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## Modulation of the Proteostasis Network Promotes Tumor Resistance to Oncogenic KRAS Inhibitors

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## Abstract

Despite significant advances in targeting mutant KRAS, tumor resistance to KRAS inhibitors (KRASi) remains a major barrier to progress. Here, we report proteostasis reprogramming as a key convergence point of multiple KRASi-resistance mechanisms. Inactivation of oncogenic KRAS downregulated both the heat shock response and the IRE1 $\alpha$  branch of the unfolded protein response, causing severe proteostasis disturbances. However, IRE1 $\alpha$  was selectively reactivated in an ER stress-independent manner in acquired KRASi-resistant tumors, restoring proteostasis. Oncogenic KRAS promoted IRE1 $\alpha$  protein stability via ERK-dependent phosphorylation of IRE1 $\alpha$ , leading to IRE1 $\alpha$  disassociation from HRD1 E3-ligase. In KRASi-resistant tumors, both reactivated-ERK and hyperactivated-AKT restored IRE1 $\alpha$  phosphorylation and stability. Suppression of IRE1 $\alpha$  overcame resistance to KRASi. This study reveals a druggable mechanism that leads to proteostasis reprogramming and facilitates KRASi resistance.

## One Sentence Summary:

ER stress-independent unconventional phosphorylation of IRE1 $\alpha$  is a convergence point of multiple KRAS inhibitor resistance mechanisms.

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*KRAS* is one of the most frequently mutated genes in human cancer, especially in pancreatic ductal adenocarcinoma (PDAC), non-small cell lung cancer (NSCLC), and colorectal carcinoma (CRC) (1–5). Oncogenic *KRAS* engages multiple effector pathways to drive tumorigenesis, notably the RAF/MEK/ERK (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways (6–10). Small molecules that directly target the *KRAS*<sup>G12C</sup> mutation, including sotorasib and adagrasib (11–13), have shown encouraging therapeutic efficacies in clinical trials (14–16). The US Food and Drug Administration (FDA) granted accelerated approval for sotorasib and adagrasib to treat patients with *KRAS*<sup>G12C</sup>-mutant NSCLC. However, resistance to these *KRAS* inhibitors is almost inevitable resulting from the activation of compensatory pathways, e.g. EGF, FGF, AURKA, or SOS1, or the acquisition of new mutations, e.g. *KRAS*, *NRAS*, *BRAF*, *EGFR*, or *FGFR2* (15–35). Although inhibitors targeting *KRAS* mutations other than G12C are in clinical trials (36), similar bypass of *KRAS* dependence has been demonstrated in preclinical studies using genetically engineered mouse models (GEMMs) of lung and pancreatic cancer (37–39). Due to these clinical challenges, understanding the mechanisms that mediate the resistance to *KRAS* inhibitors is imperative to develop more effective therapies to prevent the recurrence of *KRAS*-driven cancers.

The ability to overcome an imbalanced protein homeostasis or “proteostasis” network is instrumental to maintain cancer cell survival and circumvent various insults that impair

the quality of protein synthesis and folding (40, 41). Cells use compartment-specific stress sensors to monitor and maintain a high-fidelity proteome. Cytosolic proteins are monitored by the heat shock response (HSR) (42), whereas transmembrane and secreted proteins are monitored in the endoplasmic reticulum (ER) by the unfolded protein response (UPR) (43–45). When the HSR is triggered by stress stimuli, heat shock factors (HSFs) become activated and transactivate genes encoding chaperones and other factors of the proteostasis network (46). HSF1 is a master regulator of the proteotoxic stress response and has been implicated in mediating proteome fidelity in cancer cells (47). The UPR is a three-branched stress response that is activated upon disruption of ER homeostasis. The UPR is mediated by the ER transmembrane sensors inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ), activated transcription factor 6 (ATF6), and protein kinase RNA-like ER kinase (PERK) (48). IRE1 $\alpha$  is the most ancient and conserved member of the mammalian UPR sensory triad (49, 50). Under ER stress conditions, IRE1 $\alpha$  undergoes oligomerization and *trans*-autophosphorylation to activate its RNase domain. This results in excision of 26 nucleotides from unspliced *XBPI* (*XBPIu*) mRNA, and a frame shift mutation to produce the mature, spliced *XBPI* (*XBPIs*) encoding a potent transcriptional activator (fig. S1A) (51, 52). Mutant RAS was traditionally viewed as an inducer of general UPR through stressing ER during oncogenic transformation of non-malignant cells (53). Genetic screens revealed the synthetic lethal interaction between mutant RAS and IRE1 $\alpha$  in yeast (54). Yet it remains unclear how the proteostasis network is orchestrated by oncogenic KRAS, or how the proteostasis reprogramming mechanisms occur that bypass KRAS addiction and allow for acquired resistance to KRAS inhibitors.

Here, we report that proteostasis is dynamically altered upon oncogenic KRAS inhibition. We identified the IRE1 $\alpha$ -mediated reprogramming of proteostasis as an essential mechanism that facilitates resistance to KRAS–MAPK inhibition. Importantly, we elucidated the biochemical basis for the ER stress–independent post-translational modification and regulation of IRE1 $\alpha$  by both oncogenic KRAS signaling and KRASi resistance signaling, which serves as a therapeutic vulnerability that can be targeted to overcome resistance to KRAS–MAPK inhibition.

## Oncogenic KRAS Inactivation Reprograms Proteostasis

To understand the impacts of oncogenic KRAS on proteostasis, we used primary mouse PDAC cells derived from our previously generated, doxycycline (Dox)-inducible, *Kras*<sup>G12D</sup>-driven PDAC mouse model (55), which are hereafter designated as *iKras* cells. Dox withdrawal turned off *Kras*<sup>G12D</sup> expression in *iKras* cells (fig. S1B), resulting in the inactivation of downstream MAPK signaling (fig. S1C), decreased 5-bromodeoxyuridine (BrdU) incorporation (fig. S1D) and increased cell apoptosis (fig. S1E). We monitored protein aggregation upon *Kras*<sup>G12D</sup> inactivation using PROTEOSTAT, a molecular rotor dye that specifically intercalates into the cross-beta spines of the quaternary protein structures found in misfolded and aggregated proteins (Fig. 1A). As a positive control, the treatment of *iKras* cells with the proteasome inhibitor MG132 significantly induced PROTEOSTAT signal (fig. S1F). The inactivation of *Kras*<sup>G12D</sup> by Dox withdrawal induced protein aggregation (Fig. 1, B and C). However, 30 days after Dox withdrawal, *iKras* cells displayed restored proteostasis (Fig. 1, B and C) and resumed cell growth (fig. S1D, E and G), at levels

comparable to those observed for parental cells (*iKras<sup>P</sup>*). These cells resistant to *Kras<sup>G12D</sup>* inactivation are hereafter designated as *iKras<sup>R</sup>* cells. Continuous *Kras<sup>G12D</sup>* inactivation was required to maintain the resistance phenotypes as reactivation of *Kras<sup>G12D</sup>* for extended periods (12 days) partially reversed the resistance of *iKras<sup>R</sup>* cells to Dox withdrawal (fig. S1, H to K). Dox administration had no impact on cell growth or proteostasis in *LSL-Kras<sup>G12D</sup>* cells lacking Dox-inducible *Kras<sup>G12D</sup>* expression (fig. S1, L to P). In the *iKras* cells, staining with Congo red (CR) or thioflavin T (ThT), which recognize misfolded protein aggregates (56), or detection of lysine 48 (K48)-linked polyubiquitin levels, which tag misfolded or aggregated proteins for proteasome-mediated degradation (57), independently confirmed the restoration of proteostasis upon acquired resistance to *Kras<sup>G12D</sup>* inactivation (fig. S1, Q to S).

We also used the *iKras* GEMM to examine *in vivo* proteostasis reprogramming (55). *Kras<sup>G12D</sup>* inactivation by Dox withdrawal resulted in rapid tumor regression, but 70% of tumors relapsed after 9–47 weeks (37). Notably, PROTEOSTAT staining of *iKras* GEMM tumors at different stages of *Kras<sup>G12D</sup>* inactivation revealed increased protein aggregation in regressing parental tumors and restored proteostasis in relapsed tumors (Fig. 1, D and E, and fig. S2A), which grow independently of *Kras<sup>G12D</sup>* expression.

To further investigate the impacts of oncogenic KRAS inhibition on proteostasis, we used a *KRAS<sup>G12C</sup>* inhibitor, sotorasib (12), to treat MIA-PaCa-2 human PDAC cells and H358 human NSCLC cells harboring the *KRAS<sup>G12C</sup>* mutation. Sotorasib effectively suppressed the activation of MEK/ERK (fig. S2, B and C), leading to cell growth inhibition in both MIA-PaCa-2 and H358 cell lines (fig. S2, D and E). Both MIA-PaCa-2 and H358 cells also displayed increased PROTEOSTAT staining and K48-linked polyubiquitination levels in response to sotorasib treatment, suggesting enhanced protein aggregation (Fig. 1F and fig. S2, F to H). However, after MIA-PaCa-2 and H358 cells gained resistance to sotorasib (fig. S2, D and E), they also exhibited restored proteostasis (Fig. 1F and fig. S2, F to H). Similar as *iKras<sup>R</sup>* cells, continuous sotorasib treatment was required to maintain the resistance of MIA-PaCa-2<sup>R</sup> cells to KRASi (fig. S2, I and J). MIA-PaCa-2 xenograft tumors were initially sensitive to sotorasib treatment but relapsed after 6 weeks (fig. S2K). PROTEOSTAT staining revealed increased protein aggregation after initial sotorasib treatment and restored proteostasis in relapsed tumors (Fig. 1, G and H). These data demonstrate that oncogenic KRAS inhibition induces protein aggregation and severely disrupts proteostasis. However, KRASi-resistant cancer cells able to grow in the absence of mutant KRAS gain the capacity to overcome associated proteotoxic stress and regain proteostasis.

## Oncogenic KRAS Inactivation Differentially Impacts the Key Nodes of Proteostasis Regulatory Network

Proteostasis is maintained by an integrated network that includes translation, protein quality control mechanisms that regulate the content and quality of the proteome, and protein degradation pathways, such as the ubiquitin/proteasome system and the autophagy/lysosome system, which eliminate misfolded or aggregated proteins (42, 43, 58). *Kras<sup>G12D</sup>* inactivation by Dox withdrawal in *iKras* cells did not alter overall proteasomal activity

(fig. S3, A and B). We observed increased LC3 cleavage and LAMP1 levels upon *Kras*<sup>G12D</sup> inactivation (fig. S3C), indicating elevated autophagy and lysosomal activities, which unlikely caused increased protein aggregation. Next, we examined the protein quality control and stress response pathways that monitor and regulate protein folding, including the UPR and HSR. *Kras*<sup>G12D</sup> inactivation in *iKras*<sup>P</sup> cells markedly reduced IRE1 $\alpha$  protein levels, *Xbp1* splicing, and the expression levels of the XBP1s targets *Edem1* and *Sec61a1* (Fig. 2A and fig. S3, D to F). As a control, Dox treatment had no impacts on IRE1 $\alpha$  levels in the constitutively activated *LSL-Kras*<sup>G12D</sup> cells (fig. S3G). By contrast, ATF6 was barely affected, and phospho-PERK was negatively correlated with IRE1 $\alpha$ /XBP1 in response to *Kras*<sup>G12D</sup> inactivation (Fig. 2A). Resolving protein aggregation with tauroursodeoxycholic acid (TUDCA), a chemical chaperone that promotes protein folding and stimulates molecular chaperone function (59, 60), completely blunted *Kras*<sup>G12D</sup> inactivation-induced phospho-PERK (fig. S3, H to J), indicating that PERK was activated as a result of the disrupted proteostasis by KRASi. PERK is one of the four kinases (GCN2, PERK, HRI and PKR) that phosphorylate eIF2 $\alpha$  and regulate the Integrated Stress Response (ISR) (61). Unexpectedly, eIF2 $\alpha$  and its downstream ATF4 followed an opposite pattern as that of phospho-PERK upon *Kras*<sup>G12D</sup> inactivation (Fig. 2A). *Perk* deletion had no effects on their levels (fig. S3K). Genetic deletion of each ISR kinases demonstrated that phospho-eIF2 $\alpha$  and ATF4 were dependent on GCN2 which was activated in *iKras*<sup>P</sup> cells (Fig. 2A and fig. S3K). These data establish GCN2 as a regulator of ISR in this context. Notably, IRE1 $\alpha$ /XBP1, but not GCN2 or eIF2 $\alpha$ , was selectively restored in *iKras*<sup>R</sup> cells (Fig. 2A and fig. S3, D to F). Consistently, sotorasib treatment reduced IRE1 $\alpha$  protein levels in MIA-PaCa-2 and H358 cells, but IRE1 $\alpha$  was restored following the acquisition of sotorasib resistance (Fig. 2, B and C, and fig. S2, D and E). IRE1 $\alpha$  immunostaining in the parental, *Kras*<sup>G12D</sup>-extincted, relapsed *iKras* GEMM tumors (Fig. 2D), and sotorasib-treated MIA-PaCa-2 human xenograft tumors (Fig. 2E) all independently confirmed the *in vivo* pattern of acute IRE1 $\alpha$  suppression followed by reactivation in relapsed tumors. We also confirmed the continuous *in vivo* suppression of phospho-GCN2, phospho-eIF2 $\alpha$ , and ATF4 by sotorasib treatment in MIA-PaCa-2 xenograft tumors (fig. S3L). Collectively, these data show that acute oncogenic KRAS inactivation inhibits the IRE1 $\alpha$  branch of the UPR and GCN2-regulated ISR. However, only IRE1 $\alpha$  is reactivated in the KRASi-resistant tumors.

In contrast with our findings on IRE1 $\alpha$ , sotorasib continuously suppressed HSF1 phosphorylation at serine residue 326 (S326), which is required for HSF1 activation (62), in both parental and sotorasib-resistant MIA-PaCa-2 and H358 cells (Fig. 2, B and C). The inhibitory phosphorylation of HSF1 at S121 (62) was increased in both sotorasib-resistant MIA-PaCa-2<sup>R</sup> and H358<sup>R</sup> cells (Fig. 2, B and C). As a result, sotorasib treatment markedly reduced the expression of HSF1 target genes, including *HSPA6* and *HSPA1B*, in both parental and sotorasib-resistant MIA-PaCa-2 and H358 cells (fig. S3, M and N). We confirmed the *in vivo* suppression of HSF1-S326 phosphorylation by sotorasib treatment in MIA-PaCa-2 human xenograft tumors (Fig. 2E) and marked reduction of HSF1 luciferase reporter activities in sotorasib-resistant MIA-PaCa-2<sup>R</sup> cells (fig. S3, O and P). The genetic inactivation of *Kras*<sup>G12D</sup> also resulted in the considerable reduction of HSF1 luciferase reporter activities in both *iKras*<sup>P</sup> and *iKras*<sup>R</sup> cells (Fig. 2F). Collectively, these data

demonstrate that oncogenic KRAS inactivation initially impairs both the ER and cytosolic protein quality control machinery, as well as the ISR. However, only IRE1 $\alpha$ /XBP1, but not HSF1 or ISR, is restored in the KRASi-resistant tumors.

## IRE1 $\alpha$ /XBP1 is Required for Maintaining Proteostasis in KRASi-Resistant Cancer Cells

Next, we examined the necessity of IRE1 $\alpha$ /XBP1 for proteostasis maintenance. In *iKras<sup>P</sup>* cells, *Ire1a* and *Xbp1* knockdown modestly induced protein aggregation (Fig. 2, G and H, and fig. S4A), with minimal effects on cell growth (Fig. 2I). By contrast, *Ire1a* and *Xbp1* knockdown in the *iKras<sup>R</sup>* cells led to profound protein aggregation and PERK phosphorylation (Fig. 2, G and H, and fig. S4, A to E), and significantly inhibited cell growth (Fig. 2I, and fig. S4F). These effects were rescued by TUDCA (Fig. 2, G to I, and fig. S4B), demonstrating the importance of IRE1 $\alpha$ /XBP1 in the maintenance of proteostasis and cell survival. Depletion of *Perk* did not affect proteostasis and cell growth of *iKras<sup>R</sup>* cells and had no impacts on *Ire1a*-depletion induced phenotypes (fig. S4, G to K), suggesting that PERK is dispensable for KRASi-resistant cancer cells. The kinase activity of IRE1 $\alpha$  is required for its RNase activation (63). In contrast to WT IRE1 $\alpha$ , neither kinase-dead IRE1 $\alpha$ <sup>K599A</sup> nor RNase-dead IRE1 $\alpha$ <sup>K907A</sup> mutant (63) was able to rescue *Ire1a*-depletion induced phenotypes in *iKras<sup>R</sup>* cells (fig. S5, A to D), suggesting that IRE1 $\alpha$ 's function depends on its catalytic RNase activity. In addition to XBP1s, IRE1 $\alpha$  RNase also cleaves ER-localized RNAs through Regulated IRE1 $\alpha$ -Dependent Decay (RIDD) pathway (64, 65). Although some RIDD targets were regulated by IRE1 $\alpha$  (fig. S5, E and F), restoration of XBP1s completely rescued the *Ire1a* depletion-induced protein aggregation and cell growth defects in *iKras<sup>R</sup>* cells (fig. S5, G to I). These data establish that IRE1 $\alpha$  RNase activity and RNase-dependent *XBP1* splicing drives proteostasis in KRASi-resistant cancer cells. Consistently, CRISPR-mediated *Ire1a* or *Xbp1* knockout (fig. S5J) resulted in more severe protein aggregation in *iKras<sup>R</sup>* cells than in *iKras<sup>P</sup>* cells (fig. S5K). Taken together, we demonstrate that IRE1 $\alpha$ /XBP1 is indispensable for the maintenance of balanced proteostasis in KRASi-resistant cancer cells.

## The MAPK Pathway Regulates IRE1 $\alpha$ /XBP1 in Parental KRAS-Driven Cancer Cells

We aimed to determine the mechanism through which oncogenic KRAS regulates IRE1 $\alpha$ . MAPK and PI3K are two major effector pathways downstream of oncogenic KRAS (6). The MEK inhibitor trametinib and the ERK inhibitor SCH772984 both substantially reduced IRE1 $\alpha$  protein levels in *iKras<sup>P</sup>* cells (Fig. 3A). By contrast, the PI3K inhibitor pictilisib and the AKT inhibitor MK2206 had little impact on IRE1 $\alpha$  levels in *iKras<sup>P</sup>* cells (Fig. 3B). Examination of five additional *KRAS*-mutant cell lines confirmed that MEK/ERK inhibitors, but not PI3K/AKT inhibitors, reduced IRE1 $\alpha$  protein levels (fig. S6, A to C). These effects were similar to what was observed in response to the genetic or pharmacological inhibition of oncogenic KRAS (Fig. 3F, and fig. S6D). Recent studies show that SHP2 is critical for KRAS<sup>G12C</sup> cycling and ERK activation (30, 33, 66–69). Inhibition of SHP2 with SHP099 significantly suppressed ERK activity and reduced IRE1 $\alpha$

levels in *KRAS*<sup>G12C</sup>-mutant H358 cells (fig. S6E), whereas modest effect was observed in *KRAS*<sup>G12D</sup>-mutant *iKras* cells due to the limited intrinsic GTPase activity of *KRAS*<sup>G12D</sup> (fig. S6F). SHP099 also inhibited the growth of sotorasib-resistant H358<sup>R</sup> cells and MIA-PaCa-2<sup>R</sup> cells (fig. S6, G and H). Long-term treatment of H358 cells with SHP099 led to drug resistance and recovered proteostasis (fig. S6, I to K), accompanied with IRE1 $\alpha$  restoration in the resistant cells (fig. S6L). Depletion of *IRE1 $\alpha$*  in SHP2i-resistant cells resulted in marked protein aggregation and re-sensitized the cells to SHP099 (fig. S6, M to O). Another upstream activator of RAS signaling is EGFR, suppression of EGFR with gefitinib significantly reduced ERK and IRE1 $\alpha$  levels in H358 cells (fig. S6P). In contrast, inhibition of MEK/ERK in non-malignant BEAS-2B lung epithelial cells with non-oncogenic RAS signaling barely affected IRE1 $\alpha$  levels (fig. S6, Q and R). Using tissue microarrays, we found that IRE1 $\alpha$  levels correlated with phospho-ERK levels in treatment-naïve PDAC patient samples (Fig. 3, C and D), and high expression of IRE1 $\alpha$  was associated with higher histologic tumor grade (Fig. 3E). Collectively, these data demonstrate that oncogenic *KRAS*-mediated MAPK pathway activation leads to the activation of IRE1 $\alpha$  in parental *KRAS*-mutant cancers.

### MAPK Promotes IRE1 $\alpha$ Protein Stability by Inhibiting SEL1L/HRD1-mediated IRE1 $\alpha$ Ubiquitination

Sotorasib treatment did not downregulate *IRE1 $\alpha$*  mRNA levels (fig. S7A), but it considerably reduced IRE1 $\alpha$  protein abundance in H358 cells (Fig. 3F). Treatment with the proteasome inhibitor MG132 rescued both sotorasib- and trametinib-induced reductions in IRE1 $\alpha$  protein levels in H358 cells (Fig. 3F). Similarly, the observed reduction in IRE1 $\alpha$  protein levels in response to *Kras*<sup>G12D</sup> inactivation, trametinib, or SCH772984 treatment could be rescued by MG132 in *iKras*<sup>P</sup> cells (fig. S7, B and C). These data demonstrate that the inhibition of oncogenic *KRAS* or MAPK promotes proteasome-mediated IRE1 $\alpha$  degradation in parental *KRAS*-mutant cancers.

IRE1 $\alpha$  is a bona-fide substrate of the SEL1L/HRD1 ER-associated degradation (ERAD) complex (70), which is composed of the E3 ubiquitin ligase HRD1 and its adapter protein SEL1L (71). The SEL1L/HRD1 complex ubiquitinates and targets IRE1 $\alpha$  for proteasomal degradation in multiple cell types (70, 72, 73). Sotorasib treatment significantly enhanced the association between IRE1 $\alpha$  and the SEL1L/HRD1 complex (Fig. 3G), resulting in increased IRE1 $\alpha$  ubiquitination in H358 cells (Fig. 3H). Sotorasib also promoted the interaction of IRE1 $\alpha$  with p97 and NPL4 (fig. S7, D and E), which deliver the ubiquitinated ERAD substrates to the proteasome for degradation (74). *SEL1L* knockdown reduced sotorasib- or trametinib-induced IRE1 $\alpha$  ubiquitination and restored IRE1 $\alpha$  protein levels (Fig. 3, H and I, fig. S7F), leading to the prevention of *KRAS*-MAPK inhibition induced protein aggregation (fig. S7G). Inhibition of p97 with CB5083 (75) also rescued sotorasib-induced IRE1 $\alpha$  degradation (fig. S7I). Consistently, *Sel1l* or *Hrd1* depletion in *iKras*<sup>P</sup> cells blocked the induction of IRE1 $\alpha$  degradation and prevented protein aggregation in response to *Kras*<sup>G12D</sup> extinction and trametinib or SCH772984 treatment (fig. S7, J to P). These data demonstrate that oncogenic *KRAS*-mediated MAPK activation stabilizes IRE1 $\alpha$  protein by preventing SEL1/HRD1-mediated ubiquitination and subsequent proteasomal degradation.

## ERK Directly Interacts with and Phosphorylates IRE1 $\alpha$

Oncogenic KRAS-MAPK did not directly regulate ERAD complex expression in *iKras*<sup>P</sup> or H358 cells (Fig. 3I and fig. S7, J, M and Q). It is well established that phosphorylation often interferes with protein-protein interactions and thus regulates protein ubiquitination and stability. We tested whether MAPK might promote IRE1 $\alpha$  phosphorylation, resulting in IRE1 $\alpha$  disassociation from the SEL1L/HRD1 complex. Indeed, the expression of a constitutively activated MEK construct (MEK<sup>DD</sup>) dramatically enhanced IRE1 $\alpha$  phosphorylation in 293T cells detected by an anti-phospho-MAPK substrate motif antibody (fig. S8A). In contrast, sotorasib treatment significantly reduced IRE1 $\alpha$  phosphorylation levels compared with control H358 cells (Fig. 4A). Co-immunoprecipitation (co-IP) assay demonstrated that ERK interacted with IRE1 $\alpha$  in 293T cells and H358 cells (Fig. 4B, and fig. S8B). Furthermore, GST pull-down and *in vitro* kinase assays confirmed that both ERK1 and ERK2 directly interacted with and phosphorylated IRE1 $\alpha$  *in vitro* (Fig. 4, C and D, and fig. S8, C to H). Depletion of *ERK1* or *ERK2* in H358 cells revealed that both ERK1 and ERK2 regulated IRE1 $\alpha$  phosphorylation (fig. S8I). IRE1 $\alpha$  possesses three putative ERK binding D-motifs (Fig. 4E) (76). Deletion of the D-motif at amino acids 687–701 largely disrupted the binding between ERK and IRE1 $\alpha$  (fig. S8J). Collectively, these data demonstrate that ERK directly interacts with and phosphorylates IRE1 $\alpha$ .

Sequence analysis showed that human IRE1 $\alpha$  contains four putative ERK phosphorylation sites at S525, S529, S549, and T973, consistent with the minimal ERK substrate motif pS/T\*P (Fig. 4E). Mass spectrometry analysis of IRE1 $\alpha$  protein purified from control or MEK<sup>DD</sup>-expressing 293T cells confirmed the ERK-dependent phosphorylation of IRE1 $\alpha$  at S525, S529, S549, and T973 (fig. S9, A to C). We mutated the identified phospho-serine or -threonine amino acids (aa) to alanine (A) and performed an *in vitro* kinase assay using [ $\gamma$ -<sup>32</sup>P] ATP. The kinase-dead form of IRE1 $\alpha$ <sup>K599A</sup> was used as a backbone to exclude the effects of IRE1 $\alpha$  autophosphorylation. ERK was able to directly phosphorylate kinase-dead autophosphorylation-deficient IRE1 $\alpha$ <sup>K599A</sup> (Fig. 4F). The T973A mutation significantly reduced but did not eliminate ERK-dependent IRE1 $\alpha$ <sup>K599A</sup> phosphorylation (Fig. 4F). Additional mutation of S525, S529 or S549 to A together with T973A further decreased the IRE1 $\alpha$  phosphorylation (Fig. 4F). The simultaneous mutation of all four sites largely abolished the ERK-dependent IRE1 $\alpha$ <sup>K599A</sup> phosphorylation (Fig. 4F). In agreement, mutation of these four S/T to A (designated as 4A mutant) diminished ERK-dependent phosphorylation on WT IRE1 $\alpha$  (Fig. 4G, and fig. S9D). The IRE1 $\alpha$  mutations did not affect IRE1 $\alpha$  binding with ERK (fig. S9, D and E). Collectively, these data identify S525, S529, S549 and T973 as ERK phosphorylation sites on IRE1 $\alpha$ . Importantly, analysis of Clinical Proteomic Tumor Analysis Consortium (CPTAC) datasets in patients with NSCLC (77) showed a statistically significant correlation between IRE1 $\alpha$ -S549 phosphorylation and ERK phosphorylation in treatment-naïve NSCLC patients (Fig. 4H, and fig. S9F). The peptides containing S525, S529, and T973 were not covered in the CPTAC datasets and could not be evaluated in patients.

To determine the functional significance of IRE1 $\alpha$  phosphorylation sites, we generated loss-of-function mutation for each site. Single site mutation was insufficient to promote IRE1 $\alpha$  interaction with HRD1 and did not alter IRE1 $\alpha$  levels in *iKras* cells (Fig. 4, I and



J). Simultaneous mutation of all four sites promoted IRE1 $\alpha$  interaction with HRD1 and its degradation (Fig. 4, I and J). *In vitro* pull-down assays confirmed that ERK-mediated IRE1 $\alpha$  phosphorylation reduced IRE1 $\alpha$  interaction with HRD1/SEL1L and that phospho-deficient IRE1 $\alpha^{4A}$  mutant bound to HRD1 regardless of ERK presence (fig. S10, A to E). By contrast, gain-of-function phospho-mimetic mutation for each individual site (S525D, S529D, S549D, or T973E) disrupted IRE1 $\alpha$  interaction with HRD1 (Fig. 4K), leading to the stabilization of IRE1 $\alpha$  protein in the absence of ERK (Fig. 4L). Similar effects were observed for IRE1 $\alpha^{SDTE}$  mutant with gain-of-function mutation for all four sites (Fig. 4, K and L, and fig. S10F). IRE1 $\alpha^{SDTE}$  was resistant to sotorasib- or SCH772984- promoted protein degradation in MIA-PaCa-2 cells (fig. S10, G and H). As a result, sotorasib failed to induce protein aggregation in IRE1 $\alpha^{SDTE}$  expressing MIA-PaCa-2 tumors (Fig. 5, A to C). These tumors became partially resistant to sotorasib-induced anti-tumor effects (Fig. 5D). In line with these data, the IRE1 $\alpha^{SDTE}$  mutant rescued *IRE1 $\alpha$* -depletion induced protein aggregation, phospho-PERK and cell growth defects in *iKras<sup>R</sup>* cells (fig. S10, I to L). Single-site phospho-deficient IRE1 $\alpha$  mutant also rescued these phenotypes due to the presence of the other three phosphorylated sites (fig. S10, L to O). The phospho-deficient IRE1 $\alpha^{4A}$  mutant failed to restore IRE1 $\alpha$  protein levels and was unable to rescue these phenotypes (fig. S10, L to O). Collectively, these data demonstrate that IRE1 $\alpha$  phosphorylation at S525, S529, S549, and T973 inhibits IRE1 $\alpha$  association with the ERAD complex, leading to enhanced stability, maintaining proteostasis.

A screen of 32 serine/threonine phosphatases in 293T cells revealed that expression of SCP3 significantly reduced MEK<sup>DD</sup>-induced IRE1 $\alpha$  phosphorylation (fig. S11, A to C). *In vitro* phosphatase assays and co-IP experiments confirmed that SCP3 interacted with and directly dephosphorylated IRE1 $\alpha$  (Fig. 5E, and fig. S11D). *Scp3* silencing increased IRE1 $\alpha$  phosphorylation and overexpression of SCP3 reduced IRE1 $\alpha$  phosphorylation in *iKras* cells (Fig. 5, F and G). Similarly, *SCP3* deletion significantly slowed down sotorasib-induced IRE1 $\alpha$  dephosphorylation in H358 cells (fig. S11E). These data identified SCP3 as the phosphatase regulating IRE1 $\alpha$  phosphorylation, although KRAS did not directly alter SCP3 levels or activities (fig. S11, F to I). Collectively, these analyses establish a mechanism of IRE1 $\alpha$  regulation by oncogenic KRAS.

## Multiple Pathways Converge to Reactivate IRE1 $\alpha$ in KRASi-Resistant Cancer Cells

Next, we sought to determine how IRE1 $\alpha$  evades oncogenic KRAS inhibition and determine the reactivation mechanism in KRASi-resistant cells. Oncogenic KRAS was efficiently suppressed by Dox withdrawal in *iKras<sup>R</sup>* cells (Fig. 2A, and fig. S1B), and most KRAS proteins were bound by sotorasib in sotorasib-resistant H358 (H358<sup>R</sup>) and MIA-PaCa-2 (MIA-PaCa-2<sup>R</sup>) cells, similar to observations in parental cells (Fig. 2, B and C). Furthermore, silencing of *KRAS* in H358<sup>R</sup> cells did not hinder IRE1 $\alpha$  reactivation (fig. S12A). These data exclude the possibility that the inefficient inhibition of oncogenic KRAS drives IRE1 $\alpha$  reactivation in these resistant cells. eIF2 $\alpha$  phosphorylation inhibits global protein synthesis (61, 78). Consistent with the inactivated phospho-eIF2 $\alpha$  in KRASi-resistant cells (Fig. 2A), we observed increased global protein synthesis in *iKras<sup>R</sup>* cells

evidenced by enhanced puromycin incorporation compared with that in *iKras<sup>P</sup>* cells (fig. S12B). However, inhibition of protein synthesis with cycloheximide did not affect IRE1 $\alpha$ /XBP1s in *iKras<sup>R</sup>* cells (fig. S12, C). Furthermore, ER stress sensing-deficient IRE1 $\alpha$ <sup>D2M</sup> mutant (79) was similarly restored in KRASi-resistant cells as that of WT IRE1 $\alpha$  (fig. S12, E and F), and successfully rescued *IRE1 $\alpha$* -depletion induced phenotypes (fig. S12, G to J). These data demonstrate that IRE1 $\alpha$  is reactivated in KRASi-resistant cells in an ER stress independent manner.

Recent studies report reactivated ERK and AKT as sotorasib-resistant mechanisms in patients (18, 20–23). Indeed, we observed the reactivation of phospho-ERK and the hyperactivation of phospho-AKT in sotorasib-resistant MIA-PaCa-2<sup>R</sup> and H358<sup>R</sup> cells compared with their respective parental cells (Fig. 2, B and C). Unexpectedly, the inhibition of reactivated ERK by SCH772984 treatment was insufficient to suppress IRE1 $\alpha$  levels in MIA-PaCa-2<sup>R</sup>, H358<sup>R</sup>, and *iKras<sup>R</sup>* cells (Fig. 5H), as well as in MIA-PaCa-2<sup>R</sup> and *iKras<sup>R</sup>* tumors *in vivo* (fig. S13, A and B). Similarly, the suppression of AKT by MK2206 treatment had no effect on IRE1 $\alpha$  levels in KRASi-resistant cells (Fig. 5H). However, the simultaneous suppression of both ERK and AKT successfully blunted IRE1 $\alpha$  reactivation in MIA-PaCa-2<sup>R</sup>, H358<sup>R</sup>, and *iKras<sup>R</sup>* cells (Fig. 5H), and in MIA-PaCa-2<sup>R</sup> and *iKras<sup>R</sup>* tumors *in vivo* (fig. S13, A and B). Consistently, the hyperactivation of either the MEK/ERK pathway, through expression of MEK<sup>DD</sup>, or the PI3K/AKT pathway, through the expression of constitutively active PIK3CA<sup>H1047R</sup> or myr-AKT, resulted in IRE1 $\alpha$  restoration in the absence of oncogenic KRAS in *iKras* cells (Fig. 5I and fig. S13C). Interestingly, myr-AKT also promoted WT, but not phospho-deficient 4A mutant, IRE1 $\alpha$  phosphorylation at serine and threonine residues (Fig. 5J), suggesting that these phosphorylation sites are regulated by both ERK and hyperactivated AKT in KRASi-resistant cells. In agreement, the activation of either MEK/ERK, through MEK<sup>DD</sup> expression, or the hyperactivation of AKT, through myr-AKT expression, was sufficient to disrupt the interaction of the SEL1L/HRD1 E3 ligase complex with IRE1 $\alpha$  (fig. S13D). By contrast, the simultaneous suppression of both ERK and AKT, but not ERK or AKT alone, significantly promoted IRE1 $\alpha$  interaction with the SEL1L/HRD1 E3 ligase complex in sotorasib-resistant MIA-PaCa-2<sup>R</sup> cells (fig. S13E). YAP1 also drives resistance of certain tumors to KRASi (37, 38). However, deletion of *Yap1* in *iKras<sup>R</sup>-YAP1* cells derived from YAP1-amplified GEMM tumors escaping KRAS<sup>G12D</sup> addiction did not impact IRE1 $\alpha$  and proteostasis (fig. S14, A to F). Instead, IRE1 $\alpha$  and proteostasis was dependent on ERK and AKT in these cells despite reduced ERK activity compared with that in parental *iKras* cells (fig. S14, G to J). Collectively, these data demonstrate that both reactivated ERK and hyperactivated AKT converge through IRE1 $\alpha$  phosphorylation at S525, S529, S549, and T973 to prevent ubiquitination-mediated proteasomal degradation of IRE1 $\alpha$  in KRASi-resistant cancer cells. Blocking either individual pathway is not sufficient to inhibit IRE1 $\alpha$  due to functional redundancy and compensation by the other pathway.

Next, we sought to understand the mechanism that activates ERK and AKT in the KRASi-resistant cells. Receptor tyrosine kinases (RTKs) activation is one of the most common mechanisms driving sotorasib resistance in patients (19–21) and RTKs are known to activate ERK and AKT (17, 18, 30, 80). Array analysis of 49 RTKs in parental and sotorasib-resistant H358 and MIA-PaCa-2 models revealed the induction of multiple and distinct sets

of RTKs in each model (fig. S15, A, B, and E). In H358 model, EGFR, ErbB2, ErbB3, FGFR3, and VEGFR2 were significantly induced in the resistant cells (fig. S15, A and B). Inhibiting these RTKs with combined sapitinib, AZD4547 and axitinib, but not individual inhibitor alone, completely suppressed ERK reactivation in H358<sup>R</sup> cells (fig. S15, C and D). Blocking FGFR3 with AZD4547 largely abolished AKT hyperactivation (fig. S15, C and D). These upregulated RTKs had to be simultaneously suppressed to completely blunt both ERK and AKT, resulting in the abrogation of IRE1 $\alpha$  restoration in H358<sup>R</sup> cells (fig. S15D). Similar to the H358 model, blocking multiple, but not individual, upregulated RTKs (including EGFR, ErbB2, VEGFR, PDGFRb and DDR2) completely suppressed both ERK and AKT, leading to the abrogation of IRE1 $\alpha$  restoration in MIA-PaCa-2<sup>R</sup> cell (fig. S15, F and G). Treatment of MIA-PaCa-2<sup>R</sup> tumors with combined RTK inhibitors, including sapitinib, axitinib and VU6015929, confirmed the inactivation of ERK and AKT *in vivo*, leading to the suppression of IRE1 $\alpha$ , marked induction of protein aggregation, and reduced tumor growth (fig. S15, H to K). However, they were not well tolerated in the tumor-bearing mice, causing rapid drop of body weight and early lethality (fig. S15L). Collectively, these data demonstrate that multiple and diverse sets of RTKs drive ERK and AKT activation in different KRASi-resistant tumors, which subsequently converge on IRE1 $\alpha$  to re-establish proteostasis.

## IRE1 $\alpha$ Inhibition Sensitizes Oncogenic KRAS-Driven Tumors to a MEK Inhibitor

Although the simultaneous suppression of the MAPK and PI3K pathways, or diverse upstream RTKs, effectively inhibits IRE1 $\alpha$ , the heterogeneous resistance mechanisms in different patients (18–23) and dose-limiting, on-target toxicity of these inhibitors (81, 82) limits their clinical applications for intervening in IRE1 $\alpha$ -mediated proteostasis reprogramming in treatment-resistant tumors. Therefore, we directly targeted the IRE1 $\alpha$ /XBP1 pathway in KRAS-driven cancers in combination with KRAS<sup>G12C</sup> or MEK inhibitor. Although MEK inhibitor trametinib or *Xbp1* knockout alone both modestly impeded *iKras* tumor growth *in vivo*, the loss of *Xbp1* significantly enhanced the response of *iKras* xenograft tumors to trametinib and induced marked protein aggregation (fig. S16, A to D). Treatment of *iKras* tumors with a highly selective IRE1 $\alpha$  RNase inhibitor, ORIN1001 (83–86), recapitulated the effects of the *Xbp1* knockout and markedly enhanced the sensitivity of the *iKras* tumors to trametinib treatment with significant induction of protein aggregation (fig. S16, A, and E to G). In a cohort of PDAC patient-derived xenograft (PDX) models (fig. S16N), ORIN1001 also significantly enhanced the sensitivity of the *KRAS*<sup>G12D</sup>-mutant PATC53 (Fig. 6, A to D), PATC148 (Fig. 6E, and fig. S16, H and I), PDAC35 (Fig. 6F, and fig. S16, J and K), SW1990 (Fig. 6, G and H, and fig. S16, L and M), and *KRAS*<sup>G12V</sup>-mutant PDAC19 PDX (Fig. 6I) tumors to trametinib treatment and potently induced protein aggregation in the combination therapy-treated tumors. Collectively, these *in vivo* data demonstrate that IRE1 $\alpha$ /XBP1 inhibition dramatically enhanced the response of *KRAS*-mutant PDAC tumors to trametinib treatment.

## IRE1 $\alpha$ Inhibition Enhances the Responses of KRAS<sup>G12C</sup>-Driven Tumors to Sotorasib

Next, we examined the effects of IRE1 $\alpha$  inhibition on the response to sotorasib treatment in *KRAS*<sup>G12C</sup>-driven tumors. *IRE1 $\alpha$*  silencing modestly reduced MIA-PaCa-2 xenograft tumor growth but not to the extent observed with sotorasib treatment (Fig. 7A and fig. S17A). However, IRE1 $\alpha$  deficiency significantly enhanced the response of these tumors to sotorasib treatment and considerably suppressed tumor relapse (Fig. 7A and fig. S17A). In contrast, *PERK* depletion had little impact on MIA-PaCa-2 tumor growth and response to sotorasib, as well as *IRE1 $\alpha$* -depletion induced tumor sensitivity to sotorasib (fig. S17, B to E), excluding the involvement of PERK in KRASi resistance. IRE1 $\alpha$  inhibition with ORIN1001 treatment combined with sotorasib treatment also resulted in complete MIA-PaCa-2 tumor regression and long-term remission (Fig. 7B, and fig. S17, F and G). We did not observe significant bodyweight changes or signs of toxicity in the combination treatment group (fig. S17, H and I). Treatment of non-KRAS addicted MIA-PaCa-2<sup>R</sup> tumors with ORIN1001 alone also substantially impeded the tumor growth (Fig. 7C), but did not result in complete response. The reduced efficacy with ORIN1001 alone was likely due to the absence of sotorasib which was required for long-term inhibition of KRAS<sup>G12C</sup> (87) and rewiring of the proteostasis network to be IRE1 $\alpha$ -centered.

ORIN1001 has over 100-fold mammalian enzyme selectivity over its yeast ortholog (83). Structure analysis of the mammalian and yeast enzymes revealed a critical residue (V918) in mammalian IRE1 $\alpha$  that differs from the yeast enzyme and could be critical for ORIN1001 binding (Fig. 7D). Binding of ORIN1001 to IRE1 $\alpha$  results in the formation of an imine that could be reduced and detected by UV-excited fluorescence (Fig. 7E). Indeed, whereas purified WT IRE1 $\alpha$  protein directly bound to ORIN1001, mutation of valine to phenylalanine at V918 (V918F) abolished the binding (Fig. 7F). The V918F mutation did not affect the ability of ER stressor tunicamycin to induce *XBPI* splicing, but completely abolished the response of IRE1 $\alpha$  to ORIN1001 (Fig. 7G). Treatment of IRE1 $\alpha$ <sup>V918F</sup>-expressing MIA-PaCa-2<sup>R</sup> tumors with ORIN1001 failed to inhibit XBP1s *in vivo* (Fig. 7H). These data identify IRE1 $\alpha$ <sup>V918F</sup> as a drug-resistant mutant that retains intact RNase activity but is immune to ORIN1001. Importantly, the IRE1 $\alpha$ <sup>V918</sup>-expressing, but not WT IRE1 $\alpha$ -expressing, MIA-PaCa-2<sup>R</sup> tumors were completely immune to ORIN1001-induced sensitivity to sotorasib (Fig. 7I) and protein aggregation (Fig. 7, J and K). Collectively, these data confirm the on-target effects of ORIN1001 *in vivo*.

We also tested the therapeutic efficacy of combined ORIN1001 and sotorasib treatment in the H358 NSCLC model. The H358 tumors were highly sensitive to sotorasib treatment, and complete regression was observed within 70 days (Fig. 8A). However, the termination of sotorasib treatment resulted in rapid tumor relapse (Fig. 8A). Remarkably, tumors treated with combined sotorasib and ORIN1001 did not relapse after treatment termination (Fig. 8, A and B). KRAS inhibitor resistance mechanisms are heterogeneous in human patients (18–23). To assess the human relevance, we treated five *KRAS*<sup>G12C</sup>-mutant NSCLC PDX models with sotorasib and ORIN1001. As shown in Fig. 8, C to G and fig. S18 A to E, ORIN1001 significantly sensitized all five PDX models to sotorasib. In three PDX models

(J000096652, TM00186 and TC303AR), the tumors were initially sensitive to sotorasib treatment, but eventually all relapsed. Addition of ORIN1001 to sotorasib led to complete responses and prevented tumor relapse (Fig. 8, C to E). In the other two PDX models (J000093018 and TM00192), ORIN1001 also significantly enhanced the tumor responses to sotorasib, but the responses were not as striking as the other models (Fig. 8, F and G). Analysis of these five PDX models showed that sotorasib did not effectively inhibit MAPK in the J000093018 and TM00192 PDX models (fig. S18, F to J), leading to incomplete reprogramming of the proteostasis network and reduced efficacy (fig. S18, K and L). Collectively, these *in vivo* data demonstrate that IRE1 $\alpha$  inhibition is effective in enhancing the response of *KRAS*<sup>G12C</sup>-driven tumors to sotorasib. Combination therapy with ORIN1001 and sotorasib achieves complete responses and prevents tumor relapse in a significant portion of *KRAS*<sup>G12C</sup>-driven tumors.

## Concluding remarks

Most cancers require a balanced proteostasis network to maintain oncogenic growth. Therapeutic insults often disrupt proteostasis and induce proteotoxic stresses (88). How proteostasis network is orchestrated by driver oncogenes and the proteostasis reprogramming mechanisms that bypass oncogene addiction and allow for acquired resistance to targeted therapies remain largely unknown. We show that oncogenic *KRAS* is critical for protein quality control in tumor cells. Inhibition of oncogenic *KRAS* inactivates both cytosolic and ER protein quality control machinery by inhibiting the master regulators HSF1 and IRE1 $\alpha$ . However, residual cancer cells that survive *KRAS* inhibition directly restore IRE1 $\alpha$  through an ER stress-independent unconventional phosphorylation mechanism that re-establishes proteostasis and sustains acquired resistance to *KRAS* inhibition.

In contrast to what occurs in non-malignant cells (53), oncogenic *KRAS* activation resolves, rather than induces, ER stress in transformed cancer cells through oncogenic kinase-dependent phosphorylation of IRE1 $\alpha$ . We identified four phosphorylation sites in IRE1 $\alpha$  that are distinct from IRE1 $\alpha$  autophosphorylation sites. The phosphorylation of IRE1 $\alpha$  at these sites prevents IRE1 $\alpha$  binding with the SEL1L/HRD1 E3 ligase complex, thus impairing the ubiquitination-dependent degradation of IRE1 $\alpha$  and stabilizing the protein. Importantly, these sites are convergence points for multiple resistance pathways and function as central gatekeeper of the rewired proteostasis network in the *KRAS*<sup>i</sup>-resistant tumors. Inactivation of these sites is sufficient to abolish the direct regulation of IRE1 $\alpha$  by oncogenic signaling and collapse the re-established proteostasis to overcome resistance to *KRAS* inhibitor.

Despite the approval of sotorasib and adagrasib for the treatment of *KRAS*<sup>G12C</sup>-mutant NSCLC patients, resistance to these inhibitors is rapid and almost inevitable (15, 18–29). The heterogeneous resistance mechanisms in patients and dose-limiting toxicity associated with targeting multiple resistance pathways, such as RTKs, MAPK and PI3K, remain a major barrier to progress. Our mechanistic study directly addressed these clinical challenges by revealing IRE1 $\alpha$ -mediated proteostasis reprogramming as a convergence point for multiple heterogeneous resistance mechanisms in response to *KRAS*–MAPK inhibition.

ORIN1001 is a highly specific IRE1 $\alpha$  RNase inhibitor (83) that demonstrates safety and tolerability in Phase I clinical trial (NCT03950570) despite the occurrence of some adverse effects (86, 89). ORIN1001 substantially enhanced the responses of *KRAS*-mutant lung or pancreatic cancer PDX models to sotorasib or trametinib. These data demonstrate that direct targeting IRE1 $\alpha$  is a more effective and well-tolerated therapeutic strategy for reversing KRASi or MEKi resistance.

In summary, our study reveals the direct crosstalk between oncogenic signaling and the protein quality control machinery. This study elucidated a molecular mechanism that accounts for the proteostasis modulation observed in response to KRAS inhibition. It is noteworthy that the mechanisms of KRAS inhibitor resistance are heterogeneous in patients. Additional studies will be required to examine what proportion of KRAS-driven cancers that develop resistance to KRAS inhibitors use IRE1 $\alpha$ -mediated mechanisms of resistance.

## Materials and Methods

### Cell culture and treatment

MIA-PaCa-2, SW1990, PaTu 8988T, 293T, H358, and BEAS-2B cells were obtained from the American Type Culture Collection (ATCC). Patient-derived PATC53 and PATC148 cells were a gift from Dr. Michael Kim at The University of Texas MD Anderson Cancer Center (90). *iKras* cells were derived from our previously generated, doxycycline (Dox)-inducible, *Kras*<sup>G12D</sup>-driven PDAC mouse model (*tetO\_LSL-Kras*<sup>G12D</sup>/*p53*<sup>flox/+</sup>/*p48-Cre/ROSA26-LSL-rtTA-IRES-GFP*) (55). *LSL-Kras*<sup>G12D</sup> cells were derived from *Kras*<sup>G12D</sup> knock-in PDAC mouse model (*LSL-Kras*<sup>G12D</sup>/*p53*<sup>flox/+</sup>) as described previously (55). The cell lines used in this study are listed in Table S1. BEAS-2B cells were maintained in BEBM Bronchial Epithelial Cell Growth Basal Medium (Lonza, CC-3171) with growth factors and supplements from BEGM Bronchial Epithelial SingleQuots Kit (Lonza, CC-4175). Insulin and hEGF were withdrawn from the medium 48 hours before sample collection. H358, *iKras*, *LSL-Kras*<sup>G12D</sup>, and PATC148 cells were maintained in RPMI supplemented with 10% FBS serum (Gibco, 10437028) and 100 $\mu$ g/mL penicillin/streptomycin (Invitrogen, 15140163). H358<sup>R</sup> cells were generated by *in vitro* culture of H358 cells with increasing dose of sotorasib for 6 months until the cells acquired resistance to 30 nM sotorasib. Doxycycline (VWR, AAJ60579–22, 1 $\mu$ g/mL) was added to the *iKras* cell culture medium to maintain *KRAS*<sup>G12D</sup> expression. 10% charcoal stripped FBS (VWR, 97065–304) was used to culture *iKras* cells for doxycycline-withdrawal experiments as previously described (55). *iKras*<sup>R</sup> cells were generated by *in vitro* culture of parental *iKras* cells in the absence of Dox until the cells acquired resistance to *KRAS*<sup>G12D</sup> inactivation. MIA-PaCa-2, SW1990, PaTu 8988T, 293T and PATC53 cells were maintained in DMEM supplemented with 10% FBS (Gibco, 10437028) and 100 $\mu$ g/mL penicillin and streptomycin (Invitrogen, 15140163). MIA-PaCa-2<sup>R</sup> cells were generated by *in vitro* culture of parental MIA-PaCa-2 cells with 30 nM sotorasib until the cells acquired resistance to KRAS inhibition. The chemicals used in this study are listed in Table S2.

## Tumor inoculation and treatment

The inoculation and establishment of PDX or xenograft tumors was described previously (90). PDAC19 and PDAC35 PDX models were generated by Baylor College of Medicine PDAC PDX Core. TC303AR PDX was generated by MD Anderson Cancer Center PDX Core. J000096652, TM00186, J00093018, and TM00192 PDX models were purchased from Jackson Laboratory. For tumor fragments transplantation of PDX, 1mm<sup>3</sup> fresh tumor fragments were transplanted into the lower flanks of 6-week-old immune-compromised SCID/Beige mice (Charles river, strain code 250, CB17.Cg-*Prkdc*<sup>scid</sup>*Lysf*<sup>bg-J</sup>/*Ctrl*, both female and male) or NSG mice (Jackson Laboratory, stain code 005557, NOD.Cg-*Prkdc*<sup>scid</sup>*Il2rg*<sup>tm1Wjl/SzJ</sup>, both female and male). For subcutaneous xenograft experiments, 1 × 10<sup>6</sup> PATC53, SW1990, MIA-PaCa-2, H358 or *iKras* cells suspended in 100 µL 50% Matrigel (Corning, #354230, in PBS) were injected subcutaneously into the lower flanks of 6-week-old SCID/Beige mice or Athymic Nude mice (Envigo, strain code 69, Hsd:Athymic Nude-*Foxn1*<sup>nu</sup>, female). Tumor growth was monitored using calipers and tumor volumes were calculated by the equation  $V(\text{mm}^3) = L \times W^2/2$ , where  $L$  is the largest diameter and  $W$  is the perpendicular diameter. When tumors reached a volume of approximately 50–500 mm<sup>3</sup>, mice were randomized and treated with drugs as indicated. ORIN1001 was provided by Orinove Inc. and suspended in 1% microcrystalline cellulose in 50% sucrose and sonicated for 90 min in water bath sonicator (VWR Ultrasonic Cleaner, Model 97043–964) before dosing at 150 mg/kg or 300 mg/kg body weight via daily oral gavage (83).

Trametinib (1mg/kg) or sotorasib (30, 50 or 100 mg/kg) was formulated in the hydroxypropylmethylcellulose (HPMC)-Tween 80 buffer solution (0.5% HPMC and 0.2% Tween 80, pH 8.0) and administered via daily oral gavage as described previously (12). SCH772984 (50mg/kg) was formulated in 45% saline, 50% PEG 400 and 5% DMSO, and administered via daily intraperitoneal injection. MK2206 (120mg/kg) was formulated in 30% Captisol (Cydex) and administered by oral gavage every other day. All mice were maintained in accordance with Baylor College of Medicine Animal Care and Use Committee procedures and guidelines.

## Protein aggregation detection assay

The PROTEOSTAT Aggresome detection kit (Enzo Life Sciences, ENZ-51035-K100), Congo red dye (CR, Sigma, 234610) or thioflavin T dye (ThT, Sigma, T3516) was used to detect misfolded or aggregated proteins in cells or tumor tissues (56, 72). The PROTEOSTAT aggresome detection assay was performed according to the manufacturer's instructions. Briefly, the *iKras* cells seeded on glass slides were washed with PBS, fixed with 4% formaldehyde for 30 min at room temperature (RT), permeabilized with Permeabilizing Solution (0.5% Triton X-100, 3mM EDTA) for 30 min on ice with gentle shake, and stained with the PROTEOSTAT dye (1:20,000 dilution) for 30 min at RT. MIA-PaCa-2 or H358 cells were trypsinized from culture dishes followed by washing, fixation, permeabilization and staining as described above. Tumor sections were deparaffinized and rehydrated before staining. Samples were incubated with PROTEOSTAT dye (1:20,000 dilution) for 30 min at RT. Nuclei were counterstained with DAPI. Cells treated with 10 µM MG132 (provided in the PROTEOSTAT Aggresome detection kit) for 16 h was used as positive control. Samples stained with DAPI only were used as negative control. Congo red (CR) and thioflavin T

(ThT) staining were performed as described previously (56). Briefly, the *iKras* cells seeded on glass slides were washed with PBS, fixed with 4% formaldehyde for 30 min at RT, permeabilized with Permeabilizing Solution (0.5% Triton X-100, 3mM EDTA) for 30 min on ice with gentle shake, and stained with 20  $\mu$ M ThT or 50 nM CR dissolved in PBS for 30min at RT, followed by rinsing in PBS for 3 times. Nuclei were stained with DAPI.

Images (16-bit greyscale TIFFs) were analyzed using CellProfiler v2.2 (Broad Institute) as described previously (72). Briefly, the DAPI channel images were first smoothed with a median filter and nuclei were identified with automatic thresholding and a fixed diameter. The cell nuclei that touch the border of the image were eliminated for quantification. The cell nuclei that touch each other were separated with a watershed algorithm. Then, cell boundaries were identified by watershed gradient based on the dye signal of PROTEOSTAT, ThT or CR, using nuclei as a seed. Metrics for PROTEOSTAT, ThT or CR were extracted from the cells.

### Cell viability assay

200 cells were seeded in 96-well plate and treated with different inhibitors as indicated in the figures. Cell viability was measured daily with CCK-8 kit (APExBIO, # K1018). Briefly, 10 $\mu$ L CCK-8 solution was added to each well and incubated for 1 hour at 37 °C. The absorbance at 450 nm was measured using BioTek Synergy HTX Multi-Mode microplate reader.

### Colony formation assay

200 cells were seeded in 12-well plate and cultured for 5 days. Cells were washed with PBS, fixed with methanol and stained with 0.5% crystal violet. The colony number was counted and quantified.

### BrdU incorporation Assay

For BrdU staining, cells in the logarithmic phase of proliferation were first labeled with BrdU at a final concentration of 10  $\mu$ M for 30 min at 37 °C, followed by intracellular staining using the BrdU staining kit according to the manufacturer's instructions (BD Biosciences, 559619). Flow cytometry data were collected using BD FACS Diva 8 on a BD LSR II or BD Fortessa analyzer. The acquired data were analyzed using the FlowJo 10 software.

### Luciferase assay

For *iKras* cells, the HSF1 firefly luciferase reporter was constructed by cloning 4 copies of the heat shock element (HSE) followed by a minimal promoter sequence (5'-AGAGGGTATATAATGGAAGCTCGACTTCCAG-3') (Promega, E375A) (all primer sequences are listed in Table S3) into the SacI and HindIII sites of the pGL3-basic luciferase reporter (Promega, E1751). The construct was verified by DNA sequencing. The *iKras<sup>P</sup>* or *iKras<sup>R</sup>* cells were seeded in 96-well plate at 800 cells/well and co-transfected with 100ng *firefly* luciferase reporter and 5ng *Renilla* luciferase plasmid (pRL-TK, Promega, E2241, used as internal control) using Lipofectamine 3000 (Thermo Fisher, L300008). The transfected *iKras<sup>P</sup>* cells cultured in the presence or absence of Dox for 48 h or the



transfected *iKras<sup>R</sup>* cells cultured in the absence of Dox were heat shocked at 43 °C for 1 h and recovered overnight before measuring the luciferase activities using the Dual-luciferase Reporter Assay System (Promega, #E1910) according to the manufacturer's instructions. For MIA-PaCa-2 cells, the same sequence (Promega, E375A) was cloned into the XhoI and BamHI sites of the pRRL-Luciferase plasmid (Addgene, 120798). The construct was verified by DNA sequencing. The *firefly* or pLenti.PGK.blast-Renilla\_Luciferase (Addgene, 74444) plasmid was packaged into lenti-viruses and infected MIA-PaCa-2<sup>P</sup> or MIA-PaCa-2<sup>R</sup> cells. After selection with blasticidine (20 µg/mL), the cells were seeded in 96-well plate at 800 cells/well. The infected MIA-PaCa-2<sup>P</sup> cells cultured in the presence or absence of sotorasib for 48 h were heat shocked at 43 °C for 1 h and recovered overnight before measuring the luciferase activities using the Dual-luciferase Reporter Assay System (Promega, #E1910) according to the manufacturer's instructions.

### Proteasome activity assay

The Proteasome Activity Fluorometric Assay Kit (BioVision, K245) was used to detect proteasome activity according to the manufacturer's instructions. Briefly,  $1 \times 10^6$  *iKras* cells from different treatments were homogenized in a tight-fitting bounce homogenizer (Thomas Scientific, 1176F27) with 500µl 0.5% NP-40 in PBS. 10 µL Proteasome Substrate (AMC-peptide, provided in the kit) was added to 10 µL cell lysate from each treatment group or 10 µL positive control lysate (provided in the kit) and mixed. The reaction was performed at 37 °C and protected from light. The kinetics of fluorescence at excitation/emission = 350/440 nm were measured every 30 min using BioTek Synergy HTX Multi-Mode Plate Reader. Cells treated with 10µM MG132 (provided in the detection kit) for 16 h were used as negative control. The fluorescence signals were normalized against total protein abundance detected with BCA Protein Assay Kit (Thermo Fisher Scientific, 23225).

### Plasmids, virus production, and infection

The plasmids used are listed in Table S4. The pRK5-Flag-IRE1α or pCDH-Flag-IRE1α was generated by cloning the full-length human IRE1α into pRK5 (Genentech) or pCDH (System Biosciences, CD511B-1) vector. The point mutations of IRE1α were introduced with Q5 Site-Directed Mutagenesis Kit (New England Biolabs, E0554). All the IRE1α plasmids are shRNA-resistant and listed in Table S4. Primers used for cloning are listed in Table S3. pHAGE-BRAF<sup>V600E</sup>, pHAGE-MEK<sup>DD</sup>, and pHAGE-PIK3CA<sup>H1047R</sup> plasmids were generated as described previously (91). The pCDH-HA-Myr-AKT plasmid was purchased from Addgene (# 46969) (92). pLVX-Flag-HA-*HRD1* was generated as described previously (73). The shRNAs targeting mouse *Xbp1*, *Ire1a*, *Perk*, *Gcn2*, *Hri*, *Pkr* and *Scp3* were cloned into pLKO.1-TRC (Addgene, 10878). The shRNAs targeting human *XBPI*, *IRE1α*, *NcK*, and *PERK* were cloned in pLKO.1-TRC (Addgene 10878) or pLKO-Tet-On (Addgene 21915) vector to generate constitutive or inducible constructs. The shRNA sequences are listed in Table S3. The pLKO.1 shScramble (Addgene 1864) or pLKO.1 Tet-On shScramble (same shRNA sequence as in pLKO.1 shScramble) was used as control. The p-GIPZ non-silencing shRNA control, p-GIPZ-MAPK1, p-GIPZ-MAPK3 were from Dharmacon Reagents. The *Cas9*-expressing plasmid lentiCas9-Blast was purchased from Addgene (#52962) (93). The gRNAs targeting *Ire1a* or *Xbp1* were cloned into lentiGuide-Puro vector (Addgene, #52963). All gRNA sequences are listed in Table S3. Plasmids

containing coding sequence of different phosphatases are listed in Table S4. To generate lentiviruses, 293T cells were co-transfected with psPAX2 and pMD2.G using Lipofectamine 3000 (Thermo Fisher Scientific, # L3000008). Lentiviruses were collected 48 and 72 hours after transfection and used for infecting cells in the presence of 8 µg/ml polybrene (Millipore Sigma, TR-1003-G) prior to puromycin selection (2 µg/ml, Millipore Sigma, P8833).

### Generation of knock-out (KO) cells

To generate *Irela* or *Xbp1* KO cells, *iKras* cells were first infected with lentiviruses encoding *Cas9* (lentiCas9-Blast, Addgene 52962) (93) and selected with 10 µg/mL blasticidin (Santa Cruz, 3513-03-09). The *Cas9*-expressing cells were then infected with lentiviruses expressing two gRNAs targeting the same exon and selected with puromycin (2 µg/mL, MilliporeSigma, P8833) to generate pooled KO cells. The gRNA sequences are listed in Table S3.

### Immunohistochemical (IHC) Staining

Tumor specimens were fixed with freshly made 4% paraformaldehyde for 24 h, washed with PBS and stored in 70% ethanol until paraffin embedding. IHC staining was performed on 5 µm-thick paraffin sections. For p-ERK, p-HSF1, p-GCN2, p-eIF2α and YAP1 staining, 10mM sodium citrate buffer (pH 6.0) was used for antigen retrieval. For IRE1α, p-PERK, ATF4 and p-AKT IHC, 1mM EDTA buffer (pH 9.0) was used for antigen retrieval. Endogenous peroxidase was quenched with 3% H<sub>2</sub>O<sub>2</sub> for 20 min followed by blocking with 3% normal goat serum. The following primary antibodies were used: IRE1α (1:20, Cell Signaling Technology, 3294); p-ERK (1:200, Cell Signaling Technology, 4376); and p-HSF1 (1:200, Life Technologies, BSM-52166R); p-PERK (1:25, Cell Signaling Technology, 3179); ATF4 (1:50, Santa Cruz, 390063); p-GCN2 (1:200, Thermo Fisher, PA5-105886); p-AKT (1:50, Cell Signaling Technology, 4060); p-eIF2α(1:50, Cell Signaling Technology, 9721); YAP1 (1:400, Cell Signaling Technology, 14074). Slides were incubated with ImmPRESS Excel HRP Goat Anti-Rabbit Polymer Reagent (Vector labs, MP-7451-15) for 30 minutes. Sections were developed with DAB+ solution (Dako, K3468) and counterstained with Harris Hematoxylin. The antibody used are listed in Table S5.

### Tissue microarray (TMA) analysis

For quantifications of TMA staining, TMAs stained with anti-IRE1α or anti-p-ERK antibody were scanned using the Aperio scanner and analyzed with QuPath software (94). Detailed tutorials of the software can be found at <https://qupath.readthedocs.io/en/stable/index.html>. Briefly, images were preprocessed by automated 'TMA dearraying' and 'stain' vector estimation. Tissue sections were identified by running 'simple tissue detection'. The 'positive cell detection' command was used to detect DAB staining intensity. The score compartment was set as 'DAB OD mean'. Tumor cells and stromal cells were classified by 'training object classifier' based on annotations. The fraction score was calculated as the proportion of positively stained tumor cells (0%–100%). The intensity and fraction scores were then multiplied to obtain the H-score.

### RNA extraction and real-time quantitative reverse-transcriptase PCR

Total RNA was extracted using TRIzol (Thermo Fisher Scientific, 15596026). Total RNA (1  $\mu$ g) was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368813), followed by qPCR on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). The sequences of all primers are listed in Table S3.

### Detergent-insoluble aggregates detection

Detergent-insoluble aggregates detection was performed as described previously (47). Briefly, cells with different treatments were harvested by trypsinization. After washing with cold PBS,  $1 \times 10^6$  cells were lysed with RIPA buffer (25 mM Tris-HCl pH7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1.5 mM EDTA) supplemented with protease inhibitor cocktail (Roche, 14826500), phosphatase inhibitor cocktail (Sigma, 4906845001) and 10 mM N-Ethylmaleimide (Sigma, E3876). Protein lysates were cleared by centrifugation at 16,000 g for 20 min at 4 °C. The remaining insoluble pellets were then washed with RIPA buffer for three times to remove any remaining detergent-soluble proteins. The pellet containing detergent-insoluble aggregates was solubilized with urea buffer (8 M urea, 2% SDS, 50 mM DTT, 50 mM Tris-HCl pH7.4) for western blot analysis.  $3 \times 10^5$  cells were directly lysed in urea buffer (8 M urea, 2% SDS, 50 mM DTT, 50 mM Tris-HCl pH7.4) serving as loading control.

### Co-immunoprecipitation

The co-immunoprecipitation (co-IP) assay was performed as previously described (73). Briefly, 293T cells transfected with different plasmids or H358 cells infected with indicated viruses were lysed with lysis buffer (50mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche, 14826500) and phosphatase inhibitor cocktail (Sigma, 4906845001). Protein lysates were cleared by centrifugation at 12,000 g for 20 min at 4 °C. Supernatant was incubated with anti-Flag M2 beads (Sigma, F-2426) or anti-Myc beads (Sigma, E-6654) for 4h to overnight at 4 °C with gentle rotating. The beads were then washed once with lysis buffer, followed by additional three washes with wash buffer (50mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol). The proteins were eluted by boiling in  $2 \times$  Laemmli sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 355 mM b-mercaptoethanol, 0.01% bromophenol blue) and analyzed by SDS-PAGE and western blot.

### Ubiquitination and phosphorylation assay

The ubiquitination and phosphorylation assays were performed to detect IRE1 $\alpha$  ubiquitination and phosphorylation. MIA-PaCa-2 or H358 cells infected with lentiviruses encoding control or Flag-IRE1 $\alpha$  were treated with DMSO, sotorasib or trametinib as described in figure legend and lysed with RIPA buffer (25 mM Tris-HCl pH7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1.5 mM EDTA) supplemented with protease inhibitor cocktail (Roche, 14826500), phosphatase inhibitor cocktail (Sigma, 4906845001) and 10 mM N-Ethylmaleimide (Sigma, E3876). Protein lysates were sonicated for 30s and cleared by centrifugation at 12,000 g for 20 min at 4

°C. Supernatant was incubated with anti-Flag M2 beads (Sigma, F-2426) for 4h at 4 °C with gentle rotating. The beads were then washed with RIPA buffer for three times. The immunoprecipitates were eluted and denatured by boiling for 10 min in denature buffer (50 mM Tris-HCl pH7.6, 1% SDS, 0.5 mM EDTA, 1 mM DTT) to disrupt the interactions between immunoprecipitated IRE1 $\alpha$  and its interacting proteins. The denatured elutes were diluted 1:10 with lysis buffer (50mM Tris-HCl pH7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) and subjected to a second-round immunoprecipitation with anti-Flag M2 beads (Sigma, F-2426) (4h at 4 °C) to remove all the interacting proteins and selectively pull down only IRE1 $\alpha$  protein which allows for the specific analysis IRE1 $\alpha$  ubiquitination and phosphorylation. The beads were then washed with RIPA buffer for three times. The proteins were eluted by boiling in 2  $\times$  Laemmli sample buffer (65.8 mM Tris-HCl pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 355 mM  $\beta$ -mercaptoethanol, 0.01% bromophenol blue) and analyzed by SDS-PAGE and western blot.

### GST pull-down assay

The GST pull-down assay was performed to detect interaction between ERK and IRE1 $\alpha$ . Briefly, recombinant GST or GST-ERK2 protein purified from *E.coli* (SignalChem, M28–10G-20) was incubated with recombinant His-IRE1 $\alpha$  protein purified from Sf9 cells (83) in RIPA buffer (25 mM Tris-HCl pH7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1.5 mM EDTA) for 30 min before the GSH-Sepharose beads (GE Healthcare, 17075601) were added and rotation for 1 h at 4 °C. The beads were washed with RIPA buffer for three times. The proteins were eluted by boiling in 2  $\times$  Laemmli sample buffer and analyzed by SDS-PAGE and western blot.

### Flag pull-down assay

293T cells transfected with plasmid expressing Flag-GFP or Flag-IRE1 $\alpha$  were lysed in RIPA buffer (25 mM Tris-HCl pH7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1.5 mM EDTA, supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail) 48 hours after transfection. Protein lysates were cleared by centrifugation at 12,000 g for 20 min at 4 °C. Supernatant was incubated with anti-Flag M2 beads (Sigma, F-2426) overnight at 4 °C with gentle rotating. The beads were then washed with RIPA buffer for three times. These preloaded Flag M2 beads with Flag-GFP or Flag-IRE1 $\alpha$  were then incubated with purified GST-ERK2 (SignalChem, M28–10G-20) for 30 min before washing with wash buffer (50mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) for three times and elution by boiling in 2  $\times$  Laemmli sample buffer for 10 minutes. The eluents were analyzed by SDS-PAGE and western blot.

### *In vitro* kinase assay

Flag-GFP or Flag-IRE1 $\alpha$  proteins were purified from 293T cells as described above. The Flag M2 beads pre-loaded with Flag-GFP or Flag-IRE1 $\alpha$  proteins were rinsed with kinase assay buffer I (SignalChem, K01–09, 25 mM MOPS pH7.2, 12.5 mM beta-glycerol-phosphate, 25 mM MgCl<sub>2</sub>, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT) and subjected to *in vitro* kinase assay in the kinase assay buffer I plus 0.4 mM cold ATP and 1.0  $\mu$ g GST-ERK2 activated by MEK1 *in vitro* (SignalChem, M28–10G-20). The reaction was carried out at 30 °C for 30 min. The beads were then washed for three times with RIPA buffer (25 mM

Tris-HCl pH7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1.5 mM EDTA). The proteins were eluted by boiling in 2 × Laemmli sample buffer and analyzed by SDS-PAGE and western blot.

For *in vitro* kinase assay labeled with [ $\gamma$ - $^{32}$ P] ATP, Flag-IRE1 $\alpha$ <sup>K599A</sup> (kinase-dead form) or different mutated Flag-IRE1 $\alpha$ <sup>K599A</sup> proteins expressed in 293T cells were bound to anti-Flag M2 beads as described above and incubated with 0.5  $\mu$ g GST-ERK2 activated by MEK1 *in vitro* (SignalChem, M28–10G-20) in the presence of 1  $\mu$ Ci of [ $\gamma$ - $^{32}$ P] ATP (PerkinElmer, NEG002A100UC) and 0.4 mM cold ATP in the kinase buffer I (SignalChem, K01–09) for 30 min at 30 °C. The reaction was stopped by boiling in 2 × Laemmli sample buffer for 10 minutes. The eluted proteins were resolved by SDS-PAGE and detected by autoradiography.

For *in vitro* kinase assay with recombinant His-IRE1 $\alpha$ , 1.0  $\mu$ g protein purified from Sf9 cells (83) and recombinant GST-ERK2 protein, the reaction was carried out in the kinase assay buffer I (SignalChem, K01–09) plus 0.4 mM cold ATP for 30 min at 30 °C. The reaction was stopped by adding 8 M urea buffer followed by purification of His-IRE1 $\alpha$  with Ni-NTA agarose (QIAGEN, 30210). The proteins were eluted with 2 × Laemmli sample buffer by boiling for 10 minutes and analyzed by SDS-PAGE and western blot.

### ***In vitro* phosphatase assay**

The *in vitro* phosphatase assay was performed as described previously (95). Phosphorylated Flag-IRE1 $\alpha$  proteins were purified from 293T cells expressing MEK<sup>DD</sup> as described above. The Flag M2 beads pre-loaded with phosphorylated Flag-IRE1 $\alpha$  proteins were rinsed with phosphatase assay buffer (40 mM Tris-HCl pH7.5, 20 mM KCl, 10 mM MnCl<sub>2</sub>, and 2 mM DTT) and subjected to *in vitro* phosphatase assay in the phosphatase assay buffer and 1.0  $\mu$ g recombinant His-SCP3 protein (NOVUS, NBP1–99109). The reaction was carried out at 30 °C for 30 min. The beads were then washed for three times with RIPA buffer (25 mM Tris-HCl pH7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1.5 mM EDTA). The proteins were eluted by boiling in 2 × Laemmli sample buffer and analyzed by SDS-PAGE and western blot. For *in vitro* kinase assay with recombinant His-IRE1 $\alpha$ , 1.0  $\mu$ g His-IRE1 $\alpha$  protein was phosphorylated by recombinant GST-ERK2 protein *in vitro* as described above, and then subjected to *in vitro* phosphatase assay with SCP3 protein. 1.0  $\mu$ g recombinant His-SCP3 protein (NOVUS, NBP1–99109) or Myc-SCP3 protein purified from H358 cells treated with DMSO or sotorasib (30 nM) were used for the *in vitro* phosphatase assay as indicated in the figures. The proteins were boiled in 2 × Laemmli sample buffer and analyzed by SDS-PAGE and western blot.

### **Phospho-RTK array**

The Proteome Profiler Human Phospho-RTK Array Kit (R&D systems, ARY001B) was used to assess the phosphorylation status of 49 RTKs in H358 cells or MIA-PaCa-2 tumors treated with vehicle or sotorasib. The phospho-RTK array assay was performed according to the manufacturer's instructions. Briefly, the H358 cells or MIA-PaCa-2 tumors were lysed in the Lysis Buffer 17 (provided in the kit) with protease inhibitors. The protein concentration was measured with Pierce<sup>TM</sup> BCA Protein Assay Kit (ThermoFisher Scientific,

23225). 1000 µg total protein lysate was incubated with the RTK array membrane overnight at 4°C. After washing, the anti-phospho-tyrosin-HRP detection antibody was incubated with the membrane, and chemiluminescence signal was detected with Amersham Imager 600 (GE Healthcare Lifer Sciences) and quantified with Image J.

### **Biochemical fluorescence assay to detect IRE1α interaction with ORIN1001**

The *in vitro* binding assay to detect IRE1α with ORIN1001 was performed as described previously (96). Briefly, WT or V918F mutant Flag-IRE1α proteins were purified from 293T cells as described above. The Flag M2 beads pre-loaded with equal amount of Flag-IRE1α<sup>WT</sup> or Flag-IRE1α<sup>V918F</sup> proteins were incubated with 200 µM ORIN1001 in binding buffer (50 mM Tris-HCl pH7.5, 100 mM NaCl, 10% Glycerol, 1% Triton X-100, 50 mM EDTA) for 3 hours at 4°C. After that, 6 mM NaBH<sub>4</sub> was added to reduce the imine (Schiff base) between IRE1α and ORIN1001 to stable amine. The beads were then extensively washed for three times with binding buffer and three times with RIPA buffer (25 mM Tris-HCl pH7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1.5 mM EDTA). The proteins were eluted by boiling in 2 × Laemmli sample buffer and analyzed by SDS-PAGE. UV transmission (GE Healthcare Lifer Sciences) was used to detect ORIN1001 that is covalently bound to IRE1α protein in the SDS-PAGE.

### **Modelling of the IRE1α-ORIN1001 complex**

Murine IRE1α and MKC9989 (PDB 4PL3) was used as a template to model ORIN1001 bound to murine IRE1α with Schrodinger software. Homology modelling of yeast IRE1α (PDB 3FBV) was also achieved and was superimposed manually onto the model of ORIN1001 with murine IRE1α.

### **Clinical Proteomic Tumor Analysis Consortium (CPTAC) data analysis**

Correlation between IRE1α phosphorylation (S549) and ERK phosphorylation (Y204) in CPTAC NSCLC tumors were analyzed using the LinkedOmics platform (97) (<http://www.linkedomics.org>).

### **Western Blot**

Whole cell lysates or immunoprecipitation samples were separated by SDS-PAGE and transferred to nitrocellulose membrane (Biorad, 1620112). The antibody used are listed in Table S5. The reagents and kits used in this study are listed in Table S2.

### **Statistics and reproducibility**

Data are expressed as the mean ± SD or mean ± SEM as indicated in the figure legends; *n* is the number of independent biological replicates, unless specifically indicated otherwise in the figure legend. The respective *n* values are shown in the figure legends. No statistical method was used to pre-determine the sample sizes. For animal experiments, at least 4 biological replicates were included based on previously published work, preliminary studies as standard for this field of research. See figures legends for each experiment. Treatments were performed in a non-blinded manner by a research technician who was not aware of the objectives of the study because the colors of the drugs used are different from that of vehicle

control. The results were quantified using GraphPad Prism 8. 2-tailed, unpaired Student's *t* test with or without Welch's correction was utilized to compare the differences between 2 groups as indicated in figure legends. One-way ANOVA with Dunnett's or Tukey's multiple comparison test was used to compare the differences among 3 or more groups as indicated in figure legends. Two-way ANOVA with Bonferroni's multiple comparison test was used to calculate the significance difference for cell growth, tumor volume and body weight measurement over time. The log-rank (Mantel-Cox) test was used to test for the significant differences of survival between the groups.  $P < 0.05$  was considered statistically significant. No samples or animals were excluded from the analysis.

### Study approval

All protocols described in this study were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee (AN6813).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### Competing interests:

M.F.R. receives research funding from Pfizer and Genentech. M.F.R. is a consultant and receives consulting fees from: Novartis, Seagen, MacroGenics, and AstraZeneca. M.F.R. is the Principal Investigator of the clinical trial [NCT03950570](#). X.C. reports previous research funding from Fosun Pharma. J.A.R. reports receiving a commercial research grant from The University of Texas MD Anderson Cancer Center, sponsored research agreement from Genprex, Inc. has ownership interest (including stock, patents, etc.) in Genprex, Inc., and is a consultant/advisory board member for Genprex, Inc. M.J.E. reports full time employment with AstraZeneca from March 7<sup>th</sup> 2022. M.J.E. holds patents and receives income from Veracyte on the PAM50-based products, Prosigna.

### Data and materials availability:

All data are available in the manuscript or the supplementary material. Requests for materials should be addressed to X.C.

## References

1. Scheidig AJ, Burmester C, Goody RS, The pre-hydrolysis state of p21(ras) in complex with GTP: new insights into the role of water molecules in the GTP hydrolysis reaction of ras-like proteins. *Structure* 7, 1311–1324 (1999). [PubMed: 10574788]
2. Scheffzek K. et al. , The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science* 277, 333–338 (1997). [PubMed: 9219684]
3. Bollag G, McCormick F, Differential regulation of rasGAP and neurofibromatosis gene product activities. *Nature* 351, 576–579 (1991). [PubMed: 1904555]
4. Li C. et al. , The G protein signaling regulator RGS3 enhances the GTPase activity of KRAS. *Science* 374, 197–201 (2021). [PubMed: 34618566]
5. McGrath JP, Capon DJ, Goeddel DV, Levinson AD, Comparative biochemical properties of normal and activated human ras p21 protein. *Nature* 310, 644–649 (1984). [PubMed: 6147754]
6. Downward J, Targeting RAS signalling pathways in cancer therapy. *Nat. Rev. Cancer* 3, 11–22 (2003). [PubMed: 12509763]
7. Simanshu DK, Nissley DV, McCormick F, RAS proteins and their regulators in human disease. *Cell* 170, 17–33 (2017). [PubMed: 28666118]
8. Cox AD, Fesik SW, Kimmelman AC, Luo J, Der CJ, Drugging the undruggable RAS: Mission possible? *Nature reviews. Drug discovery* 13, 828–851 (2014). [PubMed: 25323927]
9. Encarnación-Rosado J, Kimmelman AC, Harnessing metabolic dependencies in pancreatic cancers. *Nat. Rev. Gastroenterol. Hepatol.* 18, 482–492 (2021). [PubMed: 33742165]
10. Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D, RAS oncogenes: weaving a tumorigenic web. *Nat. Rev. Cancer* 11, 761–774 (2011). [PubMed: 21993244]
11. Ostrem JM, Peters U, Sos ML, Wells JA, Shokat KM, K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions. *Nature* 503, 548–551 (2013). [PubMed: 24256730]
12. Canon J. et al. , The clinical KRAS(G12C) inhibitor AMG 510 drives anti-tumour immunity. *Nature* 575, 217–223 (2019). [PubMed: 31666701]
13. Hallin J. et al. , The KRAS(G12C) Inhibitor MRTX849 Provides Insight toward Therapeutic Susceptibility of KRAS-Mutant Cancers in Mouse Models and Patients. *Cancer Discov.* 10, 54–71 (2020). [PubMed: 31658955]
14. Skoulidis F. et al. , Sotorasib for Lung Cancers with KRAS p.G12C Mutation. *N. Engl. J. Med.* 384, 2371–2381 (2021). [PubMed: 34096690]
15. Hong DS et al. , KRAS(G12C) Inhibition with Sotorasib in Advanced Solid Tumors. *N. Engl. J. Med.* 383, 1207–1217 (2020). [PubMed: 32955176]
16. Janne PA et al. , Adagrasib in Non-Small-Cell Lung Cancer Harboring a KRAS(G12C) Mutation. *N. Engl. J. Med.* 387, 120–131 (2022). [PubMed: 35658005]
17. Akhave NS, Biter AB, Hong DS, Mechanisms of Resistance to KRAS(G12C)-Targeted Therapy. *Cancer Discov.* 11, 1345–1352 (2021). [PubMed: 33820777]
18. Awad MM et al. , Acquired Resistance to KRASG12C Inhibition in Cancer. *N. Engl. J. Med.* 384, 2382–2393 (2021). [PubMed: 34161704]
19. Li BT et al. , Largest evaluation of acquired resistance to sotorasib in KRAS p.G12C-mutated non-small cell lung cancer (NSCLC) and colorectal cancer (CRC): Plasma biomarker analysis of CodeBreaK100. *J. Clin. Oncol.* 40, 102–102 (2022).
20. Zhao Y. et al. , Diverse alterations associated with resistance to KRAS(G12C) inhibition. *Nature* 599, 679–683 (2021). [PubMed: 34759319]
21. Yaeger R. et al. , Molecular Characterization of Acquired Resistance to KRASG12C-EGFR Inhibition in Colorectal Cancer. *Cancer Discov.* 13, 41–55 (2023). [PubMed: 36355783]
22. Tanaka N. et al. , Clinical Acquired Resistance to KRAS(G12C) Inhibition through a Novel KRAS Switch-II Pocket Mutation and Polyclonal Alterations Converging on RAS-MAPK Reactivation. *Cancer Discov.* 11, 1913–1922 (2021). [PubMed: 33824136]
23. Tsai YS et al. , Rapid idiosyncratic mechanisms of clinical resistance to KRAS G12C inhibition. *J. Clin. Invest.* 132, (2022).

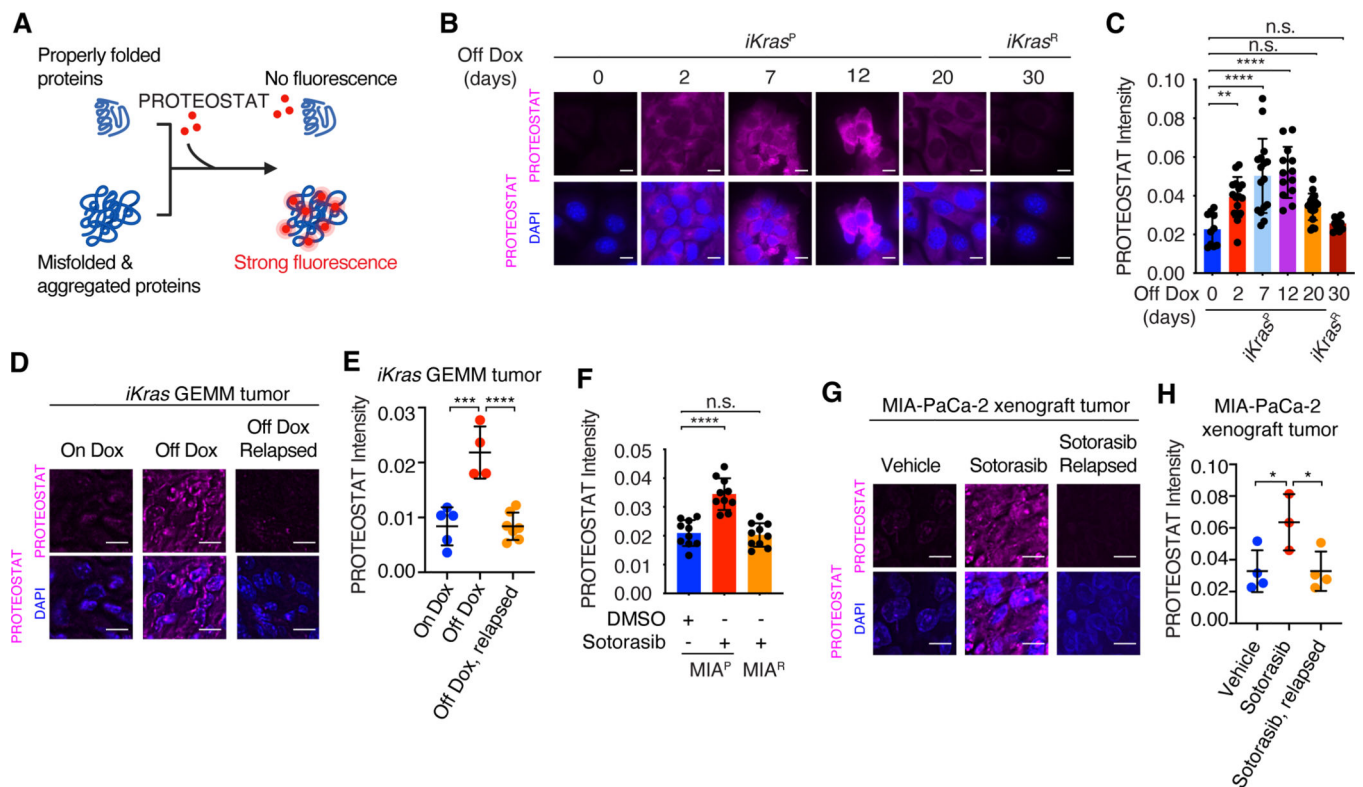


24. Amodio V. et al. , EGFR blockade reverts resistance to KRAS G12C inhibition in colorectal cancer. *Cancer Discov*, (2020).
25. Xue JY et al. , Rapid non-uniform adaptation to conformation-specific KRAS(G12C) inhibition. *Nature* 577, 421–425 (2020). [PubMed: 31915379]
26. Ryan MB et al. , Vertical Pathway Inhibition Overcomes Adaptive Feedback Resistance to KRAS(G12C) Inhibition. *Clin. Cancer Res.* 26, 1633–1643 (2020). [PubMed: 31776128]
27. Brown WS et al. , Overcoming Adaptive Resistance to KRAS and MEK Inhibitors by Co-targeting mTORC1/2 Complexes in Pancreatic Cancer. *Cell Rep Med* 1, 100131 (2020).
28. Molina-Arcas M. et al. , Development of combination therapies to maximize the impact of KRAS-G12C inhibitors in lung cancer. *Sci. Transl. Med.* 11, eaaw7999 (2019).
29. Suzuki S. et al. , KRAS Inhibitor Resistance in MET-Amplified KRAS (G12C) Non-Small Cell Lung Cancer Induced By RAS- and Non-RAS-Mediated Cell Signaling Mechanisms. *Clin. Cancer Res.* 27, 5697–5707 (2021). [PubMed: 34365406]
30. Puneekar SR, Velcheti V, Neel BG, Wong KK, The current state of the art and future trends in RAS-targeted cancer therapies. *Nat. Rev. Clin. Oncol.* 19, 637–655 (2022). [PubMed: 36028717]
31. Longo DL, Rosen N, Targeting Oncogenic RAS Protein. *N. Engl. J. Med.* 387, 184–186 (2022). [PubMed: 35830646]
32. Thatikonda V. et al. (Cold Spring Harbor Laboratory, 2023).
33. Fedele C. et al. , SHP2 inhibition diminishes KRASG12C cycling and promotes tumor microenvironment remodeling. *J. Exp. Med.* 218, (2021).
34. Ho CSL et al. , HER2 mediates clinical resistance to the KRAS(G12C) inhibitor sotorasib, which is overcome by co-targeting SHP2. *Eur. J. Cancer* 159, 16–23 (2021). [PubMed: 34715459]
35. Ryan MB et al. , KRAS(G12C)-independent feedback activation of wild-type RAS constrains KRAS(G12C) inhibitor efficacy. *Cell reports* 39, 110993 (2022).
36. Sattler M, Mohanty A, Kulkarni P, Salgia R, Precision oncology provides opportunities for targeting KRAS-inhibitor resistance. *Trends in Cancer* 9, 42–54 (2023). [PubMed: 36751115]
37. Kapoor A. et al. , Yap1 activation enables bypass of oncogenic Kras addiction in pancreatic cancer. *Cell* 158, 185–197 (2014). [PubMed: 24954535]
38. Shao DD et al. , KRAS and YAP1 converge to regulate EMT and tumor survival. *Cell* 158, 171–184 (2014). [PubMed: 24954536]
39. Fisher GH et al. , Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. *Genes Dev.* 15, 3249–3262 (2001). [PubMed: 11751631]
40. Joshi S. et al. , Adapting to stress - chaperome networks in cancer. *Nat. Rev. Cancer* 18, 562–575 (2018). [PubMed: 29795326]
41. Chen X, Cubillos-Ruiz JR, Endoplasmic reticulum stress signals in the tumour and its microenvironment. *Nat. Rev. Cancer* 21, 71–88 (2021). [PubMed: 33214692]
42. Labbadia J, Morimoto RI, The biology of proteostasis in aging and disease. *Annu. Rev. Biochem.* 84, 435–464 (2015). [PubMed: 25784053]
43. Walter P, Ron D, The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 334, 1081–1086 (2011). [PubMed: 22116877]
44. Wang M, Kaufman RJ, Protein misfolding in the endoplasmic reticulum as a conduit to human disease. *Nature* 529, 326–335 (2016). [PubMed: 26791723]
45. Almanza A. et al. , Endoplasmic reticulum stress signalling - from basic mechanisms to clinical applications. *FEBS J* 286, 241–278 (2019). [PubMed: 30027602]
46. Gomez-Pastor R, Burchfiel ET, Thiele DJ, Regulation of heat shock transcription factors and their roles in physiology and disease. *Nature reviews Molecular cell biology* 19, 4–19 (2018). [PubMed: 28852220]
47. Tang Z. et al. , MEK guards proteome stability and inhibits tumor-suppressive amyloidogenesis via HSF1. *Cell* 160, 729–744 (2015). [PubMed: 25679764]
48. Clarke HJ, Chambers JE, Liniker E, Marciniak SJ, Endoplasmic reticulum stress in malignancy. *Cancer Cell* 25, 563–573 (2014). [PubMed: 24823636]

49. Cox JS, Shamu CE, Walter P, Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. *Cell* 73, 1197–1206 (1993). [PubMed: 8513503]
50. Mori K, Ma W, Gething M-J, Sambrook J, A transmembrane protein with a cdc2+ CDC28-related kinase activity is required for signaling from the ER to the nucleus. *Cell* 74, 743–756 (1993). [PubMed: 8358794]
51. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K, XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 107, 881–891 (2001). [PubMed: 11779464]
52. Calton M. et al. , IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* 415, 92–96 (2002). [PubMed: 11780124]
53. Denoyelle C. et al. , Anti-oncogenic role of the endoplasmic reticulum differentially activated by mutations in the MAPK pathway. *Nat. Cell Biol.* 8, 1053–1063 (2006). [PubMed: 16964246]
54. Sustic T. et al. , A role for the unfolded protein response stress sensor ERN1 in regulating the response to MEK inhibitors in KRAS mutant colon cancers. *Genome Med.* 10, 90 (2018). [PubMed: 30482246]
55. Ying H. et al. , Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell* 149, 656–670 (2012). [PubMed: 22541435]
56. Nilsson MR, Techniques to study amyloid fibril formation in vitro. *Methods* 34, 151–160 (2004). [PubMed: 15283924]
57. Galves M, Rathi R, Prag G, Ashkenazi A, Ubiquitin signaling and degradation of aggregate-prone proteins. *Trends Biochem. Sci.* 44, 872–884 (2019). [PubMed: 31079890]
58. Klaipts CL, Jayaraj GG, Hartl FU, Pathways of cellular proteostasis in aging and disease. *J. Cell Biol.* 217, 51–63 (2017). [PubMed: 29127110]
59. Cortez L, Sim V, The therapeutic potential of chemical chaperones in protein folding diseases. *Prion* 8, 197–202 (2014). [PubMed: 24818993]
60. Engin F. et al. , Restoration of the unfolded protein response in pancreatic beta cells protects mice against type 1 diabetes. *Sci. Transl. Med.* 5, 211ra156 (2013).
61. Costa-Mattoli M, Walter P, The integrated stress response: From mechanism to disease. *Science* 368, eaat5314 (2020).
62. Guettouche T, Boellmann F, Lane WS, Voellmy R, Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. *BMC Biochem.* 6, 4 (2005). [PubMed: 15760475]
63. Tirasophon W, Welihinda AA, Kaufman RJ, A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. *Genes Dev.* 12, 1812–1824 (1998). [PubMed: 9637683]
64. Hollien J, Weissman JS, Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science* 313, 104–107 (2006). [PubMed: 16825573]
65. Lhomond S. et al. , Dual IRE1 RNase functions dictate glioblastoma development. *EMBO Mol. Med.* 10, e7929 (2018). [PubMed: 29311133]
66. Fedele C. et al. , SHP2 Inhibition Prevents Adaptive Resistance to MEK Inhibitors in Multiple Cancer Models. *Cancer Discov.* 8, 1237–1249 (2018). [PubMed: 30045908]
67. Mainardi S. et al. , SHP2 is required for growth of KRAS-mutant non-small-cell lung cancer in vivo. *Nat. Med.* 24, 961–967 (2018). [PubMed: 29808006]
68. Nichols RJ et al. , RAS nucleotide cycling underlies the SHP2 phosphatase dependence of mutant BRAF-, NF1- and RAS-driven cancers. *Nat. Cell Biol.* 20, 1064–1073 (2018). [PubMed: 30104724]
69. Ruess DA et al. , Mutant KRAS-driven cancers depend on PTPN11/SHP2 phosphatase. *Nat. Med.* 24, 954–960 (2018). [PubMed: 29808009]
70. Sun S. et al. , IRE1 $\alpha$  is an endogenous substrate of endoplasmic-reticulum-associated degradation. *Nat. Cell Biol.* 17, 1546–1555 (2015). [PubMed: 26551274]
71. Brodsky JL, Cleaning up: ER-associated degradation to the rescue. *Cell* 151, 1163–1167 (2012). [PubMed: 23217703]

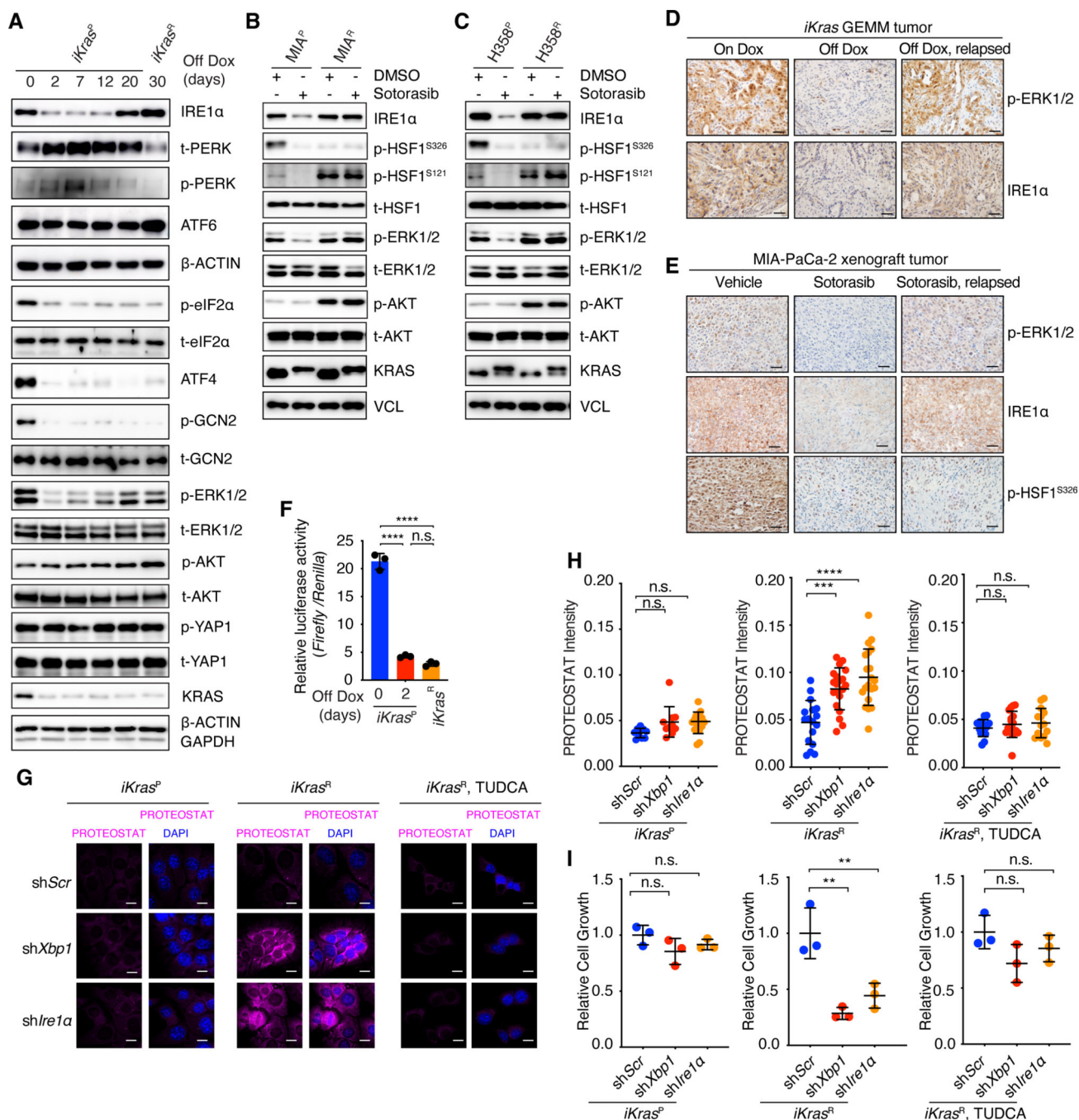
72. Liu X. et al. , Notch-induced endoplasmic reticulum-associated degradation governs mouse thymocyte beta-selection. *Elife* 10, e69975 (2021).
73. Xu L. et al. , Protein quality control through endoplasmic reticulum-associated degradation maintains haematopoietic stem cell identity and niche interactions. *Nat. Cell Biol.* 22, 1162–1169 (2020). [PubMed: 32958856]
74. Meyer H, Bug M, Bremer S, Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. *Nat. Cell Biol.* 14, 117–123 (2012). [PubMed: 22298039]
75. Anderson J, Daniel et al. , Targeting the AAA ATPase p97 as an Approach to Treat Cancer through Disruption of Protein Homeostasis. *Cancer Cell* 28, 653–665 (2015). [PubMed: 26555175]
76. Roskoski R Jr., ERK1/2 MAP kinases: structure, function, and regulation. *Pharmacol. Res.* 66, 105–143 (2012). [PubMed: 22569528]
77. Satpathy S. et al. , A proteogenomic portrait of lung squamous cell carcinoma. *Cell* 184, 4348–4371 e4340 (2021). [PubMed: 34358469]
78. You K. et al. , QRICH1 dictates the outcome of ER stress through transcriptional control of proteostasis. *Science* 371, eabb6896 (2021).
79. Liu CY, Xu Z, Kaufman RJ, Structure and intermolecular interactions of the luminal dimerization domain of human IRE1alpha. *J. Biol. Chem.* 278, 17680–17687 (2003). [PubMed: 12637535]
80. Buscail L, Bourmet B, Cordelier P, Role of oncogenic KRAS in the diagnosis, prognosis and treatment of pancreatic cancer. *Nat. Rev. Gastroenterol. Hepatol.* 17, 153–168 (2020). [PubMed: 32005945]
81. Tolcher AW et al. , Antitumor activity in RAS-driven tumors by blocking AKT and MEK. *Clin. Cancer Res.* 21, 739–748 (2015). [PubMed: 25516890]
82. Chung V. et al. , Effect of Selumetinib and MK-2206 vs Oxaliplatin and Fluorouracil in Patients With Metastatic Pancreatic Cancer After Prior Therapy: SWOG S1115 Study Randomized Clinical Trial. *JAMA Oncol* 3, 516–522 (2017). [PubMed: 27978579]
83. Zhao N. et al. , Pharmacological targeting of MYC-regulated IRE1/XBP1 pathway suppresses MYC-driven breast cancer. *J. Clin. Invest.* 128, 1283–1299 (2018). [PubMed: 29480818]
84. Le Reste PJ et al. , Local intracerebral inhibition of IRE1 by MKC8866 sensitizes glioblastoma to irradiation/chemotherapy in vivo. *Cancer Lett.* 494, 73–83 (2020). [PubMed: 32882336]
85. Logue SE et al. , Inhibition of IRE1 RNase activity modulates the tumor cell secretome and enhances response to chemotherapy. *Nature communications* 9, 3267 (2018).
86. Gabrail NY et al. , A phase 1/2 trial of ORIN1001, a first-in-class IRE1 inhibitor, in patients with advanced solid tumors. *J. Clin. Oncol.* 39, 3080–3080 (2021).
87. Salmon M. et al. , Kras oncogene ablation prevents resistance in advanced lung adenocarcinomas. *J. Clin. Invest.* 133, (2023).
88. Mahler HC, Friess W, Grauschopf U, Kiese S, Protein aggregation: pathways, induction factors and analysis. *J. Pharm. Sci.* 98, 2909–2934 (2009). [PubMed: 18823031]
89. Rimawi MF et al. , Early efficacy evaluation of ORIN1001, a first in class IRE1 alpha inhibitor, in advanced solid tumors. *J. Clin. Oncol.* 41, 1092–1092 (2023). [PubMed: 36493335]
90. Kim MP et al. , Generation of orthotopic and heterotopic human pancreatic cancer xenografts in immunodeficient mice. *Nat. Protoc.* 4, 1670–1680 (2009). [PubMed: 19876027]
91. Yan L. et al. , Targeting Glucose Metabolism Sensitizes Pancreatic Cancer to MEK Inhibition. *Cancer Res.* 81, 4054–4065 (2021). [PubMed: 34117030]
92. Cheng Z. et al. , Inhibition of BET bromodomain targets genetically diverse glioblastoma. *Clin. Cancer Res.* 19, 1748–1759 (2013). [PubMed: 23403638]
93. Sanjana NE, Shalem O, Zhang F, Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* 11, 783–784 (2014). [PubMed: 25075903]
94. Bankhead P. et al. , QuPath: Open source software for digital pathology image analysis. *Sci. Rep.* 7, 1–7 (2017). [PubMed: 28127051]
95. Wrighton KH et al. , Small C-terminal domain phosphatases dephosphorylate the regulatory linker regions of Smad2 and Smad3 to enhance transforming growth factor-beta signaling. *J. Biol. Chem.* 281, 38365–38375 (2006). [PubMed: 17035229]

96. Cross BC et al. . The molecular basis for selective inhibition of unconventional mRNA splicing by an IRE1-binding small molecule. *Proc. Natl. Acad. Sci. U. S. A.* 109, E869–878 (2012). [PubMed: 22315414]
97. Vasaikar SV, Straub P, Wang J, Zhang B, LinkedOmics: analyzing multi-omics data within and across 32 cancer types. *Nucleic Acids Res.* 46, D956–D963 (2018). [PubMed: 29136207]



**Fig. 1. Oncogenic KRAS inactivation reprograms proteostasis.**

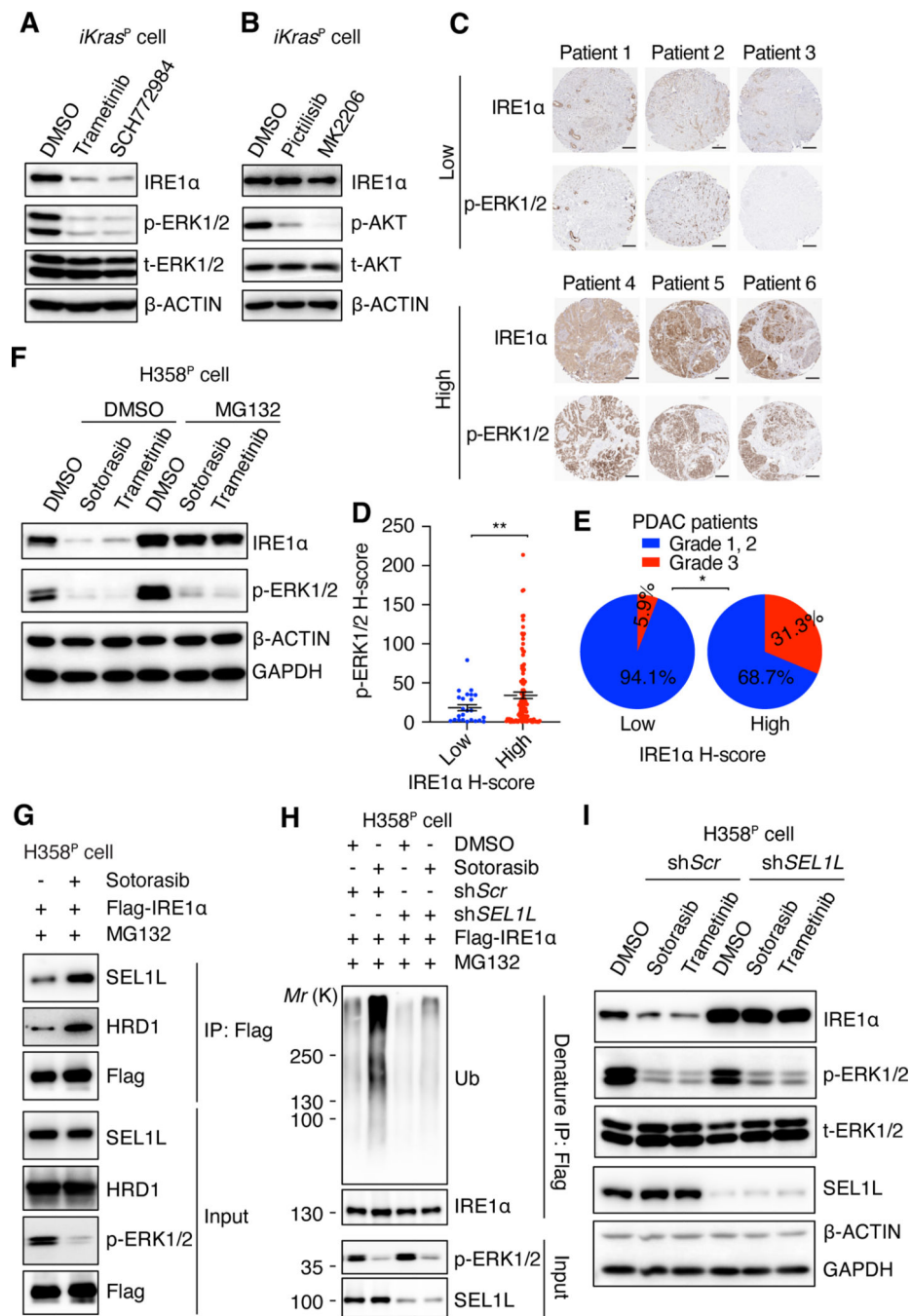
(A) Schematic illustration of labeling and detection of misfolded and aggregated proteins with PROTEOSTAT dye. Upon intercalation into the cross-beta spine typically found in misfolded and aggregated proteins, PROTEOSTAT dye emits strong fluorescence. (B and C) Representative images (B) and quantification (C) of PROTEOSTAT (magenta) and DAPI (blue) staining in *iKras<sup>P</sup>* cells at different time points after *Kras<sup>G12D</sup>* inactivation by Dox-withdrawal (Off Dox) until the cells acquired resistance to *Kras<sup>G12D</sup>* inactivation (*iKras<sup>R</sup>* cell). (D and E) Representative images (D) and quantification (E) of PROTEOSTAT (magenta) staining in spontaneous tumors from the Dox-inducible, *Kras<sup>G12D</sup>*-driven PDAC mouse model (*iKras* GEMM) treated with doxycycline (Dox, 2g/L,  $n=5$ ), Dox withdrawal for 3 days ( $n=4$ ) or relapsed after 30 weeks of Dox-withdrawal ( $n=7$ ). (F) Quantification of PROTEOSTAT intensity in parental MIA-PaCa-2 (MIA<sup>P</sup>) cells treated with DMSO or 30nM sotorasib for 2 days or in sotorasib-resistant MIA-PaCa-2 (MIA<sup>R</sup>) cells treated with 30nM sotorasib. MIA<sup>R</sup> cells were generated *in vitro* by continued sotorasib treatment until the cells acquired resistance. (G and H) Representative images (G) and quantification (H) of PROTEOSTAT (magenta) and DAPI (blue) staining in MIA-PaCa-2 xenograft tumors treated with vehicle ( $n=4$ ), sotorasib (30mg/kg for 1 day,  $n=3$ ), or relapsed after 9 weeks of sotorasib treatment (30mg/kg,  $n=4$ ). Data represent average fluorescence intensity of PROTEOSTAT/cell from each image (C and F) or tumor (E and H) and are presented as mean  $\pm$  SD from  $n = 10$  images. Scale bar: 20 $\mu$ m. Ordinary one-way ANOVA with Dunnett's multiple comparisons test (C, E, F and H) was used to calculate  $P$  values. n.s., not significant, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ .



**Fig. 2. Oncogenic KRAS inactivation differentially impacts the key nodes of the proteostasis regulatory network.**

(A) Immunoblot with indicated antibodies in whole-cell lysates of *iKras<sup>P</sup>* at different time points after *Kras<sup>G12D</sup>* inactivation by Dox-withdrawal (Off Dox) until the cells acquired resistance to *Kras<sup>G12D</sup>* inactivation (*iKras<sup>R</sup>* cell). (B and C) Immunoblot with indicated antibodies in whole-cell lysates of parental or sotorasib-resistant MIA-PaCa-2 (B) or H358 (C) cells treated with DMSO or 30 nM sotorasib. (D) Immunohistochemical (IHC) staining with indicated antibodies in *iKras* GEMM tumors treated with doxycycline (On Dox), Dox

withdrawal for 3 days (Off Dox), or relapsed after 30 weeks of Dox-withdrawal (Off Dox, relapsed). **(E)** IHC staining with indicated antibodies in MIA-PaCa-2 xenograft tumors treated with vehicle, sotorasib (30mg/kg for 1 day), or relapsed after 9 weeks of sotorasib treatment (30mg/kg). **(F)** Relative HSE luciferase activity in *iKras<sup>P</sup>* or *iKras<sup>R</sup>* cells cultured in the presence or absence of Dox for 2 days. Data are shown as mean  $\pm$  SD,  $n=3$ . **(G and H)** Representative images **(G)** and quantification **(H)** of PROTEOSTAT (magenta) and DAPI (blue) staining in *iKras<sup>P</sup>* or *iKras<sup>R</sup>* cells infected with lentiviruses encoding *scramble* shRNA (sh*Scr*), *Xbp1* shRNA (sh*Xbp1*) or *Irel*  $\alpha$  shRNA (sh*Irel* $\alpha$ ). Cells were treated with 2.5mM TUDCA dissolved in water for 2 days as indicated. Data represent the average fluorescence intensity of PROTEOSTAT/cell from each image acquired and presented as mean  $\pm$  SD from  $n=10$  (On Dox),  $n=17$  (Off Dox), or  $n=17$  (Off Dox + TUDCA) images. **(I)** CCK-8 assay was used to quantify cell viability of *iKras* cells treated as in **G** and **H**. Data are presented as mean  $\pm$  SD relative to sh*Scr*,  $n=3$ . Ordinary one-way ANOVA with Dunnett's multiple comparisons test (**H** and **I**) or ordinary one-way ANOVA with Tukey's multiple comparisons test (**F**) was used to calculate *P* values. n.s., not significant, \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . Scale bar: 40 $\mu$ m (**D** and **E**) or 20 $\mu$ m (**G**).



**Fig. 3. KRAS-MAPK signaling stabilizes IRE1α through inhibiting SEL1L-HRD1 mediated IRE1α ubiquitination.**

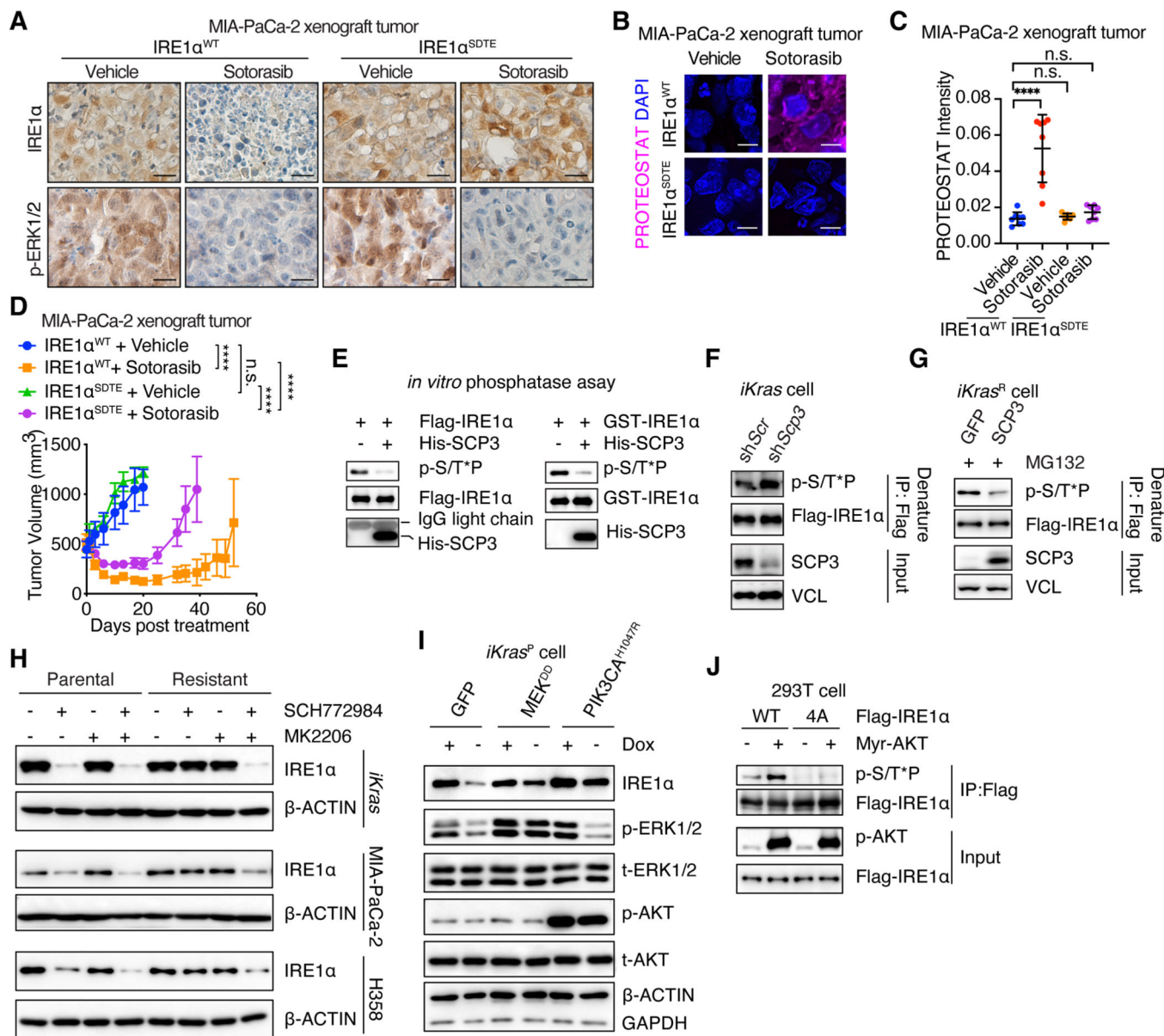
(A and B) Immunoblot with indicated antibodies in whole-cell lysates of *iKras<sup>P</sup>* cells treated with DMSO, trametinib (MEK inhibitor, 20 nM), SCH772984 (ERK inhibitor, 1 μM), pictilisib (PI3K inhibitor, 1 μM), or MK2206 (AKT inhibitor, 2 μM) as indicated for 2 days. (C) Representative images of IHC staining of IRE1α and p-ERK1/2 in tissue microarray of treatment naïve tumors from PDAC patients. Scale bar: 200μm. (D) H-score of p-ERK1/2 in tissue microarray samples with distinct IRE1α intensities. Data are presented as mean



± SEM. **(E)** Proportion of patients with different tumor grades in PDAC patients with low or high IRE1 $\alpha$  H-score. **(F)** Immunoblot with indicated antibodies in whole-cell lysates of H358<sup>P</sup> cells treated with DMSO, 30 nM sotorasib or 20 nM trametinib for 2 days. Cells were treated with DMSO or 1  $\mu$ M MG132 for 12h before harvest. **(G)** Sotorasib promotes the interaction between IRE1 $\alpha$  and SEL1L/HRD1. H358<sup>P</sup> cells expressing Flag-IRE1 $\alpha$  were treated with DMSO or 30 nM sotorasib for 2 days and subjected to immunoprecipitation (IP) with anti-Flag M2 agarose beads. **(H)** Sotorasib promotes SEL1L-dependent IRE1 $\alpha$  ubiquitination. H358<sup>P</sup> cells expressing Flag-IRE1 $\alpha$  and sh*Scr* or sh*SEL1L* were treated with DMSO or 30 nM sotorasib for 2 days and subjected to denature IP with anti-Flag M2 agarose beads. The immunoblot was probed with anti-ubiquitin (Ub) antibody to detect IRE1 $\alpha$  ubiquitination. MG132 (1  $\mu$ M) was added into the culture medium 12 h before harvest **(G and H)**. **(I)** Immunoblot with indicated antibodies in whole-cell lysates of H358<sup>P</sup> cells infected with lentiviruses encoding sh*Scr* or sh*SEL1L* and treated with DMSO, 30 nM sotorasib, or 20 nM trametinib for 2 days. 2-tailed, unpaired Student's t test with Welch's correction **(D)** or Fisher's exact test **(E)** was used to calculate *P* values. \* *P*<0.05, \*\**P*<0.01.

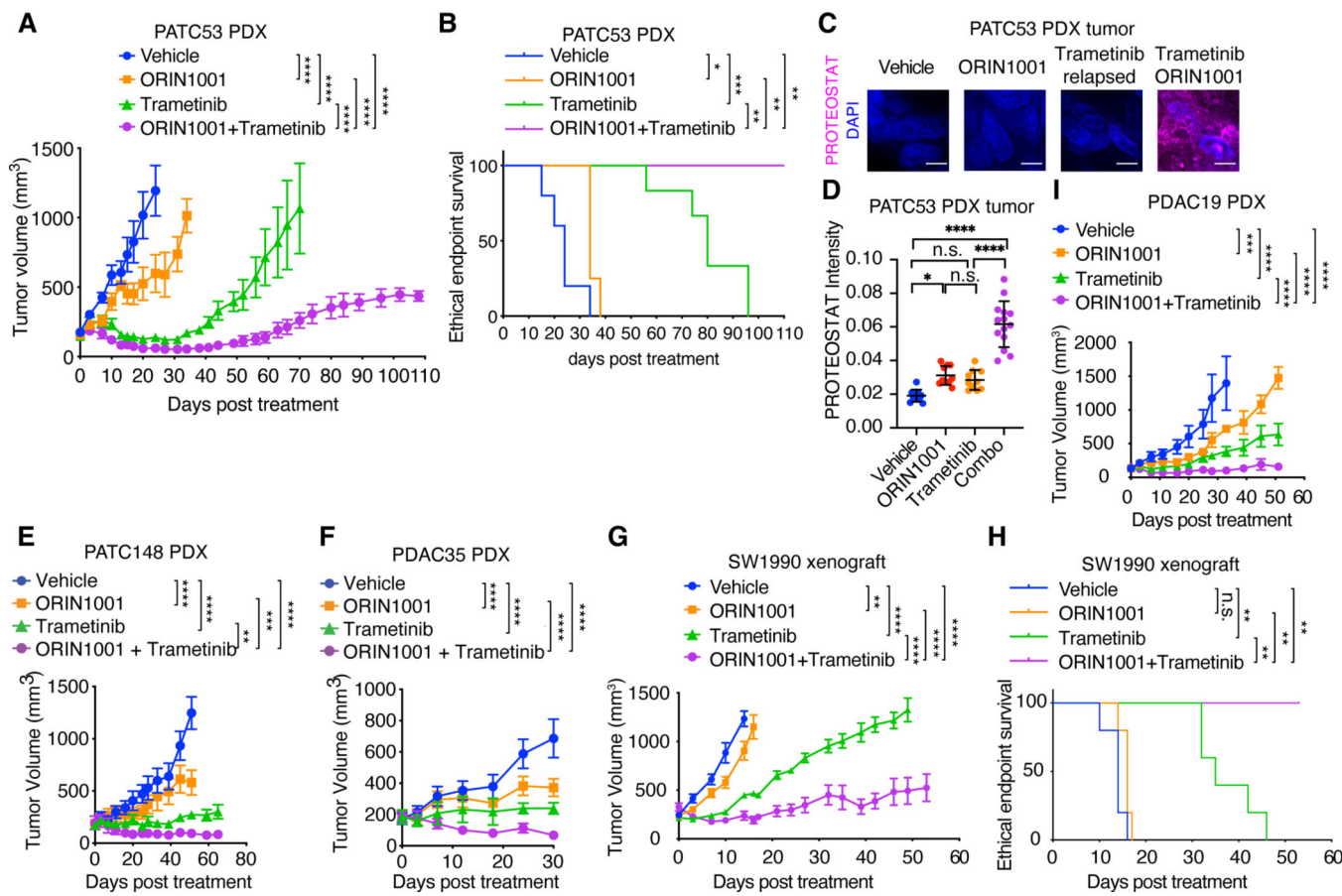


binding D-motifs and ERK phosphorylation sites at Ser525, Ser529, Ser549, and Thr973 (red). Phospho-deficient (4A) and phospho-mimetic (SDTE) mutations of IRE1 $\alpha$  are shown. LD: luminal domain. TM: transmembrane domain. **(F)** *In vitro* [ $g\text{-}^{32}\text{P}$ ] ATP kinase assay using different Flag-tagged IRE1 $\alpha$  mutants and GST-ERK2. The IRE1 $\alpha$  phosphorylation was detected by autoradiography. One-Step Blue Protein Stain was used to detect IRE1 $\alpha$  protein loading. **(G)** *In vitro* kinase assay using equal amount of Flag-tagged WT or phospho-deficient IRE1 $\alpha$  mutant proteins (4A) and GST-ERK2. **(H)** Spearman correlation between p-IRE1 $\alpha$  (at S549) and p-ERK1 (at Y204) in 55 patients with non-small cell lung cancer. **(I and K)** Whole-cell lysates of *iKras* cells expressing HA-HRD1 together with WT or mutant IRE1 $\alpha$  cultured in the absence of Dox were subjected to IP with anti-HA agarose beads to detect IRE1 $\alpha$  interaction with HRD1. MG132 (1  $\mu\text{M}$ ) was added into the culture medium 12h before harvest **(A, I and K)**. **(J and L)** Immunoblot of WT or mutant IRE1 $\alpha$  in whole-cell lysates of *iKras* cells cultured in the presence or absence of Dox for 2 days.



**Fig. 5. Multiple pathways converge to restore IRE1 $\alpha$  in KRASi-resistant cancer cells.** (A) IHC staining of p-ERK1/2 and IRE1 $\alpha$  in shRNA-resistant IRE1 $\alpha$ <sup>WT</sup>- or IRE1 $\alpha$ <sup>SDTE</sup>-transduced, endogenous IRE1 $\alpha$ -depleted MIA-PaCa-2 tumors treated with vehicle or sotorasib (100mg/kg) for 4 days. Scale bar: 40 $\mu$ m. (B-C) Representative images (B) and quantification (C) of PROTEOSTAT (magenta) and DAPI (blue) staining in MIA-PaCa-2 tumors as in (A). Data represent average fluorescence intensity of PROTEOSTAT/cell from each image acquired and are presented as mean  $\pm$  SD from  $n=8$  independent images. Scale bar: 20 $\mu$ m. (D) Tumor volume quantification of MIA-PaCa-2 tumors as in (A). (E) *In vitro* phosphatase assay. Phosphorylated Flag-IRE1 $\alpha$  protein purified from MEK<sup>DD</sup>-expressing 293T cells (Left panels) or recombinant IRE1 $\alpha$  protein phosphorylated by recombinant ERK2 *in vitro* (Right panels) were subjected to *in vitro* phosphatase assay with recombinant SCP3. (F-G) *iKras* cells expressing Flag-IRE1 $\alpha$  were infected with shRNA targeting *Scr* or

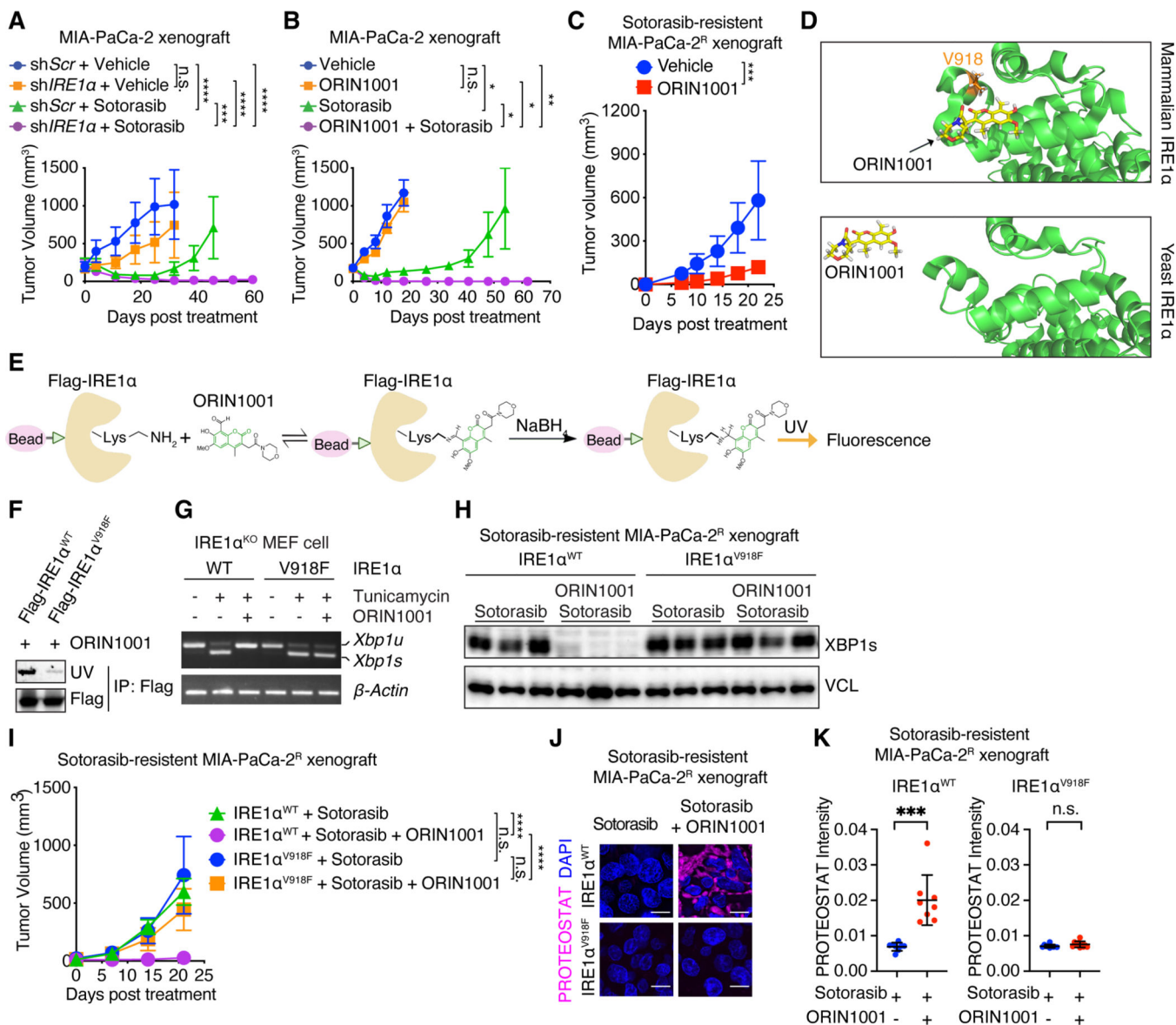
*Scp3* (F), GFP- or SCP3- expressing lentivirus (G). The whole cell lysates were subjected to denature IP with anti-Flag M2 agarose beads, followed by immunoblot with anti-pS/T\*P antibody to detect IRE1 $\alpha$  phosphorylation. (H) Immunoblot of IRE1 $\alpha$  in whole-cell lysates of parental and KRAS inhibition-resistant cells treated with DMSO, 2 $\mu$ M MK2206 and/or 1  $\mu$ M SCH772984 for 2 days (MIA-PaCa-2 and H358 cells) or 14 days (*iKras* cells). (I) Immunoblot of IRE1 $\alpha$  in whole-cell lysates of *iKras*<sup>P</sup> cells expressing GFP, MEK<sup>DD</sup>, or PIK3CA<sup>H1047R</sup> in the presence or absence of Dox for 2 days. (J) 293T cells expressing IRE1 $\alpha$ <sup>WT</sup> or IRE1 $\alpha$ <sup>4A</sup> in the presence or absence of myr-AKT were subjected to IP with anti-Flag M2 agarose beads followed by immunoblot to detect IRE1 $\alpha$  phosphorylation. Ordinary one-way ANOVA with Dunnett's multiple comparisons test (C), and Two-way ANOVA with Bonferroni's multiple comparisons test (D) were used to calculate *P* values. n.s., not significant, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001.



**Fig. 6. IRE1 $\alpha$  inhibition sensitizes oncogenic KRAS-driven tumors to MEK inhibition.**

(A) Tumor volume quantification of established PATC53 PDX tumors in SCID/beige mice treated with vehicle ( $n=5$ ), IRE1 $\alpha$  RNase inhibitor ORIN1001 ( $n=4$ ), MEK inhibitor trametinib ( $n=6$ ), or ORIN1001 plus trametinib ( $n=4$ ). (B) Kaplan-Meier survival curve of PATC53 PDX tumor-bearing mice under treatment as indicated in (A). (C-D) Representative images (C) and quantification (D) of PROTEOSTAT (magenta) and DAPI (blue) staining in endpoint PATC53 xenograft tumors treated as in (A). Data represent average fluorescence intensity of PROTEOSTAT/cell from each image acquired and are presented as mean  $\pm$  SD from  $n=10$  independent images. Scale bar: 20 $\mu$ m. (E) Tumor volume quantification of established PATC148 PDX tumors in SCID/beige mice treated with vehicle ( $n=6$ ), ORIN1001 ( $n=6$ ), trametinib ( $n=4$ ), or ORIN1001 plus trametinib ( $n=4$ ). (F) Tumor volume quantification of established PDAC35 PDX tumors in SCID/beige mice treated with vehicle ( $n=5$ ), ORIN1001 ( $n=4$ ), trametinib ( $n=4$ ), or ORIN1001 plus trametinib ( $n=4$ ). (G) Tumor volume quantification of established SW1990 PDAC xenograft tumors in SCID/beige mice treated with vehicle ( $n=6$ ), ORIN1001 ( $n=6$ ), trametinib ( $n=5$ ), or ORIN1001 plus trametinib ( $n=4$ ). (H) Kaplan-Meier survival curve of SW1990 PDAC xenograft tumor-bearing mice under treatment as indicated in (G). (I) Tumor volume quantification of established PDAC19 PDX tumors in SCID/beige mice treated with vehicle, ORIN1001, trametinib, or ORIN1001 plus trametinib ( $n=4$ ). ORIN1001: 150mg/kg. Trametinib: 1mg/kg. Data are presented as mean  $\pm$  SEM (A, E to G, I) or mean  $\pm$  SD (D). Two-way ANOVA with Bonferroni's

multiple comparisons test (**A**, **E** to **G**, **I**), log-rank (Mantel-Cox) test (**B** and **H**), or ordinary one-way ANOVA with Dunnett's multiple comparisons test (**D**) was used to calculate *P* values. n.s., not significant, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ .

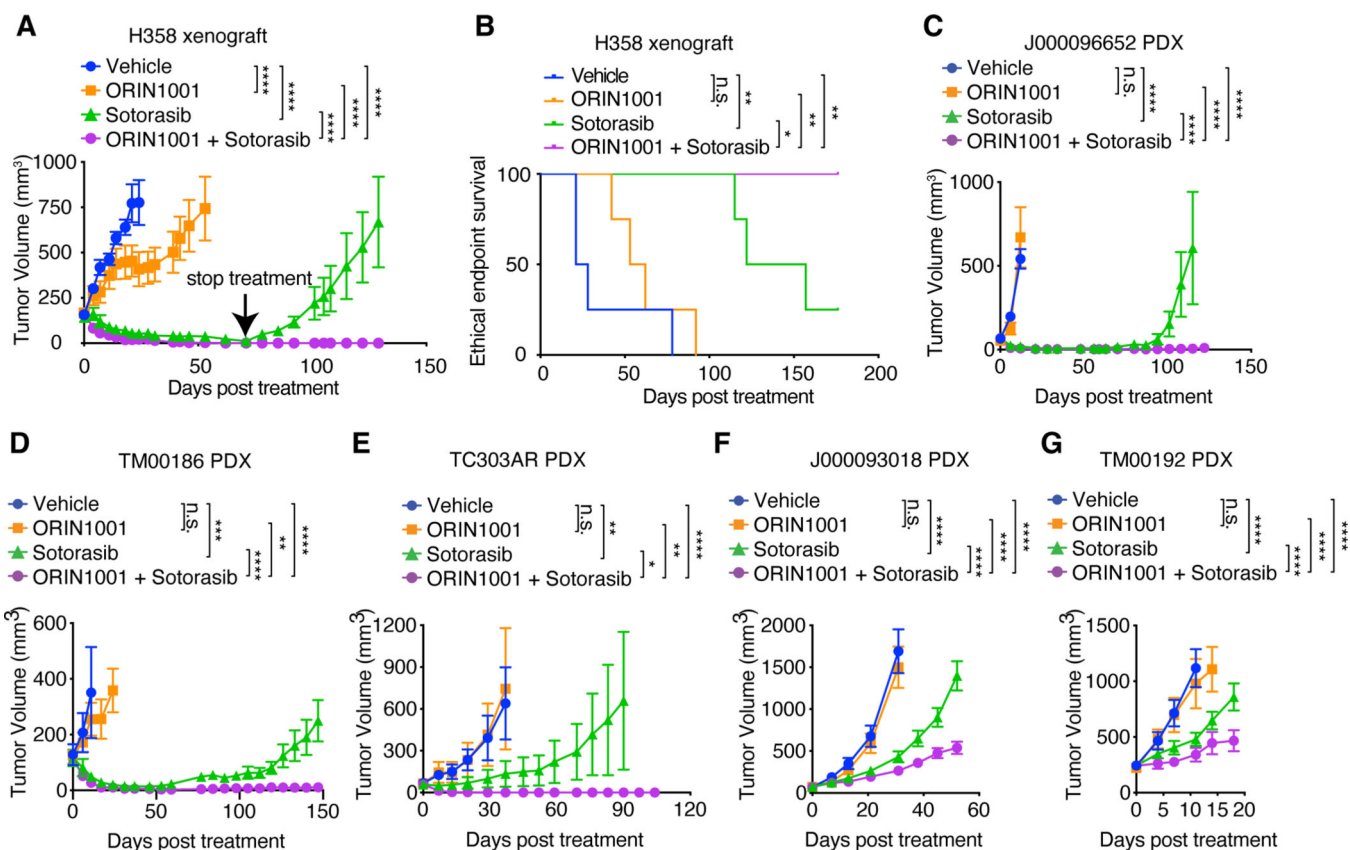


**Fig. 7. IRE1a inhibition enhances tumor responses to sotorasib.**

(A) Tumor volume of MIA-PaCa-2 tumors transduced with dox-inducible shScr or shIRE1a and treated with doxycycline water, vehicle ( $n=5$ ) or sotorasib ( $n=6$ ). (B) Tumor volume of MIA-PaCa-2 tumors treated with vehicle ( $n=5$ ), ORIN1001 ( $n=4$ ), sotorasib ( $n=4$ ), or both ( $n=5$ ). (C) Tumor volume of sotorasib-resistant MIA-PaCa-2<sup>R</sup> tumors treated with vehicle ( $n=4$ ) or ORIN1001 ( $n=7$ ). (D) Docking modeling of ORIN1001 with IRE1a. V918 is critical for the formation of the shallow pocket at mammalian IRE1a RNase-active site for ORIN1001 binding. (E) Biochemical fluorescence assay detecting the binding between ORIN1001 and IRE1a *in vitro*. (F) Equal amount of Flag-IRE1a<sup>WT</sup> or Flag-IRE1a<sup>V918F</sup> protein purified from 293T cells was used to pull down ORIN1001 *in vitro*. UV transmission was used to detect ORIN1001 that is covalently bound to IRE1a protein in the SDS-PAGE. (G) *Xbp1* splicing in *Ire1a*-knock out MEF cells expressing IRE1a<sup>WT</sup> or IRE1a<sup>V918F</sup> and treated with tunicamycin (5  $\mu$ g/mL) and/or ORIN1001 (5



$\mu\text{M}$ ) for 6 hours as indicated. **(H)** Immunoblot of XBP1s in shRNA-resistant  $\text{IRE1}\alpha^{\text{WT}}$  or  $\text{IRE1}\alpha^{\text{V918F}}$ -transduced, endogenous *IRE1* $\alpha$ -depleted MIA-PaCa-2<sup>R</sup> tumors treated as indicated. **(I)** Tumor volume of established shRNA-resistant  $\text{IRE1}\alpha^{\text{WT}}$ - or  $\text{IRE1}\alpha^{\text{V918F}}$ -expressing, endogenous *IRE1* $\alpha$ -depleted, sotorasib-resistant MIA-PaCa-2<sup>R</sup> tumors treated as indicated.  $n=10$ . **(J-K)** Representative images **(J)** and quantification **(K)** of PROTEOSTAT and DAPI staining in MIA-PaCa-2<sup>R</sup> tumors treated as in **(I)**. Data represent average fluorescence intensity of PROTEOSTAT/cell from each image and are presented as mean  $\pm$  SD from  $n>10$  independent images. Scale bar: 20 $\mu\text{m}$ . Sotorasib: 100mg/kg. ORIN1001: 300mg/kg. Data are presented as mean  $\pm$  SEM **(A, B, C, and I)**. Two-way ANOVA test with Bonferroni's multiple comparisons test **(A, B, C, and I)** or ordinary one-way ANOVA with Dunnett's multiple comparisons test **(K)** was used to calculate *P* values. n.s., not significant, \*  $P<0.05$ , \*\*  $P<0.01$ , \*\*\*  $P<0.001$ , \*\*\*\*  $P<0.0001$ .



**Fig. 8. IRE1 $\alpha$  inhibition enhances the response of KRAS<sup>G12C</sup>-driven tumors to sotorasib.**

(A) Tumor volume quantification of established H358 tumors in SCID/beige mice treated with vehicle, ORIN1001 (150mg/kg), sotorasib (30mg/kg), or ORIN1001 plus sotorasib. ( $n=4$ ). Treatment was stopped at day 71. (B) Kaplan-Meier survival curve of H358 tumor-bearing mice under different treatments as indicated in (A) from treatment start time. (C) Tumor volume quantification of established J000096652 PDX tumors in NSG mice treated with vehicle ( $n=6$ ), ORIN1001 (300mg/kg,  $n=4$ ), sotorasib (100mg/kg,  $n=7$ ), or ORIN1001 plus sotorasib ( $n=8$ ). Treatment was stopped at day 65. (D) Tumor volume quantification of established TM00186 PDX tumors in NSG mice treated with vehicle ( $n=6$ ), ORIN1001 (300mg/kg,  $n=6$ ), sotorasib (100mg/kg,  $n=7$ ), or ORIN1001 plus sotorasib ( $n=9$ ). (E) Tumor volume quantification of established TC303AR PDX tumors in NSG mice treated with vehicle ( $n=5$ ), ORIN1001 (300mg/kg,  $n=5$ ), sotorasib (100mg/kg,  $n=9$ ), or ORIN1001 plus sotorasib ( $n=9$ ). Treatment was stopped at day 53. (F) Tumor volume quantification of established J000093018 PDX tumors in NSG mice treated with vehicle ( $n=6$ ), ORIN1001 (300mg/kg,  $n=6$ ), sotorasib (100mg/kg,  $n=9$ ), or ORIN1001 plus sotorasib ( $n=9$ ). (G) Tumor volume quantification of established TM00192 PDX tumors in NSG mice treated with vehicle ( $n=6$ ), ORIN1001 (300mg/kg,  $n=5$ ), sotorasib (100mg/kg,  $n=9$ ), or ORIN1001 plus sotorasib ( $n=9$ ). Data are presented as mean  $\pm$  SEM (A, C to G). Two-way ANOVA test with Bonferroni's multiple comparisons test (A, C to G), log-rank (Mantel-Cox) test (B) was used to calculate  $P$  values. n.s., not significant, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ .