane. Bax inhibitor-1 (BI-1) forms a complex with the cytosolic domain of IRE1α and inhibits IRE1α signaling. BAX, BAK, ASK1-interacting protein (AIP1), and Hsp72 are also associated with the cytoplasmic domain of IRE1α and enhance its activation. Thus, the IRE1α binding partners are key regulators of selective activation of its signaling pathways and determination of cell fate upon ER stress.

Protein kinase C substrate 80K-H (PRKCSH/Hepatocystin) normally resides in the ER lumen, where it functions as the sensor of the UPR. Among various protein kinases, IRE1α has been proposed as a major contributor to tumor progression. IRE1α is the ancestral branch of the ER stress response and is linked to cell survival and tumor progression under stress. Indeed, the IRE1α pathway is linked to the expression of several tumor-promoting factors. IRE1α is activated by autophosphorylation and oligomerization, resulting in activation of its endoribonuclease (RNase) to cleavage and initiation of the splicing of the X-box binding protein 1 (XBP1) mRNA. XBP1 is a unique transcription factor that regulates genes responsible for ER-associated degradation (ERAD) and protein folding. Aside from RNase activity of IRE1α, phosphorylation of its kinase domain recruits TRAF2 to facilitate JNK and p38 MAPK activation under ER stress. Activated IRE1α also induces ERK MAPK activation through dissociation from the SH2/SH3 domain-containing adaptor Nck upon ER stress. The activation of IRE1α is regulated by the UProsome, a complex protein platform at the ER membrane. Bax inhibitor-1 (BI-1) forms a complex with the cytosolic domain of IRE1α and inhibits IRE1α signaling. BAX, BAK, ASK1-interacting protein (AIP1), and Hsp72 are also associated with the cytoplasmic domain of IRE1α and enhance its activation. Thus, the IRE1α binding partners are key regulators of selective activation of its signaling pathways and determination of cell fate upon ER stress.

PRKCSH regulates the IRE1α-XBP1 and -MAPK pathways. To define the possible function of PRKCSH in the regulation of UPR, we investigated the involvement of PRKCSH in the IRE1α-XBP1 pathway by using PRKCSH overexpression (L02-PRK) and knockout (L02-PRK KO) L02 normal liver cells. The ER localization of ectopically expressed PRKCSH was confirmed by immunocytochemistry (Supplementary Fig. 2a). Upon treatment with tunicamycin (TM), a typical ER stress inducer, the level of spliced XBP1 mRNA was increased in L02-PRK cells compared to control cells (L02-Mock), whereas the levels of total XBP1 mRNA were similar (Fig. 2a, Supplementary Fig. 3a, b). Similar results were obtained for the level of spliced XBP1 protein (Fig. 2b). Also, the level of spliced XBP1 protein was increased in a dose-dependent manner by TM treatment; this increase was stronger in L02-PRK cells than in control cells (Fig. 2c). Upon glucose deprivation, the level of spliced XBP1 protein was also significantly upregulated in various cancer tissues such as glioblastoma multiforme, esophageal carcinoma, lymphoid neoplasm diffuse large B-cell lymphoma, thymoma, liver hepatocellular carcinoma, pancreatic adenocarcinoma, stomach adenocarcinoma, and skin cutaneous melanoma. (Fig. 1a, b; Supplementary Fig. 1a). Subsequently, we also analyzed PRKCSH expression in human tumor tissues using the data available from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database. The expression of the PRKCSH gene was significantly upregulated in liver, colon, gastric, breast, and lung cancer tissues (Supplementary Fig. 1b). Immunohistochemical (IHC) analysis of an liver cancer tissue microarray also revealed that the incidence of PRKCSH positivity was higher in tumor tissues (positive samples: 45 out of 58; 77.6%) than in nontumor tissues (positive samples: 10 out of 59; 16.9%) (Fig. 1c, d; Supplementary Fig. 1c). Immunoblot analysis also showed that the expression level of PRKCSH is increased in hepatoma cell lines (HepG2 and Huh-7) compared to that of normal liver cell lines (CCL-13 and L02) (Fig. 1e). These data indicated that an increased level of PRKCSH is implicated in tumorigenesis; hence, we further analyzed the relationship between PRKCSH expression and clinicopathological parameters by using the same IHC data set. PRKCSH expression was significantly correlated with both extrahepatic metastasis (chi-square test, P = 0.029) and TNM classification of malignant tumors (TNM) stage (chi-square test, P = 0.028) (Fig. 1f, Supplementary Table 1). Analysis of the prognostic association of patient survival with PRKCSH mRNA level by using the data from TCGA and the European Bioinformatics Institute of the European Molecular Biology Laboratories data (EMBL-EBI) revealed that patients with high expression showed poor survival rate (Fig. 1g; Supplementary Fig. 1d). These results suggest that a potential function of PRKCSH is closely related to HCC tumorigenesis and progression.
higher in L02-PRK cells than in L02-Mock cells (Fig. 2d). Conversely, the levels of spliced XBP1 mRNA and its protein were attenuated in L02-PRK KO cells under ER stress (Fig. 2c, f). Subsequently, we assessed the role of PRKCSH in the ER stress-mediated activation of MAPKs. Activation of ERK1/2 and JNK1/ 2 was increased in L02-PRK cells compared to L02-Mock cells (Fig. 2g), but was attenuated in L02-PRK KO cells upon TM treatment (Fig. 2h) or glucose deprivation (Fig. 2i). Finally, we examined the effect of PRKCSH on the expression of the XBP1 target genes ERDJ4, GRP78, Sec61A1, and p58IPK (Supplementary Fig. 3a)\(^\text{39,40}\). Upon TM treatment, the expression of these genes was significantly higher in L02-PRK cells than in L02-Mock cells, but was lower in L02-PRK KO (Fig. 2j). Overall, these results indicate that PRKCSH boosts the activation of both IRE1α–XBPI and IRE1α–MAPK pathways under ER stress.

**PRKCSH contributes to selective activation of IRE1α pathway.** Some investigators have reported that PRKCSH is localized in the
nucleus in breast cancer models. However, we did not observe nuclear localization of PRKCSH in liver tumor tissues (Supplementary Fig. 2b), indicating that the PRKCSH level is increased in the ER of these cells in comparison with normal liver cells. To investigate the potential role of PRKCSH in the regulation of the ER stress response in HCC cell models, we compared the levels of spliced XBP1 and activated MAPKs between L02-Mock and L02-PRK KO cells treated with 10 μg/mL TM for the indicated time (Fig. 2). Immunoblot analysis of spliced XBP1 levels in L02-WT and L02-PRK KO cells showed increased spliced XBP1 levels in L02-PRK KO cells treated with 10 μg/mL TM for 5 h (Supplementary Fig. 2b), indicating that the PRKCSH level is increased in the ER of these cells in comparison with normal liver cells. To investigate the potential role of PRKCSH in the regulation of the ER stress response in HCC cell models, we compared the levels of spliced XBP1 and activated MAPKs between
PRKCSH-silenced and control hepatoma cells. The splicing of XBP1 mRNA was significantly downregulated in PRKCSH-knockdown HepG2 cells in comparison with control cells upon TM treatment, whereas the expression of total XBP1 mRNA was not different between these cells (Fig. 3a). Comparable results were also observed in Huh-7 hepatoma cells (Fig. 3b, Supplementary Fig. 3c). Silencing PRKCSH in both hepatoma cell lines decreased the level of spliced XBP1 protein (Fig. 3c, d). To further confirm the potential role of PRKCSH in XBP1 splicing under ER stress, we used Huh-PRK KO cells. The level of spliced XBP1 protein was also decreased in these cells upon TM treatment (Fig. 3e). Consistent with the effect of TM on XBP1 splicing, activation of ERK1/2, and JNK1/2 MAPKs upon TM treatment was decreased by PRKCSH silencing in HepG2 cells (Fig. 3f).

Furthermore, the expression levels of IRE1α–XBP1 target genes were also decreased by PRKCSH silencing in Huh-7 hepatoma cells (Fig. 3g). PRKCSH knockout in Huh-7 cells also markedly reduced the expression levels these genes (Fig. 3g). Lastly, since we showed that the levels of PRKCSH mRNA were elevated in several tumor tissues (Fig. 1b), we checked whether the expression levels of XBP1 target genes were elevated in human tumors. We found that all the tested XBP1 target genes were upregulated in tumor tissues compared to nontumor tissues (Fig. 3h, Supplementary Fig. 4). The expression level of each of these genes was positively correlated with the level of PRKCSH mRNA (Fig. 3h, Supplementary Fig. 4). These results suggest that the upregulated PRKCSH boosts the activation of the IRE1α pathway during tumorigenesis.

We then examined the effect of PRKCSH on the activation of the other UPR branches, the PERK and ATF6 pathways. PRKCSH-overexpression, -silencing, or knockout had little effect on PERK phosphorylation or ATF4 expression (Supplementary Fig. 5a–d). In agreement with this result, there were no significant changes in the expression level of ERO1LB, a target gene of the PERK pathway, in L02-PRK, L02-PRK KO, PRKCSH-silenced, and Huh-PRK KO cells in comparison with control cells (Supplementary Fig. 5e). Furthermore, silencing or overexpression of PRKCSH resulted in no meaningful difference in the levels of ATF6 activation (Supplementary Fig. 5g, h) or the expression of its target genes, as indicated by the total level of XBP1 mRNA (Figs 2a, 3a, b). Taken together, our results indicate that PRKCSH has a specific function in the regulation of the IRE1α pathway in the ER stress response.

PRKCSH promotes phosphorylation and oligomerization of IRE1α. Under ER stress, IRE1α can be phosphorylated in the linker region, kinase activation domain, and the RNase domain. Phosphorylation of the kinase domain activates the downstream MAPKs and RNase domain of IRE1α. Thus, to define the molecular mechanism by which PRKCSH activates the IRE1α pathway, we determined the phosphorylation of IRE1α. Upon TM treatment, the level of IRE1α phosphorylation was higher in L02-PRK cells than in L02-Mock cells (Fig. 4a). In contrast, L02-PRK KO cells showed a decreased level of IRE1α phosphorylation compared to wild-type (WT) cells after either TM treatment (Fig. 4b) or glucose starvation (Fig. 4c). PRKCSH-silenced hepatoma cells and Huh-PRK KO cells showed a remarkable reduction in IRE1α phosphorylation upon TM treatment (Fig. 4d–f). Since IRE1α oligomerization is a key step for UPRosome formation and triggering the subsequent XBP1 splicing, we then investigated the effect of PRKCSH on IRE1α oligomerization under ER stress. The oligomerization of IRE1α was monitored as the formation of foci in cells transfected with a Flag and Venus-tagged fluorescent human IRE1α fusion construct (IRE1α-FV). To examine whether ectopic overexpression of IRE1α results in formation of inclusion bodies containing proteasome components, we performed immunocytochemical analysis. The IRE1α-FV protein was not associated with proteasome under either resting or ER stress condition, suggesting that the fluorescent foci were derived from oligomerized IRE1α in the ER (Supplementary Fig. 6). Upon TM treatment, IRE1α oligomerization was higher in L02-PRK cells (up to 64% foci-positive cells) than in L02-Mock cells (up to 42% foci-positive cells) (Fig. 4g). In Huh-7 hepatoma cells, the oligomerization of IRE1α was drastically increased upon TM treatment (up to 65% foci-positive cells), whereas silencing PRKCSH in this cell line considerably reduced it under ER stress (up to 30% foci-positive cells) (Fig. 4h). These results indicate that PRKCSH promotes activation of IRE1α under ER stress.

Finally, we investigated whether IRE1α is necessary for PRKCSH-mediated XBP1 splicing and MAPK activation. Silencing of IRE1α in L02-PRK cells markedly reduced XBP1 splicing and ERK1/2 MAPK activation (Fig. 4i–k), suggesting that IRE1α is required for PRKCSH-mediated XBP1 splicing and MAPK activation. To confirm this result, we investigated the impact of IRE1α overexpression on XBP1 splicing and MAPK activation in PRKCSH-deficient cells. Overexpression of IRE1α increased XBP1 splicing and MAPK activation in L02-PRK KO cells (Fig. 4i–k). Taken together, our results indicate that IRE1α is a downstream target of PRKCSH during ER stress response.

PRKCSH specifically interacts with IRE1α under ER stress. To determine how PRKCSH promotes the activation of IRE1α under ER stress, we investigated whether the two proteins physically interact under ER stress by using co-immunoprecipitation (co-IP) from lysates of L02 normal liver cells. Association between PRKCSH and IRE1α was not observed under resting conditions; however, strong association was observed at 60 min of TM treatment, concomitant with the dissociation of GRP78 from IRE1α (Fig. 5a). Interestingly, this association was disappeared at 120 min of TM treatment, although dissociation of GRP78 from IRE1α persisted, consistent with previously reported observations. Similar results were obtained under glucose deprivation (Fig. 5a). TM treatment also increased the association between endogenous PRKCSH and IRE1α in Huh-7 hepatoma cells (Fig. 5a). Interestingly, the interaction was more sustained than that in L02 cells. To confirm the interaction of PRKCSH with IRE1α, we performed co-IP using lysates of L02 cells transfected with a Flag-tagged PRKCSH construct (PRK-Flag). Interaction between PRK-Flag and endogenous IRE1α was increased upon TM treatment (Fig. 5b). We further investigated this interaction using lysates of L02 cells transfected with IRE1α-FV. Association of endogenous PRKCSH with IRE1α-FV was marginal in resting cells but was increased upon TM treatment (Fig. 5c). These results indicated that ER stress induces a physical association between PRKCSH and IRE1α. We further determined whether PRKCSH interacts with PERK under TM treatment. PRKCSH did not interact with PERK under either resting or ER stress conditions (Supplementary Fig. 5f), although PERK was activated under the same ER stress conditions (Supplementary Fig. 5a, b). Overall, our data demonstrate that PRKCSH specifically interacts with IRE1α under ER stress.

PRKCSH E/P domain is essential for boosting IRE1α activation. To determine whether the interaction between PRKCSH and IRE1α is direct, we performed an in vitro pull-down assay with GST-PRKCSH deletion mutants and purified IRE1α. IRE1α interacted with the C-terminal domain of PRKCSH, but not with its N-terminal domain (Fig. 5d), indicating that PRKCSH directly interacts with IRE1α. To further determine the region of
Fig. 3 PRKCSH is required for boosting activation of the IRE1α pathway in hepatoma cells under ER stress. a, b Quantitative real-time PCR analysis of total XBP1 and spliced XBP1 expression in PRKCSH-silenced HepG2 (a) and Huh-7 (b) hepatoma cells treated with 10 μg/mL TM for the indicated time.

c, d Immunoblot analysis of sXBP1 protein levels in PRKCSH-silenced HepG2 (c) and Huh-7 (d) cells treated with 10 μg/mL TM for the indicated time.

e, f Immunoblot analysis of ERK and JNK phosphorylation in PRKCSH-silenced HepG2 cells treated with 10 μg/mL TM for the indicated time.

For quantitative real-time PCR analysis, Data are represented as mean ± SEM from three independent experiments. One-way ANOVA; **P < 0.01. Expression of Sec61A1 as representative XBP1 target gene and its correlation with the levels of PRKCSH mRNA in glioblastoma multiforme (GBM), esophageal carcinoma (ESCA), lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), thymoma (THYM), and liver hepatocellular carcinoma (LIHC) tissues. TCGA data were analyzed by using the GEPIA web tool. Scatter plots show relative expression of Sec61A1 mRNA in non-tumor and tumor tissues. The median expression levels in each group are indicated by horizontal lines. Significance of the differences between nontumor and tumor (left) was determined by one-way ANOVA (P < 0.001). Correlation between Sec61A1 and PRKCSH (right) was determined by Pearson’s rank correlation coefficient. r Pearson correlation coefficient, P Pearson p value.
PRKCSH responsible for association with IRE1α, we performed co-IP from lysates cells transfected with Flag-tagged PRKCSH mutants. We first confirmed that all these mutant proteins except ΔS/G2B are localized in the ER, similar to WT (Fig. 5f, Supplementary Fig. 7a). Co-IP assay revealed that the internal E/P domain of PRKCSH is required for the interaction with IRE1α upon ER stress (Fig. 5e, f, Supplementary Fig. 7b). Unlike the ΔG2B mutant, the ΔS/G2B mutant, which lack the N-terminal ER localization signal, did not interact with IRE1α (Fig. 5f). This result indicates that PRKCSH interacts with IRE1α in the ER but not in the cytoplasm. Interestingly, unlike WT PRKCSH, mutants lacking the G2B region (ΔG2B and E/P) interacted with IRE1α even under resting conditions (Fig. 5e, f, Supplementary Fig. 7b). The inhibitory effect of the G2B domain on PRKCSH binding to IRE1α
IRE1α suggests that it regulates the specific binding of PRKCSH to IRE1α only when ER stress is present. Because the interaction between the GIα subunit and PRKCSH is necessary for GI activity, we examined the potential impact of ER stress on GI activity. Co-IP assay revealed that ER stress induced the dissociation of the GIα–PRKCSH complex in both L02 and Huh-7 cells (Fig. 5g). This result suggests that GI activity is reduced during ER stress response. To investigate the impact of interaction between IRE1α and PRKCSH on IRE1α activation, we evaluated the levels of IRE1α phosphorylation, XBP1 splicing, and ERK activation. Expression of WT PRKCSH enhanced the ER stress-induced phosphorylation of IRE1α, whereas its level in cells expressing the ΔE/P mutant was similar to that of control cells (Fig. 6a). Consistent with this result, the levels of spliced XBP1 protein and ERK activation were also increased in cells transfected with WT PRKCSH but not with the ΔE/P mutant (Fig. 6b, c). Expression of the ΔG2B or E/P mutant increased IRE1α phosphorylation and XBP1 splicing upon TM treatment, whereas expression of the ΔS/G2B mutant showed little effect in comparison with control cells (Fig. 6d–g). Both the ΔG2B and E/P mutants interacted with but not activate IRE1α under resting conditions (Fig. 5f). These results indicate that PRKCSH–IRE1α interaction as such does not trigger IRE1α activation but boosts it upon ER stress.

Taken together, our results suggest that PRKCSH is involved in regulation of GI activity through interaction with the GIα subunit under resting condition; however, it regulates IRE1α activation through the E/P domain-mediated interaction with IRE1α under ER stress (Fig. 5h).

**PRKCSH is crucial for tumor resistance to ER stress.** The IRE1 signaling branch is responsible for tumor resistance against ER stress-induced cell death and for the regulation of the expression of tumor-promoting factors. To investigate the effect of PRKCSH on ER stress-induced cell death, we monitored apoptotic cell death and PARP1 cleavage in PRKCSH-overexpressing and -silenced cells. Importantly, overexpression of PRKCSH rendered L02 cells strongly resistant to ER stress-induced cell death upon treatment with TM or another ER stressor, thapsigargin (TG), and reduced PARP1 cleavage (Fig. 7a, Supplementary Fig. 8a). PRKCSH silencing in Huh-7 cells significantly sensitized them to ER stress-induced cell death and increased PARP1 cleavage upon TM or TG treatment (Fig. 7b, c, Supplementary Fig. 8b, c). Similar results were also obtained in Huh-PRK KO cells upon TM treatment (Fig. 7d, Supplementary Fig. 8d). To examine the effect of PRKCSH on long-term overall survival upon ER stress, we performed cell survival assay using TM-treated L02-PRK and Huh-PRK KO cells. Overexpression of PRKCSH increased survival of L02 cells under ER stress. Conversely, knockout of PRKCSH reduced survival of Huh-7 cells under ER stress (Fig. 7e). In addition, we determined whether PRKCSH contributes to cytoprotection against other stress condition such as nutrient starvation. Similar to the results for TM treatment, overexpression of PRKCSH also increased survival of L02 cells upon nutrient starvation, whereas its knockout reduced survival of Huh-7 cells (Fig. 7e). To evaluate the in vivo relevance of our study, we performed in vivo tumor growth experiment in xenograft nude mouse model using Huh-PRK KO and WT cells. Loss of PRKCSH reduced in vivo tumor growth under physiological stress condition (Fig. 7f, Supplementary Fig. 9). To investigate whether the E/P domain is required for the cytoprotective effect of PRKCSH against ER stress, we monitored ER stress-induced apoptotic cell death using cells overexpressing WT or mutant PRKCSH (ΔE/P and MRH). Deletion of the E/P domain resulted in a loss of cytoprotective function of PRKCSH (Fig. 7g, Supplementary Fig. 8e). We investigated whether IRE1α is required for PRKCSH-mediated cell survival under ER stress. IRE1α silencing attenuated the resistance of L02-PRK cells to ER stress-induced cell death (Fig. 7h, Supplementary Fig. 8f). This effect was confirmed by an increase in ER stress-induced PARP1 cleavage in IRE1α-knockdown cells (Fig. 7h). Next, we investigated the effect of PRKCSH on the regulation of ER stress-induced expression of tumor-promoting factors (Supplementary Fig. 3d). Overexpression of PRKCSH increased the levels of TNFA, IL8, and VEGF mRNAs upon TM treatment (Fig. 8a). Conversely, PRKCSH silencing in HepG2 and Huh-7 hepatoma cells significantly attenuated TM-induced expression of these genes (Fig. 8b, c). Finally, we investigated whether IRE1α is required for PRKCSH-mediated expression of tumor-promoting cytokines under ER stress. The increased expression of tumor-promoting factors in PRK cells was reduced by IRE1α silencing (Fig. 8d). Overall, these results suggest that the PRKCSH–IRE1α signaling axis is crucial for adaptation of tumor cells to ER stress.

**Discussion**

Previous studies have shown that PRKCSH is the β subunit of GII; PRKCSH determines the fate of glycoproteins and is needed for optimal quality control of glycoproteins in the ER. In the present study, we demonstrate a function of PRKCSH as a specific regulator of IRE1α signaling. The function is derived from the physical interaction between PRKCSH and IRE1α under ER stress. Our data provide strong evidence that PRKCSH can be a useful tumor marker in various cancer tissues and protects tumor cells from ER stress by promoting IRE1α activity (Fig. 9). In the ER, PRKCSH is essential for GI activity, which ensures secretion properly folded glycoproteins and targeting the improperly folded ones for degradation by the ERAD pathway. Thus, we expected that alteration of PRKCSH expression would
Under ER stress, GRP78 dissociates from these sensors, leading to interaction between the ER luminal domain of each sensor and unfolded proteins, which promotes the autophosphorylation and oligomerization of IRE1α and PERK, or export of ATF6 from the ER to Golgi18. Since the IRE1α and PERK sensors share functionally similar luminal sensing domains47, it has been assumed that ER stress may activate these sensors nonselectively. However, it turned out that they are selectively activated by ER stress according to cell types and ER stress inducers7,18,48. The selective activation of each UPR sensor is mediated by sensor specific signaling mechanisms and the nature of ER stress. Therefore, the sensors nonselectively, but only promote phosphorylation of IRE1α, leading to its oligomerization followed by XBP1 splicing and export of ATF6 from the ER to Golgi18. Since the IRE1α and PERK sensors share functionally similar luminal sensing domains47, it has been assumed that ER stress may activate these sensors nonselectively. However, it turned out that they are selectively activated by ER stress according to cell types and ER stress inducers7,18,48. The selective activation of each UPR sensor is mediated by sensor specific signaling mechanisms and the nature of ER stress.
regulators. In the present study, we showed that PRKCSH interacts only with IRE1α but not with PERK upon ER stress. Because the luminal domain of ATF6 has no structural similarity with that of IRE1α, PRKCSH is likely to be a specific binding partner of IRE1α. Our findings may explain how PRKCSH selectively boosts activation of the IRE1α pathway under ER stress.

Two domains of PRKCSH contribute to its glucosidase activity: G2B, the N-terminal Glia-binding domain, and MRH, the C-terminal N-glycan mannone recognition domain. Binding of the G2B domain of PRKCSH to the Glia subunit is required for maintaining the Glia level in the ER and GII enzymatic activity. The proline-rich E/P segment of PRKCSH is positioned between the G2B and MRH domains. Several studies have proposed that this segment in PRKCSH has not been elucidated. In the current study, we investigated how the interaction between PRKCSH and IRE1α is required for boosting IRE1α activation. Interestingly, deletion of the G2B domain results in low, the interaction between PRKCSH and IRE1α is transient, which leads to a temporary phosphorylation of IRE1α and MAPKs, whereas overexpression of PRKCSH in the same cells results in sustained phosphorylation of IRE1α and MAPKs. In hepatoma cells, where the expression level of PRKCSH is low, the interaction between PRKCSH and IRE1α is transient, which leads to a temporary phosphorylation of IRE1α and MAPKs, whereas overexpression of PRKCSH in the same cells results in sustained phosphorylation of IRE1α and MAPKs. PRKCSH silencing in both cell lines diminished these effects. Although in present study we have not determined how other components of the UPRosome contribute to the association or
were treated with 10 μM TG for 72 h. Cell death was determined from PI staining followed by FACS analysis. Immunoblot analysis of PARP1 cleavage (right) in these cells. In cell death analysis and cell survival analysis, data are shown as mean ± SEM from three or four independent experiments. One-way ANOVA; *P < 0.05, **P < 0.01.

Fig. 7 PRKCSH mitigates ER stress-induced cell death. a Cell death analysis (left) in L02-Mock and L02-PRK cells treated with 10 μg/mL TM or 1 μM TG for 72 h. Cell death was determined from PI staining followed by FACS analysis. Immunoblot analysis of PARP1 cleavage (right) in these cells. b Cell death analysis (left) in PRKCSH-silenced Huh-7 cells treated with 10 μg/mL TM or 1 μM TG for 48 h. Immunoblot analysis of PARP1 cleavage (right) in these cells. c Cell death analysis (left) in PRKCSH-silenced Huh-7 cells treated with 10 μg/mL TM or 1 μM TG for 72 h. Immunoblot analysis of PARP1 cleavage (right) in these cells. d Cell death analysis (left) in Huh-WT and Huh-PRK KO cells treated with 10 μg/mL TM for 72 h. Immunoblot analysis of PARP1 cleavage (right) in these cells. e Cell survival analysis in L02-Mock and L02-PRK cells (left), and Huh-WT and Huh-PRK KO cells (right). Cells were exposed to 10 μg/mL TM or EBSS (for nutrient starvation) for 48 h, and then replated in normal cell culture medium. After 5 days, cell viability was determined by staining with crystal violet. Number of cells per area (mm²) was calculated. Scale bars represent 100 μm. f In vivo tumor growth analysis in xenograft nude mice injected with Huh-PRK KO or WT cells. Balb/c nude mice were subcutaneous transplanted with each tumor cells in the right groin (four mice in each group). The volume of tumors was monitored for 35 days. Representative images of tumor-bearing mice (top) and tumor volumes (bottom) were presented. Scale bars represent 10 mm. g Cell death analysis in L02 cells transfected with the control, WT PRKCSH, ΔE/P, or MRH mutant plasmids; cells were treated with 10 μg/mL TM for 72 h. h Cell death analysis (left) in IRE1α-silenced L02-PRK cells treated with 10 μg/mL TM for 72 h. Immunoblot analysis of PARP1 cleavage (right) in these cells. In cell death analysis and cell survival analysis, data are shown as mean ± SEM from three or four independent experiments. One-way ANOVA; *P < 0.05, **P < 0.01.

disassociation of the PRKCSH–IRE1α complex, our results suggest that the endogenous level of PRKCSH in each cell type determines the extent of its interaction with IRE1α and the activity of IRE1α, which is important for adaptation of tumor cells to ER stress.

Activated RNase domain of IRE1α not only induces the splicing of XBP1 mRNA, but also contributes to the regulated IRE1-dependent decay (RIDD) pathway that cleaves ER-associated RNAs. The splicing of XBP1 mRNA promotes cell survival, whereas RIDD leads to cell death61. IRE1α oligomerization is
Selective activation of IRE1α

PRKCSH enhances expression of tumor-promoting cytokines under ER stress. a Quantitative real-time PCR analysis of tumor-promoting cytokines in L02-Mock and L02-PRK cells treated with 10 μg/mL TM for 5 h. b Quantitative real-time PCR analysis of tumor-promoting cytokines in PRKCSH-silenced Huh-7 cells treated with 10 μg/mL TM for 5 h. c Quantitative real-time PCR analysis of tumor-promoting cytokines in PRKCSH-silenced HepG2 cells treated with 10 μg/mL TM for 5 h. d Quantitative real-time PCR analysis of tumor-promoting cytokines in IRE1α-silenced L02-PRK cells treated with 10 μg/mL TM for 5 h. Data are shown as mean ± SEM from three or four independent experiments. One-way ANOVA; *P < 0.05, **P < 0.01

Fig. 8

A proposed model for the dual function of PRKCSH in ER protein quality control. Under resting conditions, PRKCSH functions as the noncatalytic beta subunit of glucosidase II, which catalyzes glucose trimming from newly synthesized glycoproteins and ensures secretion of properly folded glycoproteins and targeting improperly folded glycoproteins for degradation by the ERAD pathway. This is mediated by the interaction of PRKCSH with glucosidase II alpha subunit (Glxα) through the G2B domain and with mannose residues of glycoproteins through the MRH domain of PRKCSH. Under ER stress, PRKCSH dissociates from the Glxα subunit and subsequently associates with IRE1α through its internal E/P domain, which leads to autophosphorylation and oligomerization of IRE1α followed by RNase-mediated splicing of XBP1 and kinase domain-mediated phosphorylation of MAPKs, thereby selectively promoting activation of the IRE1α signaling branch. Findings from this study are shown on a yellow background, whereas the known function of PRKCSH is shown on a white background.

Fig. 9

required for maximal splicing of XBP1 mRNA, which leads to cell survival under ER stress. Although it has been reported that nonmuscle myosin heavy chain IIIB-mediated oligomerization of IRE1α is caused by direct interaction between both proteins, irrespective of IRE1α phosphorylation, it is well-known that autophosphorylation of IRE1α is required for its optimal oligomerization. In the present study, PRKCSH induced phosphorylation of IRE1α followed by its oligomerization. Under ER stress in L02 cells, PRKCSH–IRE1α interaction was transient and the two proteins rapidly dissociated. Nevertheless, IRE1α clustering was promoted after its dissociation from PRKCSH. This fact indicates that association between both proteins does not directly contribute to IRE1α clustering, but increases IRE1α phosphorylation, which in turn promotes IRE1α oligomerization. Consequently, we firmly believe that PRKCSH is required for optimal oligomerization of IRE1α under ER stress, which may paradoxically affect cell fate. In numerous tumors, IRE1α–XBP1 signaling has cytoprotective activity, allowing tumor cells to adapt to ER stress. PRKCSH expression was positively correlated with the expression of XBP1 target genes such as ER chaperones and ERAD components in various cancer tissues. We found that PRKCSH overexpression in normal liver cells increases the levels of XBP1 splicing and expression of ER chaperones, leading to resistance to ER stress-induced cell death; whereas PRKCSH silencing sensitizes hepatoma cells to ER stress-induced cell death with downregulation of XBP1 splicing and expression of its downstream target genes. Accordingly, IRE1α silencing in L02-PRK cells restores PRKCSH-mediated resistance to ER stress. Overall, the upregulation of PRKCSH in tumor tissues suggests a molecular rationale for altered IRE1α–XBP1 signaling in and adaption of tumor cells to ER stress.

It is accepted that UPR signaling is important for generating malignancy through induction of tumor-promoting factors such as TNF-α, IL-8, and VEGF. These factors are also involved in tumor growth because they stimulate proliferation and survival of
tumor cells under stress\textsuperscript{5,6}. Indeed, these factors are closely associated with epithelial–mesenchymal transition, angiogenesis, and tumor metastasis\textsuperscript{6}. PRKCSH expression was positively associated with the rate of extracellular metastasis and TNM stage in HCC tissues. Our findings suggest that PRKCSH contributes to the regulation of ER stress-induced expression of these factors by promoting IRE1α activity. Thus, the PRKCSH–IRE1α signaling axis may be crucial for tumorigenesis through promoting the expression of these factors. We have previously reported that TNF-α induces the expression of PRKCSH, which is involved in a cytokine-mediated response against hepatitis B virus infection\textsuperscript{6,9}. Thus, it is likely that IRE1α activity may be positively linked to PRKCSH expression through the induction of TNF-α expression.

Together with the results of other studies, our data suggest that PRKCSH has a dual function in the ER protein quality control as regulator of GII activity under resting conditions and as a selective regulator of IRE1α under ER stress. Furthermore, we provide a potential mechanism by which different UPR branches can be selectively regulated in tumor cells and how tumor cells can adapt under environmental stress conditions.

Methods
Reagents and antibodies. Propidium iodide (PI)/RNase staining solution (4087) was purchased from Cell Signaling Technology. Tunicamycin (TM; BML-CC104) was obtained from Enzo Life Science. Thapsigargin (TG; 586005) was obtained from Merck. Doxycycline hyclate (D9891) was purchased from Sigma-Aldrich. All stock solutions were prepared according to the manufacturers’ instructions.

For immunoblotting, following antibodies were used: PRKCSH (Proteintech, Cat:12418-1-AP, 1:2000), GAPDH (Santa Cruz Biotechnology, Cat:sc-7272, 1:5000), PARP1 (Santa Cruz Biotechnology, Cat:s-7150, 1:1000), GST (Santa Cruz Biotechnology, Cat:sc-205821), and knockout was confirmed by PCR.

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Gene silencing by siRNA. All small interfering RNA (siRNA) duplexes were synthesized by ST Pharm Co. (Seoul, Republic of Korea). Two siRNAs targeting PRKCSH (siPRK #1, 5'-AGGA AGA GAG CUC CUG AAG ATT-3' and siPRK #2, 5'-AGGA AGA GAG CGG UGA AGA ATT-3') were used. One siRNA targeting IRE1α (sire1α #2, 5'-GAA UCC UCU ACG UGG GUA AAA AGG ATT-3') was used. One nonspecific scrambled control siRNA (scCon, 5'-AUG AAG GAG AAU UGC UCA ATT-3') was used. Cells were transfected with siRNAs (final concentration, 40 nM) using Lipofectamine 2000 (ThermoFisher Scientific, 11668-09), according to the manufacturer’s instructions. At 48 h post-transfection, cells were subjected to immunoblotting to confirm transfection.

Cell treatment with ER stressors. Individual PRKCSH constructs or siRNAs were transiently transfected by using Lipofectamine 2000. At 48 h post-transfection, cells were treated with (final concentrations) 1 μM TG or 10 μg/mL TM for the indicated times. Stable cell lines (L02-PRK, L02-PRK KO, or Huh-PRK KO) were cultured in DMEM without G418 or puromycin for 7 days before treatment with an ER stressor, and were then seeded into a 6-well plate (2 x 10\(^3\) cells/mL). After 24 h, medium was changed to fresh medium with TG or TM. Glucose deprivation was induced by replacing medium with fresh DMEM without glucose (Wolgene, LM001-56) supplemented with 10% dilaoyl bovine sevom (Wolgene, S0001-08) for the indicated times.

Expression in human tumors. Following expression analysis of PRKCSH was performed by using RNA-seq data from TCGA. Gene Expression Profiling Interactive Analysis (GEPIA), a web-based tool (http://gepia.cancer-pku.cn/), was used for analysis of differential expression of PRKCSH in patients of the human anatomy clipart, analysis of correlation with the expression of XBP1 downstream target genes, and patient survival analysis\textsuperscript{65}. Gene expression datasets for liver cancers (GSE25097 and GSE20140), colon cancer (GSE10990), gastric cancer (GSE13861), breast cancer (GSE41244), and lung cancer (GSE27262) were downloaded from the NCBI GEO database. A profile graph was used to extract the expression values of specific genes by entering the corresponding identifier from the ID column. For survival analysis of patients with liver cancer, a gene expression dataset for hepatocellular carcinoma samples (E-TABM-36) was downloaded from the ArrayExpress database of EMBL-EBI. Processed data were used to extract the expression values of the PRKCSH gene and clinical data.

IHC analysis of liver tissues. Liver tissue array slides were purchased from SuperBioChips Laboratories and used to determine the levels of PRKCSH by IHC analysis. The slides contained non-tumor (CSN3, 59 cases) and tumor (CS3, 58 cases) liver tissue specimens. Clinicopathological information is available at the manufacturer’s website (http://www.tissue-array.co.kr/). The slides were baked at 60 °C for 30 min, deparaffinized with xylene and dehydrated with ethanol. The slides were subjected to antigen retrieval and incubated with blocking solution to prevent nonspecific antibody binding, followed by incubation with anti-PRKCSH antibody overnight at 4 °C. After counterstaining with hematoxylin, the sections were dehydrated and mounted. Staining intensity of the PRKCSH protein was measured using the NIH ImageJ software with the IHC Profiler plugin (http://rsb.info.nih.gov/ij/\textsuperscript{166}). Staining intensity of PRKCSH is shown as the inverted median pixel value (IMPV). The IMPV of PRKCSH was determined by HCS analysis of liver tissues. Liver tissue array slides were purchased from SuperBioChips Laboratories and used to determine the levels of PRKCSH by IHC analysis. The slides contained non-tumor (CSN3, 59 cases) and tumor (CS3, 58 cases) liver tissue specimens. Clinicopathological information is available at the manufacturer’s website (http://www.tissue-array.co.kr/). The slides were baked at 60 °C for 30 min, deparaffinized with xylene and dehydrated with ethanol. The slides were subjected to antigen retrieval and incubated with blocking solution to prevent nonspecific antibody binding, followed by incubation with anti-PRKCSH antibody overnight at 4 °C. After counterstaining with hematoxylin, the sections were dehydrated and mounted. Staining intensity of the PRKCSH protein was measured using the NIH ImageJ software with the IHC Profiler plugin (http://rsb.info.nih.gov/ij/\textsuperscript{166}). Staining intensity of PRKCSH is shown as the inverted median pixel value (IMPV). The IMPV of PRKCSH was determined by HCS analysis.
(ThermoFisher Scientific, 4309155) following the manufacturers’ protocols. To investigate splicing pattern of XBP1 mRNA, we also performed semiquantitative PCR on an XPer Thermal Cycler System (BIOER Technology, Hangzhou, China). Unprocessed images of all agarose gels are provided in Supplementary Fig. 10. All investigate splicing pattern of XBP1 mRNA, we also performed semiquantitative scopy46. For PRKCSH silencing, cells were transfected with siPRK or siCon. At 24 h post-transfection, cells were treated with doxycycline (5 μM) for 24 h to induce expression of IRE1α-FV, followed by treatment with TM for 4 h. Cells were fixed with 3.7% paraformaldehyde (Electron Microscopy Sciences, 15710) for 15 min at room temperature. Knockdown of PRKCSH in this cell was evaluated by immunocytochemistry with anti-PRKCSH antibody. For stable L02-PRK cells, cells grown on coverslips were transfected with IRE1α-FV. At 24 h post-transfection, cells were treated with doxycycline (5 μM) for 24 h to induce expression of IRE1α-FV, followed by treatment with TM for 4 h. Cells were fixed with 3.7% paraformaldehyde for 15 min at room temperature. Oligomerization of IRE1α was observed using a Carl Zeiss Axiovert 200 fluorescence microscope and analyzed with the software supplied by the manufacturer (Carl Zeiss). The number of IRE1α foci per cell was determined by counting more than 100 cells with fluorescent IRE1α. Data were presented as percentage of cells with IRE1α foci among all cells with fluorescent IRE1α.

To examine the association of free or oligomerized IRE1α with the proteasome, cells grown on coverslips were transfected with IRE1α-FV. At 16 h post-transfection, cells were treated with doxycycline (5 μM) for 24 h to induce IRE1α-FV expression, followed by treatment with TM for 4 h. Cells were fixed with 3.7% paraformaldehyde for 15 min at room temperature. After washing with PBS, cells were probed with anti-205 proteasome α4 antibody overnight at 4 °C. After three washes with PBS, the cells were incubated with Alexa Fluor 546-conjugated anti-mouse IgG secondary antibody at room temperature for 40 min.

**Immunocytochemistry.** To determine PRKCSH expression upon its knockdown, cells were seeded on coverslips and transfected with siPRK or siCon. At 48 h post-transfection, cells were fixed with 3.7% paraformaldehyde for 15 min at room temperature. After washing with PBS, cells were probed with anti-PRKCSH antibody at 4 °C overnight. After three washes with PBS, the cells were incubated with Alexa Fluor 546-conjugated anti-mouse IgG secondary antibody at room temperature for 40 min. Fluorescence images were obtained using a Carl Zeiss Axiovert 200 microscope and analyzed by using the supplier’s software. The intracellular localization of WT PRKCSH and its mutants were determined by indirect immunofluorescence analysis using anti-Calnexin (Cell signaling Technology, 2679) anti-Flag M2 antibodies (Sigma-Aldrich), or anti-PRKCSH (Santa Cruz Biotechnology).

**Subcellular fractionation.** Cells were lysed in buffer A (25 mM HEPES, pH 7.4, 50 mM KCl, 1 mM EDTA, 0.5% NP-40, 1 mM DTT) supplemented with a protease inhibitor cocktail (ThermoFisher Scientific). After incubation on ice for 30 min, the samples were separated by centrifugation. Supernatants were collected as cytosolic fraction. The pellets were washed with buffer B (buffer A without NP-40) and the recovered nuclear fraction was washed with buffer C (buffer B containing 450 mM KCl and 50% glycerol). Tubulin (Cell Signaling Technology, 2144) was used as a cytosolic marker and lamin A/C (Cell Signaling Technology, 2032) as a nuclear marker.

**Co-IP assay.** Nontransfected cells or cells transiently transfected with individual constructs were lysed with immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 7.2, 10% NP-40, 50 mM NaCl), and a protease inhibitor cocktail (ThermoFisher Scientific, 78441)). The lysate was precleared with protein A agarose (Sigma-Aldrich, 11314515001) at 4 °C for 1 h and incubated with anti-IRE1α (Cell Signaling, 1:50), anti-Flag M2 (Sigma-Aldrich, 1:50), or normal rabbit IgG antibody (Cell Signaling, Cat.2729, 1:50) at 4 °C overnight, and then with protein A agarose at 4 °C for 4 h. The agarose was washed three times with lysis buffer and boiled in 1× sample buffer. Boiled samples were submitted to immunoblot analysis. The manufacturer’s instructions. Cells were analyzed with a FACScalibur flow cytometer (BD Biosciences), and cellular DNA content and forward scatter were analyzed with FCS Express 6 Plus software (De Novo Software) or WinMDI2.9 software. To determine PARP1 cleavage, cells were prepared as above, and then immunoblots analyses were performed with anti-PARP1 antibody as recommended by the supplier.

**Cell survival and in vivo tumor growth assay.** Cell survival assay was performed for determining the long-term overall survival of L02-PRK or Huh-PRK KO cells9,70. Totals, 2 × 10⁶ cells/mL were seeded in 6-well plate and treated with 10 μg/mL TM, or exposed to Earle’s balanced salt solution for 48 h. Cells were washed and trypsinized. A total of 1 × 10⁶ cells/mL were replated into 6-well plate and cultured for 5 days in complete DMEM media supplemented with 10% fetal bovine serum. Cells were washed three times in PBS, fixed with 3.7% paraformaldehyde, stained with crystal violet for 30 min, and then washed three times with water. Survival rate was calculated by cell number/mm³.

In vivo tumor growth experiment was performed in accordance with the guidelines of the Committee for Animal Experiments of Konkuk University. All animal protocols were approved by the Institutional Animal Care and Use Committee of Konkuk University (Protocol no. KU19032). Male BALb/c nude mice (6–7 weeks old, 25 g) were purchased from Orient Bio (Seongnam, Republic of Korea). The Huh-PRK KO or WT cells (5 × 10⁵ cells per 0.1 mL Hank’s Balanced Salt Solution) were injected subcutaneously in the right groin. The mice were monitored daily and tumor sizes were measured every 2–3 days by a digital caliper, and tumor volumes were calculated using the formula V = π/6 (L × l²), where W and l are tumor width and length, respectively.

**Statistical analysis.** All experiments were repeated at least three times. Data were expressed as mean ± SEM. Differences between two groups were analyzed by Student’s t test. Multigroup comparisons were performed by one-way analysis of variance (ANOVA). Immunohistochemistry staining of tissue array was assessed using the chi-square test. Prognoses for patients with hepatocellular carcinoma were estimated by using Kaplan–Meier survival analyses with the log-rank test. Correlations between PRKCSH and UPR gene expression in various cancer tissues were assessed using Pearson’s rank correlation coefficient. The differences between the groups in cancer tissues were compared using the unpaired or paired two-tailed Student’s t test. Statistical analyses were performed and graphs were plotted using the GraphPad Prism software (version 6, GraphPad Software, Inc.). P values <0.05 were considered statistically significant.

**Data availability**

All data supporting the findings of this study are available within the article and its Supplementary Information files and from the corresponding author upon reasonable request. TCGA datasets of gene expression in human tumors are accessible using the GEO web server. Additional gene expression datasets were obtained for liver cancers (GSE25097 and GSE20140), colon cancer (GSE10950), gastric cancer (GSE13861), breast cancer (GSE24124), and lung cancer (GSE27262). A reporting summary for this article is available as a Supplementary Information file.

Received: 1 February 2018 Accepted: 12 June 2019

Published online: 18 July 2019

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Acknowledgements
The authors gratefully acknowledge Dr. Nozomu Kono (University of Tokyo) for providing the IRE1-FV construct. We also gratefully acknowledge Dr. Randal Kaufman for providing the hIRE1a.pCD construct. This study was supported by the National Research Foundation of Korea (NRF) grants funded by the Korea government (NRF-2015R1D1A1A01057281, NRF-2017R1A2B3006335, NRF-2018D1A1B07044129, and NRF-2016R1A5A202284), and by the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare (No. HI17C0874).

Author contributions
G.C.S. and K.H.K. designed the experiments and analyzed the data; G.C.S., S.U.M., H.S.C., and H.D.H. performed the experiments; H.S.K. analyzed the bioinformatic and statistical data. All authors discussed the results and analysis; G.C.S. and K.H.K. co-wrote the paper, with contributions from all authors.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-11019-w.

Competing interests: The authors declare no competing interests.

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Peer review information: Nature Communications thanks Constantinos Koumenis and other anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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