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## GLI3-dependent repression of DR4 mediates hedgehog antagonism of TRAIL-induced apoptosis

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### Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis through its cognate receptors death receptor 4 (DR4) and death receptor 5 (DR5), preferentially in malignant cells. However, many malignant cells remain resistant to TRAIL cytotoxicity by poorly characterized mechanisms. Here, using cholangiocarcinoma cells, as a model for TRAIL resistance, we identified a role for the oncogenic Hedgehog (Hh)-GLI pathway in the regulation of TRAIL cytotoxicity. Blockade of Hh using pharmacological and genetic tools sensitizes the cells to TRAIL cytotoxicity. Restoration of apoptosis sensitivity coincided with upregulation of DR4 expression, while expression of other death effector proteins remained unaltered. Knockdown of DR4 mimics Hh-mediated resistance to TRAIL cytotoxicity. Hh regulates the expression of DR4 by modulating the activity of its promoter. Luciferase, chromatin immunoprecipitation and expression assays show that the transcription factor GLI3 binds to the DR4 promoter and Hh requires an intact GLI3-repression activity to silence DR4 expression. Finally, small interfering RNA (siRNA)-targeted knockdown of *GLI3*, but not *GLI1* or *GLI2*, restores DR4 expression and TRAIL sensitivity, indicating that the Hh effect is exclusively mediated by this transcription factor. In conclusion, these data provide evidence of a regulatory mechanism, which modulates TRAIL signaling in cancer cells and suggest new therapeutic approaches for TRAIL-resistant neoplasms.

### Keywords

apoptosis; cholangiocarcinoma; GLI transcription factors; TRAIL

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#### Conflict of interest

The authors declare no conflict of interest.

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## Introduction

Tumor necrosis factor–related apoptosis-inducing ligand or TRAIL (TNSF10) is a ligand that initiates signaling cascades by binding to two cognate receptors termed death receptor 4, DR4 (TNSF10A, also referred to as TRAIL receptor 1), and death receptor 5, DR5 (TNSF10B, also referred to as TRAIL receptor 2/KILLER/TRICK-2) (Johnstone *et al.*, 2008). TRAIL can signal both apoptosis, especially in transformed cells (Johnstone *et al.*, 2008), and non-apoptotic signaling cascades (Ishimura *et al.*, 2006). Pro-apoptotic signaling by TRAIL and its receptors has been best characterized. Clustering and oligomerization of DR4 and DR5 by TRAIL results in conformational changes of the death domains within their cytoplasmic tails, facilitating recruitment and activation of caspases 8 and 10 within a death-inducing signaling complex (Kischkel *et al.*, 1995; Ashkenazi and Dixit, 1999; Falschlehner *et al.*, 2007). If activation of these initiator caspases is sufficiently robust, they directly activate caspase 3, which in turn results in cellular demise (Daniel *et al.*, 2001; Eggert *et al.*, 2001). This is referred to as type I death receptor signaling. However, if the magnitude of caspase 8 and 10 activation is not sufficient to directly activate caspase 3, TRAIL may still induce apoptosis by a pathway involving mitochondrial dysfunction, type II death receptor signaling (Daniel *et al.*, 2001). In this pathway, caspase 8 and 10 result in the cleavage of the pro-apoptotic BH3-only protein Bid to generate a truncated Bid (tBID). This pro-apoptotic protein triggers the mitochondrial death pathway, a pathway of apoptosis, which is regulated by members of the mitochondrial B-cell leukemia 2 protein family (Liu *et al.*, 1996; Kluck *et al.*, 1997). Although these aspects of the cascade including its mitochondrial inhibitory mechanism are well characterized, the non-mitochondrial regulatory events modulating the response to TRAIL are still poorly understood.

Here, we used cholangiocarcinoma cells as a model to study TRAIL resistance. Given the emerging data implicating Hedgehog (Hh) signaling in gastrointestinal tumor biology (Lees *et al.*, 2005; Parkin and Ingham, 2008), we explored Hh signaling as a mechanism contributing to TRAIL resistance by cancer cells. The data support a regulatory mechanism independent of mitochondria modulating cell-death response to TRAIL. We show that activation of the oncogenic Hh-GLI pathway represses the expression of DR4 receptor through regulation of its promoter. Mechanistically, we have shown that silencing of DR4 by Hh requires an intact GLI3 activity. Consistent with these data, the inhibition of Hh pathway sensitizes cholangiocarcinoma cells to TRAIL-induced apoptosis. Thus, our data define a mechanism by which oncogenic signaling could prevent TRAIL-induced cell death in cancer cells.

## Results

### Blockade of the Hh pathway sensitizes cholangiocarcinoma cells to TRAIL-induced apoptosis by upregulating DR4

We first ascertained whether inhibiting Smoothed, the signaling component of the Hh receptor complex, using cyclopamine (Taipale *et al.*, 2000; Chen *et al.*, 2002), sensitizes cholangiocarcinoma cells to TRAIL-induced cytotoxicity. Neither TRAIL nor cyclopamine alone induced significant apoptosis in cholangiocarcinoma cells such as KMCH, HuCCT-1 and Mz-ChA-1. However, cyclopamine sensitized all the three human cholangiocarcinoma cell lines to TRAIL cytotoxicity (Figures 1a and b). This effect of cyclopamine was concentration- and time-dependent with sensitization as low as 500 nm, a maximal effect at 5  $\mu\text{M}$  and utmost toxicity by 8 h of incubating KMCH cells with cyclopamine plus TRAIL (Supplementary Figure 1). In contrast, cyclopamine did not sensitize the TRAIL-resistant human hepatoblastoma cell line, human hepatocellular liver carcinoma cell line or the non-malignant normal rat cholangiocyte cell line to TRAIL cytotoxicity (Supplementary Figure 2). Therefore, sensitization to TRAIL-mediated apoptosis by cyclopamine was concentration- and cell-specific.

Validation using short hairpin RNA (shRNA)-targeted knockdown of Smoothened also sensitizes KMCH cells to TRAIL cytotoxicity (Figures 1c and e), showing that the relevant effect of cyclopamine is on Smoothened inhibition. Similarly, overexpression of a constitutively active, cyclopamine-insensitive, Smoothened was able to rescue the cyclopamine effect and restore the resistance to TRAIL cytotoxicity in KMCH cells (Figures 1f and g). Next, we sought to identify potential changes in expression of apoptotic mediators in cyclopamine treated cells, which could explain this sensitization to TRAIL-mediated apoptosis. After 24 h of cyclopamine treatment, DR4, but not DR5 protein and mRNA were upregulated in KMCH cells, whereas protein levels of other apoptotic mediators including Fas, TRAIL decoy receptors 1 and 2 (DcR1, DcR2), cellular FLICE-inhibitory protein, caspase 8 and members of the B-cell leukemia 2 protein family were unaltered (Figures 2a and c). In addition, validation using shRNA-targeted knockdown of Smoothened also upregulated DR4 expression at both mRNA and protein level (Figures 2d and e). This increase in total cellular DR4 protein levels was also associated with an increase in cell surface expression of the death receptor (Figure 2f). Increased DR4 mRNA and protein expression on cyclopamine treatment or knockdown of Smoothened suggested that sensitization to TRAIL cytotoxicity could be mediated specifically through DR4. Consistent with this interpretation, a DR4-specific agonistic antibody, but not a DR5-specific agonistic antibody, triggered apoptosis in cyclopamine-treated cells (Figure 3a). In addition, cells stably expressing shRNA-targeting DR4 were not sensitized to TRAIL cytotoxicity by cyclopamine (Figure 3b). Finally, DR4 overexpression sensitized the cells to a TRAIL-mediated cell death (Figure 3c). Taken together, these observations implicate DR4 upregulation as a likely mechanism responsible for TRAIL cytotoxicity in cyclopamine-treated cholangiocarcinoma cells.

### Cholangiocarcinoma cells have canonical Hh pathway

Sensitization to TRAIL-mediated apoptosis by cyclopamine suggests cholangiocarcinoma cells express and respond to Hh signaling ligands. To confirm this hypothesis, expression of the mediators of Hh-GLI signaling pathway was examined by quantitative RT-PCR (Figure 4). All three Hh ligands (Sonic, Indian and Desert) could be identified at the mRNA level in the three human cholangiocarcinoma cell lines. However, both Sonic Hedgehog (Shh) and Indian Hedgehog (Ihh) were predominantly expressed by KMCH cells whereas Indian Hedgehog was predominantly expressed only by HuCCT-1 and Mz-ChA-1 cells (Figure 4a). As acceptable commercial anti-sera for Hh ligands at the protein level are not available, protein expression for the ligands could not be confirmed. Expression for the cell surface proteins, Smoothened and Patched1, was confirmed by both RT-PCR and immunoblot analysis (Figure 4b); the specificity of the anti-sera for Smoothened was confirmed by using appropriate positive and negative controls (Supplementary Figure 3). The cell lines expressed the transcription factors regulated by Hh, *GLI1*, *GLI2* and *GLI3*. (Figure 4c). Finally, in all the 47 human cholangiocarcinoma samples (containing cancer plus admixed stromal cells), mRNA transcripts were also identified for all the Hh pathway mediators (Supplementary Figure 4), thus supporting the biological relevance of our findings. To address whether the components of the Hh cascade are signaling through the canonical pathway, the cholangiocarcinoma cells were treated with cyclopamine, and the mRNA expression for *GLI1* and Patched1, established transcriptional targets of canonical Hh pathway, were assayed by RT-PCR. *GLI1* and Patched1 were downregulated by cyclopamine treatment as well as shRNA targeting the knockdown of Smoothened (Figure 4d). Conversely, both *GLI1* and Patched1 were upregulated in KMCH cell treated with recombinant Shh ligand (Figure 4e). Collectively, these data suggest that the Hh signaling is constitutively active and acts through the canonical ligand activated pathway in human cholangiocarcinoma cells. Interestingly, cyclopamine alone did not alter cell proliferation or survival over 96 h (data not shown), suggesting the major effect of Hh inhibition is sensitization to TRAIL cytotoxicity.

### Hh silencing of the DR4 promoter requires intact GLI-binding sites

To determine if DR4 is transcriptionally regulated by GLI, we searched the 8 kb 5'-flanking region of the human and mouse DR4 gene for the 5'-GACCACCCAXXXG-3' GLI consensus-binding motif using the Enhancer Element Locator software (<http://www.cs.helsinki.fi/u/kpalin/EEL/>). In all, four candidate-binding sites (Figure 5a) were identified; all with the same sequence 5'-GGCCTCCCAAAGTG-3', each had two mismatches (underlined) as compared with the consensus sequence. Using an earlier described luciferase reporter driven by the DR4-promoter fragment (-586/+63; Guan *et al.*, 2002), we inserted a tandem repeat (five copies) of the DR4 candidate GLI-binding sites. To confirm whether GLI sites are required to modulate the DR4-promoter activity, we performed a luciferase-based assay using either the DR4-promoter construct alone (parental) or the DR4 promoter plus GLI-enhancer sequences. The parental construct was not responsive to cyclopamine, consistent with a lack of identified GLI-binding sites. However, when GLI-binding sites were inserted, the construct was Hh-responsive, as luciferase activity was increased by cyclopamine treatment (Figure 5b). In contrast, when mutant GLI-binding sites (four tandem copies) were incorporated into the reporter gene construct instead, luciferase activity was not enhanced by cyclopamine treatment. Consistent with these data, reporter gene activity was actually enhanced in the DR4-promoter construct containing mutated GLI-enhancer sequences compared with that containing intact GLI-binding sites, presumably because of the release from tonic GLI-mediated suppression (Figure 5b). Additionally, we assayed promoter function using an unrelated Hh-responsive construct containing eight tandem GLI-binding sites (Chen *et al.*, 2009). This additional promoter was also stimulated by the cyclopamine treatment (Figure 5b). These data suggest that Hh may regulate DR4 gene expression through GLI-binding sites in the DR4 promoter.

### GLI3 knockdown restores DR4 levels and sensitizes cholangiocarcinoma cells to TRAIL-mediated apoptosis

Canonical Hh pathway signaling uses the GLI1, GLI2 and GLI3 proteins as transcriptional effectors of its cellular functions. To ascertain which of these GLI proteins likely suppresses the expression of DR4; *GLI1*, *GLI2* and *GLI3* were selectively knocked down by a small interfering RNA (siRNA) approach in KMCH cells (Figure 6a). DR4 expression was increased by gene silencing of *GLI3*, while gene silencing of *GLI1* or *GLI2* did not alter DR4 expression (Figures 6b and c). In contrast, gene silencing of *GLI1*, *GLI2* or *GLI3* did not alter the DR5 expression. These data suggest that the transcriptional factor, GLI3, downregulates DR4 expression in human cholangiocarcinoma cells by actively repressing its promoter. If this interpretation is correct, knockdown of *GLI3* should sensitize cells to TRAIL cytotoxicity. Indeed, KMCH cells stably expressing shRNA-targeting *GLI3* were sensitized to TRAIL-mediated apoptosis, while cells co-transfected with shRNA-targeting *GLI3* plus an shRNA-resistant *GLI3* were resistant to TRAIL cytotoxicity (Figure 6d). A parsimonious interpretation of these data is that *GLI3* represses DR4 expression. Based on this assessment, we used the luciferase reporter assay to measure the effect of *GLI3* silencing or overexpression on DR4-promoter function. Similar to cyclopamine, *GLI3* gene silencing increased luciferase activity (Figure 6e). Consistent with repression by *GLI3*, DR4-reporter activity was decreased in *GLI3* overexpressing cells (Figure 6e), as was the activity of an earlier described GLI-responsive promoter (Supplementary Figure 5). However, the reporter containing mutant GLI-binding sites did not have increased activity on *GLI3* silencing, nor decreased function when *GLI3* was overexpressed (Figure 6e). Finally, chromatin immunoprecipitation assay showed that only *GLI3* was found to bind consistently to the DR4 promoter (Figure 6f). We note that on occasion *GLI2* binding was observed, suggesting that Gli dimers may participate in suppression of DR4 expression, but this observation could not be reliably replicated. Given that *Gli3* siRNA alone relieved Hh repression of DR4, and the consistent results of the chromatin immunoprecipitation assay, we can only confidently implicate *Gli3* in DR4 regulation.

## Discussion

Our study is consistent with previous reports that Hh signaling components are constitutively expressed by cholangiocarcinoma cells (Berman *et al.*, 2003). However, this study extends these observations by showing that the Hh pathway imparts survival signals by inhibiting TRAIL cytotoxicity. This observation is quite relevant to human cholangiocarcinoma which paradoxically expresses TRAIL *in vivo* (Ishimura *et al.*, 2006), and is, therefore, reliant on mechanisms to prevent TRAIL death signaling for survival. Given the role of immune surveillance in cancer biology by natural killer and natural killer T cells which eradicate target cells by TRAIL-mediated apoptosis (Johnstone *et al.*, 2008), this function of Hh signaling in cancer biology could be pertinent to other cancers as well as cholangiocarcinoma.

The Hh signaling has been shown previously to regulate apoptotic pathways. For example, in basal cell carcinoma and central nervous system tumors, Hh regulates expression of the anti-apoptotic protein B-cell leukemia 2 and cellular FLICE-inhibitory protein (Bigelow *et al.*, 2004; Regl *et al.*, 2004; Kump *et al.*, 2008). Despite its ability to promote apoptosis, cyclopamine treatment did not alter the B-cell leukemia 2 or myeloid cell leukemia 1 protein expression in human cholangiocarcinoma cells. This was unexpected given that B-cell leukemia 2 family proteins, especially myeloid cell leukemia 1, are pivotal in inhibiting death ligand-induced apoptosis in cholangiocarcinoma cells (Taniai *et al.*, 2004; Kobayashi *et al.*, 2005; Mott *et al.*, 2007). Cyclopamine did, however, result in enhanced DR4 expression. The upregulation of DR4 appears to be the mechanism for cyclopamine sensitization to TRAIL as cells in which DR4 expression was suppressed by targeted-shRNA, retained TRAIL resistance despite cyclopamine treatment. Inhibition of Hh signaling with enhanced DR expression also appears to overcome myeloid cell leukemia 1-mediated resistance to TRAIL cytotoxicity in cholangiocarcinoma cells. This observation suggests cyclopamine switches TRAIL signaling from a Type II pathway (dependent on mitochondrial dysfunction for apoptosis) to a Type I pathway (mitochondrial-independent pathway of apoptosis). Further studies will be required to examine this concept.

Why DR5, which is expressed by these cells, does not compensate for low-level DR4 expression in TRAIL killing is unclear and requires further investigation. TRAIL receptors undergo post-translational modification by O-glycosylation and perhaps other processes modulating their cytotoxicity (Wagner *et al.*, 2007). Selective inactivation of DR5 through such modifications is a possible mechanism for this finding.

Previous studies reported that DNA-damaging agents, such as VP-16 and doxorubicin, induce DR4 expression by either p53-dependent or -independent mechanisms (Guan *et al.*, 2001). There are few reports dealing with the issue of p53-independent regulation of DR4 expression. Nuclear factor- $\kappa$ B and activator protein-1 were recently reported to regulate DR4 expression at the transcriptional level (Guan *et al.*, 2002; Mendoza *et al.*, 2008). In this study, we have shown that gene silencing of *GLI3* increased the expression of DR4, indicating that *GLI3* suppresses DR4 expression. The inhibition of DR4 expression by *GLI3* appears to be direct, as multiple *GLI*-binding elements are present in the promoter, and mutation of these elements increases the activity of the DR4 promoter. In addition, our data show a physical interaction of *GLI3* protein with the endogenous DR4 promoter, which further supports regulation through direct binding. As our studies were performed in KMCH cells with mutant p53 (Wehbe *et al.*, 2006), our observations provide mechanistic insight into the p53-independent regulation of this death receptor gene. Tonic suppression by *GLI3* may be an important mechanism for its regulation.

In conclusion, our studies implicate Hh suppression of DR4 expression as a mechanism for TRAIL resistance in human cholangiocarcinoma cells. As these cancers express TRAIL *in*

*vivo*, pharmacological inhibition of Hh signaling may promote autocrine and/or paracrine cholangiocarcinoma autonomous cytotoxicity. Collectively, these concepts suggest Hh pathway inhibitors, which are under clinical development (Rubin and de Sauvage, 2006; Rudin *et al.*, 2009), could have therapeutic efficacy in the treatment of human cholangiocarcinoma.

## Materials and methods

### Cell culture

The human cholangiocarcinoma cell lines such as KMCH, HuCCT-1 and Mz-ChA-1 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 000 U/l penicillin, 100 mg/l streptomycin and 100 mg/l gentamycin as previously described (Kobayashi *et al.*, 2005; Isomoto *et al.*, 2007).

### Quantitative RT-PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and complementary DNA was prepared using random primers and moloney murine leukemia virus-reverse transcriptase as previously described in detail (Higuchi *et al.*, 2001). The complementary DNA product was amplified by polymerase chain reaction with Taq DNA polymerase using standard protocols. Polymerase chain reaction primers are depicted in Supplementary Table 1. Primers for 18S ribosomal RNA (rRNA) were purchased from Ambion (Austin, TX, USA). RT-PCR was performed with the Roche LightCycler using SYBR green as the fluorophore (Invitrogen) as previously described (Kobayashi *et al.*, 2005). The copy number of the target mRNA in each sample was normalized as a ratio using the copy number for 18S rRNA in the denominator.

### Immunoblot analysis

We obtained whole-cell lysates as previously described (Isomoto *et al.*, 2007). SDS-PAGE resolved protein samples, transferred to nitrocellulose membranes and blotted with the indicated primary antibodies at a dilution of 1:500 to 1:1000. Peroxidase-conjugated secondary antibodies (Biosource International, Camarillo, CA, USA) were incubated at a dilution of 1:3000 to 1:5000. Bound antibodies were visualized using enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL, USA) and Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA). Primary anti-sera taken, were those raised to Smoothed (Santa Cruz Biotechnology, Santa Cruz, CA, USA: H-300), patched-1 (Cell Signaling, Danvers, MA, USA), Bcl-2 and Mcl-1 (Santa Cruz Biotechnology: B19 for Bcl-2, S19 for Mcl-1), Bak (Upstate, Charlottesville, VA, USA), Bax (Santa Cruz Biotechnology: C-12), Bid (R&D Systems, Minneapolis, MN, USA), Bim (BD Biosciences, San Jose, CA, USA), Bik and Bad (Santa Cruz Biotechnology: N-19), Puma (Abcam, Cambridge, MA, USA), Noxa (ProSci, Poway, CA, USA), caspase 8 (BD Biosciences), Fas (Santa Cruz Biotechnology: C-20), DR4 (Alexis, San Diego, CA, USA), DR5 and cFLIP (ProSci), DcR1 and DcR2 (Enzo Life Science, Plymouth Meeting, PA, USA), and actin (Santa Cruz Biotechnology: C-11).

### Apoptosis

Apoptosis was quantified morphologically by staining the nuclei with 4',6-diamino-2-phenylindole dihydrochloride and assessing the characteristic nuclear changes of apoptosis (that is, chromatin condensation and nuclear fragmentation) by fluorescence microscopy using excitation and emission wavelengths of 380 and 430 nm, respectively. Caspase-3/7 activity in cell cultures was used to assess apoptosis biochemically and was measured by quantifying rhodamine release from the caspase-3/7 substrate rhodamine-110, bis (*N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide), using the Apo-ONE homogeneous caspase-3/7 kit (Promega, Madison, WI, USA) following the manufacturer's instructions.

## RNA interference

A siRNA was employed to silence *GLI1*, -2 or -3 expression. All siRNAs were designed and synthesized using the software available at <http://www.ambion.com> and the Silencer siRNA Construction kit from Ambion (Austin, TX, USA). The siRNA sequences used to inhibit *GLI1*, -2 and -3 expression were as follows: *GLI1*, 5'-AACTTTGATCCTTACCTCCACCTGTCTC-3'; *GLI2*, 5'-AACTTTGAGGTCTCTAACATCCTGTCTC-3'; *GLI3*, 5'-AAGGCATAATGTTGTCACAGACCTGTCTC-3' (partial T7-promoter sequence is bold). As control, cells were transfected with a scrambled RNA duplex with the following sequence: 5'-AACGTGATTTATGTCACCAGA-3'. Inhibition of target mRNA expression was assessed after transient transfection of cells with siRNA by RT-PCR. Cells grown in six-well dishes were transiently transfected with 20 nm siRNA using 2.5  $\mu$ l/ml LipofectAMINE 2000 (Invitrogen) in a total transfection volume of 2 ml of OPTI-MEM I (Gibco-Invitrogen, Carlsbad, CA, USA). After incubation at 37 °C and 5% CO<sub>2</sub> for 4 h, the medium was exchanged with 2 ml of medium.

The shRNA for DR4 was from Sigma-Aldrich (St Louis, MO, USA) (MISSION short hairpin RNA lentiviral plasmid, targeting nucleotides 1499–1519, Genebank accession # NM 003844 for DR4-shRNA). To generate the *GLI3*-targeted shRNA construct, the pSSH1 plasmid containing the human H1 promoter for the expression of shRNA was used. A double-stranded DNA template (5'-GATCCCCGCAATAGGCTTTAGGAAAATTCAAGAGATTTTCCTAAAGCCTATTGCTTTTTGGAAA-3') was inserted into the pSSH1 plasmid after the H1 RNA promoter. The DNA insert contains both sense and anti-sense sequences (bold type) for the *GLI3* mRNA and a 9-nucleotide linker sequence, yielding transcription of an shRNA-targeting *GLI3*. For stable transfection, KMCH cells were transfected using OPTI-MEM I (Gibco-Invitrogen) containing 5  $\mu$ l/ml LipofectAMINE 2000 (Invitrogen) and 3  $\mu$ g/ml plasmid DNA. Forty-eight hours after transfection, fresh medium containing 1  $\mu$ g/ml puromycin or 1.2 g/l G418 was added. Surviving clones were separated using cloning rings and individually cultured. lentiviral particles containing shRNA to Smoothened were constructed using the BLOCK-iT Inducible H1 Lentiviral RNAi System (Invitrogen), following the manufacturer's protocol. Sequences (inserted into pLenti4/BLOCK-iT-DEST) are depicted in Supplementary Table 2. Cells were transduced with lentiviral particles containing pLenti6/TR 1-day before transduction with the inducible constructs as described above by following manufacturer's protocol and the expression was induced with 1 mg/ml tetracycline (Sigma-Aldrich). After an additional 48 h, cells were used for the experiments. Target suppression was assessed by immunoblot analysis (DR4 and Smoothened) or by RT-PCR (*GLI3*).

## DR4-surface expression

The KMCH cells were cultured on glass cover slip and allowed to grow to ~50% confluency. Following treatment with vehicle or cyclophamide, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline containing 0.1 M PIPES, 1 mM ethylene glycol tetra acetic acid and 3 mM magnesium sulfate. Cells were then blocked for 1 h at room temperature with phosphate-buffered saline containing 5% bovine serum albumin, 5% glycerol and 0.04% sodium azide. After incubation with polyclonal rabbit anti-DR4 (ProSci) in blocking buffer at 4 °C overnight, the cells were washed three times with phosphate-buffered saline, incubated with Alexa Flour 488-conjugated chicken anti-rabbit IgG (Molecular Probes, Carlsbad, CA, USA) at a dilution of 1:500 in blocking buffer for 1 h at 37 °C. Cells were then washed three times in phosphate-buffered saline and three times in H<sub>2</sub>O, mounted onto slides using ProLong Antifade Kit (Molecular Probes), and imaged by confocal microscopy, with excitation and emission wavelengths of 488 and 507 nm, respectively. Cell surface fluorescence was

quantified using the LSM210 imaging software (Carl Zeiss Microimaging Inc., Thornwood, NJ, USA).

### DR4 overexpression

The KMCH cells were transfected with green fluorescent protein (pEGFP-N1) or DR4-GFP (Akazawa *et al.*, 2009) using FuGene HD. Twenty-four hours after transfection, fresh medium was added (untreated) or cells were treated with TRAIL (5 ng/ml) for 6 h, followed by 4',6-diamino-2-phenylindole dihydrochloride staining. Green fluorescent protein - positive cells were then analyzed for apoptotic nuclear morphology.

### Expression constructs for GLI3 and Smoothened

The GLI3 expression construct included the *GLI3* cDNA in a pRK5 backbone, and was a kind gift from Dr Steven Yan Cheng (University of California, San Francisco, CA, USA). Cells were transfected using OPTI-MEM I (Gibco-Invitrogen) containing 5 µl/ml LipofectAMINE 2000 (Invitrogen) and 3 µg/ml plasmid DNA. Transfected cells were used for experiments 72 h after transfection. The coding sequence for Smoothened containing the M2 activating mutation and HSV glycoprotein D-amino terminal epitope tag (CA-SMO) was subcloned from pRK7 (Xie *et al.*, 1998) into pCDNA 3.1 + by digestion at *HindIII* and *EcoRI* using standard recombinant DNA methodology. Cells were transfected using OPTI-MEM I (Gibco-Invitrogen) containing 5 µl/ml LipofectAMINE 2000 (Invitrogen), and 3 µg/ml plasmid DNA. Forty-eight hours after transfection, fresh medium containing 1.2 g/l G418 was added. Surviving clones were separated using cloning rings and individually cultured.

### Reporter constructs and DR4 promoter-reporter assay

The luciferase reporter plasmid containing 0.65 kb DR4 5'-flanking region, pGL3 (-586/+63), has been described previously (Guan *et al.*, 2002). Double-stranded oligonucleotides corresponding to the candidate GLI-binding sites in the DR4 promoter region were synthesized (Mayo DNA Synthesis Core Facility, Rochester, MN, USA) and ligated in tandem (five copies) at the *BamHI* site of the pGL3 (-586/+63) vector. As a negative control, double-stranded oligonucleotides with two C-to-G mutations in the DR4 GLI-binding site (Figure 5a) were synthesized and ligated (four tandem copies) at the *BamHI* site of the pGL3 (-586/+63) vector. The mutations were positioned within the consensus-binding region to disrupt GLI-binding. The vectors containing the correct and mutant inserts were sequenced to confirm that the GLI-binding site was as expected and that the luciferase gene did not contain cloning artifacts. As a positive control to determine GLI activity, a reporter containing eight consecutive GLI-binding sites downstream of the luciferase gene (8×-GLI) was included in the experimental paradigm (Sasaki *et al.*, 1997). The 8×-GLI reporter was kindly provided by Dr Chi-chung Hui (Research Institute, Toronto, ON, Canada). The plasmid with the TRAIL-R1/DR4 (luciferase + DR4 promoter), the TRAIL-R1/DR4 GLI-binding site (luciferase + DR4 promoter + GLI-binding sites), the mutant binding site (luciferase + DR4 promoter + mutant GLI-binding sites) or 8×-GLI (luciferase + 8× GLI-binding sites) were transfected into KMCH cells using FuGene HD (Roche Diagnosis, Basel, Switzerland). Cells were co-transfected with 25 ng of a plasmid expressing *Renilla* luciferase (pRL-CMV) (Promega). Twenty-four hours after transfection, cell lysates were prepared, and both firefly and *Renilla* luciferase activities were quantified using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalized to *Renilla* luciferase activity to control for transfection efficiency and cell number.

### Chromatin immunoprecipitation assay

The KMCH cells were treated with 1% formaldehyde for 15 min at room temperature, harvested in lysis buffer (R&D Systems) and sheared to fragment DNA of ~500 bp. Samples



were then immunoprecipitated using GLI1, GLI2 or GLI3 anti-sera (R&D Systems) or beads alone at 4 °C overnight. Following immunoprecipitation, samples were washed and eluted using the chromatin immunoprecipitation kit (R&D systems) following manufacturer's instructions. Cross-links were removed at 65 °C for 4 h and immunoprecipitated DNA was purified (Qiagen, Valencia, CA, USA) and subsequently amplified by polymerase chain reaction using a primer set for an area containing potential GLI-binding site I in DR4 promoter sequence: forward 5'-AGCGCAATGGCTCCATCTCGGCTC-3', reverse 5'-AGTCACGGTCCTGCGAAGAA-3'. Polymerase chain reaction products were visualized by ethidium bromide staining after electrophoresis in a 2% agarose gel.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

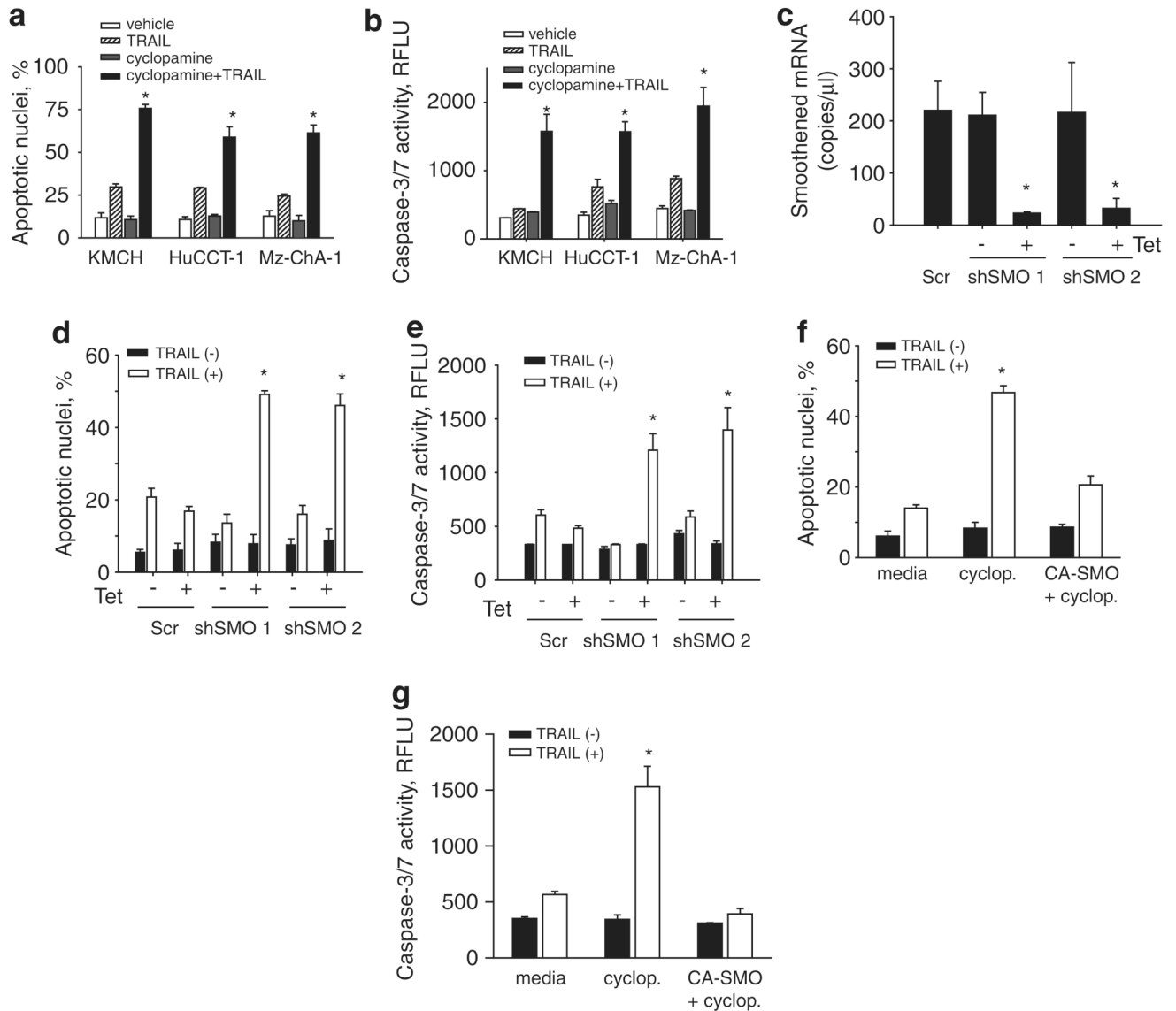
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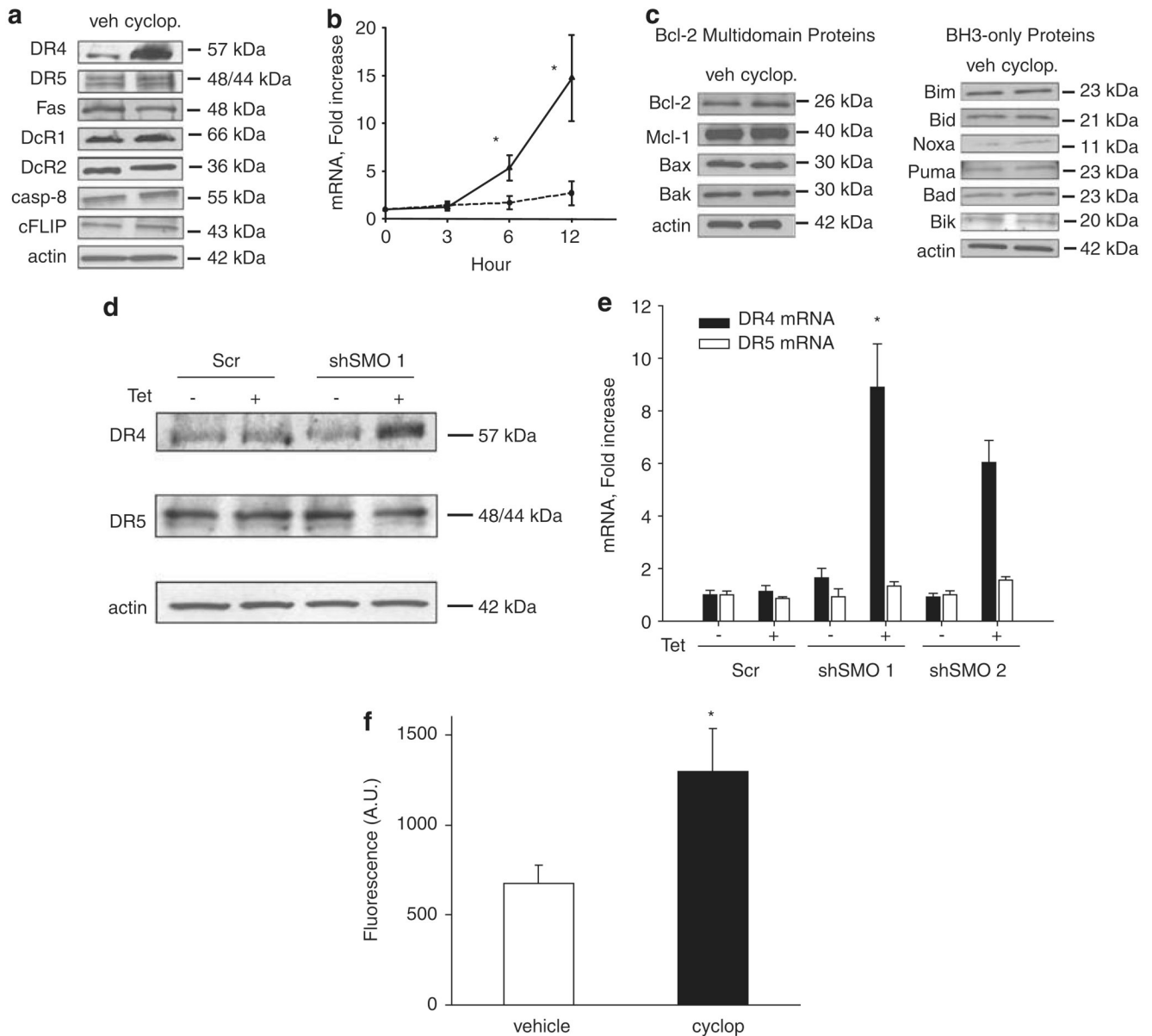
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**Figure 1.**

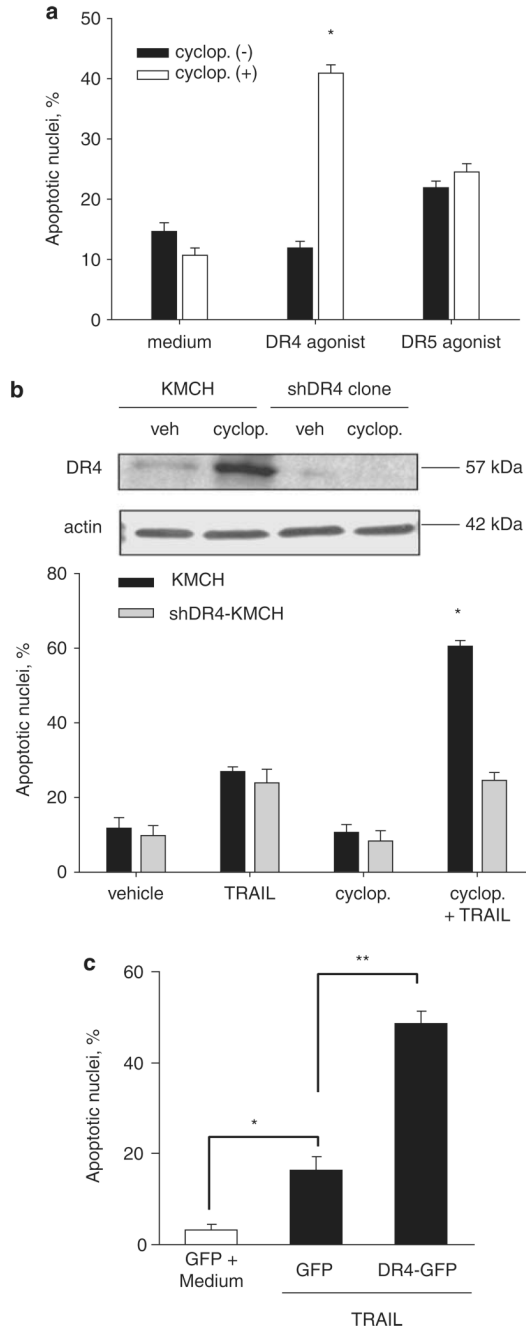
Cyclopamine sensitizes cholangiocarcinoma cells to Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated cytotoxicity. **(a)** Cells were pretreated with vehicle (ethanol, 0.01% v/v) or cyclopamine (5  $\mu$ M) for 24 h. After pretreatment, human recombinant TRAIL was added where indicated at 5 ng/ml in fresh medium and the cells were incubated for an additional 5 h. Cells were then stained with 4',6-diamino-2-phenylindole dihydrochloride (DAPI) and cells with apoptotic morphology were counted. Mean  $\pm$  s.e.m., \* $P$ <0.001. **(b)** In parallel, cells were pretreated with vehicle or cyclopamine and treated with TRAIL as in panel **(a)** but after 6 h caspase-3/7 activity was measured. Mean  $\pm$  s.e.m., \* $P$ <0.001. **(c)** KMCH cells were transfected with either of two independent short hairpin RNA (shRNA) expression vectors targeting Smoothed (shSMO 1 and shSMO 2) or a scrambled shRNA vector, followed by quantitative RT-PCR for Smoothed using total RNA. Results expressed as copies of each complementary DNA. Mean  $\pm$  s.e.m., \* $P$ <0.001. **(d)** Cells transfected as in panel **(c)** were then treated with TRAIL (5 ng/ml) for 6 h, then stained with DAPI and cells with apoptotic morphology were counted. **(e)** Caspase-3/7 activity was measured 6 h after TRAIL treatment

of cells transfected as in panel (c). Mean  $\pm$  s.e.m., \* $P$ <0.001. (f, g) KMCH cells were pretreated with vehicle or cyclopamine as above, or transfected with a constitutively active Smoothed vector (CA-SMO). Cells were then stained with DAPI and cells with apoptotic morphology were counted (f) or caspase-3/7 activity was measured 6 h after TRAIL treatment (g). Mean  $\pm$  s.e.m., \* $P$ <0.001.

**Figure 2.**

Cyclopamine upregulates death receptor 4 (DR4) expression. **(a)** Immunoblotting of cell lysates from KMCH cells treated with vehicle or cyclopamine ( $5 \mu\text{M}$ ) for Fas, TRAIL receptors DR4, DR5, DcR1 and DcR2, and proximal signaling proteins caspase 8 and cellular FLICE-inhibitory protein. Actin was used as a loading control. **(b)** Quantitative RT-PCR for DR4 and DR5 using total RNA from KMCH cells after treatment with cyclopamine for the indicated times. Expression was normalized to 18S rRNA levels and presented as fold increase over time. Mean  $\pm$  s.e.m., \* $P < 0.01$ . **(c)** Whole-cell lysates of KMCH cells treated with vehicle or cyclopamine ( $5 \mu\text{M}$ ) were prepared for immunoblotting for B-cell leukemia 2 family proteins, including BH3-only proteins. **(d)** KMCH cells transfected with scrambled control short hairpin RNA (shRNA) or two shRNA sequences targeting Smoothed (SMO shRNA 1 and SMO shRNA 2) were treated with tetracycline (1 mg/ml) to induce shRNA expression and whole-cell lysates prepared for immunoblotting. **(e)** In parallel, total RNA was prepared from KMCH

cells transfected as indicated and RT-PCR for DR4 or DR5 was performed. Mean  $\pm$  s.e.m., presented as fold increase over scrambled. \* $P$ <0.01. (f) DR4 surface expression was performed by using confocal microscopy. KMCH cells treated with cyclopamine (5  $\mu$ M) for 24 h. Quantification of DR4-green fluorescent protein (GFP) localization was accomplished using the LSM210 imaging software. Fluorescence intensity for the green channel was quantified. Mean  $\pm$  s.e.m. \* $P$ <0.05.

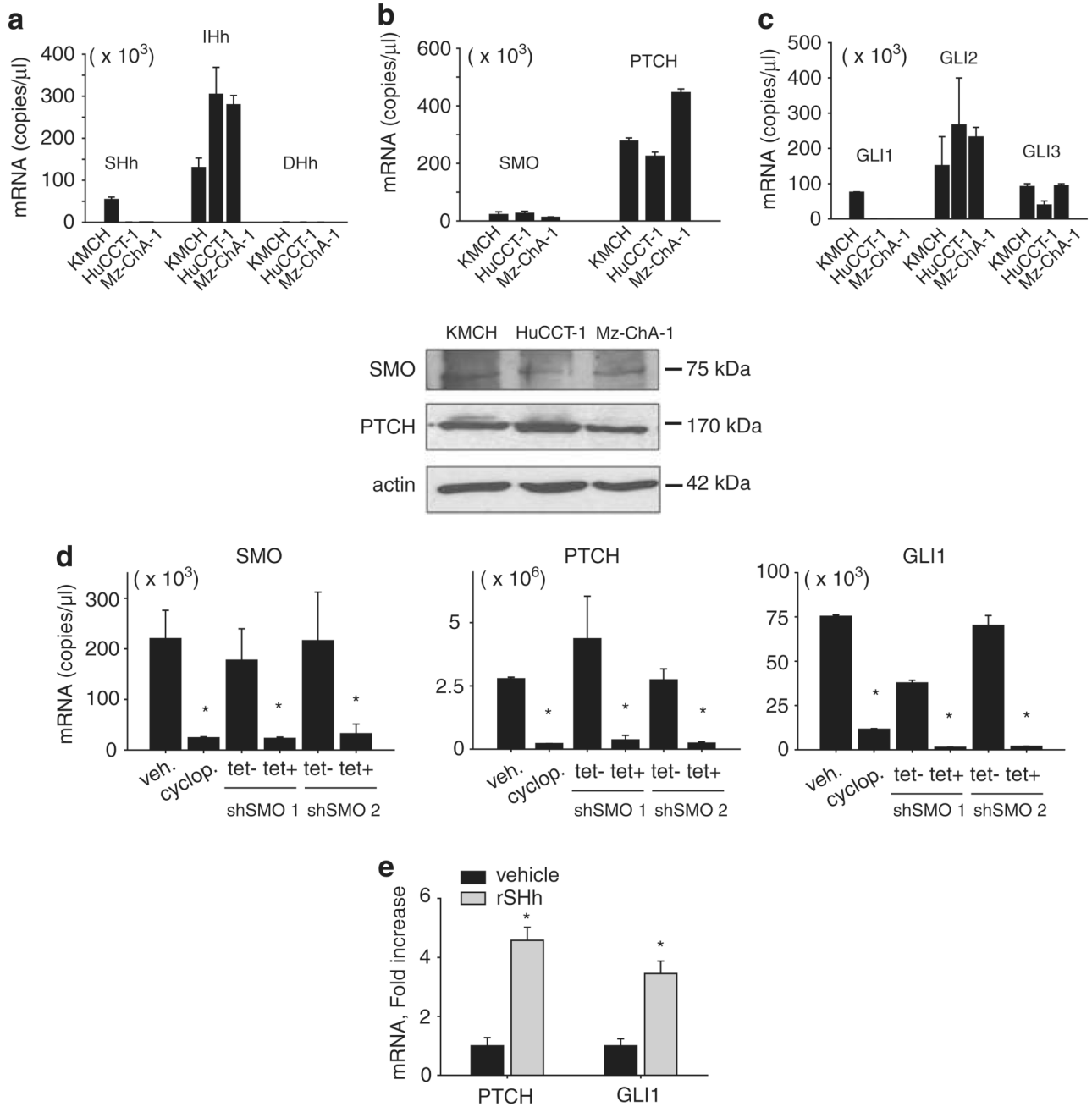


**Figure 3.**

Death receptor 4 (DR4) receptor activation is necessary and sufficient for apoptosis in cyclopamine-sensitized cells. **(a)** Cells were pretreated with vehicle or cyclopamine ( $5 \mu\text{M}$ ) for 24 h. DR4 agonist ( $0.1 \mu\text{g/ml}$ ) or DR5 agonist ( $1 \mu\text{g/ml}$ ) was then added in fresh medium and the cells were further incubated for the indicated time-intervals. Cells were then stained with 4',6-diamino-2-phenylindole dihydrochloride (DAPI) and cells with apoptotic morphology were counted. Mean  $\pm$  s.e.m., \* $P < 0.001$ . **(b)** KMCH cells stably transfected with DR4-shRNA and parent KMCH cells were incubated with vehicle or  $5 \mu\text{M}$  cyclopamine for 24 h. *Upper*, Immunoblotting of whole cell lysates was performed using antibody against DR4. Actin was used as a loading control. *Lower*, Cells were pretreated where indicated with cyclopamine then

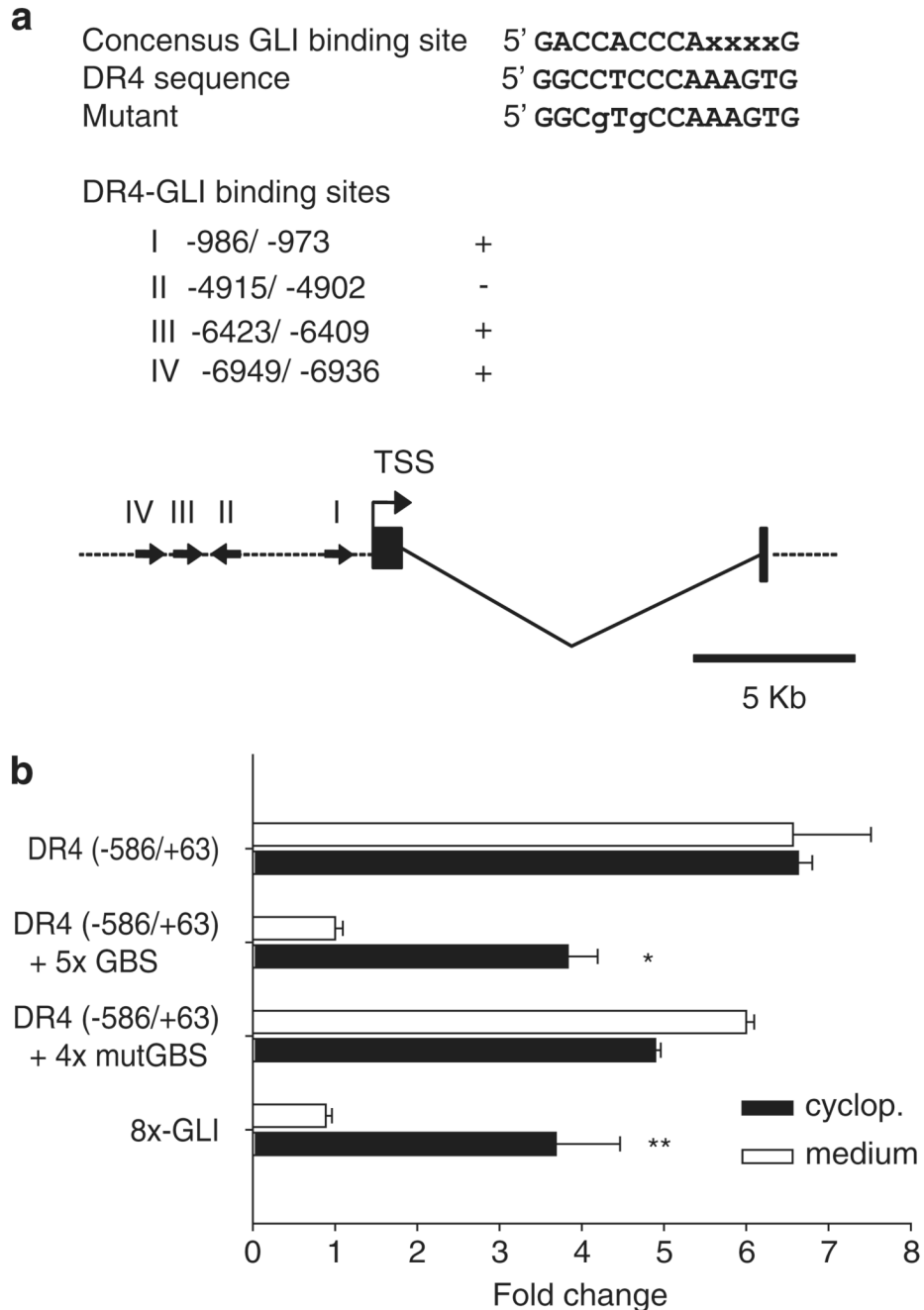


treated with TRAIL (5 ng/ml) for 5 h followed by DAPI staining. Cells with apoptotic morphology were counted. Mean  $\pm$  s.e.m., \* $P$ <0.001 compared with parent cells treated with TRAIL and cyclopamine. (c) KMCH cells transiently transfected with green fluorescent protein (GFP) (control) or DR4-GFP were treated where indicated with TRAIL (5 ng/ml) for 6 h. The number of GFP-positive cells with apoptotic nuclear morphology is presented as a percent of total GFP-positive cells. Data are mean  $\pm$  standard error, \* $P$ <0.05; \*\* $P$ <0.01.

**Figure 4.**

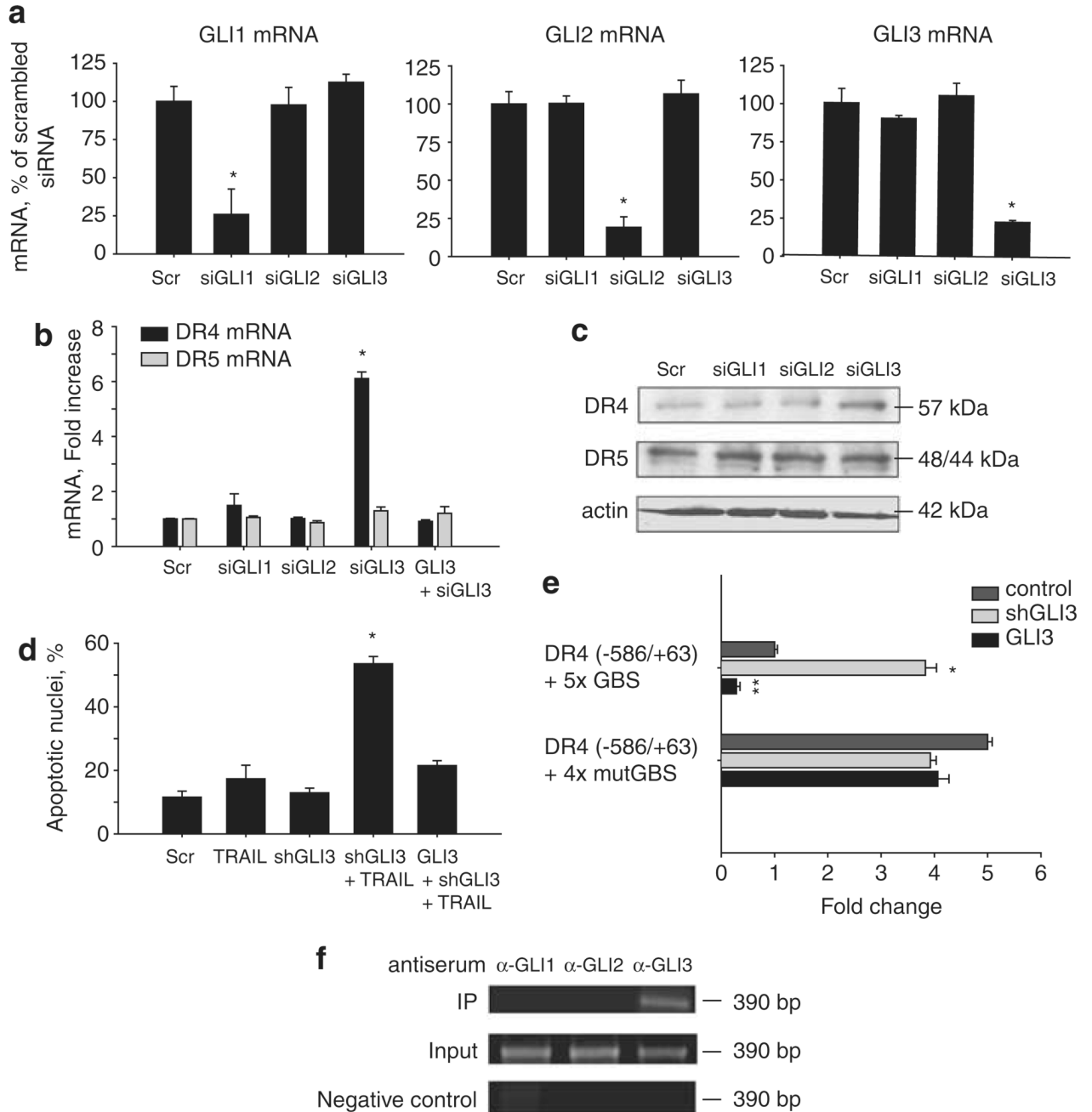
Cholangiocarcinoma cell lines expressed Hedgehog pathway mediators and respond to Hedgehog pathway activation and inhibition. (a) Quantitative RT-PCR for Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh) using total RNA from KMCH, HuCCCT-1 and Mz-ChA-1 cells. Results are expressed as copies of each complementary DNA (cDNA) (Mean  $\pm$  s.e.m.). (b) *Upper*, Quantitative RT-PCR for Smoothed and Patched-1 using total RNA from KMCH, HuCCCT-1 and Mz-ChA-1 cells. Results are expressed as copies of each cDNA (Mean  $\pm$  s.e.m.). *Lower*, Whole cell lysates from KMCH, HuCCCT-1 and Mz-ChA-1 cells were probed using a Smoothed or a Patched-1 antibody. Actin was used as a loading control. (c) Quantitative RT-PCR for Hedgehog transcription factors, *GLI1*, -2 and

-3, using total RNA from KMCH, HuCCT-1 and Mz-ChA-1 cells. Results expressed as copies of each cDNA (Mean  $\pm$  s.e.m.). **(d)** Quantitative RT-PCR for Smoothed, Patched-1 and *GLII* using total RNA from KMCH cells after treatment with cyclopamine or gene silencing of Smoothed by a tetracycline-inducible shRNA technique. Results are expressed as copies of each cDNA (Mean  $\pm$  s.e.m.). Cells were treated with cyclopamine 5  $\mu$ M for 24 h before isolating mRNA or were transfected with Smoothed shRNA for 5 days before isolating mRNA. \* $P$ <0.001. **(e)** Quantitative RT-PCR for Patched-1, and *GLII* using total RNA from KMCH cells after treatment with recombinant sonic hedgehog ligand (rShh). Results were normalized to 18S and expressed as fold increase over vehicle. Mean  $\pm$  s.e.m., \* $P$ <0.01.



**Figure 5.** GLI3 directly repressed death receptor 4 (DR4) promoter function. **(a)** Putative GLI-binding sites in the DR4 promoter region. Nucleotide positions (I, II, III and IV) were counted from the transcription start site (TSS). Potential binding sites were observed in both the forward and the reverse direction; (+, sense; -, anti-sense) as indicated also by arrow on the schematic. **(b)** A DR4 promoter fragment (-586/+63) in pGL3, the same promoter plus five copies of the GLI-binding sequence (DR4 (-586/+63) + 5 × GBS), and a reporter construct containing 8 × GLI-binding sites (8 × -GLI) were used to evaluate the promoter activity on cyclopamine treatment. KMCH cells were co-transfected with 25 ng of pRL-CMV and 0.5 μg of indicated pGL3-based DR4 promoter reporter plasmids or 8 × -GLI reporter plasmids. Twenty-four hours

after transfection, medium or cyclopamine ( $5 \mu\text{M}$ ) was added where indicated for 24 h. Both firefly and *Renilla* luciferase activities were quantified and data (firefly/*Renilla* luciferase activity) are expressed as fold increase over DR4 promoter-reporter constructs containing 5 × GLI-binding sites treated with medium. Mean  $\pm$  s.e.m., \* $P < 0.001$ , \*\* $P < 0.05$ .

**Figure 6.**

Gene silencing of *GLI3* sensitizes cholangiocarcinoma cells to TRAIL-mediated apoptosis through death receptor 4 (DR4) upregulation. (a) Quantitative RT-PCR for *GLI1*, -2 and -3 using total RNA from KMCH cells transfected with the specific siRNA for *GLI1*, -2 or -3. Forty-eight hours after transfection, total RNA was isolated from cells. Cells transfected with scramble small interfering RNA (siRNA) were used as a control. Results normalized to 18S and expressed as percentage of scrambled. Mean  $\pm$  s.e.m., \* $P$ <0.001. (b) Quantitative RT-PCR for DR4 and DR5 using total RNA from KMCH cells after the specific GLI-siRNA transfection. Forty-eight hours after transfection, total RNA was isolated from cells. Cells transfected with scramble siRNA were used as a control. Results normalized to 18S and

expressed as fold increase over scramble. *GLI3* silencing was rescued where indicated by co-transfection with a *GLI3* expression plasmid resistant to the siRNA. Mean  $\pm$  s.e.m., \* $P$ <0.001. (c) Immunoblotting of whole cell lysates obtained from KMCH cells transfected with the specific siRNA targeting *GLI1*, -2, or -3. Seventy-two hours after transfection, total cellular protein was isolated from the cells. Membranes were probed using a DR4 and a DR5 antibody. Actin was used as a loading control. (d) KMCH cells transfected with *GLI3* shRNA or co-transfected with sh*GLI3* plus a *GLI3*-expression-plasmid resistant to the shRNA were incubated with 5 ng/ml human recombinant TRAIL for 5 h. Cells were then stained with 4',6-diamino-2-phenylindole dihydrochloride (DAPI) and cells with apoptotic morphology were counted. Mean  $\pm$  s.e.m., \* $P$ <0.001 compared with vehicle. (e) DR4 promoter constructs (-586/+63) with a tandem repeat of the candidate GLI-binding site (DR4 (-586/+63) + 5  $\times$  GBS) or a tandem repeat of a mutated GLI-binding site (DR4 (-586/+63) + 4  $\times$  mutGBS) were assayed in KMCH cells. Cells were co-transfected with sh*GLI3* or a *GLI3*-expression construct as indicated. Mean  $\pm$  s.e.m. \* $P$ <0.001 compared with medium; \*\* $P$ <0.005 compared with medium. (f) Chromatin immunoprecipitation was performed on KMCH cells using anti-*GLI1*, -*GLI2* or -*GLI3* anti-serum and the pull down used for DR4 promoter polymerase chain reaction using primers flanking the candidate GLI-binding site I. The polymerase chain reaction amplicon was visualized by ethidium bromide staining after agarose electrophoresis.