Hedgehog-EGFR cooperation response genes determine the oncogenic phenotype of basal cell carcinoma and tumour-initiating pancreatic cancer cells

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Inhibition of Hedgehog (HH)/GLI signalling in cancer is a promising therapeutic approach. Interactions between HH/GLI and other oncogenic pathways affect the strength and tumourigenicity of HH/GLI. Cooperation of HH/GLI with epidermal growth factor receptor (EGFR) signalling promotes transformation and cancer cell proliferation *in vitro*. However, the *in vivo* relevance of HH-EGFR signal integration and the critical downstream mediators are largely undefined. In this report we show that genetic and pharmacologic inhibition of EGFR signalling reduces tumour growth in mouse models of HH/GLI driven basal cell carcinoma (BCC). We describe HH-EGFR cooperation response genes including SOX2, SOX9, JUN, CXCR4 and FGF19 that are synergistically activated by HH-EGFR signal integration and required for *in vivo* growth of BCC cells and tumour-initiating pancreatic cancer cells. The data validate EGFR signalling as drug target in HH/GLI driven cancers and shed light on the molecular processes controlled by HH-EGFR signal cooperation, providing new therapeutic strategies based on combined targeting of HH-EGFR signalling and selected downstream target genes.

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INTRODUCTION

Malignant transformation is a multistep process that involves cooperation (i.e. synergistic interaction) of oncogenic signals to determine the malignant state of a cell (Hanahan & Weinberg, 2000). A detailed understanding of these interactions and the downstream processes induced by oncogene cooperation is important for the identification of novel drug targets and the development of rational-based combination therapies.

The Hedgehog (HH)/GLI signalling pathway has been implicated in a variety of human malignancies such as basal cell carcinoma (BCC), melanoma, medulloblastoma, glioblastoma, cancers of the prostate, lung, pancreas, breast as well as certain haematopoietic malignancies (reviewed in, Ng & Curran, 2011; Teglund & Toftgard, 2010). First clinical studies using selective HH/GLI pathway inhibitors have demonstrated a

remarkable therapeutic efficacy in advanced BCC and medulloblastoma, suggesting that targeted HH/GLI inhibition may hold promise also in other malignant diseases (Rudin et al, 2009; Skvara et al, 2011; Von Hoff et al, 2009). Furthermore, distinct tumour-initiating and metastatic cancer cells are sensitive to HH/GLI pathway inhibition, raising the hope that interfering with HH/GLI signalling may also improve relapse rates and metastasis (Clement et al, 2007; Dierks et al, 2008; Mueller et al, 2009; Peacock et al, 2007; Varnat et al, 2009, 2010; Zhao et al, 2009).

Activation of HH/GLI signalling is initiated by binding of HH proteins to the trans-membrane protein patched (PTCH), which in the absence of ligand represses the pathway by preventing the activation of the essential pathway effector smoothened (SMO). Binding of HH to PTCH allows activation of SMO, leading to the formation of activator forms of the GLI zinc finger transcription factors GLI2 and GLI3. Direct transcriptional activation of GLI1 by GLI2/3 enhances the level of GLI activators (Jiang & Hui, 2008; Rohatgi & Scott, 2007) and high-level expression of GLI1 is considered a reliable indicator of HH pathway activity. GLI1 and GLI2 act as main mediators of HH signalling in cancer by controlling the expression of target genes involved in proliferation, metastasis, survival and stemness (reviewed in, Kasper et al, 2006a; Ruiz i Altaba et al, 2007; Stecca & Ruiz, 2010).

Current therapeutic strategies are targeting the essential HH effector SMO. Despite first promising results in BCC and medulloblastoma (Rudin et al, 2009; Skvara et al, 2011; Von Hoff et al, 2009), limited therapeutic efficiency and drug resistance pose major challenges for targeting HH/GLI signalling by SMO-inhibition (Buonamici et al, 2010; Rudin et al, 2009; Von Hoff et al, 2009; Yauch et al, 2009). In addition, SMO-independent stimulation of GLI activity via interactions with RAS-MEK/ERK, PI3K/AKT (Riobo et al, 2006a; Riobo et al, 2006b; Stecca et al, 2007) or growth factor pathways such as TGFβ (Dennler et al, 2009; Nolan-Stevaux et al, 2009) and epidermal growth factor receptor (EGFR) signalling (Kasper et al, 2006a; Palma et al, 2005; Palma & Ruiz i Altaba, 2004; Schnidar et al, 2009) may bypass or attenuate the therapeutic efficacy of SMO antagonists in malignant diseases such as melanoma or pancreatic cancer.

In previous studies, our own group has analysed at a molecular level the cooperative interaction of the HH/GLI and EGFR pathway, which results in synergistic regulation of selected HH/GLI target genes and oncogenic transformation *in vitro* (Schnidar et al, 2009). Integration of EGFR and HH/GLI signalling involves activation of RAS/MEK/ERK and JUN/AP1 signalling in response to EGFR activation (Kasper et al, 2006b; Schnidar et al, 2009). *In vivo* evidence for the therapeutic relevance of HH/GLI and EGFR signal cooperation in HH-associated cancers is lacking and key mediators acting downstream of HH/GLI and EGFR signal cooperation are still unknown.

Here, we demonstrate an essential *in vivo* requirement of EGFR in HH/GLI-driven BCC and identify a set of HH/GLI-EGFR cooperation response genes critical for the determination of the oncogenic phenotype of BCC and tumour-initiating pancreatic cancer cells. The data shed light on the molecular mechanisms underlying *in vivo* tumour growth in response to HH-EGFR signal cooperation.

RESULTS

In vivo requirement of EGFR in Hh/Gli-driven skin cancer

Having shown that HH/GLI and EGFR cooperate in oncogenic transformation *in vitro*, we aimed to evaluate the *in vivo* role of EGFR in Hh/Gli driven cancers. To do so, we first tested genetically the requirement of EGFR in a mouse model of BCC. Using tamoxifen-regulated Cre/loxP technology to accomplish skin-specific expression of an oncogenic Smo variant (SmoM2) (Xie et al, 1998; Supporting Information Fig S1), we addressed whether concomitant epidermal deletion of EGFR affects SmoM2-driven BCC development. Activation of SmoM2 in $K5cre^{ER}$;*SmoM2*;$EGFR^{+/+}$ mice resulted in focal epidermal hyperplasia and numerous BCC-like lesions that were most prominent on the ears (Fig 1A (right), B and B′). Of note, epidermal-specific deletion of EGFR in $K5cre^{ER}$;*SmoM2*;$EGFR^{fl/fl}$ mice reduced both the number and size of tumours (Fig 1A, C and C′). Similarly, EGFR deletion reduced basaloid hyperplasia and basaloid hamartoma-like lesions in the dorsal skin of transgenic mice (Supporting Information Fig S2). Compared to $K5cre^{ER}$;*SmoM2*;$EGFR^{+/+}$ mice, $K5cre^{ER}$;*SmoM2*;$EGFR^{fl/fl}$ mice showed a 70 percent decrease in tumour multiplicity on the ears (Fig 1D). Those lesions that still developed on the ears of $K5cre^{ER}$;*SmoM2*;$EGFR^{fl/fl}$ mice were significantly smaller in size compared to those found in $K5cre^{ER}$;*SmoM2*;$EGFR^{+/+}$ mice (Fig 1E), but still expressed the BCC-markers K17 and Sox9 (Supporting Information Fig S3). Together, these data suggest a functional requirement of EGFR for tumour initiation and growth in SmoM2-driven skin cancer.

We next addressed whether systemic administration of afatinib (BIBW2992), a highly efficient irreversible EGFR/erbB family inhibitor (Li et al, 2008), is able to affect BCC development *in vivo*. Therefore, we established an allograft model that allows *in vivo* tumour growth of $Ptch^{-/-}$ mouse BCC cells (ASZ001) (Aszterbaum et al, 1999; So et al, 2006). Mice grafted with ASZ001 BCC cells were allowed to grow palpable tumours before the start of treatment with afatinib or solvent. Notably, afatinib at a dose of 15 mg/kg/day efficiently arrested tumour growth, while control treated mice (solvent only) showed a rapid increase in tumour volume (Fig 2A). To confirm the cell-autonomous requirement of EGFR in BCC cells, we performed knockdown of EGFR expression in $Ptch^{-/-}$ BCC cells. shRNA against EGFR (see Fig 2C) significantly reduced tumour growth (Fig 2B), confirming the cell-autonomous *in vivo* requirement of EGFR in BCC tumour cells.

HH/GLI-EGFR cooperation response genes as mediators of synergistic signal integration

Oncogenic signal cooperation has been shown to synergistically regulate so called cooperation response gene (CRG) sets enriched for determinants of the malignant phenotype (McMurray et al, 2008). We therefore set out to identify

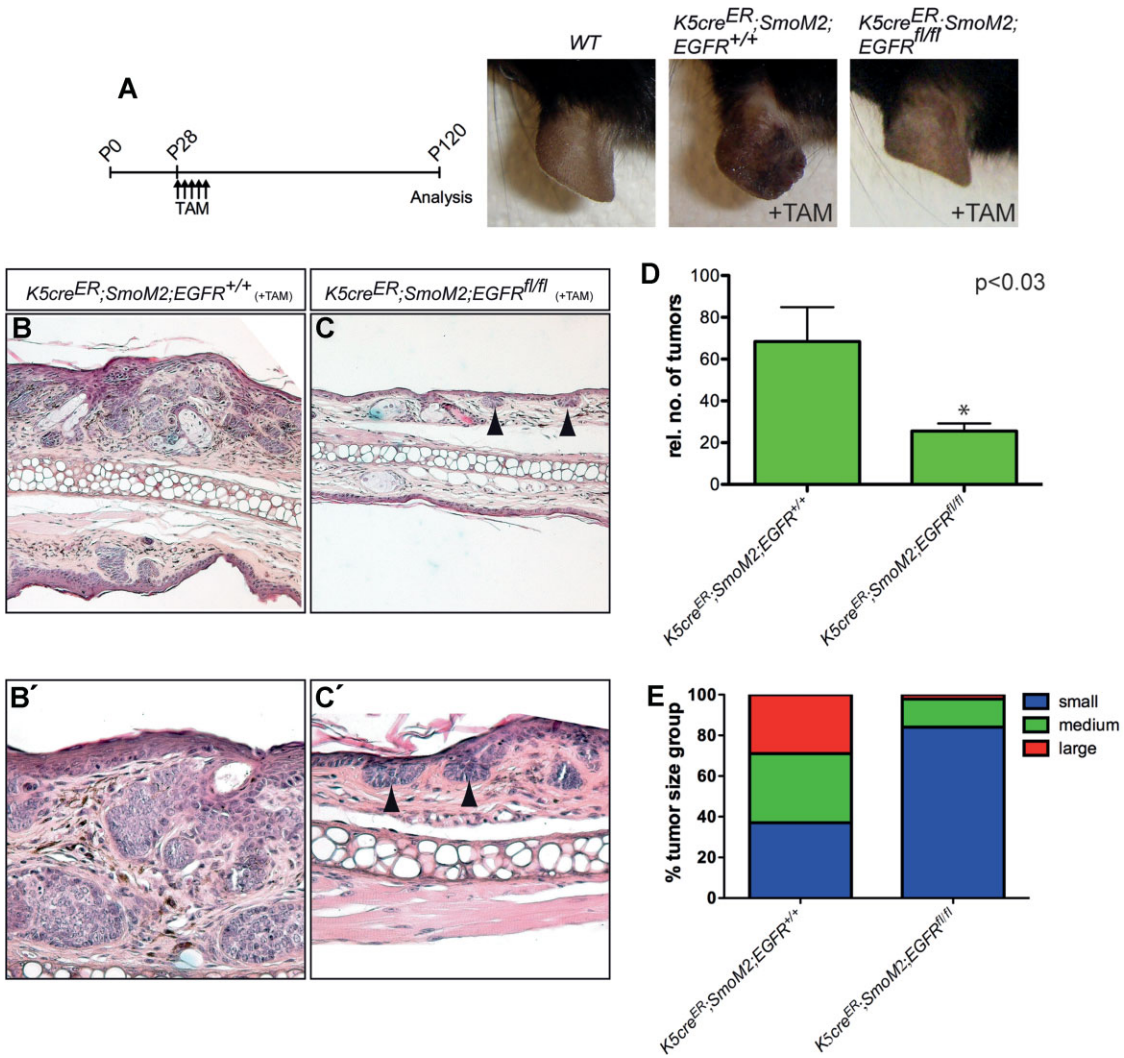

Figure 1. Epidermal-specific deletion of EGFR inhibits SmoM2-driven growth of BCC-like lesions.

A. Genetic mouse BCC model testing EGFR function. To induce SmoM2 expression and delete EGFR, transgenic mice were injected with tamoxifen (TAM) 28 days after birth (P28) and analysed three months later at post-natal day P120. Left: ears of wild-type (*WT*), *K5cre^{ER};SmoM2;EGFR$^{+/+}$* and *K5cre^{ER};SmoM2;EGFR$^{fl/fl}$* mice injected with tamoxifen (+TAM). In *K5cre^{ER};SmoM2;EGFR$^{+/+}$*, but not in EGFR-deficient mice, pigmented BCC-like lesions are readily visible on ears.

B-B′. Phenotype of SmoM2 BCC mice. Histological analysis of ears from TAM injected *K5cre^{ER};SmoM2;EGFR$^{+/+}$* mice. Basaloid BCC-like lesions with pronounced downgrowths are clearly visible.

C-C′. Phenotype of BCC mice lacking EGFR. Histology of ears from TAM injected *K5cre^{ER};SmoM2;EGFR$^{fl/fl}$* mice. Arrowheads point at small basaloid hyperproliferations.

D. Quantification of BCC lesions. Tumour multiplicity represented as mean number of lesions in TAM treated *K5cre^{ER};SmoM2;EGFR$^{+/+}$* ($n = 10$) and *K5cre^{ER};SmoM2;EGFR$^{fl/fl}$* mice ($n = 10$). Error bars represent SEM.

E. Quantification of BCC tumour size. To quantify the effect of epidermal EGFR deletion on tumour size, lesions were categorized into small ($<1000\ \mu m^2$), medium ($1000–2999\ \mu m^2$) and large sized tumours ($3000–15{,}000\ \mu m^2$) (for details see Material and Methods) and plotted as percentage fraction of all tumours analysed per genotype ($n_{(EGFR\ +/+)} = 602$, $n_{(EGFR\ fl/fl)} = 255$).

HH-EGFR cooperation response genes that mediate HH/GLI and EGFR cooperation in tumourigenesis.

Given the potent mitogenic activity of combined HH/GLI and EGFR, e.g. in neural stem cells (Palma et al, 2005; Palma & Ruiz i Altaba, 2004), we first tested whether HH-EGFR cooperation promotes oncogenic transformation by cooperative induction of cell cycle regulators. As HH-EGFR signalling converges at the promoters of selected direct GLI target genes (Kasper et al, 2006b), we first screened for cell cycle regulators directly controlled by GLI and then tested whether these factors can be activated synergistically by GLI/EGFR (Supporting Information Tables S1 and S2). We identified CCND1, CDT1, FOXM1, KNTC1 and TOPBP1 as direct GLI targets (Supporting Information Fig S4A and B). However, none of these target genes was

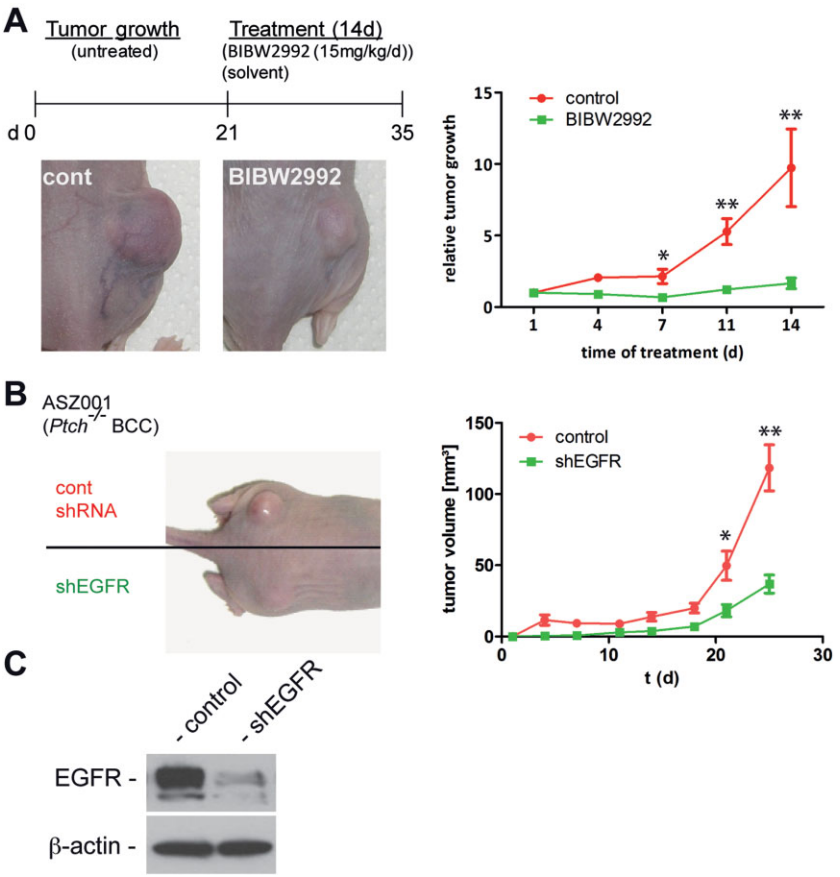

Figure 2. Genetic and pharmacological inhibition of EGFR in BCC cells reduces tumour growth *in vivo*.

A. Effect of pharmacologic EGFR inhibition on BCC growth. Mouse Ptch$^{-/-}$ BCC cells (ASZ001) were grafted subcutaneously onto nude mice and allowed to form palpable tumours. 21 days after the inoculation of BCC cells, mice received orally BIBW2992 at 15mg/kg/day or solvent only (control) for two weeks. Right graph shows the increase in tumour volume of BIBW2992 ($n = 10$) and control treated mice ($n = 10$) during the 14-day treatment period.

B. RNAi knockdown of EGFR in ASZ001 BCC cells. Control cells transduced with non-target shRNA (cont shRNA) were grafted subcutaneously onto the left and EGFR knockdown cells (shEGFR) onto the right lower flank of nude mice ($n = 10$). Tumour growth was measured over a period of 25 days.

C. RNAi knockdown of EGFR. Western blot analysis of control shRNA (control) and shEGFR transduced ASZ001 cells showing reduced EGFR expression upon RNAi mediated knockdown. β-actin served as loading control. Error bars represent SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

synergistically induced by combined GLI/EGFR activation (Supporting Information Fig S4C). In addition, deletion of EGFR did not promote apoptosis of SmoM2-expressing keratinocytes (Supporting Information Fig S4D), suggesting that enhanced proliferation and survival are not the main reason for the tumour promoting effect of HH-EGFR cooperation.

To shed light on the molecular processes controlled by HH-EGFR cooperation, we screened for downstream mediators of signal cooperation by genome-wide expression profiling of human HaCaT keratinocytes with either single activation of GLI1, EGFR or a combination of both signals. Synergistically induced GLI1-EGFR cooperation response genes were selected based on their synergy score (see Materials and Methods) and filtered for (i) early synergistic activation by HH/GLI-EGFR (i.e. within 4–18 h post stimulation), (ii) a documented role in the regulation of tumour growth, cancer stem cell activation and/or metastasis and (iii) the presence of putative GLI binding sites in their *cis*-regulatory region. This approach identified JUN, TGFA, FGF19 (criterion: tumour growth), SOX9, SOX2 (criterion: stemness), and SPP1/osteopontin and CXCR4 (criterion: metastasis) as putative candidate mediators of HH-EGFR signal cooperation. qPCR validation of chip data confirmed that GLI1 and EGFR stimulation (by EGF treatment (10 ng/ml)) synergistically activated transcription of JUN, FGF19 and CXCR4 already after 4.5 h. Activation of SOX9 and TGFA at the 4.5-h time point is mainly induced by EGF but at later time points becomes dependent on combined GLI1 and EGFR activation. Combined GLI1-EGFR signalling also induced synergistic activation of SOX2 and SPP1 transcription at the 9 and 18-h time points (Fig 3A).

To address if these genes represent immediate early HH/GLI-EGFR response genes, we tested them for direct regulation by GLI. All response genes, including the previously identified GLI targets JUN and SPP1 (Laner-Plamberger et al, 2009; Yoon et al, 2002) (data not shown), contained at least two clustered GLI binding sites within 5 kb of their upstream *cis*-regulatory sequences (Fig. 3B). Luciferase reporter assays in combination with site-directed mutagenesis of predicted GLI binding sites confirmed direct regulation of FGF19, TGFA, CXCR4 and SOX9 by GLI1 and dominant active GLI2 (GLI2deltaN) (Fig. 3C and Fig S5 of Supporting Information). GLI1 and GLI2deltaN also induced the promoter activity of the stemness transcription factor SOX2 (Fig 3C, and Fig S5 of Supporting Information), in line with a previous study (Takanaga et al, 2009). In summary, the data suggest that FGF19, TGFA, CXCR4, SOX9, SOX2—and the known GLI targets JUN and SPP1—represent immediate-early targets of HH/GLI-EGFR cooperation.

Activation of cooperation response genes requires the specific combination of GLI and EGFR signalling

SMO-independent signal inputs such as RAS-MEK/ERK have been shown to modify the transcriptional activity of GLI proteins (Riobo et al, 2006a; Stecca et al, 2007). We have previously

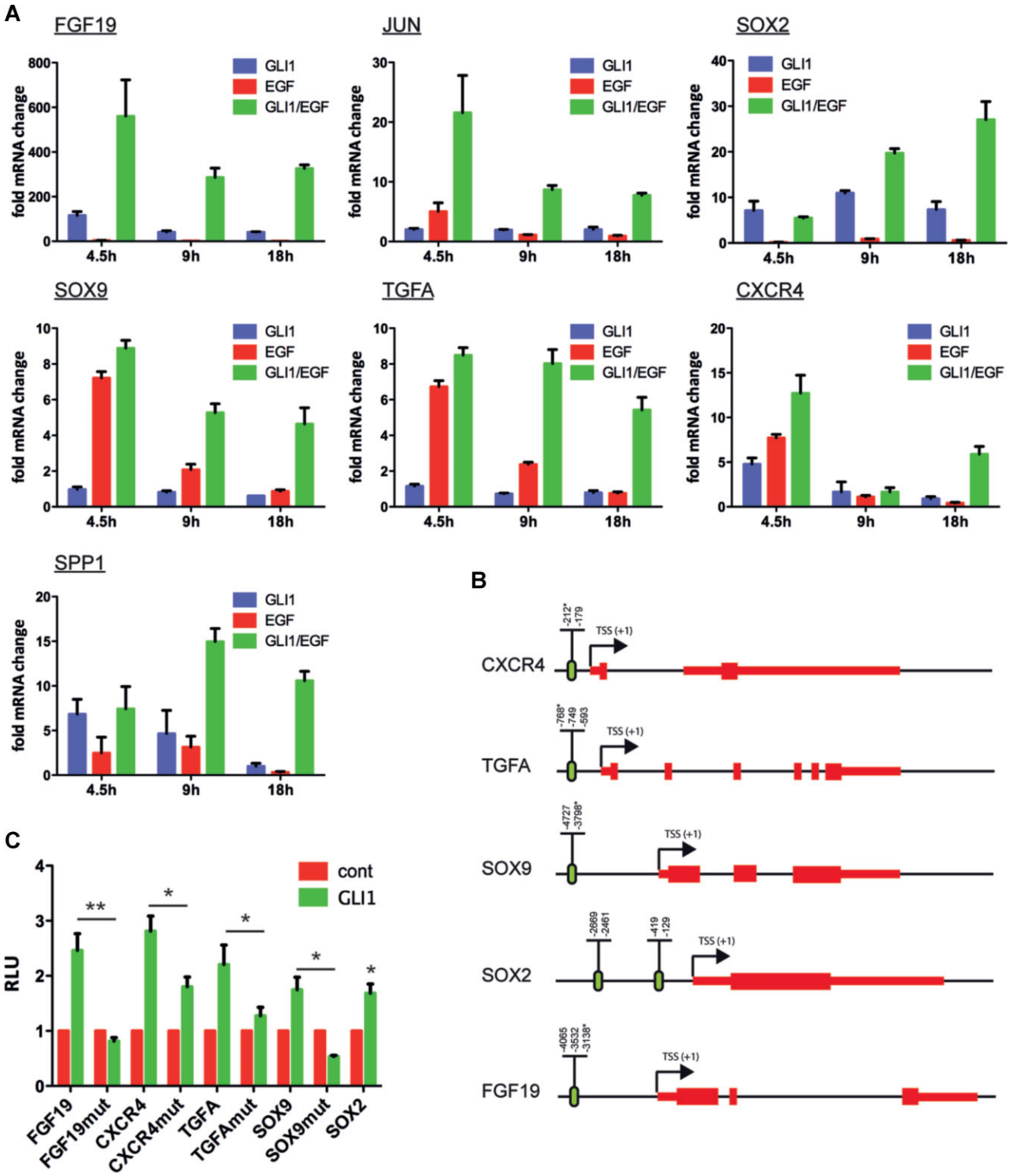

Figure 3. Identification of HH/GLI-EGFR cooperation response genes (CRG).

A. CRG regulation by GLI1 and EGFR signalling. qPCR analysis of CRG mRNA expression in response to GLI1 expression, EGFR signalling (EGF) or a combination of both stimuli (GLI1/EGF) after 4.5, 9 and 18 h of single or combined activation (three biological replicates measured in duplicate, values are fold change compared to untreated controls).

B. Location of GLI binding sites in CRG promoters. Genomic locus of CRG with *in silico* predicted GLI binding site clusters located in their 5′ upstream regulatory regions. Numbers indicate the position of binding sites relative to the transcriptional start site (TSS).

C. Direct transcriptional regulation of CRG by GLI. Luciferase reporter assays showing activation of CRG promoters in response to GLI1 expression. For FGF19, CXCR4, TGFA and SOX9 selected GLI binding sites were mutated (FGF19mut, CXCR4mut, TGFAmut, SOX9mut). The mutated GLI binding sites are indicated by asterisks in B. Error bars represent SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

shown that EGFR-dependent activation of RAS-MEK/ERK and JUN/AP1 activation is critical for mediating HH/GLI-EGFR signal cooperation (Schnidar et al, 2009). Since activation of RAS-MEK/ERK is a common event in response to receptor tyrosine kinase (RTK) activation, we addressed whether the activation of HH/GLI-EGFR response genes is specifically mediated by combined GLI and EGFR signalling, or whether other RTK pathways can also cooperate with GLI and induce a similar cooperation response gene profile. Therefore, we tested various RTK ligands including hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), fibroblast growth factor 7 (FGF7) and basic FGF (bFGF) for their ability to induce RAS/RAF/MEK/ERK and JUN/AP1 activation in human HaCaT keratinocytes. As shown in Fig 4A, EGF, FGF7, HGF and to a lower extent also VEGF stimulated MEK/ERK, though only EGF was able to induce JUN activation (pJUN). Analysis of HH-EGFR cooperation response gene expression in GLI1 expressing keratinocytes simultaneously treated either with EGF, HGF, FGF7, or VEGF revealed that only the combination of GLI1 and EGF led to synergistic induction of JUN, SOX9, FGF19, SPP1, TGFA and SOX2. Only SOX2 expression was also enhanced by GLI1 and FGF7 treatment (Fig 4B). The canonical GLI targets PTCH and BCL2 (Kasper et al, 2006b) were not hyper-activated by any of the combinations, rather we observed an unexplained reduction of PTCH mRNA expression by combined GLI1 and VEGF, HGF or FGF7 treatment (Fig 4B). As expression of oncogenic RAS has been shown to promote the transcriptional and oncogenic activity of GLI (Pasca di Magliano et al, 2006; Stecca et al, 2007), we studied the effect of combined GLI1/RAS activation on the expression of HH-EGFR response genes. As shown in Fig 4C, co-expression of GLI1 and oncogenic $KRAS^{G12V}$ ($KRAS^{*}$) did not lead to increased expression of HH-EGFR cooperation response genes. Rather, $KRAS^{*}$ attenuated the expression of JUN, FGF19, CXCR4, TGFA and SPP1 in the presence of GLI1. By contrast and consistent with previous reports showing enhancement of GLI activity by oncogenic RAS (Stecca et al, 2007), expression of the EGF-independent GLI targets PTCH and HHIP (Kasper et al, 2006b) (data not shown) was significantly increased by $GLI1/KRAS^{*}$. Thus, the regulation of HH-EGFR cooperation response genes requires the specific combination of HH/GLI and EGFR activation. Whether MEK/ERK-dependent activation of JUN/AP1 is the critical determinant for cooperation of HH/GLI and RTK pathways such as EGFR signalling remains to be addressed.

HH-EGFR cooperation response genes mediate synergistic HH/GLI-EGFR signal integration in cancer

Having demonstrated a critical function of EGFR in HH/GLI-driven BCC, we next focused on the possible role of selected HH-EGFR cooperation response genes in mediating the oncogenic effect of HH-EGFR cooperation. Therefore, we first analysed the expression of cooperation response genes (i.e. Sox2, Sox9, Spp1, Jun, Tgfa and Cxcr4; note that closely related FGF19 homologues do not exist in mouse) in BCC-like tumours of mice with epidermis-specific expression of a dominant active form of GLI2 (*K5cre;Cleg2*, Grachtchouk et al, 2011; Pasca di Magliano et al, 2006) as well as in allograft tumours established from $Ptch^{-/-}$ mouse BCC cells (ASZ001). We found elevated mRNA levels of the HH-EGFR targets Sox2, Sox9, Spp1, Jun, Tgfa and Cxcr4 in GLI2-induced BCC-like tumours compared to normal skin of control mice (Fig 5A). Likewise, allograft tumours established from grafted $Ptch^{-/-}$ BCC cells expressed elevated mRNA levels of Spp1, Tgfa, Sox9, Jun, Sox2, Cxcr4 and the known HH-EGFR target gene Il1r2 (Kasper et al, 2006b), when compared to levels in $Ptch^{-/-}$ BCC cells cultured *in vitro* before grafting (Fig 5B). By contrast, levels of Gli1 and the EGF-independent GLI target Bcl2 (Kasper et al, 2006b) did not differ between allografts and *in vitro* cultured BCC cells. These data suggest activation of EGFR signalling during *in vivo* tumour growth of ASZ001 BCC cells. Indeed, only allograft tumours from $Ptch^{-/-}$ BCC cells showed high levels of activated EGFR (pEGFR), while *in vitro* cultured BCC cells did not (Fig 5C). Allograft tumours established from $Ptch^{-/-}$ BCC cells also showed activation of Mek/Erk and Jun, similar to $Ptch^{-/-}$ BCC cells treated with EGF *in vitro* (Supporting Information Fig S6). To show *in vivo* regulation of cooperation response genes by HH-EGFR signalling, we analysed the expression of Jun, Sox2, Sox9, Tgfa, Cxcr4 and Spp1 in epidermal cells of tamoxifen-treated $K5cre^{ER}$;SmoM2;$EGFR^{+/+}$ and $K5cre^{ER}$;SmoM2;$EGFR^{fl/fl}$ mice ($n=3$ for each genotype). As shown in Fig 5D, SmoM2 expression led to enhanced levels of Jun, Sox9, Sox2, Tgfa, Cxcr4 and Spp1 mRNA in $K5cre^{ER}$;SmoM2;$EGFR^{+/+(+TAM)}$ mice. Deletion of EGFR resulted in decreased levels of HH-EGFR response genes ($K5cre^{ER}$;SmoM2;$EGFR^{fl/fl\ (+TAM)}$). Together with the finding that HH-EGFR response genes are expressed in mouse BCC-like tumours and human BCC (Supporting Information Fig S7), these results suggest that combined HH/GLI and EGFR signalling control the activation of cooperation response genes in BCC.

To address the role of HH-EGFR cooperation response genes in BCC, we analysed the effect of RNAi-mediated inhibition of Jun, Sox2 and Cxcr4 on tumour growth of $Ptch^{-/-}$ BCC cells. Fig 5E shows that individual inhibition of Jun, Sox2 or Cxcr4 in BCC cells significantly reduced tumour take of nude mice, suggesting that Jun, Sox2 and Cxcr4 constitute critical downstream mediators of oncogenic HH-EGFR signalling important for *in vivo* tumour growth of BCC cells.

HH/GLI-EGFR cooperation response genes define the phenotype of tumour-initiating pancreatic cancer cells

Given the substantial overlap of HH/GLI and EGFR signalling in many human cancers we set out to address whether HH-EGFR cooperation and the respective cooperation response gene signature are also deployed in cancers other than BCC. Here, we studied the role of HH-EGFR cooperation in pancreatic adenocarcinoma cells, which represent one of the most aggressive and still incurable malignancies with a documented role of HH/GLI and EGFR signalling (Li et al, 2007; Nolan-Stevaux et al, 2009; Pasca di Magliano et al, 2006; Yauch et al, 2008). Given the critical role of HH/GLI in cancer stem cells (Clement et al, 2007; Dierks et al, 2008; Mueller et al, 2009; Peacock et al, 2007; Varnat et al, 2009; Zhao et al, 2009), we focused on the role of HH-EGFR cooperation in tumour-initiating pancreatic cancer cells. For this purpose we developed

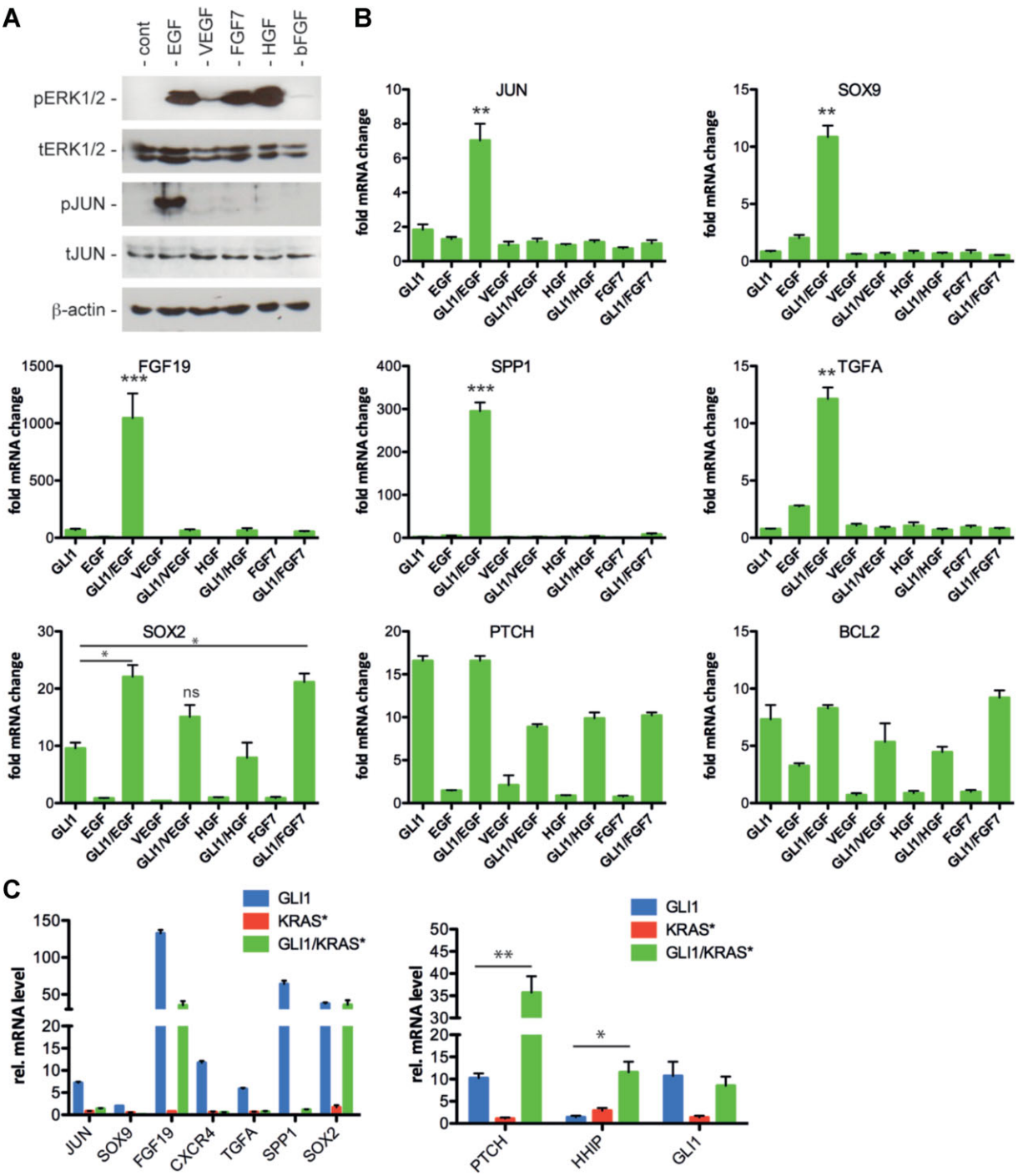

Figure 4. Activation of cooperation response genes requires the specific combination of HH/GLI and EGFR signalling.

A. Analysis of RAS/MEK/ERK signalling and JUN activation in human HaCaT keratinocytes in response to various receptor tyrosine kinase (RTK) ligands. Note that although EGF (10 ng/ml), FGF7 (50 ng/ml), HGF (50 ng/ml) and to a lower extent also VEGF (50 ng/ml) induce ERK1/2 activation (pERK1/2), only EGF treatment is able to stimulate JUN activation (phosphorylated JUN, pJUN).

B. CRG regulation by RTK pathways. qPCR analysis of HH/GLI-EGFR cooperation response gene expression in response to either GLI1 activation, treatment with RTK ligands EGF, VEGF, HGF and FGF7, or a combination of GLI1 and the RTK ligands (see A). PTCH and BCL2 served as reference for direct GLI target genes whose expression is independent of parallel EGFR signalling (Kasper et al, 2006b). As bFGF treatment did not induce activation of MEK/ERK in HaCaT cells (see A), we did not analyse the combination of GLI1/bFGF.

C. CRG and canonical GLI target regulation by GLI-RAS signalling. Expression of JUN, SOX9, FGF19, CXCR4, TGFA, SPP1 and SOX2 (left), and PTCH and HHIP (right) in response to combined GLI1 and oncogenic KRAS (KRAS*). GLI1 transgene levels are unaffected by KRAS* expression. Error bars represent SEM. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$, ns: not significant ($p > 0.05$).

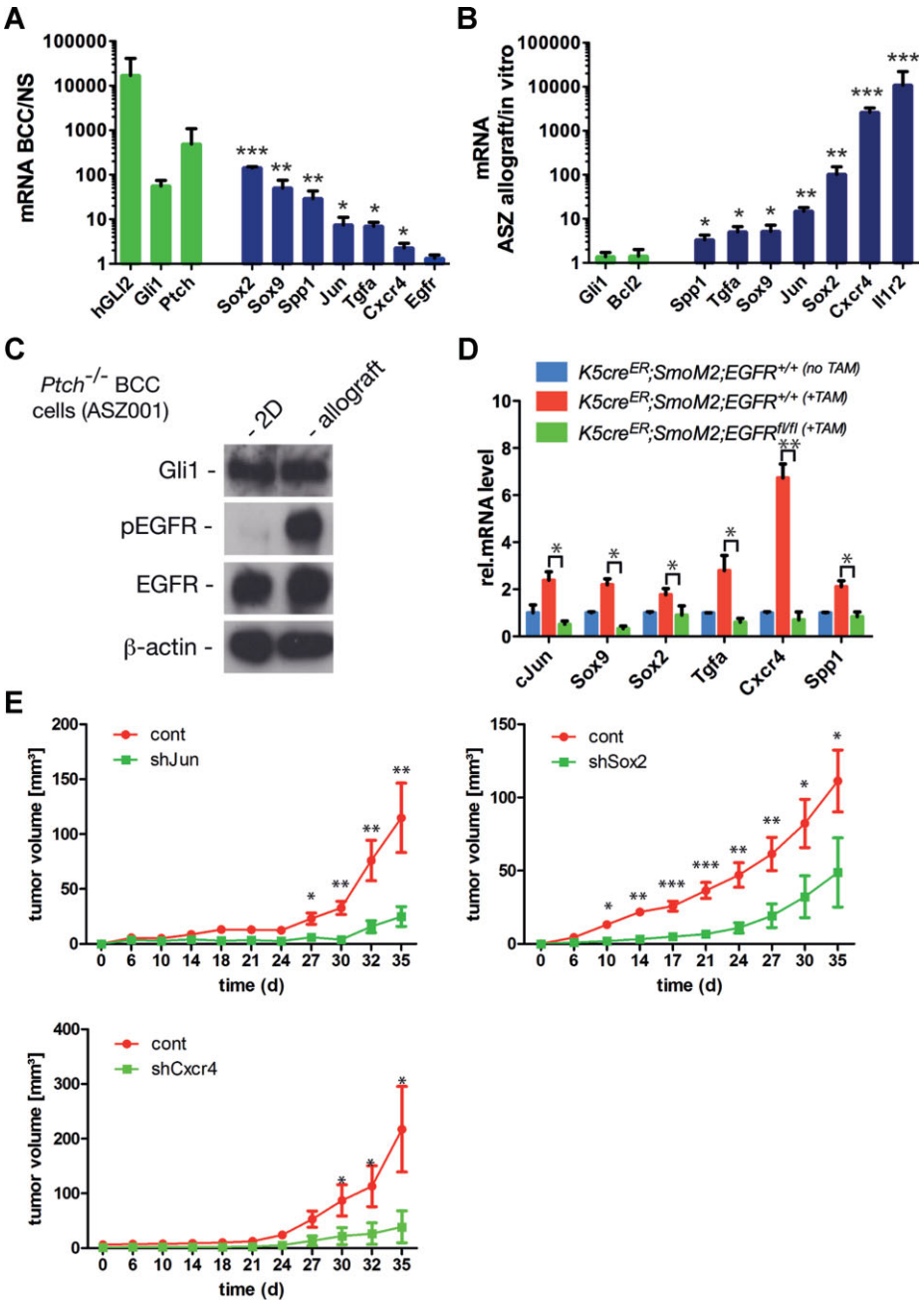

Figure 5. HH-EGFR cooperation response genes are expressed in mouse BCC models and required for *in vivo* BCC growth.

A. CRG expression in GLI-induced skin tumours. qPCR analysis of gene expression in tumours of *K5cre;Cleg2* mice (BCC) compared to normal skin (NS). Tumours express high levels of the human GLI2 transgene as well as its targets Gli1 and Ptch (green bars). Likewise, Sox2, Sox9, Spp1, Jun, Tgfa and Cxcr4 mRNA levels are significantly elevated in tumours compared to normal skin (blue bars). EGFR expression itself is not significantly changed between tumour and normal skin samples.

B. CRG expression in BCC allograft tumours. Activation of the HH-EGFR targets Sox2, Sox9, Spp1, Jun, Tgfa, Cxcr4 and Il1r2 during *in vivo* tumour growth of mouse BCC cells. Ptch$^{-/-}$ ASZ001 BCC cells were grafted onto nude mice and tumours harvested after 4 weeks (ASZ allograft). Values represent the ratio of mRNA levels in ASZ allograft samples to those in ASZ cells grown *in vitro* before grafting. Note that data in A. and B. are plotted on a log10 scale.

C. EGFR activation in BCC allograft tumours. Western blot analysis showing activation of EGFR (pEGFR) in ASZ allografts compared to ASZ cells grown *in vitro* before grafting.

D. EGFR-dependent CRG expression in mouse BCC. qPCR analysis of SmoM2-induced activation of Jun, Sox9, Sox2, Tgfa, Cxcr4 and Spp1 in primary mouse keratinocytes isolated from the skin of transgenic mice with the indicated genotype.

E. Role of CRG in BCC growth. RNAi knockdown of Jun (shJun), Sox2 (shSox2) and Cxcr4 (shCxcr4) reduces *in vivo* tumour growth of ASZ001 BCC cells in nude mice ($n = 6$). For RNAi knockdown efficiencies see Supporting Information Fig S10. Cont: grafted ASZ001 BCC cells transduced with non-target control shRNA. Error bars represent SEM. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$.

a modified 3D tumoursphere assay that selects for rare yet highly clonogenic, tumour-initiating pancreatic cancer cells that form large spheres under non-adherent conditions. We hitherto refer to these spheres as macrospheres (Fig 6A, B, for more details see Supporting Information, Materials and Methods and Fig S8 of Supporting Information). qPCR analysis of single, tumour-initiating macrospheres isolated from 3D cultures of human pancreatic cancer cells (L3.6sl, Panc-1 and L3.6pl) revealed high-level expression of stem cell genes such as OCT4, Nanog and Prominin-1/CD133 (Fig 6C). When compared to corresponding pancreatic cancer cells grown under standard 2D culture conditions, tumour-initiating macrospheres also

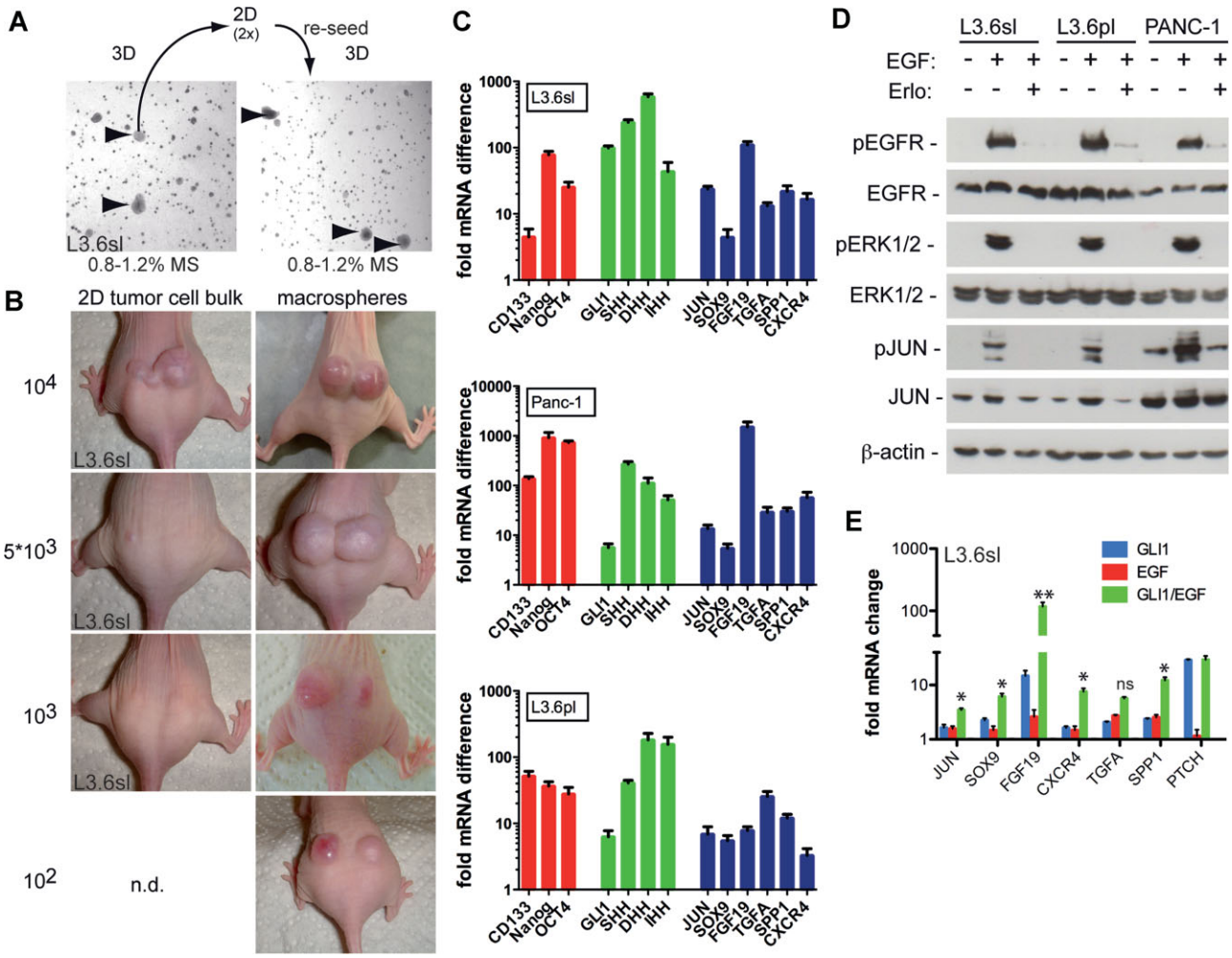

Figure 6. High-level expression of HH-EGFR CRG in tumour-initiating pancreatic cancer cells.

A. Sphere growth of rare pancreatic cancer cells. If grown in 3D culture, human pancreatic cancer cells (here: L3.6sl) form macrospheres (MS) at low frequency (0.8–1.2%). If single macrospheres are isolated from 3D cultures (arrows in left image) and grown for serial passages in 2D, the cells will again form macrospheres at a constant low frequency if re-seeded in 3D cultures (right image, also see Fig S8 and Supporting Information).

B. Tumour-initiation by sphere-forming pancreatic cancer cells. Limiting dilution xenograft experiments showing that as few as 100 pancreatic cancer cells (here L3.6sl) derived from single isolated macrospheres are sufficient to initiate tumour growth *in vivo* ($n = 4/4$). By contrast, at least 10^4 pancreatic cancer cells grown in 2D culture need to be grafted to establish tumours *in vivo* ($n = 4$ for each dilution experiment), showing that macrospheres isolated from 3D cultures of pancreatic cancer cells are highly enriched for tumour-initiating cells.

C. CRG expression in tumour-initiating pancreatic cancer cells. qPCR analysis ($n = 4$) showing elevated mRNA levels of stemness genes (CD133, Nanog, OCT4, red bars), HH pathway genes (SHH, DHH, IHH, GLI1) (green bars) and HH-EGFR cooperation response genes (JUN, SOX9, FGF19, TGFA, SPP1, CXCR4, blue bars) in tumour-initiating macrosphere cells compared to the corresponding cancer cells grown in 2D cultures. Graphs show results for three human pancreatic cancer cells lines (L3.6sl, Panc-1 and L3.6pl).

D. EGFR signalling in pancreatic cancer cells. Western blot analysis of pancreatic cancer cells (L3.6sl, Panc-1 and L3.6pl) showing activation of EGFR/ERK/JUN signalling in response to EGF treatment. Pharmacological inhibition of EGFR by Erlotinib (Erlo) prevented activation of the EGFR/MEK/JUN cascade.

E. Regulation of CRG expression by GLI-EGFR signalling. Induction of HH-EGFR cooperation response genes (JUN, SOX9, FGF19, CXCR4, TGFA, SPP1) by combined GLI1/EGFR activation in L3.6sl pancreatic cancer cells. Unlike HH-EGFR response genes, transcriptional activation of the canonical GLI target PTCH is independent of EGF treatment. Error bars represent SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, ns: not significant ($p > 0.05$).

expressed elevated mRNA levels of HH/GLI signalling components (GLI1, SHH, DHH and IHH) and notably, increased levels of HH-EGFR cooperation response genes (JUN, SOX9, FGF19, TGFA, SPP1 and CXCR4; Fig 6C). Furthermore and similar to our data of human keratinocytes and murine BCC cells, activation of EGFR led to activation of MEK/ERK/JUN signalling in pancreatic cancer cells (Fig 6D). Importantly, combined activation of GLI1 and EGFR signalling resulted in enhanced activation of the HH-EGFR cooperation response genes JUN, SOX9, FGF19, CXCR4 and SPP1. TGFA was also enhanced by GLI1/EGF but its increase did not reach statistical significance (Fig 6E). In agreement with data from keratinocytes and BCC cells, PTCH expression was induced by GLI1 but unaffected by concomitant EGFR activation (Fig 6E). Taken together, these findings demonstrate increased expression of HH-EGFR cooperation response genes in tumour-initiating pancreatic cancer cells and suggest a common molecular mechanism HH-EGFR cooperation response gene regulation in BCC and pancreatic cancer cells.

To address a putative functional role of HH/GLI-EGFR cooperation in the tumour-initiating subpopulation of pancreatic cancer cells, we monitored the effect of single and combined targeted inhibition of HH/GLI and EGFR signalling on the growth of tumour-initiating macrospheres. Single perturbation of GLI1, JUN or EGFR function by RNAi or pharmacological inhibitor treatment resulted in a moderate reduction of tumour-initiating spheres (Fig 7A, B). By contrast, combined inhibition

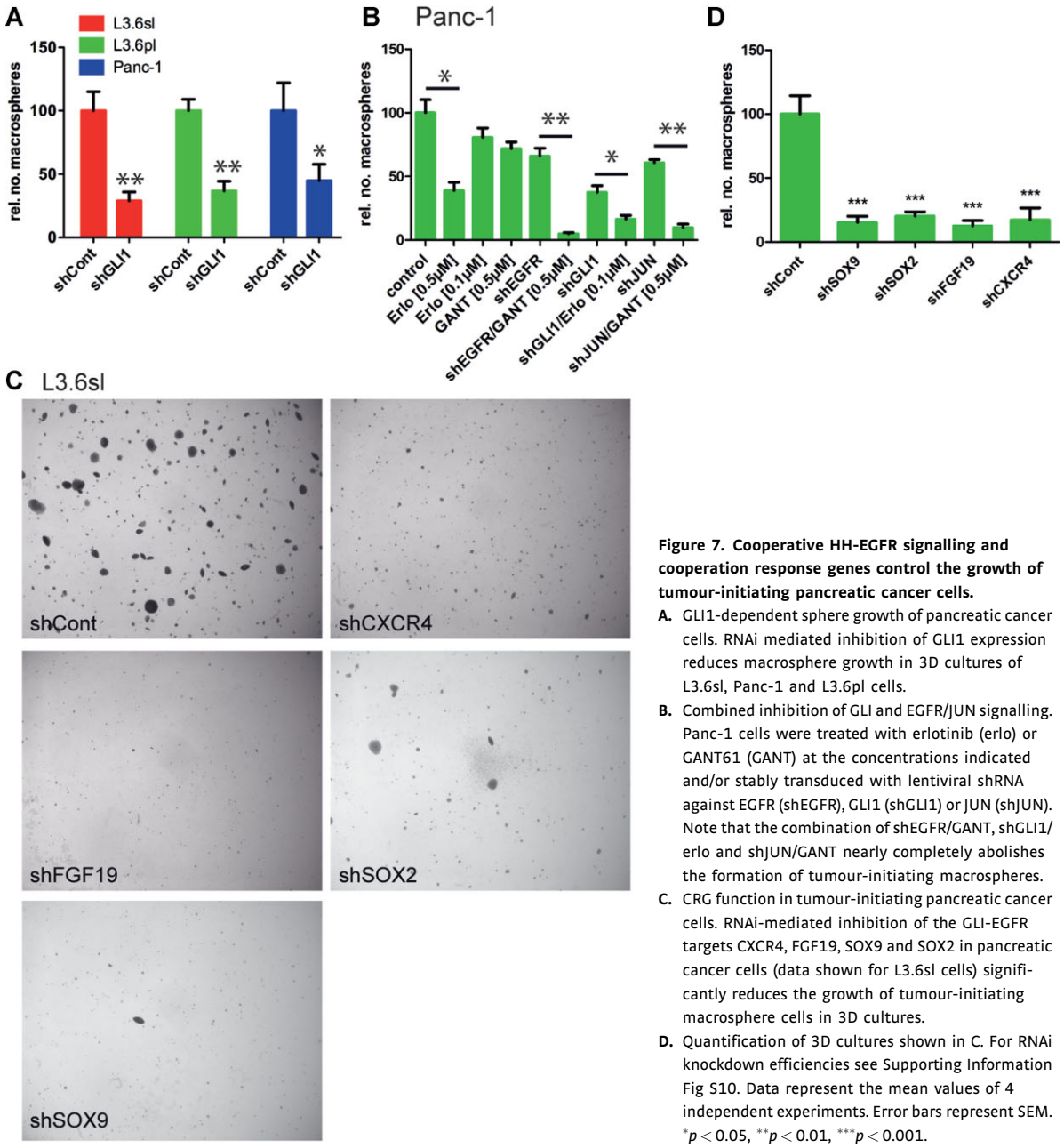

Figure 7. Cooperative HH-EGFR signalling and cooperation response genes control the growth of tumour-initiating pancreatic cancer cells.

A. GLI1-dependent sphere growth of pancreatic cancer cells. RNAi mediated inhibition of GLI1 expression reduces macrosphere growth in 3D cultures of L3.6sl, Panc-1 and L3.6pl cells.

B. Combined inhibition of GLI and EGFR/JUN signalling. Panc-1 cells were treated with erlotinib (erlo) or GANT61 (GANT) at the concentrations indicated and/or stably transduced with lentiviral shRNA against EGFR (shEGFR), GLI1 (shGLI1) or JUN (shJUN). Note that the combination of shEGFR/GANT, shGLI1/erlo and shJUN/GANT nearly completely abolishes the formation of tumour-initiating macrospheres.

C. CRG function in tumour-initiating pancreatic cancer cells. RNAi-mediated inhibition of the GLI-EGFR targets CXCR4, FGF19, SOX9 and SOX2 in pancreatic cancer cells (data shown for L3.6sl cells) significantly reduces the growth of tumour-initiating macrosphere cells in 3D cultures.

D. Quantification of 3D cultures shown in C. For RNAi knockdown efficiencies see Supporting Information Fig S10. Data represent the mean values of 4 independent experiments. Error bars represent SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

of GLI1/EGFR or GLI1/JUN strongly reduced the formation of tumour-initiating macrospheres. Intriguingly, knockdown of GLI1 and EGFR/JUN sensitized the cells to lower concentrations of the EGFR inhibitor erlotinib and the GLI antagonist GANT61, respectively (Fig 7B). These data suggest that cooperation of EGFR/JUN signalling with GLI1 function is critical for the growth of tumour-initiating pancreatic cancer cells.

Next, we addressed the role of HH-EGFR cooperation response genes in tumour-initiating pancreatic cancer cells, by RNAi knockdown of CXCR4, FGF19, SOX2 and SOX9. As shown in Fig 7C and D, RNAi against each of the HH-EGFR response genes efficiently prevented macrosphere growth. As FGF signalling has previously been shown to activate Gli2 expression (Brewster et al, 2000), the effect of FGF19 inhibition may be due to a decrease in GLI levels in response to diminished FGF19/FGFR4 signalling (Xie et al, 1999). However, neither GLI2 nor GLI1 levels were significantly reduced by knockdown of FGF19 (Supporting Information Fig S9A). Like inhibition of FGF19, FGFR inhibitor treatment dramatically reduced the formation of tumour-initiating macrospheres (Supporting Information Fig S9B), supporting the autocrine mode of FGF19/FGFR4 signalling.

To analyse the *in vivo* relevance of these data, we performed xenograft assays using pancreatic cancer cells (L3.6sl) with RNAi knockdown of GLI1, JUN, SOX9, FGF19 or CXCR4. We found that inhibition of each target had a striking inhibitory effect on the *in vivo* tumour growth of pancreatic cancer cells compared to control knockdown cells (Fig 8A–E). By contrast and in line with previous reports (Nolan-Stevaux et al, 2009; Yauch et al, 2008), RNAi against SMOH did not affect *in vivo* tumour growth (Fig 8F).

The finding that each of the HH-EGFR cooperation response genes tested reduced *in vivo* growth of pancreatic cancer cells prompted us to address the possibility of positive regulatory interactions between HH-EGFR response genes. We therefore inhibited the expression of JUN, SOX9, FGF19 or CXCR4 and then measured the effect on the expression of the unperturbed HH-EGFR cooperation response genes including SPP1. Inhibition of SOX9 significantly reduced JUN, CXCR4 and SPP1 mRNA levels, while inhibition of JUN reduced CXCR4 and SPP1 levels. Intriguingly, shRNA against FGF19 strongly inhibits CXCR4 and SPP1 expression, while inhibition of CXCR4 had a dramatic reciprocal negative impact on FGF19 expression. By contrast, PTCH mRNA levels were not significantly affected by any of the perturbation experiments (Fig 9A).

DISCUSSION

Previous studies by our group and others have pointed to a role of HH-EGFR signal cross-talk in the growth of cancer cell lines, though the therapeutic relevance of these results remained unclear mainly because of the *in vitro* nature of the studies and the possible off-target effects of the inhibitors used (Mimeault et al, 2006; Schnidar et al, 2009). Using preclinical *in vivo* models of cancer development, we now provide genetic and pharmacological evidence for a critical function of EGFR signalling in HH/GLI-driven cancers such as BCC. Therefore, these results identify EGFR is a valid therapeutic target for HH-driven BCC, supporting the concept of combination treatments with SMO and EGFR inhibitors as efficient therapeutic option.

An intriguing finding of this study was that our systematic screen for downstream mediators of the synergistic transformation effect identified a set of HH-EGFR cooperation response genes (CRG) that is directly regulated by GLI, synergistically enhanced by EGFR signalling and enriched for critical determinants of the oncogenic phenotype of both BCC and tumour-initiating pancreatic cancer cells (i.e. JUN, SOX9, SOX2, FGF19 and CXCR4). The striking inhibitory effect of HH-EGFR CRG inhibition on *in vivo* tumour growth of BCC and tumour-initiating pancreatic cancer cells suggest that EGFR cooperates with HH/GLI to initiate and promote tumour growth by synergistic activation of HH-EGFR CRG. Intriguingly, this effect appears specific to the combination of EGFR-GLI activation, as HGF/MET, FGF7/FGFR, VEGF and oncogenic KRAS signalling failed to cooperate with GLI1 in HH-EGFR CRG regulation.

Notably, HH/GLI-EGFR cooperation synergistically induced the expression of SOX2 and SOX9, two transcription factors involved in the regulation of stem cell fate (Vidal et al, 2005; Yamanaka, 2007), as well as of CXCR4, which not only marks metastatic CD133+ pancreatic cancer stem cells (CSC) but also controls their metastatic behaviour (Hermann et al, 2007). It is therefore tempting to speculate that selected HH-EGFR response genes together with GLI1 and the GLI target and stemness gene Nanog (Po et al, 2010; Zbinden et al, 2010) play a critical role in the determination of stem-like characteristics of tumour-initiating cancer cells, consistent with the identification of stem cells as cellular origin of HH-induced cancers and the documented role of HH/GLI in cancer stem cells (Clement et al, 2007; Dierks et al, 2008; Grachtchouk et al, 2011; Kasper et al, 2011; Mueller et al, 2009; Peacock et al, 2007; Varnat et al, 2009; Wang et al, 2011; Wong & Reiter, 2011; Zhao et al, 2009).

In summary, our data support a model where cooperation of HH/GLI and EGFR signalling induces high-level expression of cooperation response genes including SOX9, JUN, FGF19, CXCR4 and SOX2, thereby promoting tumour initiation and tumour growth. Positive regulatory interactions between FGF19, CXCR4, JUN and SOX9 are likely to contribute to tumourigenesis by selectively amplifying the expression of HH-EGFR regulated genes and/or by maintaining high level expression of HH-EGFR response genes required for sustained tumour growth (Fig 9B).

Finally, our study also underlines the power of analysing oncogenic pathway cooperation to identify gene sets with a critical role in tumour-initiation and cancer growth that may be exploited for novel therapeutic approaches. The identification of FGF19 and CXCR4 as druggable HH/GLI-EGFR target genes provides proof of concept for such an approach. Intriguingly, as some HH-EGFR cooperation response genes are linked within regulatory networks downstream of HH-EGFR cooperation, it will be important to evaluate the therapeutic

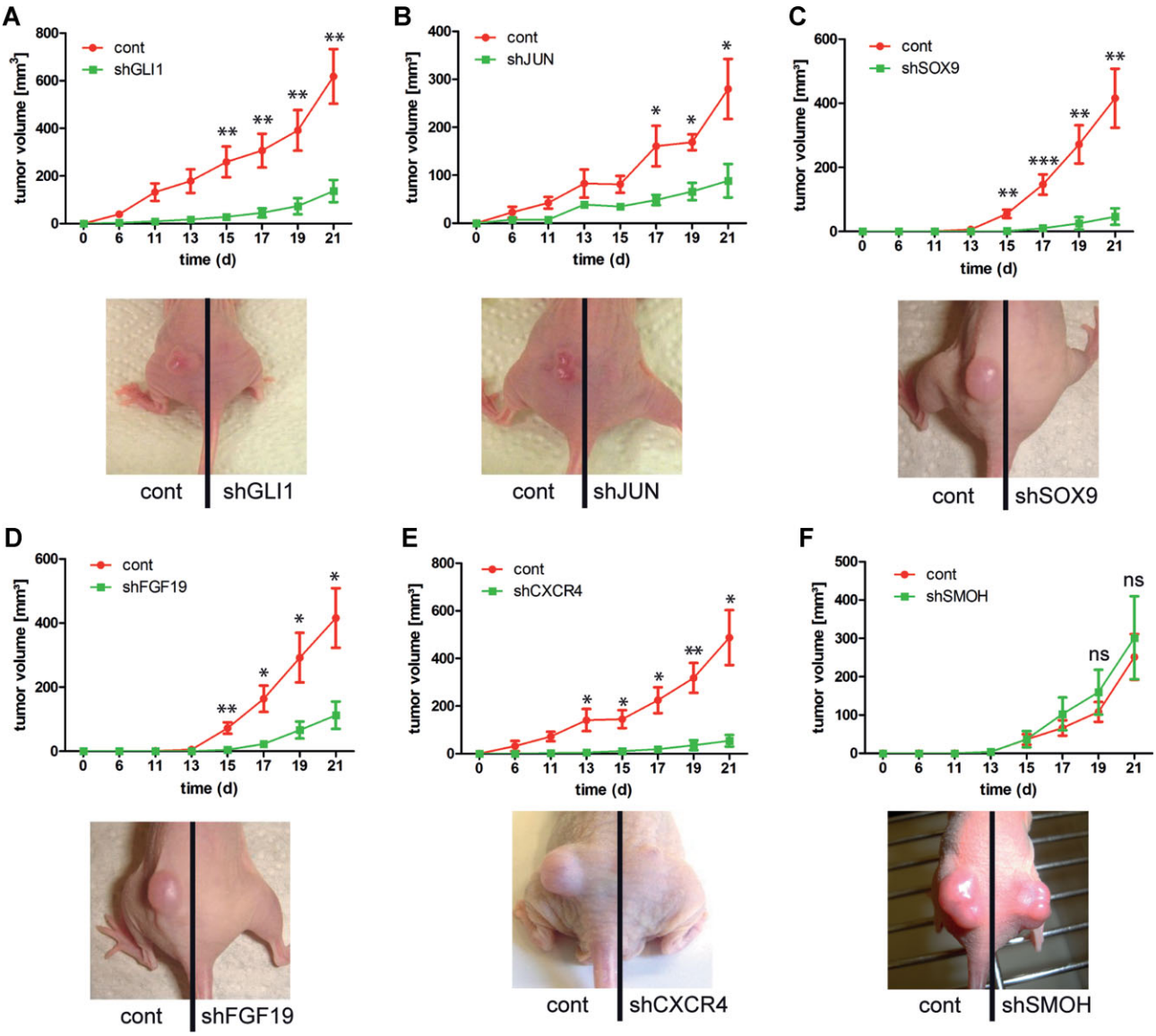

Figure 8. Inhibition of HH/GLI-EGFR target genes in pancreatic cancer cells prevents *in vivo* tumourigenesis.

A-F. Effect of RNAi knockdown of GLI1, JUN, SOX9, FGF19, CXCR4 and SMOH on *in vivo* growth of pancreatic cancer cells. 1×10^6 pancreatic cancer cells (L3.6sl) either transduced lentivirally with non-target control shRNA (cont) or with shRNA specific for the respective target were grafted contralaterally onto the lower flank of nude mice ($n = 8$ for each group). Similar to RNAi-mediated GLI1 inhibition (**A**), shRNA against the HH/GLI-EGFR targets JUN (**B**), SOX9 (**C**), FGF19 (**D**) and CXCR4 (**E**) interfere with *in vivo* tumour growth. By contrast, RNAi against SMOH (**F**) does not significantly affect *in vivo* growth of pancreatic cancer cells. Error bars represent SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant ($p > 0.05$).

efficacy of selected combinations of HH/GLI, EGFR and HH-EGFR response gene inhibitors in BCC and pancreatic cancer.

MATERIALS AND METHODS

Transgenic mice and xenografts

SmoM2 (R26SmoM2) (Jeong et al, 2004; Mao et al, 2006), *EGFR*$^{fl/fl}$ (Natarajan et al, 2007), *K5cre*^{ER} (*K5cre*^{ERT}) (Indra et al, 1999), *K5cre* (Ramirez et al, 2004) and *Cleg2* (Pasca di Magliano et al, 2006) mice were maintained on a C57BL/6 background. Activation of SmoM2 expression and epidermal deletion of EGFR in *K5cre*^{ER};*SmoM2*;*EGFR*$^{+/+}$ and *K5cre*^{ER};*SmoM2*;*EGFR*$^{fl/fl}$ mice, respectively, was accomplished by *i.p.* injection of 1 mg tamoxifen (Sigma, USA) per day. Mice were sacrificed 120 days post birth and subsequently analysed for tumour development and gene expression.

For *in vivo* tumour growth studies, 1×10^6 murine BCC or human pancreatic cancer cells in 25% Matrigel (BD Laboratories) were injected subcutaneously into the lower flanks of Foxn1$^{nu/nu}$ nude mice (Charles River Laboratories, USA). For oral administration, afatinib (Boehringer Ingelheim) was dissolved in 0.5% natrosol (Aqualon)/

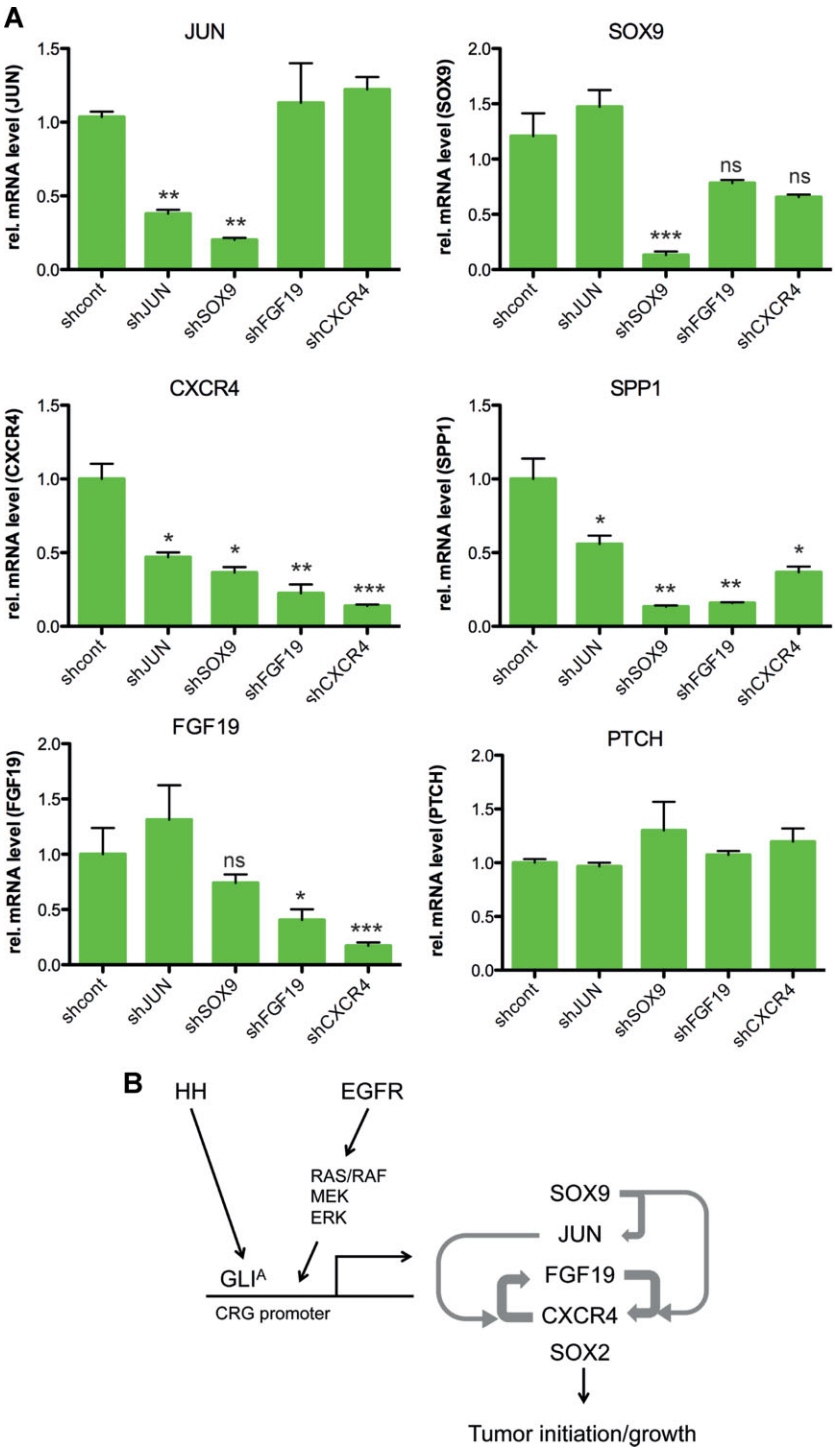

Figure 9. Regulatory interactions between HH/GLI-EGFR target genes in pancreatic cancer cells.

A. CRG expression in reciprocal RNAi knockdown studies. Pancreatic cancer cells were lentivirally transduced with non-target control shRNA (shcont) or with shRNA against JUN (shJUN), SOX9 (shSOX9), FGF19 (shFGF19) or CXCR4 (shCXCR4). 72 h post transduction, transduced cells were analysed by qPCR for the expression of the HH/GLI-EGFR cooperation response genes JUN, SOX9, FGF19, CXCR4 and SPP1, as well as for expression of the canonical GLI target PTCH. Measurement of the mRNA of the respective shRNA target served as control for successful RNAi knockdown (e.g. JUN mRNA level in shJUN cells, SOX9 mRNA level in shSOX9 cells, etc). Error bars represent SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ns: not significant ($p > 0.05$)

B. Model of HH/GLI-EGFR signal integration and activation of CRG expression in HH/GLI-associated tumourigenesis. Arrows illustrate positive regulatory interactions of gene expression between selected cooperation response genes. Width of arrows is proportional to the regulatory effect shown in A.

20 mg/ml 2-hydroxypropyl-beta-cyclodextrine (Sigma Aldrich, USA) at a final concentration of 2 mg/ml and administered orally at a concentration of 15 mg/kg/day.

Tumour volume was calculated according to the formula [$4/3 \times \pi \times$ (length/2) $\times$ (width/2) $\times$ (height/2)].

All animal experiments and mouse husbandry were done in the animal facility at the University of Salzburg in accordance with institutional and federal guidelines.

RNA interference and lentiviral transduction

For lentiviral RNAi knockdown experiments, the following shRNA constructs (Sigma-Aldrich mission TRC library) were used: shEGFR (TRCN0000055220), shRNA GLI1 (TRCN0000020486), shRNA SMOH (TRCN0000014363), shRNA human and mouse SOX2 (TRCN0000010753), shRNA SOX9 (TRCN0000020386), shRNA FGF19 (TRCN0000040258), shRNA mouse Cxcr4 (TRCN0000028750), shRNA JUN (TRCN0000039590), shRNA mouse Jun (TRCN0000055207), control

The paper explained

PROBLEM:

The Hedgehog pathway is currently very intensely studied in the field of oncology and molecular medicine. The therapeutic efficacy of several new Hedgehog antagonists developed by different biotech and pharmaceutical companies is currently evaluated in clinical trials. First results from these studies are promising, though limited response rates, insufficient efficacy and development of drug resistance underline the need for improved regimens. Combining Hedgehog antagonists with inhibitors targeting cooperative signals may prove an efficient therapeutic strategy. Detailed knowledge about such synergistic interactions driving cancer and rigorous evaluation of the therapeutic benefit in appropriate *in vivo* models is an important prerequisite for the development of novel rationale-based combination therapies simultaneously targeting such cooperative signals.

RESULTS:

In the present study, we show that genetic and pharmacologic inhibition of EGFR signalling—one of the most prominent targets in today's oncology, reduces tumour development in clinically relevant mouse models of Hedgehog/GLI driven skin cancer. Furthermore, we present a gene set that is synergistically regulated by Hedgehog-EGFR signal cooperation and essential for *in vivo* growth of Hedgehog/GLI dependent basal cell carcinoma and tumour-initiating pancreatic cancer cells. Thus, the work identifies novel downstream mechanisms mediating the oncogenic effect of synergistic Hedgehog-EGFR signal interactions.

IMPACT:

Our results confirm EGFR signalling as valid drug target in Hedgehog dependent cancers and provide a rationale for combination therapies based on simultaneous targeting of Hedgehog, EGFR and cooperation response genes including CXCR4 or FGF19. As antagonists against most of these effectors are already available and validated in clinical settings, such a drug regimen can be rapidly transferred to clinical settings to evaluate its therapeutic benefit compared to current single treatment approaches.

scrambled shRNA (SHC002) and shRNA against human CXCR4 (clone 12272, Addgene; Orimo et al, 2005). The functionality of the shRNAs was validated by qPCR and/or Western blot analysis (Supporting Information Fig S10 and Fig 2C). Lentivirus production and transductions were done as described previously (Kasper et al, 2007). Transduced cells were selected for puromycin resistance prior to further analysis.

Cell culture and inhibitor treatments

Panc-1, L3.6sl and L3.6pl (Bruns et al, 1999) cells were grown in DMEM media (PAA) with 10% fetal bovine serum (FBS) (PAA), penicillin (62.5 μg/ml), and streptomycin (100 mg/ml), at 37°C in 5% CO_2. ASZ001 cells were grown in 154CF media (Invitrogen) with 2% chelexed FBS, penicillin (62.5 μg/ml), and streptomycin (100 mg/ml), at 37°C in 5% CO_2 (So et al, 2006).

For 3-dimensional (3D) cultures, 1×10^4 cells were seeded in 12-well plates in 0.4% select agar on top of 0.5% bottom select agar (Invitrogen) according to standard protocols of anchorage independent growth assays. Cultures were grown for 4 weeks at 37°C in a humidified atmosphere containing 5% CO_2. Pancreatic cancer spheres grown in 3D cultures were isolated, purified by centrifugation and single cell suspensions generated by subsequent trypsin digest for further analysis (for details see Supporting Information). GANT61 (Merck), erlotinib (LC Laboratories) and afatinib (Boehringer-Ingelheim) were dissolved in DMSO. Spheres were documented on a stereomicroscope with Cell^D Image capture system and quantified using Colony Counter Software (Microtech Nition).

RNA Isolation, qPCR analysis and promoter studies

Total RNA was isolated using TRI-reagent (Molecular Research Center Inc.) followed by a LiCl purification step. Total RNA of sphere cultures and 2D cultured reference cells was isolated with the RNAqueous kit (Ambion Applied Biosystems) and cDNA synthesized with Superscript II reverse transcriptase (Invitrogen). qPCR was done on a Rotorgene 3000 (Qiagen) using iQ Sybr Green Supermix reagent (Bio-Rad).

In silico prediction of putative GLI binding sites was done using the ScanAce algorithm (Roth et al, 1998). Fragments with high scoring binding sites were PCR amplified and cloned into the pGL4 luciferase reporter vector (Promega). Luciferase reporter assays were carried out as described previously (Kasper et al, 2006b; for details see Supporting Information). Site-directed mutagenesis of GLI binding sites was done using the QuickChange XL site-directed mutagenesis kit (Stratagene) according to manufacturer's instructions. All constructs were confirmed by sequencing.

Chromatin immunoprecipitation was carried out with SimpleChIP Enzymatic ChIP kit (Cell Signaling Technology) according to the manufacturer's instructions. Chromatin was isolated from HaCaT keratinocytes expressing GLI1 for 48h (Regl et al, 2002) and precipitated with anti-Gli1 antibody (sc-6152, Santa Cruz Biotechnology) as described previously (Laner-Plamberger et al, 2009). Primer sequences used for amplification of promoter fragments harbouring GLI binding sites are listed in Supporting Information Table S3.

Identification of cooperation response genes

Gene expression profiling was done on a bead array technology platform (Illumina Inc., USA). For each time point human HaCaT keratinocytes either expressing GLI1, treated with EGF, or stimulated with a combination of both were analysed and compared to untreated control cells. Corresponding RNA samples were taken after 4.5, 9 and 18 h of single or combined treatment. To identify cooperation response genes, we calculated synergy scores according to (McMurray et al, 2008).

Scores < 0.9 were considered to represent targets synergistically activated in response to GLI/EGF stimulation. Synergistic regulation of HH-EGFR cooperation response genes was verified by qPCR analysis.

Author contributions

ME and SK analysed EGFR function and HH-EGFR response genes in basal cell carcinoma and pancreatic cancer cells. DM and AL characterised tumour-initiating cells, HD, HS, DM, KZ and H-CB identified direct GLI target genes. MS, FS, CH-K, ANE, MEV, CKB and AAD performed immunohistochemistry and provided material before publication and analysed data. HH, WN and CW contributed to HH-EGFR target gene identification. HL, CW and FA designed the experiments and analysed data. ME, SK and FA wrote the manuscript.

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Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

References

Aszterbaum M, Epstein J, Oro A, Douglas V, LeBoit PE, Scott MP, Epstein EH Jr (1999) Ultraviolet and ionizing radiation enhance the growth of BCCs and trichoblastomas in patched heterozygous knockout mice. Nat Med 5: 1285-1291

Brewster R, Mullor JL, Ruiz I, Altaba A (2000) Gli2 functions in FGF signaling during antero-posterior patterning. Development 127: 4395-4405

Bruns CJ, Harbison MT, Kuniyasu H, Eue I, Fidler IJ (1999) In vivo selection and characterization of metastatic variants from human pancreatic adenocarcinoma by using orthotopic implantation in nude mice. Neoplasia 1: 50-62

Buonamici S, Williams J, Morrissey M, Wang A, Guo R, Vattay A, Hsiao K, Yuan J, Green J, Ospina B, *et al* (2010) Interfering with resistance to smoothened antagonists by inhibition of the PI3K pathway in medulloblastoma. Sci Transl Med 2: 51ra70

Clement V, Sanchez P, de Tribolet N, Radovanovic I, Ruiz i Altaba A (2007) HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. Curr Biol 17: 165-172

Dennler S, Andre J, Verrecchia F, Mauviel A (2009) Cloning of the human GLI2 Promoter: transcriptional activation by transforming growth factor-beta via SMAD3/beta-catenin cooperation. J Biol Chem 284: 31523-31531

Dierks C, Beigi R, Guo GR, Zirlik K, Stegert MR, Manley P, Trussell C, Schmitt-Graeff A, Landwerlin K, Veelken H, *et al* (2008) Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. Cancer Cell 14: 238-249

Grachtchouk M, Pero J, Yang SH, Ermilov AN, Michael LE, Wang A, Wilbert D, Patel RM, Ferris J, Diener J, *et al* (2011) Basal cell carcinomas in mice arise from hair follicle stem cells and multiple epithelial progenitor populations. J Clin Invest 121: 1768-1781

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100: 57-70

Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heeschen C (2007) Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell 1: 313-323

Indra AK, Warot X, Brocard J, Bornert JM, Xiao JH, Chambon P, Metzger D (1999) Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. Nucleic Acids Res 27: 4324-4327

Jeong J, Mao J, Tenzen T, Kottmann AH, McMahon AP (2004) Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. Genes Dev 18: 937-951

Jiang J, Hui CC (2008) Hedgehog signaling in development and cancer. Dev Cell 15: 801-812

Kasper M, Jaks V, Are A, Bergstrom A, Schwager A, Barker N, Toftgard R (2011) Wounding enhances epidermal tumorigenesis by recruiting hair follicle keratinocytes. Proc Natl Acad Sci USA 108: 4099-4104

Kasper M, Regl G, Eichberger T, Frischauf AM, Aberger F (2007) Efficient manipulation of Hedgehog/GLI signaling using retroviral expression systems. Methods Mol Biol 397: 67-78

Kasper M, Regl G, Frischauf AM, Aberger F (2006a) GLI transcription factors: mediators of oncogenic Hedgehog signalling. Eur J Cancer 42: 437-445

Kasper M, Schnidar H, Neill GW, Hanneder M, Klingler S, Blaas L, Schmid C, Hauser-Kronberger C, Regl G, Philpott MP, *et al* (2006b) Selective modulation of Hedgehog/GLI target gene expression by epidermal growth factor signaling in human keratinocytes. Mol Cell Biol 26: 6283-6298

Laner-Plamberger S, Kaser A, Paulischta M, Hauser-Kronberger C, Eichberger T, Frischauf AM (2009) Cooperation between GLI and JUN enhances transcription of JUN and selected GLI target genes. Oncogene 28: 1639-1651

Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM (2007) Identification of pancreatic cancer stem cells. Cancer Res 67: 1030-1037

Li D, Ambrogio L, Shimamura T, Kubo S, Takahashi M, Chirieac LR, Padera RF, Shapiro GI, Baum A, Himmelsbach F, *et al* (2008) BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. Oncogene 27: 4702-4711

Mao J, Ligon KL, Rakhlin EY, Thayer SP, Bronson RT, Rowitch D, McMahon AP (2006) A novel somatic mouse model to survey tumorigenic potential applied to the Hedgehog pathway. Cancer Res 66: 10171-10178

McMurray HR, Sampson ER, Compitello G, Kinsey C, Newman L, Smith B, Chen SR, Klebanov L, Salzman P, Yakovlev A, *et al* (2008) Synergistic response to oncogenic mutations defines gene class critical to cancer phenotype. Nature 453: 1112-1116

Mimeault M, Moore E, Moniaux N, Henichart JP, Depreux P, Lin MF, Batra SK (2006) Cytotoxic effects induced by a combination of cyclopamine and gefitinib, the selective Hedgehog and epidermal growth factor receptor signaling inhibitors, in prostate cancer cells. Int J Cancer 118: 1022-1031

Mueller MT, Hermann PC, Witthauer J, Rubio-Viqueira B, Leicht SF, Huber S, Ellwart JW, Mustafa M, Bartenstein P, D'Haese JG, *et al* (2009) Combined targeted treatment to eliminate tumorigenic cancer stem cells in human pancreatic cancer. Gastroenterology 137: 1102-1113

Natarajan A, Wagner B, Sibilia M (2007) The EGF receptor is required for efficient liver regeneration. Proc Natl Acad Sci USA 104: 17081-17086

Ng JM, Curran T (2011) The Hedgehog's tale: developing strategies for targeting cancer. Nat Rev Cancer 11: 493-501

Nolan-Stevaux O, Lau J, Truitt ML, Chu GC, Hebrok M, Fernandez-Zapico ME, Hanahan D (2009) GLI1 is regulated through Smoothened-independent mechanisms in neoplastic pancreatic ducts and mediates PDAC cell survival and transformation. Genes Dev 23: 24-36

Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 121: 335-348

Palma V, Lim DA, Dahmane N, Sanchez P, Brionne TC, Herzberg CD, Gitton Y, Carleton A, Alvarez-Buylla A, Ruiz i Altaba A (2005) Sonic Hedgehog controls stem cell behavior in the postnatal and adult brain. Development 132: 335-344

Palma V, Ruiz i Altaba A (2004) Hedgehog-GLI signaling regulates the behavior of cells with stem cell properties in the developing neocortex. Development 131: 337-345

Pasca di Magliano M, Sekine S, Ermilov A, Ferris J, Dlugosz AA, Hebrok M (2006) Hedgehog/Ras interactions regulate early stages of pancreatic cancer. Genes Dev 20: 3161-3173

Peacock CD, Wang Q, Gesell GS, Corcoran-Schwartz IM, Jones E, Kim J, Devereux WL, Rhodes JT, Huff CA, Beachy PA, *et al* (2007) Hedgehog signaling maintains a tumor stem cell compartment in multiple myeloma. Proc Natl Acad Sci USA 104: 4048-4053

Po A, Ferretti E, Miele E, De Smaele E, Paganelli A, Canettieri G, Coni S, Di Marcotullio L, Biffoni M, Massimi L, *et al* (2010) Hedgehog controls neural stem cells through p53-independent regulation of Nanog. EMBO J 29: 2646-2658

Ramirez A, Page A, Gandarillas A, Zanet J, Pibre S, Vidal M, Tusell L, Genesca A, Whitaker DA, Melton DW, *et al* (2004) A keratin K5Cre transgenic line appropriate for tissue-specific or generalized Cre-mediated recombination. Genesis 39: 52-57

Regl G, Neill GW, Eichberger T, Kasper M, Ikram MS, Koller J, Hintner H, Quinn AG, Frischauf AM, Aberger F (2002) Human GLI2 and GLI1 are part of a positive feedback mechanism in Basal cell carcinoma. Oncogene 21: 5529-5539

Riobo NA, Haines GM, Emerson CP Jr (2006a) Protein kinase C-delta and mitogen-activated protein/extracellular signal-regulated kinase-1 control GLI activation in Hedgehog signaling. Cancer Res 66: 839-845

Riobo NA, Lu K, Ai X, Haines GM, Emerson CP Jr (2006b) Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling. Proc Natl Acad Sci USA 103: 4505-4510

Rohatgi R, Scott MP (2007) Patching the gaps in Hedgehog signalling. Nat Cell Biol 9: 1005-1009

Roth FP, Hughes JD, Estep PW, Church GM (1998) Finding DNA regulatory motifs within unaligned noncoding sequences clustered by whole-genome mRNA quantitation. Nat Biotechnol 16: 939-945

Rudin CM, Hann CL, Laterra J, Yauch RL, Callahan CA, Fu L, Holcomb T, Stinson J, Gould SE, Coleman B, *et al* (2009) Treatment of medulloblastoma with Hedgehog pathway inhibitor GDC-0449. N Engl J Med 361: 1173-1178

Ruiz i Altaba A, Mas C, Stecca B (2007) The Gli code: an information nexus regulating cell fate, stemness and cancer. Trends Cell Biol 17: 438-447

Schnidar H, Eberl M, Klingler S, Mangelberger D, Kasper M, Hauser-Kronberger C, Regl G, Kroismayr R, Moriggl R, Sibilia M, *et al* (2009) Epidermal growth factor receptor signaling synergizes with Hedgehog/GLI in oncogenic transformation via activation of the MEK/ERK/JUN pathway. Cancer Res 69: 1284-1292

Skvara H, Kalthoff F, Meingassner JG, Wolff-Winiski B, Aschauer H, Kelleher JF, Wu X, Pan S, Mickel L, Schuster C, *et al* (2011) Topical treatment of Basal cell carcinomas in nevoid Basal cell carcinoma syndrome with a smoothened inhibitor. J Invest Dermatol 131: 1735-1744

So PL, Langston AW, Daniallinia N, Hebert JL, Fujimoto MA, Khaimskiy Y, Aszterbaum M, Epstein EH Jr (2006) Long-term establishment, characterization and manipulation of cell lines from mouse basal cell carcinoma tumors. Exp Dermatol 15: 742-750

Stecca B, Mas C, Clement V, Zbinden M, Correa R, Piguet V, Beermann F, Ruiz IAA (2007) Melanomas require HEDGEHOG-GLI signaling regulated by interactions between GLI1 and the RAS-MEK/AKT pathways. Proc Natl Acad Sci USA 104: 5895-5900

Stecca B, Ruiz IAA (2010) Context-dependent regulation of the GLI code in cancer by HEDGEHOG and non-HEDGEHOG signals. J Mol Cell Biol 2: 84-95

Takanaga H, Tsuchida-Straeten N, Nishide K, Watanabe A, Aburatani H, Kondo T (2009) Gli2 is a novel regulator of sox2 expression in telencephalic neuroepithelial cells. Stem Cells 27: 165-174

Teglund S, Toftgard R (2010) Hedgehog beyond medulloblastoma and basal cell carcinoma. Biochim Biophys Acta 1805: 181-208

Varnat F, Duquet A, Malerba M, Zbinden M, Mas C, Gervaz P, Ruiz i Altaba A (2009) Human colon cancer epithelial cells harbour active HEDGEHOG-GLI signalling that is essential for tumour growth, recurrence, metastasis and stem cell survival and expansion. EMBO Mol Med 1: 338-351

Varnat F, Siegl-Cachedenier I, Malerba M, Gervaz P, Ruiz i Altaba A (2010) Loss of WNT-TCF addiction and enhancement of HH-GLI1 signalling define the metastatic transition of human colon carcinomas. EMBO Mol Med 2: 440-457

Vidal VP, Chaboissier MC, Lutzkendorf S, Cotsarelis G, Mill P, Hui CC, Ortonne N, Ortonne JP, Schedl A (2005) Sox9 is essential for outer root sheath differentiation and the formation of the hair stem cell compartment. Curr Biol 15: 1340-1351

Von Hoff DD, LoRusso PM, Rudin CM, Reddy JC, Yauch RL, Tibes R, Weiss GJ, Borad MJ, Hann CL, Brahmer JR, *et al* (2009) Inhibition of the Hedgehog pathway in advanced basal-cell carcinoma. N Engl J Med 361: 1164-1172

Wang GY, Wang J, Mancianti ML, Epstein EH Jr (2011) Basal cell carcinomas arise from hair follicle stem cells in Ptch1(+/-) mice. Cancer Cell 19: 114-124

Wong SY, Reiter JF (2011) Wounding mobilizes hair follicle stem cells to form tumors. Proc Natl Acad Sci USA 108: 4093-4098

Xie J, Murone M, Luoh SM, Ryan A, Gu Q, Zhang C, Bonifas JM, Lam CW, Hynes M, Goddard A, *et al* (1998) Activating smoothened mutations in sporadic basal-cell carcinoma. Nature 391: 90-92

Xie MH, Holcomb I, Deuel B, Dowd P, Huang A, Vagts A, Foster J, Liang J, Brush J, Gu Q, *et al* (1999) FGF-19, a novel fibroblast growth factor with unique specificity for FGFR4. Cytokine 11: 729-735

Yamanaka S (2007) Strategies and new developments in the generation of patient-specific pluripotent stem cells. Cell Stem Cell 1: 39-49

Yauch RL, Dijkgraaf GJ, Alicke B, Januario T, Ahn CP, Holcomb T, Pujara K, Stinson J, Callahan CA, Tang T, *et al* (2009) Smoothened mutation confers resistance to a Hedgehog pathway inhibitor in medulloblastoma. Science 326: 572-574

Yauch RL, Gould SE, Scales SJ, Tang T, Tian H, Ahn CP, Marshall D, Fu L, Januario T, Kallop D, *et al* (2008) A paracrine requirement for Hedgehog signalling in cancer. Nature 455: 406-410

Yoon JW, Kita Y, Frank DJ, Majewski RR, Konicek BA, Nobrega MA, Jacob H, Walterhouse D, Iannaccone P (2002) Gene expression profiling leads to identification of GLI1-binding elements in target genes and a role for multiple downstream pathways in GLI1-induced cell transformation. J Biol Chem 277: 5548-5555

Zbinden M, Duquet A, Lorente-Trigos A, Ngwabyt SN, Borges I, Ruiz I, Altaba A (2010) NANOG regulates glioma stem cells and is essential in vivo acting in a cross-functional network with GLI1 and p53. EMBO J 29: 2659-2674

Zhao C, Chen A, Jamieson CH, Fereshteh M, Abrahamsson A, Blum J, Kwon HY, Kim J, Chute JP, Rizzieri D, *et al* (2009) Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. Nature 458: 776-779

