The Unfolded Protein Response Selectively Targets Active Smoothened Mutants

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The Hedgehog signaling pathway, an essential regulator of developmental patterning, has been implicated in playing causative and survival roles in a range of human cancers. The signal-transducing component of the pathway, Smoothened, has revealed itself to be an efficacious therapeutic target in combating oncogenic signaling. However, therapeutic challenges remain in cases where tumors acquire resistance to Smoothened antagonists, and also in cases where signaling is driven by active Smoothened mutants that exhibit reduced sensitivity to these compounds. We previously demonstrated that active Smoothened mutants are subjected to prolonged endoplasmic reticulum (ER) retention, likely due to their mutations triggering conformation shifts that are detected by ER quality control. We attempted to exploit this biology and demonstrate that deregulated Hedgehog signaling driven by active Smoothened mutants is specifically attenuated by ER stressors that induce the unfolded protein response (UPR). Upon UPR induction, active Smoothened mutants are targeted by ER-associated degradation, resulting in attenuation of inappropriate pathway activity. Accordingly, we found that the UPR agonist thapsigargin attenuated mutant Smoothened-induced phenotypes *in vivo* in *Drosophila melanogaster*. Wild-type Smoothened and physiological Hedgehog patterning were not affected, suggesting that UPR modulation may provide a novel therapeutic window to be evaluated for targeting active Smoothened mutants in disease.

The Hedgehog (Hh) signaling pathway provides essential patterning information during development and is frequently activated in cancer (1, 2). Inappropriate Hh signaling is causative in medulloblastoma and basal cell carcinoma and has been implicated in a number of additional cancers, including those of the lung, breast, prostate, and digestive tract (2–9). Smoothened (Smo), a member of the G-protein-coupled receptor superfamily, functions as the requisite signal-transducing molecule of the Hh pathway (10, 11). Accordingly, oncogenic mutation of Smo is one mechanism by which the Hh pathway can become inappropriately activated in cancer (3; <http://www.sanger.ac.uk>).

We recently described a set of active Smo mutants that, like oncogenic Smo, induce ligand-independent Hh pathway activity (12). These mutants, C320A and C339A in the *Drosophila melanogaster* protein and C299A and C318A in the murine protein, are predicted to break disulfide bonds that stabilize a regulated conformation of the Smo extracellular loop domain (12, 13). Consistent with the prediction that alteration of such bonds results in a misfolded protein, all of these mutants are largely retained in the endoplasmic reticulum (ER) (12). Similarly, the oncogenic Smo mutant SmoM2 has been reported to be largely ER localized (14, 15). However, a small pool of M2 escapes the ER and traffics to the primary cilium through an atypical Rab8 dependent secretory route (16, 17). This transport from the ER to the primary cilium is important for M2 oncogenic activity, as genetic ablation of the primary cilium attenuates M2-induced tumor formation in mice (16, 18).

Accumulation of misfolded protein in the ER adversely affects ER homeostasis (19, 20). This can result in high ER stress, leading to induction of the unfolded protein response (UPR), a compensatory process aimed at ameliorating ER stress and preventing stress-induced cell death (20, 21). The UPR is organized into three branches, each controlled by a unique upstream activator. The PERK branch triggers phosphorylation of elongation factor 2 α to

attenuate translation of nascent proteins bound for the ER (22). The ATF6 and IRE1 α branches activate transcription factors that drive expression of UPR target genes involved in protein quality control and ER-associated degradation (ERAD). ERAD targets misfolded proteins for retro-translocation from the ER to the cytoplasm, where they undergo proteasome-mediated degradation (20, 23–25). Persistent ER stress that cannot be corrected by the UPR will eventually result in apoptosis (20). However, the exact mechanisms by which the UPR signals induction of apoptosis under such conditions are not yet clear.

Given its ability to influence cellular homeostasis and apoptosis, it is no surprise that the UPR has become an attractive target for therapeutic intervention in cancer. Because tumor cells typically exist under nutrient-poor, hypoxic conditions that readily induce ER stress, it has been widely acknowledged that therapeutic manipulation of the UPR under such conditions may serve as an Achilles' heel for targeting tumor cells (26, 27). Accordingly, a number of small-molecule ER stress modulators, both UPR agonists and antagonists, are currently in or en route to the clinic (27).

The increased localization of active Smo mutants to the ER prompted us to test whether they might be sensitive to alteration of ER homeostasis and induction of the UPR. Here, we describe our findings, which demonstrate that active Smo mutants, including extracellular loop C-to-A mutants and the oncogenic mutant SmoM2, are specifically destabilized by the UPR under conditions

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of thermally and chemically induced ER stress. Under these conditions, signaling by active Smo mutants is attenuated by their selective degradation via ERAD. Consistent with these results, the ER stress and UPR-inducing compound thapsigargin blocks Smo-mediated Hh gain-of-function phenotypes *in vivo* in *Drosophila*. These findings suggest that ER stress modulators that trigger the UPR may represent a novel therapeutic window to be evaluated for treatment of Hh-dependent cancers. Such compounds may be particularly efficacious in cancers initiated by oncogenic Smo and/or in tumors harboring Smo mutations that demonstrate reduced sensitivities to the current cache of Smo inhibitors (28–31).

MATERIALS AND METHODS

Functional assays and biochemical analyses. Reporter assays were performed as described previously (12), with the following modifications. For rescue experiments, $\sim 1.5 \times 10^6$ Cl8 cells were transfected with 100 ng ptc Δ 136-luciferase, 10 ng pAc-Renilla, 20 ng *smo* 5' untranslated region (UTR) double-stranded RNA (dsRNA), 100 ng pAc-hh or empty vector control, and 20 ng of the indicated wild-type or mutant pAc-*smo* construct (12, 32, 33). For dominant activity assays, 20 ng of the indicated *myc-smo* expression vector was expressed in the absence of Hh, and reporter activity was assessed as described previously (12). Cells were transfected at 25°C and allowed to recover for 24 h prior to shifting to 22°C or 29°C 24 h prior to analysis. For Hsp70 inhibition, cells were treated with VER155008 (VER; Tocris Bioscience) or vehicle control (dimethyl sulfoxide [DMSO]) for ~ 16 h prior to cell lysis. Reporter assays were performed at least two times in duplicate, and all data were pooled. Reporter activity is shown as the percent activity relative to the control Hh response for each temperature, set to 100%. Error bars represent standard errors of the means.

For protein stability analysis in *Drosophila* cells, $\sim 5 \times 10^6$ Cl8 cells were transfected with 5 μ g of wild-type or mutant pAc-*smo* by using Lipofectamine 2000 (Invitrogen). Cells were transfected at 25°C and allowed to recover for 24 h before shifting to 22°C or 29°C. Lysates were prepared 24 h after the temperature shift in 2% SDS, 4% glycerol, 40 mM Tris-HCl (pH 6.8), 0.5 mM dithiothreitol (DTT), and 1 $\times$ protease inhibitor cocktail (PIC; Roche). Extracts were sheared by passing 5 times through a 26-gauge syringe. Equal amounts of protein were analyzed by SDS-PAGE and Western blotting with anti-Myc (Roche) and antikinesin (cytoskeleton) antibodies.

For protein stability analysis in mammalian cells, $\sim 1 \times 10^6$ NIH 3T3 cells were transfected with 2 μ g of wild-type or mutant pCDNA3.1-*msmo* (12). Transfected cells were maintained at 37°C for ~ 44 h and then shifted to 40°C for 4 h prior to lysis. Lysates were prepared in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, 0.1% SDS, 0.5 mM DTT, and 1 $\times$ PIC (Roche) and incubated for 30 min at 4°C (34). Extracts were cleared by centrifuging at $16,000 \times g$ at 4°C for 45 min. Supernatants were analyzed by SDS-PAGE and Western blotting using anti-GADD153/CHOP (B-3; Santa Cruz Biotechnology), anti-Smo (E5; Santa Cruz Biotechnology), and antitubulin (Cell Signaling). For Gli1 protein analysis, cells were transferred to Dulbecco's modified Eagle's medium (DMEM) plus 0.5% serum for ~ 40 h prior to lysis. Gli1 was analyzed by Western blotting with L42B10 (Cell Signaling).

For drug sensitivity analysis, NIH 3T3 cells expressing murine Smo (here referred to as mSmo) proteins at 37°C were treated with vehicle or 1 μ M thapsigargin (Sigma) or the indicated concentration of 17-AAG, SNX2112, or Bortezomib (Selleck Chemicals) in DMEM containing 0.5% fetal calf serum for 4 h prior to lysis. Whole-cell lysates were prepared and analyzed as described above. For assays requiring Sonic Hedgehog (SHH) stimulation, cells were treated with recombinant SHH (R&D Systems) at a concentration of 100 ng/ml for ~ 12 h prior to the indicated drug treatments.

For glycosylation analysis, cell lysates were prepared from Cl8 or NIH

3T3 cells, as described above. Cl8 lysates were prepared in 1% NP-40, 150 mM NaCl, 50 mM Tris, 50 mM NaF, 0.5 mM DTT, and 1 $\times$ PIC (Roche), and centrifuged for 10 min at $2,000 \times g$. NIH 3T3 cells were lysed as described above. Supernatants were treated with peptide-N-glycosidase F (PNGase), endoglycosidase H (endo H), O-glycosidase, or λ -phosphatase (NEB) for 2 h at room temperature and analyzed by SDS-PAGE and Western blotting.

Control and Hrd1 small interfering RNAs (siRNAs) were obtained from Invitrogen and transfected per the manufacturer's instructions. Hrd1 siRNA consisted of a pool of three individual siRNAs. For knock-down experiments, approximately $\sim 7 \times 10^5$ cells were transfected with the indicated mSmo expression vector and 60 nM siRNA by using Lipofectamine 2000 (Invitrogen). Cells were grown at 37°C for ~ 44 h, shifted to 40°C for 4 h prior to lysis, and processed as described above.

Reverse transcription-PCR (RT-PCR) analyses. To assess endogenous *Xbp1* splicing, nontransgenic *w¹¹¹⁸* larvae were grown at 22°C or 29°C, collected at the third-instar stage, and homogenized and extracted using TRIzol reagent (Sigma). cDNA was synthesized from 5 μ g of RNA by using the SuperScript III system (Invitrogen) and the following primers: Xbp1-RT-f, 5'-AGATGCATCAGCCAATCCAAC; Xbp1-RT-r, 5'-CAGGTGGACACACAGTCGT.

To assess *gli1* and *smo* expression by quantitative PCR (qPCR), NIH 3T3 cells were plated as described above and transfected using Fugene 6 (Promega). Approximately 30 h posttransfection, culture medium was replaced with complete medium containing 250 nM thapsigargin or the vehicle (ethanol) control. RNA was extracted with RNeasy (Qiagen) ~ 16 h after medium exchange. cDNA was synthesized from 5 μ g of RNA by using the SuperScript III first-strand synthesis system (Invitrogen). qPCRs were performed on cDNA diluted 1:10 using SYBR green PCR master mix (Applied Biosystems). *GAPDH* was used as a reference gene, and results were analyzed using the standard $2^{-\Delta\Delta CT}$ method (35) and the following gene-specific primers: Gli1-qPCR-f, 5'-GGTCTCGGGGTCTCA AACTGC; Gli1-qPCR-r, 5'-CGGCTGACTGTGTAAGCAGAG; mSmo-qPCR-f, 5'-CGCCAAGGCCTTCTCTAAGCG; mSmo-qPCR-r, 5'-CCT CTGCCTGGGCTCAGCAT; mGAPDH-qPCR-f, 5'-GTGGTGAAGCAG GCATCTGA; mGAPDH-qPCR-r, 5'-GCCATGTAGGCCATGAGGTC.

qPCR analysis was performed three times in triplicate, and all data were pooled. Error bars indicate standard errors of the means.

Fly crosses. Fly stocks were maintained at 18°C on Jazz agarose (Fisher). Crosses were performed at 22°C or 29°C as indicated. Upstream activation sequence (UAS)-*myc-smo*, UAS-*myc-smoC320A*, and UAS-*myc-smoC339A* (12) were expressed under the control of *MS1096-Gal4*, *C765-Gal4*, or *Ap-Gal4* as indicated. The *Xbp1^{k13803}* allele was used for synthetic interaction studies. Synthetic interaction crosses were performed at 25°C. For drug treatment, 1- to 24-h *MS1096-GAL4*, UAS-*myc-smoC320A* or *C765-GAL4*, UAS-*myc-smoC320A* embryos were collected and transferred to vials containing 2 ml Jazz agarose containing vehicle (ethanol) or thapsigargin (Sigma) at a final concentration of 1 μ M. Drug feeding was performed across two separate crosses, and multiple progeny were analyzed.

For wing analyses, crosses were performed at least twice, and multiple progeny were analyzed. Representative wings from adult flies were mounted on glass slides by using DPX mounting medium and imaged on a Zeiss Stemi 2000-C11 microscope with a Zeiss AxioCam ICc3 camera. In cases where wings were severely blistered, the whole fly was imaged. Images were prepared using Photoshop CS4.

For salivary gland analysis, UAS-*Xbp1-GFP* (36) and/or UAS-*myc-smoC320A* was expressed under the control of *sgs3-Gal4*. Green fluorescent protein (GFP) expression was examined in salivary glands dissected from third-instar larvae. Multiple salivary glands were examined across two independent crosses, and representative samples are shown.

Immunofluorescence. pAc-*myc-smo* constructs were expressed in S2 cells as described previously (12). For temperature sensitivity analyses, cells were transfected at 25°C and allowed to recover for 6 h prior to temperature shift. Fixation, immunostaining, and image analysis were

performed 48 h after the temperature shift, as described previously (12). Primary cilium analysis in NIH 3T3 cells was performed exactly as described previously (12). GRP94 antibody was provided by L. Hendershot (37). Anti-Ci 2A1 hybridoma was obtained from the DHSB. Imaginal disc and salivary gland staining and analyses were performed as described previously (12).

For all indirect immunofluorescence assays, the indicated primary antibodies were detected using Alexa Fluor (Invitrogen) secondary antibodies conjugated to 488 or 555 fluorophores. Data were obtained using a Zeiss LSM 510 confocal microscope and processed using LSM Image Browser and Photoshop CS4 software.

RESULTS

To determine whether activities of the ER-retained active *Drosophila* Smo mutants C320A and C339A are affected by induction of a cellular stress response, we induced thermal stress by performing crosses at a high temperature (29°C). To determine whether the UPR is induced at this temperature, we monitored ER stress by examining (i) IRE1 α -mediated splicing of endogenous *Xbp1* transcript and (ii) activity of an *Xbp1*-GFP stress sensor that expresses GFP upon stress-induced *Xbp1* splicing (36, 38). Only unspliced *Xbp1* transcript was detected in RNA harvested from nontransgenic *w¹¹¹⁸* larvae grown at 22°C (Fig. 1A). Conversely, a robust enrichment of spliced *Xbp1* transcript was evident in RNA harvested from larvae grown at 29°C (Fig. 1A), indicating the presence of ER stress. Similar results were obtained with the *Xbp1*-GFP stress sensor. Whereas minimal expression of GFP was observed in salivary glands dissected from ER stress reporter larvae grown at 22°C, a strong GFP signal was observed at 29°C (Fig. 1B and B'). Taken together, these results support that an ER stress response can be induced by performing crosses at 29°C.

We previously demonstrated that when expressed under the control of the UAS/GAL4 system at 25°C, the wild-type Smo transgene induces a modest Hh gain-of-function phenotype, and SmoC320A and SmoC339A transgenes induce strong phenotypes (12). When we expressed these same transgenes at 22° or 29°C, wild-type Myc-Smo did not trigger a robust Hh gain-of-function phenotype at 22°C but did induce mild ectopic vein formation when expressed at 29°C, likely due to higher-level UAS/GAL4 transgene expression at 29°C (39) (Fig. 1D compared to D' and C). Conversely, expression of the activating C320A and C339A Myc-Smo mutants induced mild phenotypes at 29°C and dramatic phenotypes, including dorsal wing overgrowth, wing blistering, and delayed eclosion at 22°C (Fig. 1E and F, compared to E' and F'). Additionally, whereas both C320A and C339A Myc-Smo transgenic flies eclosed in normal ratios at 29°C, expression of the more active Myc-SmoC339A mutant induced a high degree of pupal lethality at 22°C. Taken together, these results suggest that active Smo mutants induce robust signaling at the low temperature but that their activity is significantly attenuated at a temperature that induces stress response pathways, including the UPR (Fig. 1A and B).

To confirm that the strong wing phenotypes observed at the low temperature were not due to chronic ER stress resulting from high-level expression of mutant Smo protein, we expressed *myc-smoC320A* with the *Xbp1*-GFP stress reporter and monitored for GFP induction in Smo-expressing salivary glands. Despite robust accumulation of Myc-SmoC320A protein in salivary gland cells, activity of the GFP stress sensor was not detected (Fig. 1G). As a control, we treated *Xbp1*-GFP transgenic larvae with the potent ER stress-inducing compound thapsigargin, and we observed a

robust GFP signal in salivary glands dissected from thapsigargin-fed larvae (Fig. 1G'). We also tested for a genetic interaction between *smo* and the IRE1 α stress response pathway. The Myc-SmoC320A phenotype was not modified by introduction of the *Xbp1* loss-of-function allele *Xbp1^{k13803}* (Fig. 1H and H'), further supporting that the observed wing phenotypes did not result from chronic ER stress triggered by expression of active Smo mutants.

To confirm that the observed *in vivo* temperature sensitivity was a specific effect on mutant Smo proteins, and not an artifact of *in vivo* transgene expression, we switched to an *in vitro* Clone 8 (Cl8) cell culture system that allowed us to perform functional assays at permissive (22°C) and restrictive (29°C) temperatures. Cl8 cells are derived from wing imaginal disc tissue and possess an intact Hh pathway, making them an ideal cell line to perform biochemical and functional analyses for Hh pathway activity (33, 40). We assessed the ability of wild-type and mutant Smo proteins to rescue reporter gene expression in Cl8 cells in a dsRNA-mediated *smo* knockdown background at 22°C or 29°C (Fig. 2A). Knockdown of endogenous *smo* by using 5'-UTR dsRNA attenuated Hh-induced reporter gene expression at both permissive and restrictive temperatures (Fig. 2A, UTR dsRNA). Reexpression of wild-type Myc-Smo by using cDNA lacking the UTR sequence did not alter baseline signaling activity, but it rescued Hh-dependent reporter gene induction to similar levels at both temperatures, suggesting that, as was observed in flies, wild-type Myc-Smo function is not significantly altered by temperature. Consistent with our previous studies performed at 25°C (12), both C320A and C339A mutant Myc-Smo proteins significantly elevated baseline signaling and partially (C320A) or fully (C339A) rescued Hh-dependent reporter gene activity in the *smo* knockdown background at 22°C (Fig. 2A, white and light gray bars). However, both mutants were compromised in their ability to elevate baseline activity and to rescue Hh-induced reporter gene activity at the restrictive 29°C temperature (Fig. 2A, dark gray and black bars).

We next examined the abilities of wild-type or mutant Myc-Smo proteins to induce ectopic reporter gene activity at permissive or restrictive temperatures in a wild-type *smo* background. Consistent with Smo being posttranslationally regulated (41, 42), provision of exogenous wild-type Myc-Smo did not increase baseline signaling at either temperature (Fig. 2B, wtSmo). Conversely, at 22°C, both mutants increased baseline signaling to a level near to (C320A) or equal to (C339A) the control Hh response (Fig. 2B, white bars). This activity was attenuated at 29°C, suggesting that the presence of endogenous Smo does not correct the temperature sensitivity of the C320A or C339A Myc-Smo mutants (Fig. 2B, gray bars). Taken together with the *smo* rescue reporter assay results, these results suggest that the observed *in vivo* temperature sensitivity for activation of Smo mutants is triggered by a molecular mechanism that affects mutant Smo proteins, rather than by altered transgene expression.

To determine whether attenuation of mutant Smo signaling was the result of a heat shock response, we tested whether the Hsp70 inhibitor VER could restore Myc-SmoC339A activity at the restrictive temperature (Fig. 2C). We did not observe a significant effect on Myc-SmoC339A-induced reporter gene activity in response to VER treatment, suggesting that heat shock is not responsible for attenuation of signaling by active Smo mutants at 29°C.

Because high-level Hh pathway activity in *Drosophila* correlates with accumulation of Smo on the plasma membrane (PM)

FIG 1 Active Smo mutants are temperature sensitive *in vivo*. (A and B) Growth at 29°C induces the UPR. RNA was harvested from *w¹¹¹⁸* larvae and assessed for endogenous *Xbp1* splicing by RT-PCR. The stress-induced *Xbp1* splice variant was not observed in RNA from larvae grown at 22°C, but it was evident in RNA extracted from larvae grown at 29°C (A). The ER stress sensor UAS-*Xbp1*-GFP was expressed in salivary glands under the control of *sgs3-Gal4* (*sgs3>Xbp1-GFP*). Crosses were performed at 22°C or 29°C, as indicated. Individual salivary glands are outlined in white. Under conditions of low ER stress, *Xbp1* was not in frame with GFP, and minimal GFP expression was observed (B'). Upon ER stress induction, *Xbp1* was alternately spliced to place it in frame with GFP, resulting in a robust GFP signal (B). (C to F) Active Smo mutants are temperature sensitive. Wild-type, C320A, and C339A Myc-Smo proteins were expressed at 22°C (D to F) or 29°C (D' to F') under the control of the *MS1096-Gal4* driver. Wild-type Myc-Smo did not induce a significant phenotype at 22°C and induced a mild phenotype at 29°C (D', compared to panel D). Conversely, C320A and C339A Smo mutants induced mild phenotypes at 29°C and strong phenotypes at 22°C (E and F), suggesting that their activity is reduced under conditions of thermal stress. The *MS1096* driver wing served as a control (C). (G) Expression of an active Smo mutant does not induce ER stress. Salivary glands from larvae expressing the *Xbp1*-GFP stress sensor alone (G') or in combination with Myc-SmoC320A (G) under the control of *sgs3-Gal4* were grown at 22°C on vehicle- or thapsigargin-treated food. Myc-SmoC320A (blue) did not induce the GFP stress sensor (G), but the potent ER stress-inducing compound thapsigargin did (G'). The filamentous actin stain phalloidin marks the plasma membrane (F-actin [magenta]). (H and H'). The *Xbp1* mutation does not modify the Myc-SmoC320A phenotype. Wings from flies expressing Myc-SmoC320A under the control of the epithelial driver *C765-Gal4* in the absence (H) or presence (H') of the *Xbp1* loss-of-function allele *Xbp1^{k13803}* are shown. Introduction of the *Xbp1^{k13803}* allele did not modify the SmoC320A-induced wing phenotype. For all conditions, representative salivary glands or wings are shown. Bars, 50 μ m.

(41), we wanted to determine whether the robust *in vitro* signaling and strong *in vivo* phenotypes induced at 22°C might result from active Smo mutants escaping the ER and localizing to the PM at this permissive temperature. To do so, we examined subcellular localization of wild-type and mutant Myc-Smo proteins in Schneider 2 (S2) cells, an embryonic *Drosophila* cell line commonly used for imaging studies, at 22°C and 29°C (Fig. 3A to C). In the absence of Hh, Smo localizes to intracellular vesicles and recycling endosomes (14). Accordingly, wild-type Myc-Smo localized primarily to punctate structures that did not significantly

overlap the ER marker calreticulin (Cal)-GFP-KDEL (43) at either 22°C or 29°C (Fig. 3A and A'). Myc-SmoC320A and C339A demonstrated a different localization pattern, colocalizing almost completely with the Cal-GFP ER marker at both 22°C and 29°C (Fig. 3B and B' and C and C'). We did not detect obvious colocalization between Myc-SmoC320A or C339A and the sub-PM filamentous actin stain phalloidin at either temperature (Fig. 3B and C), suggesting that the increased signaling activity of these mutants at 22°C was not the result of a bulk relocation from the ER to the PM.

FIG 2 Active Smo mutants are temperature sensitive *in vitro*. (A) Cl8 cells were transfected with empty vector or wild-type, C320A, or C339A Myc-Smo expression vectors (pAc-myc-smo) in the presence of pAc-hh or empty vector and control or *smo* 5'UTR dsRNA, as indicated. Reporter gene activity was determined from cells cultured at 22°C or 29°C, as indicated. Whereas wild-type Myc-Smo rescued *ptcΔ136-luciferase* activity at both temperatures, C320A and C339A were compromised in their abilities to modulate reporter gene activity at the restrictive 29°C temperature. The control Hh response at each temperature was set to 100%. Reporter gene activity is shown as the percent activity relative to the control Hh response. Hh reporter gene activity was normalized against a pAc-*Renilla* control. Error bars indicate standard errors of the means (SEM). (B) Cl8 cells were transfected with *hh* or wild-type or mutant *myc-smo* expression vectors, as indicated. The Hh response for each temperature was set to 100%. Reporter activity induced by the indicated Myc-Smo protein in the wild-type *smo* background is shown relative to the Hh response. Hh reporter gene activity was normalized against a pAc-*Renilla* control. Error bars indicate SEM. (C) The heat shock response does not alter signaling by the active Smo mutant. Cl8 cells grown at 29°C were transfected with *hh* or *myc-smo*C339A expression vectors and treated with DMSO (vehicle) or the Hsp70 inhibitor VER, as indicated. Reporter activity induced by Myc-SmoC339A is shown relative to the Hh response, set to 100%. VER did not rescue C339A signaling at the restrictive temperature, indicating that attenuated mutant Smo signaling at 29°C was not due to the heat shock response. Hh reporter gene activity was normalized against a pAc-*Renilla* control. Error bars indicate SEM.

To biochemically confirm that the activating mutants were unable to escape the ER, even under conditions that were favorable for high-level Hh signaling, we expressed wild-type, C320A, and C339A Myc-Smo proteins in Cl8 cells at 22°C in the presence of Hh and examined processing of their N-linked glycans. To do so,

we subjected lysates from Smo-expressing cells endo H, PNGase, and λ -phosphatase treatments. Endo H cleaves high-mannose oligosaccharides that are added in the ER, but it does not cleave the more complex oligosaccharides that are processed in post-ER compartments. PNGase cleaves all N-linked oligosaccharide resi-

FIG 3 Active Smo mutants are retained in the ER. (A to C) Wild-type (A), C320A (B), and C339A (C) Myc-Smo proteins were expressed in S2 cells at permissive (A to C) or restrictive (A' to C') temperatures. Myc-Smo was visualized by indirect immunofluorescence. Smo (Myc) is red, phalloidin (PM marker) is blue, and Cal-GFP-KDEL (ER marker) is green. Wild-type Smo was vesicular at both temperatures (A and A'). Mutants overlapped with the ER marker at both temperatures (B and C). Bar, 5 μ m. (D) Activated Smo mutants are not post-ER glycosylated. Whole-cell lysates from Cl8 cells expressing Hh and wild-type, C320A, or C339A Myc-Smo proteins at 22°C were treated with λ -phosphatase and the indicated deglycosylating enzymes. Post-ER-phosphorylated and glycosylated forms of wild-type Smo were detected (lanes 1 to 3 compared to lane 4, fully collapsed). C320A and C339A Smo proteins fully collapsed upon endo H and PNGase treatments, indicative of them being retained in the ER (lanes 5 to 12). Kinesin served as a loading control.

dues. EndoH, PNGase, and λ -phosphatase treatments revealed that, whereas wild-type Smo was detectable in ER-glycosylated, post-ER-glycosylated, and Hh-induced phosphorylated forms, C320A and C339A proteins were present only in the ER resident fraction; C320A and C339A fully collapsed upon either endo H or PNGase treatments (Fig. 3D, compare lanes 1 to 4 with 7 to 12). We did observe partial endo H sensitivity of wild-type Myc-Smo protein (Fig. 3D, lane 3). This was likely the result of N-linked glycans of post-ER-processed insect proteins having hybrid or paucimannosidic characteristics (44, 45). As such, the lower band of the doublet observed upon endo H treatment of wild-type Myc-Smo likely represents the ER resident fraction and the upper band likely represents the post-ER processed protein. Taken together, these results support the conclusion that the increased activity observed for C320A and C339A mutants at 22°C is not due to a bulk relocalization to the PM at the permissive temperature.

FIG 4 Active Smo mutants are destabilized at the restrictive temperature. pAc-myc-smo vectors encoding wild-type (A and D), C320A (B and E), and C339A (C and F) Myc-Smo proteins were cotransfected into S2 cells with pAc-GFP at permissive (22°C) and restrictive (29°C) temperatures. Cells were stained for Smo by indirect immunofluorescence using anti-Myc (magenta) and imaged by confocal microscopy. Whereas wild-type Myc-Smo (A and A') and GFP (D to F and D' to F') stability and expression were not significantly affected by temperature, both active mutants were destabilized at the restrictive temperature (B and C compared to B' and C'). Multiple fields of cells were examined over two independent experiments. Representative fields are shown. Bar, 50 μ m. (G) The Smo protein is destabilized at the restrictive temperature. Western blot analysis of whole-cell lysates from Cl8 cells expressing wild-type or C339A Myc-Smo proteins revealed C339A protein levels were decreased at 29°C. Wild-type Smo was not destabilized at 29°C. Kinesin (Kin) was used as the loading control.

While examining Smo subcellular localization (Fig. 3A to C), we noticed that whereas wild-type Smo protein stability did not appear to be affected by temperature, C320A and C339A Smo proteins consistently appeared more stable at the permissive 22°C temperature (Fig. 3A to C). To determine whether this was a specific stability effect on mutant Smo proteins, and not due to effects on transfection efficiency or *in vitro* transgene expression at the different temperatures, we cotransfected pAc-GFP with each of the pAc-myc-smo expression vectors into S2 cells at permissive and restrictive temperatures and examined their expression by gain-equalized immunofluorescence (Fig. 4A to F). Wild-type Myc-Smo and the GFP tracer were not significantly affected by temperature (Fig. 4A compared to A' and D to F compared to D' to F'). Conversely, Myc-SmoC320A and Myc-SmoC339A were consistently less detectable at the higher temperature (Fig. 4B and C compared to B' and C'). To confirm these results biochemically, we prepared whole-cell lysates from Cl8 cells expressing wild-type

or the highly active C339A mutant at 22°C or 29°C and examined Myc-Smo protein by Western blotting. Whereas wild-type Myc-Smo was not destabilized at the restrictive temperature, the Myc-SmoC339A protein level was significantly reduced (Fig. 4G, lanes 3 and 4 compared to lanes 5 and 6). Taken together with the immunofluorescence data, these results suggest that the activating Smo mutants are specifically destabilized at 29°C.

We previously demonstrated that, like the fly proteins, mSmo mutants corresponding to *Drosophila* Smo C320A and C339A are also largely ER retained (12). To biochemically validate ER retention of these mSmo mutants, we expressed wild-type, mSmoC299A (C320A equivalent), and C318A (C339A equivalent) in NIH 3T3 cells and examined ER and post-ER glycosylation patterns (Fig. 5A). Whereas wild-type mSmo existed in both an endo H-sensitive ER form and an endo H-resistant/PNGase-sensitive post-ER form, only the ER form of the C299A and C318A active mutants could be detected (Fig. 5A). We noted a slow-migrating form of wild-type mSmo, even in the presence of PNGase, which cleaves all ER and post-ER N-linked glycosylation moieties (Fig. 5A, lane 2). λ phosphatase treatment confirmed that this shift was not due to phosphorylation (Fig. 5A', lanes 4 and 9 to 11). To determine whether the mobility shift observed for wild-type Smo was the result of a post-ER O-linked glycosylation event, we treated lysates from cells expressing wild-type mSmo with O-glycosidase alone and in combination with PNGase and/or endo H (Fig. 5A'). We observed a modest collapse upon O-glycosidase treatment (Fig. 5A', lane 2) and complete collapse upon treatment with all three enzymes (Fig. 5A', lane 8), confirming that the residual shift observed with the wild-type protein is likely due to an O-linked glycosylation event.

The murine equivalent of the oncogenic W535L SmoM2 mutant (SmoA1, W539L, here referred to as mSmoM2) induces robust Sonic Hedgehog-independent signaling activity (3, 28). Like C299A and C318A mSmo mutants, a significant fraction of mSmoM2 is retained in the ER (14, 15). In order to examine the ratio of ER-localized to post-ER-localized mSmoM2, we expressed mSmoM2 in NIH 3T3 cells and examined ER and post-ER glycosylation levels (Fig. 5B). Wild-type mSmo demonstrated a near equal distribution between ER and post-ER glycosylated pools (Fig. 5B, lanes 1 to 3). Conversely, the bulk of mSmoM2 protein existed in the endo H-sensitive, ER-localized fraction (Fig. 5B, lanes 4 to 6, post-ER versus deglycosylated). Although modest, an endo H-resistant post-ER form of SmoM2 was detected (Fig. 5B, lane 6) and likely represented mSmoM2 protein that was localized to or was en route to the primary cilium. Accordingly, we detected mSmoM2 in the primary cilium by using indirect immunofluorescence; however, the bulk of mSmoM2 protein colocalized with the ER resident protein GRP94 (Fig. 5B', ciliary slice [arrowhead] versus ER slice).

Given that the bulk of mSmoC318A and mSmoM2 are ER localized, we reasoned that, like the active *Drosophila* mutants, they would be affected by induction of a thermal ER stress response. Thermal ER stress has been observed in mammalian cells cultured at 40°C (46). Accordingly, we observed induction of the ER stress response transcription factor CHOP in NIH 3T3 cells incubated at this high temperature for 2 to 4 h (Fig. 5C). To assess the effect of thermal ER stress on mSmo proteins, we expressed the wild type, C318A, or mSmoM2 in NIH 3T3 cells cultured at 37°C, then shifted to 40°C for 4 h prior to whole-cell lysis (Fig. 5C'). We found that whereas wild-type mSmo was not significantly affected

by temperature shift, both C318A and M2 mSmo proteins were significantly destabilized at 40°C (Fig. 5C', lanes 3 to 6 compared to 1 and 2). This destabilization was the likely result of ERAD, as the ER-resident forms of all three Smo variants were stabilized at 40°C by the proteasome inhibitor MG132 (Fig. 5C', white arrowhead, lanes 8, 10, and 12 compared to 7, 9, and 11). The post-ER form of wild-type Smo was not altered by MG132 treatment (Fig. 5C', black arrowhead, lanes 7 and 8).

To confirm that ER-retained mSmo proteins are cleared by ERAD following UPR induction, we targeted the ERAD-specific ubiquitin ligase Hrd1 with siRNA and tested for stabilization of mSmo mutants at the restrictive temperature (Fig. 5D). We observed that whereas C318A and M2 mSmo proteins were effectively destabilized at 40°C in cells treated with control siRNA, their destabilization was attenuated following Hrd1 knockdown (Fig. 5D, lanes 3 and 4 compared to lanes 9 to 12). Consistent with these results, mSmoM2-mediated induction of endogenous Gli1 protein was attenuated by high temperature and rescued following Hrd1 knockdown at 40°C (Fig. 5D', lanes 1 and 2 compared to lane 4). Curiously, the ER-retained form of wild-type mSmo was reduced at 40°C following Hrd1 knockdown (Fig. 5D, lanes 7 and 8). Additionally, the mSmoM2-induced Gli1 protein level was consistently reduced by Hrd1 knockdown in cells cultured at 37°C (Fig. 5D', lane 3). We do not know the cause of these reductions, but we speculate that Hrd1 knockdown induces compensatory ER stress responses that impact mSmoWT stability and/or downstream signaling events. Consistent with the prediction that ERAD attenuation induces higher-level ER stress, we observed enhanced CHOP induction in cells treated with Hrd1 siRNA (Fig. 5D, lanes 7 to 12).

We next wanted to determine whether the effects observed following thermal ER stress induction could be recapitulated by an ER stress modulator that is specific to the UPR. To this end, we treated Smo-expressing cells with the potent ER stress-inducing compound thapsigargin. We expressed wild-type, C318A, or M2 mSmo proteins in NIH 3T3 cells and treated cells with vehicle or thapsigargin for 4 h prior to lysis (Fig. 6A). Whereas wild-type mSmo stability was not affected by thapsigargin treatment, both C318A and M2 mSmo proteins were destabilized (Fig. 6A, lanes 3 to 6 compared to lanes 1 and 2), suggesting that in addition to being affected by thermal stress, the active mutants are also sensitive to chemically induced ER stress. To confirm that thapsigargin-mediated mSmoM2 destabilization was sufficient to attenuate downstream signaling, we assessed endogenous *gli1* induction using qPCR and Western blotting (Fig. 6B and C). qPCR analysis revealed an approximate 40-fold increase in *gli1* expression in mSmoM2-expressing cells over that observed in mSmoWT-expressing cells (Fig. 6B, white bars). Consistent with this result, we observed a robust increase in Gli1 protein in lysates prepared from mSmoM2-expressing cells (Fig. 6C, lanes 1 and 2). Thapsigargin treatment specifically reduced mSmoM2-mediated *gli1* transcript induction approximately 50% to a level ~18-fold over the mSmoWT baseline (Fig. 6B). Accordingly, Gli1 protein levels were decreased in lysates from thapsigargin-treated cells (Fig. 6C, lanes 3 and 4 compared to lane 2). Consistent with our biochemical analyses, thapsigargin did not attenuate *gli1* expression in cells expressing wild-type mSmo and instead modestly increased *gli1* expression ~4-fold over baseline (Fig. 6B, gray bars). We do not know the exact mechanism by which *gli1* expression is increased in the wild-type mSmo background in response

FIG 5 Murine Smo mutants are ER localized and temperature sensitive. (A and A') Mammalian mSmo mutants are not post-ER glycosylated. Lysates prepared from NIH 3T3 cells expressing wild-type, C299A, and C318A mSmo proteins were treated with vehicle (–), endo H, PNGase, O-glycosidase, and/or λ-phosphatase as indicated (+) and analyzed by Western blotting. The post-ER glycosylated form, present only with wild-type mSmo, was not affected by endo H (post-ER; A and A') but was affected by PNGase and O-glycosidase (deglycosylated; A and A'). The ER-localized forms of the wild type and each of the active mutants were sensitive to endo H, demonstrating a faster mobility after treatment (deglycosylated; A and A'). Endo H- and PNGase-treated C299A and C318A

FIG 6 Small-molecule UPR modulators target oncogenic Smo. (A) Active mSmo mutants are destabilized by thapsigargin. NIH 3T3 cells expressing wild-type, C318A, or M2 mSmo proteins were treated with 1 μ M thapsigargin (Thaps, +) or vehicle control (–), as indicated for 4 h prior to lysis. Western blotting of whole-cell lysates revealed C318A and M2 Smo proteins were destabilized in response to drug treatment. Wild-type mSmo was not significantly affected. Tubulin was the loading control. (B and C). Thapsigargin attenuates mSmoM2-induced pathway activity. RNA was harvested from NIH 3T3 cells expressing either wild-type or M2 mSmo proteins. qPCR analysis revealed that thapsigargin treatment resulted in specific attenuation of mSmoM2-induced *gli1* transcript (B) and protein levels (C). *smo* expression increased modestly in response to drug treatment (B'). For qPCR analysis, expression is shown as the fold induction over the wild-type mSmo vehicle control. Expression was normalized to the *GAPDH* reference gene. Error bars indicate standard errors of the means. For protein analysis (C), tubulin served as the loading control, and results with CHOP indicate ER stress induction. (D) UPR modulators destabilize mSmoM2. NIH 3T3 cells expressing mSmoM2 protein were treated with HSP90 inhibitors 17-AAG (50 μ M and 100 μ M) and SNX-2112 (25 μ M and 50 μ M) and the proteasome inhibitor bortezomib (25 μ M and 50 μ M) for 4 h prior to lysis. DMSO was the vehicle control. Western blotting of whole-cell lysates revealed mSmoM2 protein to be destabilized in response to HSP-90 inhibitors but not in response to bortezomib. Tubulin was the loading control. CHOP results indicate ER stress.

to thapsigargin. However, we speculate it may be the result of increased transcriptional activity of mSmo expression vectors in the presence of drug; both wild-type and M2 transcripts increased modestly in response to drug treatment (Fig. 6B').

To determine whether other small-molecule ER stress/UPR modulators might have similar effects, we treated mSmoM2-expressing NIH 3T3 cells with three compounds that are currently being evaluated for clinical use: the HSP90 inhibitors 17-AAG and SNX-2112 and the proteasome inhibitor bortezomib (27). Bort-

ezomib, which induces ER stress by blocking ERAD rather than by engaging the UPR, did not destabilize mSmoM2 protein expression (Fig. 6D, lanes 6 and 7 compared to lane 1). Conversely, 17-AAG and SNX-2112, both of which induce the UPR by targeting the chaperone HSP90, decreased mSmoM2 protein levels (lanes 2 to 5 compared to lane 1), further supporting the sensitivity of active Smo mutants to UPR induction.

Our *in vivo* and *in vitro* results thus far suggest that active Smo mutants that are retained in the ER are sensitive to ER stressors

had identical mobilities, suggesting that they lack post-ER glycosylation (deglycosylated; A). λ -Phosphatase did not affect mobility, indicating that wild-type mSmo is not phosphorylated in the absence of Shh (A', lane 10). Tubulin was the loading control. (B) Oncogenic mSmoM2 is largely ER retained. Lysates from NIH 3T3 cells expressing wild-type or M2 mSmo proteins were treated with deglycosylating agents as described for panel A. A significant pool of endo H-resistant post-ER protein was evident for wild-type mSmo. The bulk of mSmoM2 was endo H sensitive. The post-ER pool of mSmoM2 was modest but detectable (lane 6, post-ER label). Tubulin was used as the loading control. (B') Indirect immunofluorescence of mSmoM2 in NIH 3T3 cells demonstrated that, whereas a pool of mSmoM2 (green) was detected in the primary cilium (ciliary slice [arrow]), the bulk of the protein colocalized with the ER-resident protein GRP94 (ER slice [red]). 4',6'-diamidino-2-phenylindole DAPI (blue) marks the nucleus. Bar, 20 μ m. (C and C') Active mSmo mutants are temperature sensitive. (C) NIH 3T3 cells were grown at 37°C for ~44 h and then maintained at 37°C or shifted to 40°C for an additional 4 h prior to lysis, as indicated. Induction of the ER stress sensor CHOP was assessed by Western blotting of whole-cell lysates. Tubulin served as a loading control. (C') NIH 3T3 cells expressing wild-type, C318A, or M2 mSmo proteins were cultured as for panel C. Whereas ER (white arrowhead) and post-ER (black arrowhead) forms of wild type Smo were not significantly affected by temperature shift, both of the mutants were destabilized at the high temperature. Destabilization of the ER-resident forms of wild type and both mutants at 40°C was attenuated by treating cells with the proteasome inhibitor MG132, suggesting that they are cleared by ERAD. The post-ER form of wild type Smo was unaffected by MG132 treatment. Tubulin was the loading control (con). (D and D'). ERAD attenuation stabilizes mutant mSmo proteins. NIH 3T3 cells expressing wild-type, C318A, or M2 mSmo proteins were transfected with control or Hrd1 siRNA as indicated. Cells were shifted to 40°C for 4 h prior to lysis. Hrd1 knockdown stabilized ER-retained mutant mSmo proteins (D, lanes 1 to 6, compared to lanes 7 to 12) and rescued mSmoM2-mediated induction of endogenous Gli1 (D', lanes 1 and 2 compared to lane 4). Vec (D', lane 5) is the empty vector control lysate. Tubulin was the loading control.

FIG 7 Thapsigargin attenuates ectopic signaling by an active Smo mutant *in vivo*. (A) Transgene expression is unaffected by thapsigargin. *MS1096-GAL4*, UAS-EGFP (*MS1096>EGFP*) larvae were grown on media containing vehicle or thapsigargin, as indicated. Wing imaginal discs from third-instar larvae under both conditions demonstrated comparable GFP expression (green). 4',6-Diamidino-2-phenylindole (DAPI; magenta) marks the nuclei. (B to E) Thapsigargin prevents Myc-SmoC320A-induced Hh gain-of-function wing phenotypes. Larvae expressing wild-type or C320A UAS-*myc-smo* under the control of *MS1096-Gal4* or *C765-Gal4* were grown at 22°C on food containing vehicle (C to E) or thapsigargin (C' to E'). Representative wings from adult flies are shown. The *MS1096-Gal4* driver wing served as a control (B). Thapsigargin did not affect wings expressing wild-type Smo (C and C'). The C320A-induced phenotype was significantly attenuated by drug, allowing for development of near-normal adult wings (D' and E', compared to D and E). (F and G) SmoC320A-induced downstream pathway activity is attenuated by thapsigargin. Wing imaginal discs were dissected from *myc-smoC320A*-expressing third-instar larvae grown on vehicle-containing (F and G) or thapsigargin-containing (G') food at 22°C. UAS-*myc-smoC320A* was expressed by using the dorsal compartment wing driver *Apterous* (*Ap*)-*Gal4*. Wing discs were immunostained for Myc-SmoC320A (anti-Smo; blue), full-length Ci (green), or β -galactosidase (*dpp-lacZ*; magenta). Note the significantly reduced SmoC320A protein level, reduced Ci stabilization, and decreased β -galactosidase signals in thapsigargin-treated discs (G compared to G'; wing pouch is shown). The *Ap-Gal4* driver wing disc (F) served as a control. For all wing disc images, discs are shown with dorsal side up and the anterior to the left. Bar, 100 μ m. (H and H') Thapsigargin does not attenuate signaling induced by aberrant Hh ligand production. *Hh^{Mrt}* larvae were grown at 22°C on food containing vehicle (H) or thapsigargin (H'). Thapsigargin did not alter the gain-of-function phenotype induced by the *Hh^{Mrt}* allele. Representative wings from adult flies are shown.

that enhance protein misfolding and induce the UPR, e.g., high temperature, thapsigargin, and HSP90 inhibitors (46, 47). Wild-type Smo is not significantly targeted by the UPR following either thermal or chemical ER stress induction, suggesting that small-molecule-mediated UPR modulation may represent a selective process by which to target active Smo mutants in disease. To test this hypothesis, we examined whether thapsigargin could alter mutant Smo-induced *Drosophila* wing phenotypes. Due to the high degree of pupal lethality induced by the Myc-SmoC339A mutant at 22°C, we chose to perform this assay using the Myc-SmoC320A mutant.

To confirm that thapsigargin treatment of transgenic *Drosophila* would not nonspecifically alter UAS/GAL4 transgene expression in wing imaginal discs, we first tested the effect of feeding thapsigargin or vehicle control to larvae expressing a GFP trans-

gene under the control of the *MS1096-Gal4* wing pouch driver (Fig. 7A). GFP expression was unaffected by thapsigargin treatment at 22°C, confirming that the drug does not nonspecifically alter transgene expression *in vivo*. We next expressed either wild-type or C320A Myc-Smo proteins at 22°C under the control of *MS1096-Gal4* or *C765-Gal4* drivers and grew larvae on food containing either vehicle or thapsigargin (Fig. 7C to E). Consistent with our *in vitro* results, wings from wild-type Myc-Smo-expressing flies were unaffected by thapsigargin treatment (Fig. 7C and C', compared to B [control]). Conversely, Myc-SmoC320A-expressing thapsigargin-fed larvae demonstrated a significantly reduced Hh gain-of-function phenotype compared to that of the vehicle-fed control (Fig. 7D and E compared to D' and E'). Consistent with the observed effects on wing phenotypes, thapsigargin treatment resulted in a significant reduction of Myc-SmoC320A

protein in wing imaginal discs dissected from drug-fed larvae (Fig. 7G compared to G' [blue]). This was sufficient to attenuate downstream signaling, as C320A-mediated ectopic stabilization of the Hh pathway transcriptional effector Ci (green) and induction of the *dpp* reporter gene (magenta) were both reduced in response to drug (Fig. 7G compared to G' and F [control]).

To determine whether thapsigargin is effective against Hh pathway activity induced by aberrant ligand production, rather than by mutant Smo signaling, we tested for attenuation of phenotypes induced by the *Hh^{mrt}* allele. *Hh^{mrt}* causes ectopic expression of the Hh ligand along the dorsal/ventral border of the developing wing imaginal disc, triggering robust overgrowth of the distal portion of the anterior wing blade (Fig. 7H) (48, 49). In contrast to what was observed with flies expressing the active Smo mutant, thapsigargin feeding did not attenuate the *Hh^{mrt}* phenotype (Fig. 7H'). Taken together, these results suggest that alteration of ER homeostasis and induction of the UPR do not block ligand-induced pathway activity but may represent a selective option for attenuating aberrant Smo signaling in disease.

DISCUSSION

We have demonstrated that signaling by active Smo mutants localizing to the ER is attenuated under conditions of hyperthermal- or small molecule-mediated ER stress. Our studies focused on Smo proteins harboring activating C-to-A mutations in extracellular loop 1 and transmembrane domain 3 and the oncogenic M2 mutation, a W-to-L alteration in transmembrane domain 7 (3, 12). Each of these mutations likely induces a Smo conformational shift that mimics the Hh-induced active conformation, thereby triggering ligand-independent signaling (12, 28). We speculate that, despite this active conformation, the mutant protein is recognized by the ER as being misfolded, resulting in prolonged ER retention. We attempted to exploit this phenomenon to specifically target active Smo mutants, and we were successful in attenuating mutant Smo protein stability and downstream signaling, both *in vitro* and *in vivo*, by inducing either thermal or chemical ER stress.

Given the significant interest in identifying novel methods of targeting aberrant Hh signaling in disease (50–52), we propose that these findings may have clinical relevance, particularly in cases where disease results from Smo mutation. Previous *in vitro* studies have demonstrated reduced sensitivity of the oncogenic M2 mutant to small-molecule Smo inhibitors (28). More significantly, acquired resistance to the only FDA-approved Smo inhibitor, GDC-0449 (vismodegib), has been observed in the clinic (29). In this case, resistance is conferred by a novel Smo mutation that attenuates the ability of the compound to bind to Smo and inhibit its signaling activity (30). As such, there remains a clinical need for additional methods of targeting the Hh pathway, either in frontline combination therapy or in salvage therapy for relapsed patients who develop resistance to current Smo antagonists. We propose that compounds affecting the normal folding environment of the ER might fill this niche by specifically targeting conformationally unstable Smo mutants. Such compounds would offer a significant advantage over Smo-specific small molecules, because UPR modulators exploit a cellular process that is distinct from the Hh signaling pathway. As such, their efficacy should be unaltered by an acquired Smo mutation.

Admittedly, the current studies were performed with overexpressed Smo, a membrane protein that, when highly expressed, is likely to accumulate in the ER and induce a baseline ER stress

response. However, we argue that the observed effects were not due solely to high-level Smo expression, as we could not detect activation of ER stress responses in transgenic *Drosophila* or in cultured cells overexpressing active Smo mutants. Furthermore, overexpressed wild-type Smo protein localizing to the ER fraction was not eliminated by the UPR in response to ER stress. As such, we speculate that upon inducing ER stress in Smo-expressing cells, wild-type Smo protein continues to fold properly, does not engage the UPR, and exits the ER through its normal secretory route. Conversely, moderately misfolded active Smo mutants are readily detected by an active UPR, leading to their elimination through ERAD. As such, the observed destabilization and signaling attenuation of active Smo mutants following ER stress induction and UPR engagement results from a molecular mechanism that is specific to mutant Smo protein, an ideal scenario for clinical efficacy. Although we do not know the relative abundance of oncogenic SmoM2 protein in a human tumor cell, we hypothesize that its altered conformation and atypical ER exit (17, 28) may result in SmoM2 having an extended ER retention time, thereby sensitizing it to an active ER stress response.

Work with rhodopsin suggests that this may be a recurring theme in G-protein-coupled receptor biology. Studies performed using the *Drosophila* model of autosomal dominant retinitis pigmentosa, which closely mimics the human disease, revealed that sustained ER stress protects against retinal degeneration triggered by ER-retained disease-causing rhodopsin mutants (53, 54). Although the mechanism by which chronic, low-level ER stress facilitates protection is not clear, a recent study suggested that mild ER stress may activate autophagy to resist apoptosis (55). Interestingly, in flies heterozygous for the rhodopsin^{G69D} disease-causing mutation, ER stress-induced ERAD specifically degraded mutant rhodopsin but did not target the wild-type protein (53). Importantly, in these studies, the mutant allele was expressed at endogenous levels from the endogenous promoter (36, 53).

Based upon the results presented here, we speculate that the UPR holds promise to rapidly expand the cache of small molecules available for treatment of Hh-dependent cancer. Notably, a number of ER stress- and UPR-modulating compounds, including a thapsigargin-derived prodrug, are either approved or currently being evaluated for clinical use in treating cancers such as leukemia, lymphoma, multiple myeloma, and prostate cancer (27, 56). As such, if future studies reveal UPR-modulating compounds to be efficacious in targeting Smo-driven malignancies, the ability to translate these findings to the clinic may be expedited. This could prove to be a significant advantage over novel Hh pathway-specific small molecules that have not yet been approved for clinical use.

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We declare no conflicts of interest.

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