Oncogene
https://doi.org/10.1038/s41388-020-01453-2

ARTICLE

Crosstalk between Hedgehog pathway and the glucocorticoid receptor pathway as a basis for combination therapy in T-cell acute lymphoblastic leukemia

Deborah Bongiovanni[1] · Valeria Tosello[2] · Valentina Saccomani[1] · Silvia Dalla Santa[1] · Alberto Amadori[1,2] · Paola Zanovello[1] · Erich Piovan[1,2]

Received: 10 March 2020 / Revised: 21 August 2020 / Accepted: 2 September 2020

Abstract

Notwithstanding intensified therapy, a considerable fraction of T-cell acute lymphoblastic leukemia (T-ALL) patients face a dismal prognosis due to primary resistance to treatment and relapse, raising the need for more efficient and targeted therapies. Hedgehog (HH) signaling is a major developmental pathway frequently deregulated in cancer, for which a role in T-ALL is emerging. Mounting evidence suggests that ligand-independent activation of HH pathway occurs in cancer including T-ALL, emphasizing the necessity of dissecting the complex interplay between HH and other signaling pathways regulating activation. In this work, we present a therapeutically relevant crosstalk between HH signaling and the glucocorticoid receptor (NR3C1) pathway acting at the level of GLI1 transcription factor. GLI inhibitor GANT61 and dexamethasone were shown to exert a synergistic anti-leukemic effect in vitro in T-ALL cell lines and patient-derived xenografts. Mechanistically, dexamethasone-activated NR3C1 impaired GLI1 function by dynamically modulating the recruitment of PCAF acetyltransferase and HDAC1 deacetylase. Increased GLI1 acetylation was associated with compromised transcriptional activity and reduced protein stability. In summary, our study identifies a novel crosstalk between GLI1 and NR3C1 signaling pathway which could be exploited in HH-dependent malignancies to increase therapeutic efficacy.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological tumor arising from the expansion of malignant immature precursors primed toward T-cell development [1]. Although outcome of T-ALL patients has improved in recent years, novel therapeutic strategies are strongly needed to improve the unfavorable prognosis of primary resistant and relapsed disease, which account for ~40% of adult and ~20% of children cases [2].

The evolutionarily conserved Hedgehog (HH) signaling pathway is a master regulator of stem cell biology during embryogenesis [3–6]—including early T-cell development [7]—as well as of tissue homeostasis and renewal in adult life [5, 8]. Mammalian cells express three different HH ligands (Sonic Hedgehog, SHH; Desert Hedgehog, DHH and Indian Hedgehog, IHH), which signal through the transmembrane receptor Patched-1 (PTCH1). In absence of the ligands, PTCH1 constitutively represses the transmembrane transducer Smoothened (SMO) [9]. Upon ligand binding to PTCH1, SMO is de-repressed and triggers a cascade of events culminating in the activation of GLI zinc finger transcription factors (GLI1, GLI2, and GLI3) [10, 11]. Activated GLI proteins translocate into the nucleus to regulate the expression of several pathway-specific or cell-specific target genes. GLI2 and GLI3 mainly act as transcriptional activator and repressor respectively [12–15], while GLI1 exclusively functions as a transcriptional activator to amplify existing HH signaling in a positive

Supplementary information The online version of this article (https://doi.org/10.1038/s41388-020-01453-2) contains supplementary material, which is available to authorized users.

✉ Erich Piovan
erich.piovan@unipd.it

[1] Dipartimento di Scienze Chirurgiche, Oncologiche e Gastroenterologiche, Università di Padova, Padova, Italy

[2] UOC Immunologia e Diagnostica Molecolare Oncologica, Istituto Oncologico Veneto IOV—IRCCS, Padova, Italy

Published online: 11 September 2020

feedback loop [16]. Ligand-dependent as well as ligand-independent mechanisms—including oncogenic mutations and "non-canonical" GLI activation—were described to aberrantly initiate HH signaling in solid and hematological tumors, contributing to tumor development, progression and cancer stem cell maintenance [17–20]. In the noncanonical activation of HH pathway, GLI1 can be activated in a SMO-independent fashion through the crosstalk with other oncogenic pathways (e.g., PI3K/Akt, TGFβ/SMAD, and mTOR/S6K1 pathway), which modulate GLI1 expression levels or induce post-translational modifications (PTMs) affecting GLI1 function [21, 22]. A role for HH signaling in T-ALL has been recently described, as rare mutations have been reported in T-ALL patients and active signaling has been observed in ~20% of T-ALL cases [23–25], which might potentially benefit from treatment with HH inhibitors. Preclinical studies suggest that the inhibition of HH pathway at the level of downstream effector GLI1, rather than at the level of SMO receptor, is much more cytotoxic to T-ALL cells [24, 26], thus highlighting the role of non-canonical activation of HH pathway. However, the characteristics of T-ALL cases that are sensitive to HH inhibitors and the therapeutic relevance for these patients is yet to be elucidated.

Here, we sought to establish the molecular pathways that could potentially crosstalk with HH signaling. We describe a clinically relevant crosstalk between HH pathway and the glucocorticoid (GC) receptor pathway, which leads to a strong cytotoxic effect of the combination between GCs and GLI inhibitor GANT61.

Results

HH pathway is active in T-ALL cells and crosstalks with NOTCH1 and GC signaling pathways

GLI1, the key transcription factor of HH signaling, was previously proposed as a preferential therapeutic target in T-ALL in comparison to upstream receptor SMO [24, 26]. We initially characterized a cohort of T-ALL samples, including cell lines ($n = 10$), patient-derived xenografts (PDX, $n = 11$) and two distinct NOTCH1-induced murine models (HD-ΔPEST and ΔE models, $n = 3$ each), for the expression of GLI1, SMO, and PTCH1. The expression of GLI1 transcript, a general readout of active HH signaling, was upregulated in the majority of the tested samples with respect to normal thymocytes, confirming a frequent activation of the pathway in T-ALL (Fig. 1a, b, c). Further, elevated GLI1 protein expression was observed in numerous T-ALL cell lines and PDX samples (Fig. 1d). Interestingly, also GLI2 protein was found to be frequently expressed, especially in GLI1 low T-ALL samples (Fig. 1d).

It has been shown that HH inhibitors only partially block the growth of T-ALL cells [24] with the GLI inhibitor GANT61 being much more cytotoxic than SMO inhibitors. We thus sought to identify signaling pathways that modulate response to GANT61 and may have therapeutic value in view of future combination treatments for patients. HD-ΔPEST murine T-ALL cells were used for initial screening, as they are NOTCH1-dependent with only few other oncogenic alterations, leading to the expansion of very homogeneous and well-characterized tumor cells [27]. HD-ΔPEST leukemia cells were treated ex vivo with GANT61 in combination with a panel of inhibitors targeting diverse oncogenic signaling pathways often de-regulated in T-ALL. The highest degree of synergism—inferred from combination index (CI) values—was observed between GANT61 and the gamma-secretase inhibitor dibenzazepine (DBZ), as well as between GANT61 and the steroid dexamethasone (dexa), suggesting that HH pathway might crosstalk with NOTCH signaling pathway and the GC receptor pathway (Fig. 1e). A link between NOTCH1 signaling and the HH pathway has already been described [28, 29]. As GCs are a cornerstone of the treatment of lymphoid tumors and poor response to prednisone is an early marker of unfavorable prognosis in T-ALL patients [30, 31], we further explored the potential connection between HH pathway and the GC receptor pathway.

Dexamethasone synergizes with the GLI inhibitor GANT61 to induce a strong cytotoxic effect in human T-ALL cell lines and PDX samples

We thus addressed the therapeutic significance of jointly targeting HH pathway and the GC receptor pathway in human T-ALL. The combination treatment dexa plus GANT61 for 48 h resulted in enhanced in vitro cytotoxicity with respect to either drug alone in all tested T-ALL cell lines, regardless of their sensitivity to GCs (Fig. 2a, b, Supplementary Fig. 1a). Indeed, all T-ALL cell lines displayed CI values <1 for most drug concentrations (Fig. 2b), indicating synergism between the two drugs. Evaluation of HH target genes (*GLI1*, *PTCH1*) and GC-responsive genes (*NR3C1*, *BIM*, *GILZ*) in DND41 T-ALL cells (GC-sensitive) disclosed that the combination treatment (GANT61 + dexa) was effective in repressing HH target PTCH1 and increasing GC-sensitivity (i.e., NR3C1 and GILZ expression) notwithstanding increased GLI1 transcript levels (Supplementary Fig. 1b). Increased GC-sensitivity (GILZ) following the combination treatment (GANT61 + dexa) was also found in additional T-ALL cell lines (Supplementary Fig. 1c).

This pronounced cytotoxic effect of the combination treatment was prevalently due to increased apoptosis (Fig. 2c) rather than cell-cycle arrest (data not shown). Similar results were observed in PDX-derived cells, where the

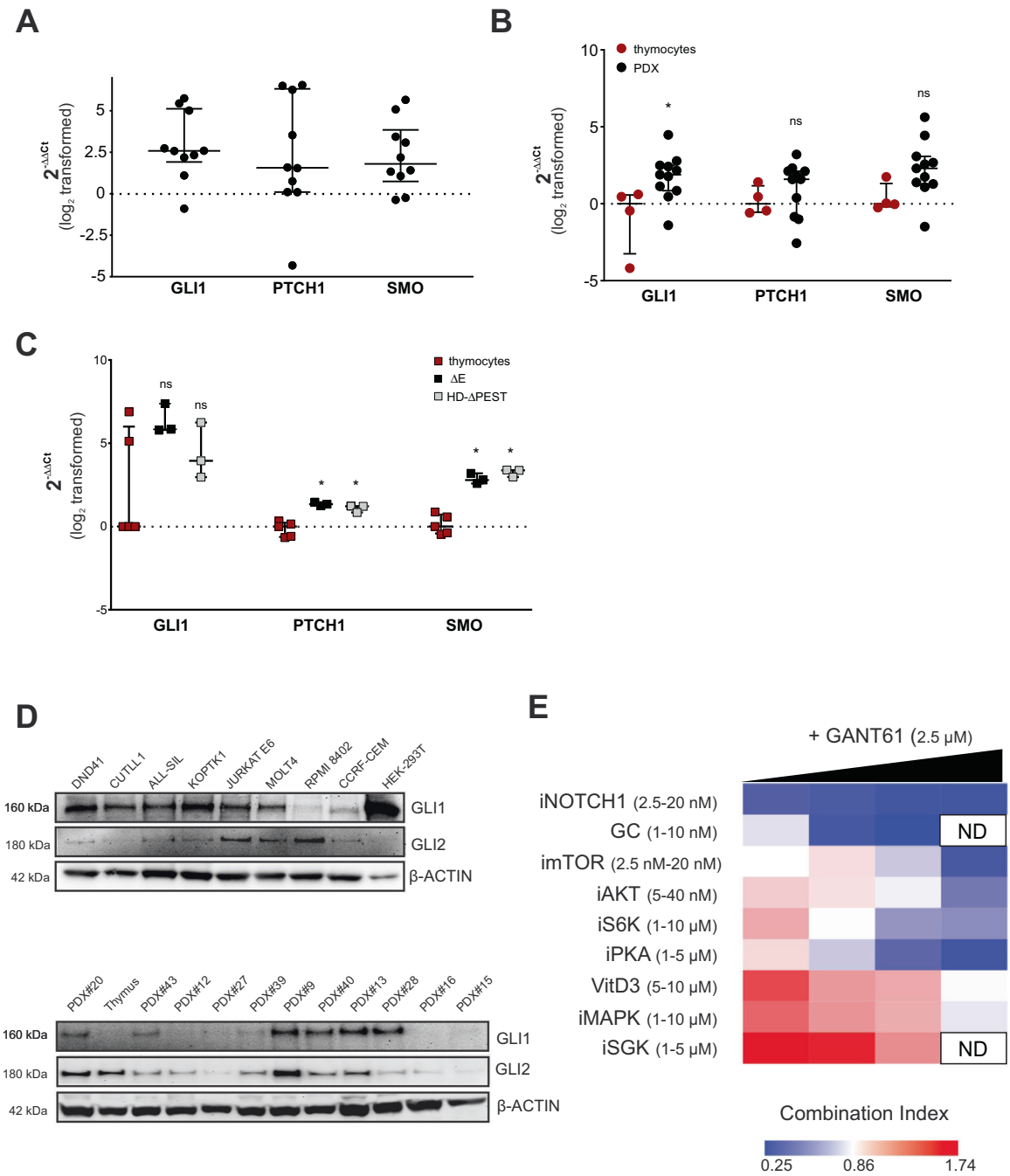
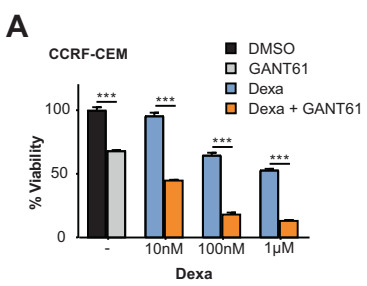


Fig. 1 NOTCH1 signaling and the glucocorticoid receptor pathways modulate sensitivity to GLI1 inhibitor GANT61 in T-ALL cells. a–c Relative expression analysis of *GLI1*, *PTCH1*, *SMO* transcripts in T-ALL cell lines (**a**, $n = 10$), PDX samples (**b**, $n = 11$), ΔE and HD-ΔPEST T-ALL murine models (**c**, $n = 3$ each). Scatter dot plots show mRNA levels as log2-transformed fold change ($2^{-\Delta\Delta CT}$) relative to normal thymocytes ($n = 1$–5). Results are shown with horizontal bars indicating the median and error bars indicate interquartile range. Dashed line indicates the median expression levels in normal thymocytes. **d** Western blot analysis shows GLI1 and GLI2 protein expression in selected T-ALL cell lines (top) and PDX samples (bottom). HEK 293T cells expressing GLI1 were used as positive control. β-actin was used as protein loading control. **e** Heatmap representation of combination index (CI) values of inhibitors targeting commonly deregulated pathways in T-ALL in combination with 2.5 μM GANT61 in HD-ΔPEST mouse-derived cells. CI > 1 indicates antagonism, CI < 1 indicates synergism. ND = not determined.

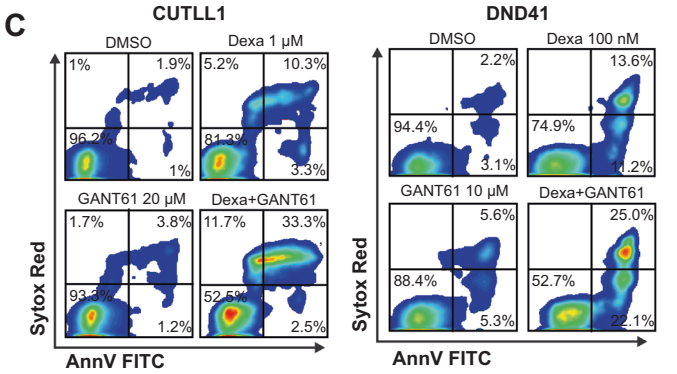
combination treatment (GANT61 + dexa) was found to be highly cytotoxic in both GC-sensitive and GC-resistant samples (Fig. 2d–f). Western blot analysis of proteins implicated in apoptotic cell death (cleaved PARP-1 and XIAP) confirmed the greater efficacy of the combination treatment compared to single agents (Fig. 2g). Overall,

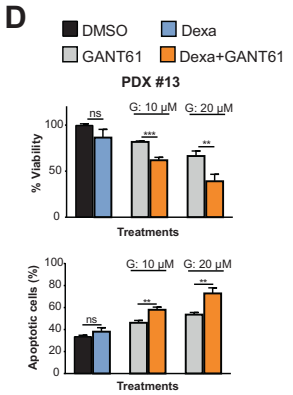


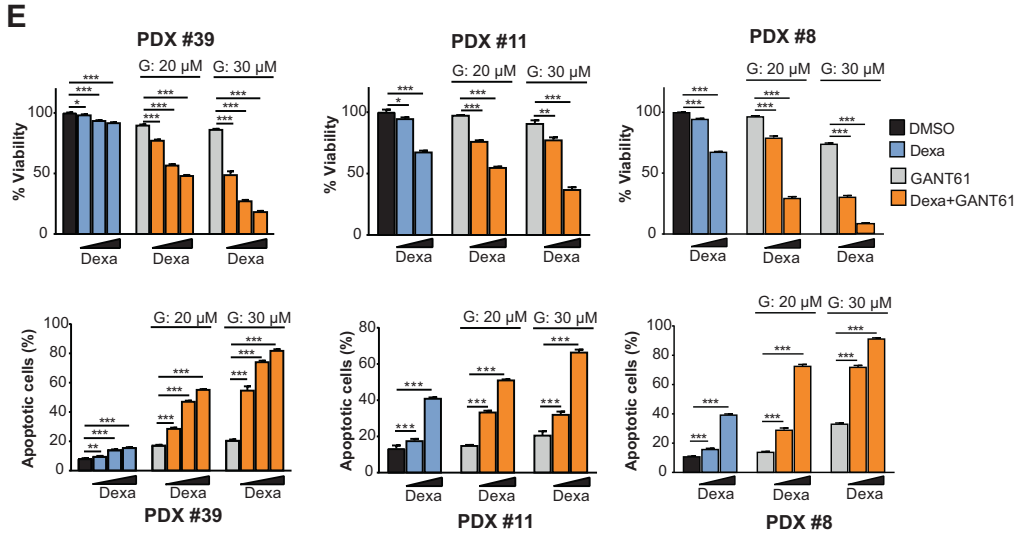
B

COMBINATION INDEX (CI)

	DEXA			GLI1	GLI2	GR	GC response
CCRF-CEM	0.19	0.18	0.20	+/-	+/-	+/-	R
DND41	0.18	0.07	0.90	++	+/-	++	S
MOLT-4	0.23	0.08	0.48	+	+	++	R
HSB-2	0.69	0.52	0.54	+	+/-	+/-	R
CUTLL1	0.95	0.57	0.70	+	+/-	+	R
KOPTK1	0.46	0.37	0.36	++	+/-	+/-	S
UP-ALL13	0.75	0.71	0.77	+	+/-	+	S
RPMI 8402	1.04	0.93	0.54	+/-	+	+/-	R
ALL SIL	0.69	0.39	0.34	+	+/-	+/-	R



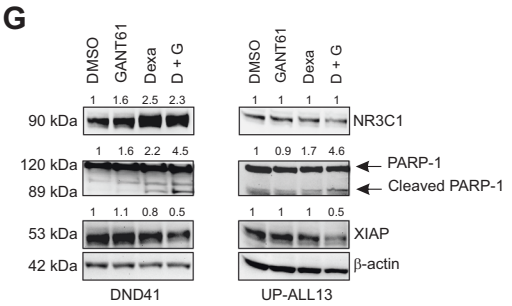




F

COMBINATION INDEX (CI)

	G \ D			
PDX 8	20 μM	0.89	0.65	-
	30 μM	0.74	0.57	-
PDX 11	20 μM	0.64	0.93	-
	30 μM	0.91	0.70	-
PDX 39	20 μM	0.32	0.10	0.07
	30 μM	0.10	0.03	0.02



◀ **Fig. 2 GANT61 and dexa synergistically reduce cell viability in vitro in T-ALL cell lines and PDX samples. a** Representative cell viability analysis in CCRF-CEM T-ALL cells treated in vitro for 48 h with DMSO (vehicle), dexa (10 nM–1 μM), GANT61 (10 μM) or dexa plus GANT61. **b** Table reporting CI values for dexa and GANT61 combinations in different T-ALL cell lines. Protein expression levels of GLI1, GLI2 and glucocorticoid receptor (GR) together with GC sensitivity is also reported. -, not expressed; +, expressed. GC-sensitivity was established as loss of viability ≥ 50% after 72 h exposure to 1 μM dexamethasone. R, resistant; S, sensitive. T-ALL cell lines were treated with increasing concentrations of dexa (0.01–0.1–1 μM) plus 10 μM or 20 μM GANT61. UP-ALL13 were treated with dexa (1-2.5 nM–5 nM) plus 10 μM GANT61. **c** Representative plots of Annexin V-FITC/ Sytox Red staining in CUTLL1 and DND41 cells. **d–e** Quantification of apoptosis and cell viability in high risk (#13) and standard/low risk (#8, #11, #39) PDX samples treated in vitro for 72 h with DMSO (vehicle), dexa only (10 nM–1 μM), GANT61 only (10–30 μM) or dexa plus GANT61. **f** Table reporting CI values for dexa and GANT61 combinations in PDX-derived cells. D = dexa, G = GANT61. **g** Western blot analysis of NR3C1, PARP-1 and XIAP expression in DND41 and UP-ALL13 cell lines treated for 24 h with DMSO (vehicle), 10 μM GANT61, dexa (1 μM or 5 nM, respectively) or GANT61 + dexa ($D + G$). β-actin was used as protein loading control. Numbers indicate results of densitometric analysis of protein bands normalized to β-actin. DMSO treated samples were set at 1. For PARP-1 blots, numbers indicate cleaved PARP-1/un-cleaved PARP-1 ratios normalized to β-actin. DMSO treated samples were set at 1. Results are shown as the mean ± SD in **a**, **d** and **e**. (ns = not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). CI > 1 indicates antagonism, CI < 1 indicates synergism.

these data highlight the therapeutic value of the combination treatment GANT61 plus dexa in T-ALL cells.

Dexamethasone acts by inhibiting GLI1 transcriptional activity

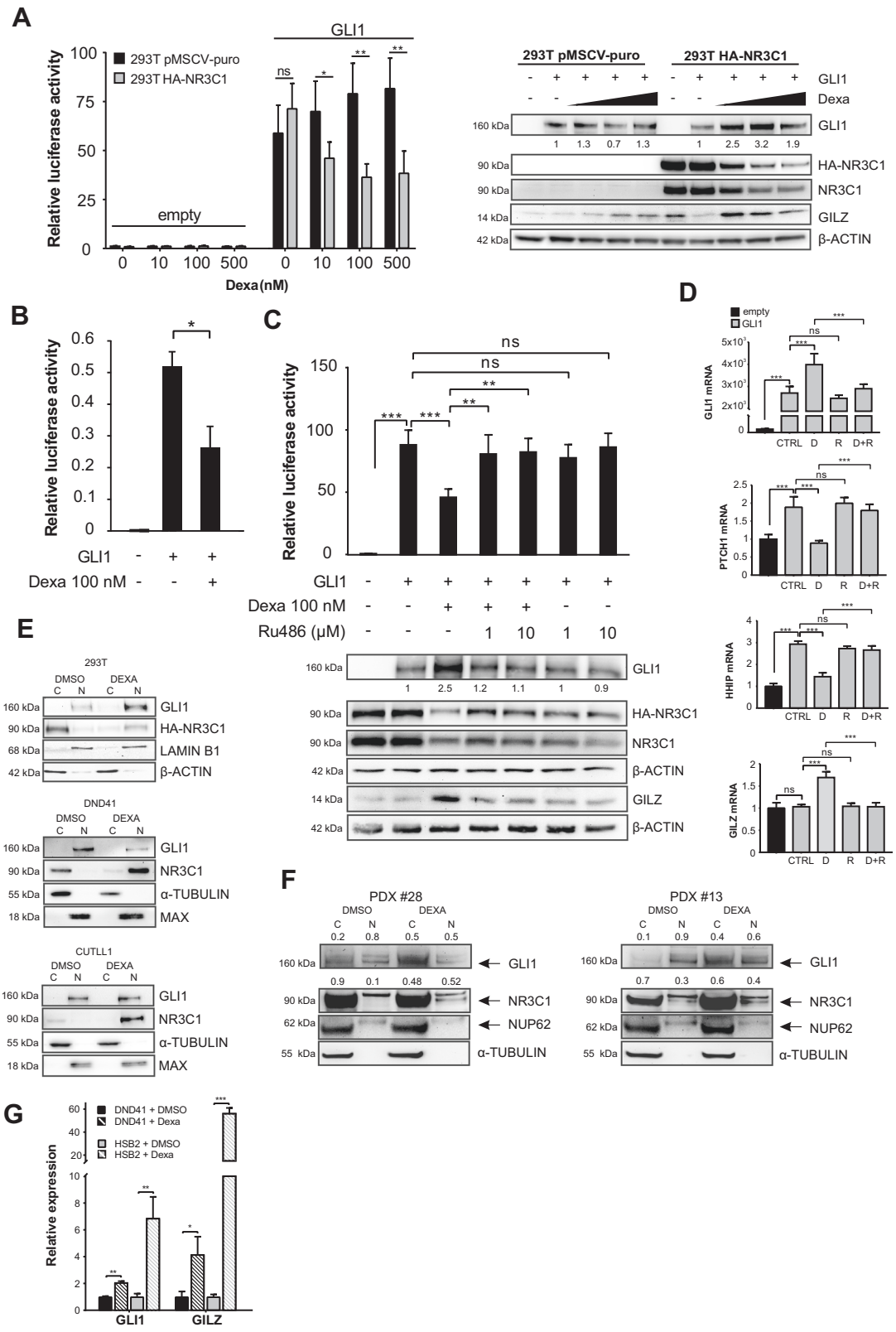
T-ALL cells are rather difficult to manipulate for mechanistic studies so we turned to HEK-293T cells stably expressing the GC receptor NR3C1 (293T HA-NR3C1), which were shown to undergo enhanced cytotoxic response following combination treatment (GANT61 + dexa), much like T-ALL cells (Supplementary Fig. 1d). To determine how GCs could affect HH pathway and elucidate their impact on GLI1 function, we tested the effects of dexa on GLI1-driven luciferase activity in 293T HA-NR3C1 cells. We found that dexa could inhibit GLI reporter activation in a dose-dependent manner (Fig. 3a), while no effect was observed in control cells not expressing NR3C1 (293T pMSCV-puro). Surprisingly, the reduced activity of GLI1 was not associated with a reduction in GLI1 protein expression, but rather an upregulation (Fig. 3a, right panel). Dexa treatment for 24 h induced several transcripts for GC-responsive genes (*NR3C1*, *BIM* and *GILZ*) and determined a G1 cell-cycle arrest, confirming that the GC receptor is functional in 293T HA-NR3C1 cells (Supplementary Fig. 1e, f). NR3C1 protein expression was reduced in response to dexa, as previously described in several nonlymphoid cells (Fig. 3a and Supplementary Fig. 1g) [32, 33]. Our data suggest that despite the reduction in NR3C1 protein expression following dexa treatment, residual NR3C1 levels are sufficient to induce a functional response (cell-cycle arrest/apoptosis) [34] and thus represent a valid model for further mechanistic studies. A similar inhibitory action of GCs on GLI1 function was also confirmed in Jurkat T-ALL cells (Fig. 3b) and HeLa cells (Supplementary Fig. 2a), as well as with another synthetic steroid, fluticasone (Supplementary Fig. 2b).

To assess the specific requirement for NR3C1 in dexa-mediated repression, we treated 293T HA-NR3C1 cells with GC antagonist mifepristone (RU486) alone or in combination with dexa. RU486 abrogated the negative modulation of GLI1 by dexa and reverted the dexa-induced upregulation of GLI1 expression (Fig. 3c, d). Coherently, RU486 could also revert the induction of NR3C1 target gene *GILZ* and the downregulation of NR3C1 protein following dexa treatment (Fig. 3c, d). Gene expression analysis confirmed the functional impairment of GLI1 by dexa, as downregulation of HH target genes *PTCH1* and *HHIP* was observed despite the higher expression levels of *GLI1* (Fig. 3d). Altogether, these data suggest a post-translational—rather than transcriptional—mechanism of regulation of GLI1 activity.

As GLI transcription factors translocate and accumulate in the nucleus upon activation, we first evaluated GLI1 cellular distribution upon dexa treatment. Cell fractionation analysis of 293T HA-NR3C1 cells indicated a preferential nuclear expression of GLI1 protein—in line with active pathway—but no changes in its distribution were observed in response to dexa (Fig. 3e). Nevertheless, GLI1 protein levels were upregulated in dexa-treated cells in comparison to control, in agreement with previous results. By contrast, dexa-activated NR3C1 was downregulated and shuttled from the cytoplasm to the nucleus. Extending our analysis to T-ALL cells, we found that GLI1 was again almost exclusively nuclear under basal conditions in T-ALL cell lines and dexa treatment did not modify its subcellular distribution (Fig. 3e). We found instead a different pattern in PDX cells (Fig. 3f), which showed a more mixed expression of GLI1 between cytoplasmic and nuclear fractions. In these cells, dexa induced an increased expression of GLI1 protein associated with increased cytoplasmic accumulation. Interestingly, dexa treatment increased GLI1 transcript levels in T-ALL cells (HSB2, DND41; Fig. 3g), but determined variable effects on total GLI1 protein levels (data not shown) suggesting important differential post-translational alterations in these cell lines.

NR3C1 interacts with GLI1 and promotes its acetylation

The activation and modulation of GLI1 function is finely tuned by PTMs, namely phosphorylation, acetylation, and ubiquitination. These modifications not only regulate GLI1



protein half-life and subcellular trafficking, but also directly affect its transactivation potential [22, 35]. Acetylation in particular was shown to inhibit the transcriptional activity of both GLI1 and GLI2 preventing their target promoter occupancy [36–38]. Activated NR3C1 can interact with histone acetyltransferases (HATs) and histone deacetylases

◀ **Fig. 3 NR3C1 signaling negatively affects GLI1 function. a** Dual luciferase reporter assay (left panel) and Western blot analysis (right panel) of 293T cells expressing HA-tagged NR3C1 or control plasmid (pMSCV-puro), transfected with GLI1 or empty vector and treated with dexa (10–100–500 nM) or DMSO (vehicle). **b** Dual luciferase reporter assay in Jurkat T-ALL cells expressing HA-tagged NR3C1, transfected with GLI1 or empty vector and treated with 100 nM dexa or DMSO (vehicle). **c–d** Dual luciferase reporter assay (**c**, upper), Western blot analysis (**c**, lower) and Real time RT-PCR (**d**) of 293T HA-NR3C1 cells transfected with GLI1 or empty vector and treated with DMSO (CTRL), 100 nM dexa (**d**), RU486 (R, 1–10 μM) or the combination dexa + RU486 (D + R). Relative luciferase activity is represented as fold change to empty vector (panels **a**, **b** and **c**). Numbers indicate results of densitometric analysis of GLI1 bands normalized to β-actin loading control (**a**, **c**). **e** Western blot analysis after cell fractionation of 293T cells stably expressing HA-tagged NR3C1 and HA-tagged GLI1 or T-ALL cells (DND41, CUTLL1) treated with dexa or DMSO (vehicle). β-actin and α-tubulin were used as loading controls for cytoplasmic fractions (*C*), lamin B1 and MAX for nuclear fractions (*N*). **f** Western blot analysis after cell fractionation of PDX samples (#28, #13) treated with dexa or DMSO (vehicle). α-tubulin was used as loading control for cytoplasmic fractions (*C*), NUP62 for nuclear fractions (*N*). Densitometric analysis of GLI1 or NR3C1 bands normalized to loading controls is reported. Numbers indicate the proportion of protein (GLI1 or NR3C1) in each cellular fraction. **g** Evaluation of GLI1 transcript in DND41 and HSB2 T-ALL cells after treatment with 1 μM dexa for 24 h. Relative mRNA expression is shown as fold change ($2^{-\Delta\Delta CT}$) to empty vector (**d**) or DMSO (**g**) normalized to *GAPDH* housekeeping gene. GILZ protein or transcript were evaluated as control for GC treatment (**a**, **c**, **d**, **g**). Results are shown as the mean ± SD. (ns = not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

(HDACs) at promoter sites. These proteins can act as transcriptional co-activators/co-repressors [39, 40] respectively. We thus determined whether dexa could alter GLI1 acetylation status. Firstly, we evaluated the physical interaction between GLI1 and NR3C1. We found that the two proteins could interact both in exogenously transfected 293T HA-NR3C1 cells and under endogenous conditions in DND41 T-ALL cells (Fig. 4a, b). Next, we addressed the acetylation status of GLI1 upon dexa treatment. In 293T HA-NR3C1 cells, we found GLI1 to be basally acetylated, with the degree of acetylation being further enhanced by dexa or HDAC pan-inhibitor Trichostatin A (TSA) (Fig. 4c). To assess the functional consequences of acetylation/deacetylation on HH signaling, we tested the effects of known modulators of GLI1 acetylation—including the HATs p300 and p300/CBP-associated factor (PCAF), and the histone deacetylase HDAC1—on GLI1-driven reporter activity (Fig. 4d). Indeed, overexpression of p300 and PCAF repressed GLI1-reporter activity much like dexa. On the other hand, HDAC1 enhanced GLI1-reporter activity, with dexa reverting this effect. Further, HDAC1 in the presence of p300 or PCAF contrasted the repressive effects of p300 or PCAF alone. Collectively, these data suggest a role for GLI1 acetylation in mediating the repressive function of dexa on GLI1 transcriptional activity.

To determine whether dexa could alter the recruitment of acetylation modulators to GLI1, we performed IP experiments (Fig. 4e–g) in transfected 293T HA-NR3C1 cells. In agreement with previous studies [36, 38, 41], we could detect both HDAC1 and PCAF in anti-HA immunoprecipitates, indicating their basal association with HA-GLI1 or HA-NR3C1. A reduction in immunoprecipitated HDAC1 as well as an enrichment in immunoprecipitated PCAF was observed after dexa treatment, consistent with our hypothesis of activated NR3C1 as a promoter of GLI1 hyper-acetylation. On the other hand p300, a well-described HAT targeting GLI1, was not recruited after dexa treatment, suggesting that PCAF may be the predominant HAT acetylating GLI1 following dexa treatment.

Dexamethasone promotes GLI1 degradation

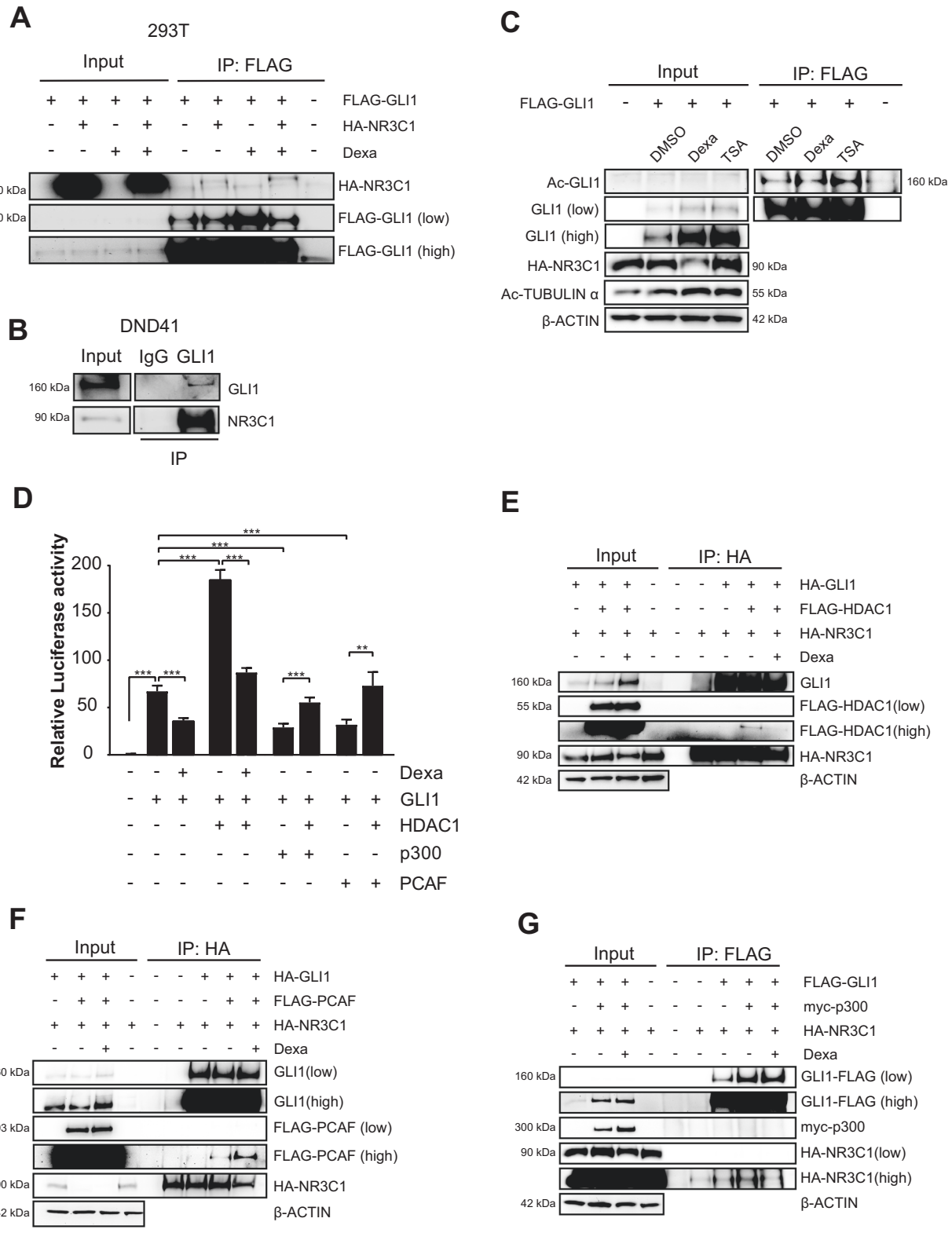
As acetylation of GLI1 homolog Ci in *Drosophila* and of GLI proteins in mammalian cells has been associated with proteasome-dependent cleavage/degradation [42], we investigated whether dexa could affect the protein stability of GLI1. We treated 293T HA-NR3C1 cells with dexa and inhibited protein synthesis with cycloheximide (CHX). We found that dexa treatment remarkably reduced GLI1 stability in 293T HA-NR3C1 cells (Fig. 5a). Similar results were obtained also in HSB2 and DND41 T-ALL cell lines (Fig. 5b).

GLI1 protein stability is often linked to its rate of ubiquitination and proteasomal degradation [35], we thus investigated whether GLI1 ubiquitination was altered following dexa treatment. To this end, 293T HA-NR3C1 cells were transfected with GLI1 and ubiquitin and treated with dexa. Proteasomal degradation was inhibited with MG132 and GLI1 was immunoprecipitated to evaluate its degree of ubiquitination. Dexa treatment determined a dramatic increase in GLI1 ubiquitination (Fig. 5c), coherent with our hypothesis of dexa reducing GLI1 stability through enhanced ubiquitination. In T-ALL cells, this reduced stability of GLI1 protein may follow faster kinetics in GC-sensitive cells expressing high levels of NR3C1 (and that undergo auto-upregulation; DND41), compared to GC-resistant cells expressing low levels of NR3C1 (that may or not undergo auto-upregulation; CUTLL1 and HSB2, respectively) (Fig. 5d).

In conclusion, our findings suggest a model of action (Fig. 5e) where active NR3C1 (through dexa) initially promotes the accumulation of acetylated GLI1, via the dynamic interaction with acetyltransferases/deacetylases, thus inhibiting its transcriptional activity. Subsequently, acetylated GLI1 undergoes ubiquitination and degradation.

Discussion

HH inhibition represents an interesting therapeutic option not merely restricted to a limited number of HH-dependent tumors, but also for a wider range of hematological



malignancies. Although all clinically approved HH inhibitors act on SMO receptor, the acquisition of drug resistance and the *a priori* insensitivity to SMO antagonists underpin the role of SMO-independent activation of HH pathway. Previous studies proposed the importance of noncanonical activation of HH signaling in T-ALL, identifying GLI1 transcription factor as the target of choice over SMO receptor in T-ALL [24, 26]. In this work, we highlight a

◀ **Fig. 4 NR3C1 interacts with GLI1 and modulates the recruitment of mediators of GLI1 acetylation/deacetylation. a** Western blot analysis for NR3C1 after GLI1 IP in 293T cells expressing HA-tagged NR3C1 and FLAG-tagged GLI1. **b** Western blot analysis for NR3C1 after GLI1 IP in DND41 T-ALL cells. **c** Western blot analysis of acetylated GLI1 (Ac-GLI1) after anti-FLAG IP in 293T HA-NR3C1 cells transfected with FLAG-tagged GLI1 or empty vector, treated with DMSO (vehicle), 100 nM dexa or HDAC pan-inhibitor Trichostatin A (TSA, 500 nM) as positive control for acetylation. Acetylation of α-tubulin (Ac-tubulin α) was evaluated as positive control for TSA treatment. **d** Dual luciferase reporter assay of GLI1-transfected 293T HA-NR3C1 cells and co-transfected with histone deacetylase HDAC1 or histone acetyltransferases p300 and PCAF. Relative luciferase activity is represented as fold change to empty vector. Results are shown as mean ± SD. (ns = not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). **e–g** Western blot analysis of FLAG-tagged HDAC1 (**e**), FLAG-tagged PCAF (**f**) and myc-tagged p300 (**g**) after anti-HA or anti-FLAG IP in 293T HA-NR3C1 cells transfected with HA-tagged or FLAG-tagged GLI1. β-actin was used as loading control for Western blot analysis. *Low* and *high* indicate shorter or longer exposures of the same membrane.

strong synergism between GLI1 inhibitor GANT61 and the GC dexa in various T-ALL models in vitro, proposing combined targeting of HH pathway and the GC receptor pathway as an effective therapeutic strategy in T-ALL. We showed that synthetic GCs like dexa could impair GLI1 transcriptional activity in 293T and Jurkat T-ALL cells and this repressive effect required the GC receptor NR3C1. The link between HH and GCs has recently gained interest as selected GC compounds were identified as SMO agonists promoting its accumulation in the primary cilium, while some others (e.g., Budesonide and Ciclesonide) were reported to impair ciliary localization and inhibit HH pathway [43, 44]. However, we should point out that in these studies HH pathway modulation was presented as an off-target effect of GCs on SMO receptor, occurring at much higher concentrations than those required for NR3C1 activation. Notwithstanding, in other works dexa failed to compete for known SMO-binding sites but was still able to inhibit HH pathway in cell-based assays [45], in agreement with our findings. Dexa has recently been shown to inhibit SHH mitogenic effect impairing the proliferation of cerebellar neuronal precursors, even if the exact mechanism was not elucidated [45–47]. Mechanistically, we propose a model of action where GC-activated NR3C1 favors the recruitment of PCAF acetyltransferase as well as the dissociation of HDAC1 deacetylase from GLI1 protein complexes, thereby affecting GLI1 function in terms of transcriptional activity and protein stability.

Our results suggest that the synergistic cytotoxic effect of the combination treatment (GC + GLI inhibitor) would result from: (i) the ability of GLI inhibitors such as GANT61 to increase sensitivity to GCs (through a mechanism that will need to be investigated in future studies) and (ii) the capacity of GCs to further attenuate the transcriptional activity of residual GLI1, possibly through increased degradation and decreased DNA-binding ability. In conclusion, we collected evidence of a crosstalk between the GC receptor signaling and HH pathway, highlighting a before undescribed role for NR3C1 as negative regulator of GLI1 and setting the therapeutic rationale for combining GLI1 inhibitors and GCs. As GLI1 inhibitors like GANT61 or arsenic trioxide (ATO) are not clinical-grade for their unsuitable pharmacokinetic profiles or limited specificity, the indirect inhibition of GLI1 function by affecting its acetylation status (through HDAC inhibitors) would represent an intriguing alternative approach when combined with GC, warranting further clinical evaluation [38, 48].

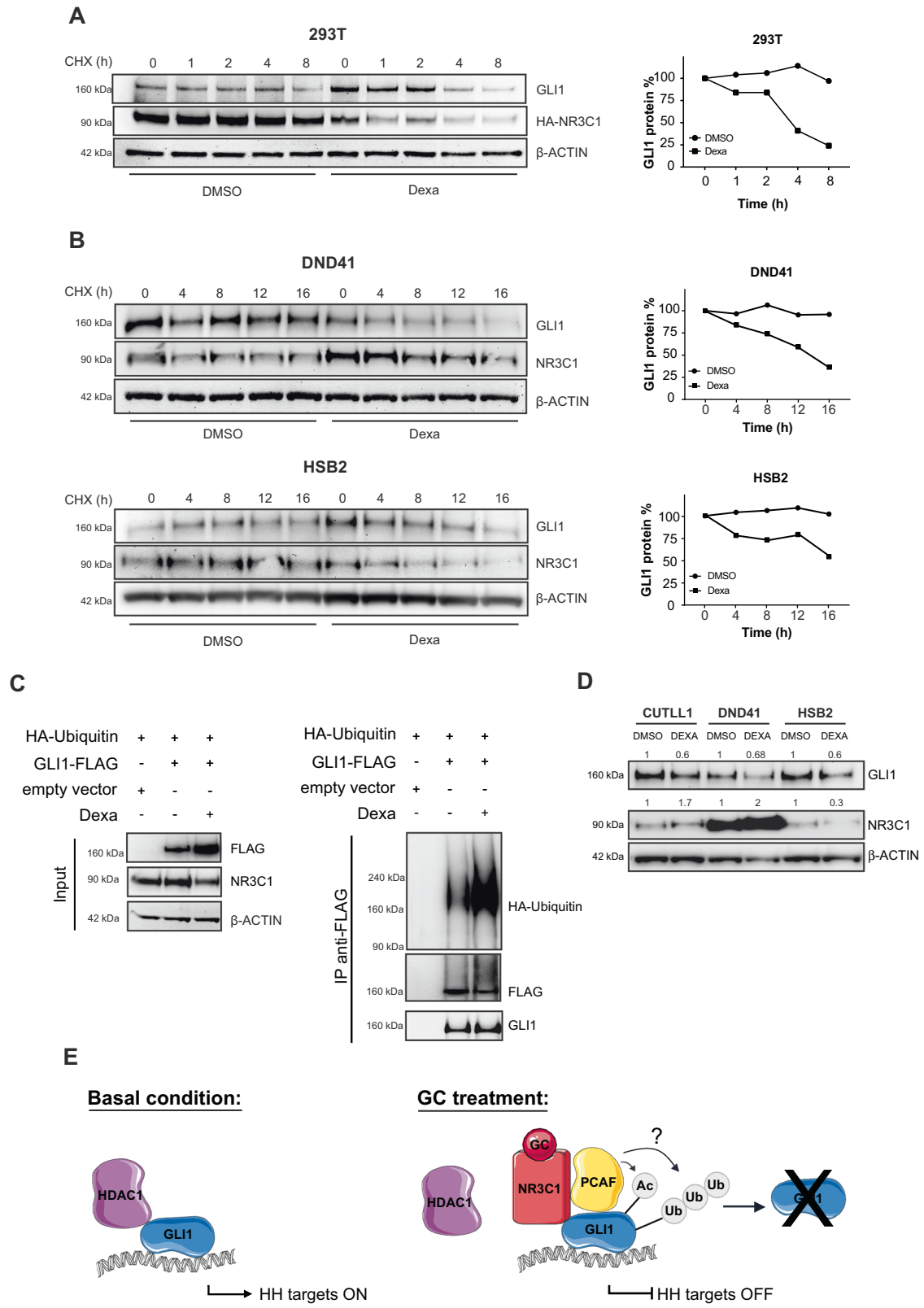
Materials and methods

Cell lines and in vitro treatments

T-ALL cell lines (CUTLL1, DND41, HSB2, MOLT4, CCRF-CEM, JURKAT E6, RPMI 8402, ALL-SIL, KOPTK1, HPB-ALL) were cultured in complete RPMI-1640 medium (Euroclone, Pero, Italy) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C under 5% CO_2. UP-ALL13 cells [49] were cultured in complete RPMI-1640 medium supplemented with 20% FBS. HEK-293T were cultured in complete DMEM medium (Euroclone), supplemented with 10% FBS. For functional studies requiring GC treatment (except for cell viability/apoptotic assays), DMEM and RPMI-1640 media were supplemented with Charcoal-stripped FBS (Invitrogen, Thermo Fisher Scientific). For cell viability and apoptosis assays, cells were treated with increasing doses of dexamethasone (1 nM–1 μM, Sigma-Aldrich, Merck, Darmstadt, Germany) alone or in combination with GANT61 (10–30 μM, Selleck Chemicals LLC, Houston, TX, United States). For the drug combination screening, the following inhibitors (Selleck Chemicals or Syncom, Groningen, the Netherlands) were used in combination with 2.5 μM GANT61: DBZ (iNOTCH1, 2.5–20 nM), BEZ235 (imTOR, 2.5–20 nM), MK2206 (iAKT, 5–40 nM), PF4708671 (iS6K, 1–10 μM), H89 (iPKA, 1–5 μM), Vitamin D3 (5–10 μM), PD98059 (iMAPK, 1–10 μM), GSK650394 (iSGK, 1–5 μM). For functional analysis of HH pathway, cells were treated with increasing concentrations of dexa (10 nM–1 μM, Sigma-Aldrich), RU486 (1–10 μM, Sigma-Aldrich) or trichostatin A (1 μM, Sigma-Aldrich).

Quantitative real time RT-PCR

Primer sequences used for Real time RT-PCR reactions are listed in Supplementary Table 1.



Primary T-ALL xenografts and NOTCH1-dependent murine models, cell viability and flow cytometry analysis, plasmids and retro/lentiviral production, reverse transcriptase and qRT-PCR, dual luciferase reporter assays, ubiquitination detection, immunoblotting, subcellular fractionation, immunoprecipitation and statistical

◀ **Fig. 5 Dexa accelerates GLI1 turnover via proteasome-dependent degradation. a–b** CHX pulse-chase experiments in 293T cells stably expressing HA-tagged NR3C1 and HA-tagged GLI1 (**a**), DND41 and HSB2 T-ALL cells (**b**) treated with dexa or DMSO (vehicle). Lysates were harvested at indicated time points after treatment by CHX (50 μg/mL) and analyzed by Western blot analysis. GLI1 protein levels (represented as % to $t = 0$) were quantified by densitometric analysis and normalized to β-actin. **c** Evaluation of GLI1 ubiquitination in 293T HA-NR3C1 cells transfected with FLAG-tagged GLI1 and HA-tagged Ubiquitin. Cells were treated with DMSO or 100 nM dexa for 18 h before being exposed to 25 μM MG132 for 5 h. GLI1 ubiquitination was detected using an anti-HA antibody following anti-FLAG IP. **d** Evaluation of GLI1 protein expression in CUTLL1, DND41 and HSB2 T-ALL cells after treatment with 1 μM dexa (24 h). β-actin was used as protein loading control. Numbers indicate results of densitometric analysis of GLI1 or NR3C1 bands normalized to β-actin. DMSO treated samples were set at 1. **e** Proposed mechanism for the crosstalk between HH signaling pathway and the GC receptor pathway. GC-activated NR3C1 promotes a hyperacetylated and transcriptionally inactive status of GLI1, putatively affecting also GLI1 protein stability. Ac acetylation, Ub ubiquitination. X, degradation.

analysis are detailed in Supplementary Materials and methods section.

Acknowledgements We are grateful to Prof Warren Pear (University of Pennsylvania, Philadelphia, U.S.A) for the MigR1vector, Prof Raphael Kopan (Washington University, St. Louis, U.S.A.) for MigR1 vectors expressing ΔE-NOTCH1 mutant allele, Prof J. Aster (Brigham and Women's Hospital, Harvard Medical School, Boston, U.S.A.) for the MigR1 HD-ΔPEST (NOTCH1 L1601P-ΔPEST) vector. Histidine-HA-Ubiquitin vector was a kind gift from Richard Baer (Columbia University). We thank Sonia Minuzzo and Marica Pinazza for providing T-ALL xenografts, Elena Laura Mazzoldi for cell sorting, Elena Masiero for technical assistance. We are grateful to Pieter Van Vlierberghe and Giorgio Arrigoni for reagents and suggestions.

Funding This work was supported by the Italian Foundation for Cancer Research (Fondazione AIRC) grants to EP (IG2018#22233); Progetto di Ricerca di Ateneo (SID19_01; Università di Padova) to EP. Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) Ex 60% to EP; Istituto Oncologico Veneto 5 × 1000 fund to EP.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. Belver L, Ferrando A. The genetics and mechanisms of T cell acute lymphoblastic leukaemia. Nat Rev Cancer 2016;16:494–507.
2. Hunger SP, Mullighan CG. Acute Lymphoblastic Leukemia in Children. N Engl J Med. 2015;373:1541–52.
3. Gallet A. Hedgehog morphogen: from secretion to reception. Trends Cell Biol. 2011;21:238–46.
4. Riddle RD, Johnson RL, Laufer E, Tabin C. Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 1993;75:1401–16.
5. Fuccillo M, Joyner AL, Fishell G. Morphogen to mitogen: the multiple roles of hedgehog signalling in vertebrate neural development. Nat Rev Neurosci. 2006;7:772–83.
6. Nieuwenhuis E, Hui CC. Hedgehog signaling and congenital malformations. Clin Genet. 2005;67:193–208.
7. Crompton T, Outram SV, Hager-Theodorides AL. Sonic hedgehog signalling in T-cell development and activation. Nat Rev Immunol. 2007;7:726–35.
8. Petrova R, Joyner AL. Roles for Hedgehog signaling in adult organ homeostasis and repair. Development 2014;141:3445–57.
9. Ramsbottom SA, Pownall ME. Regulation of hedgehog signalling inside and outside the cell. J Dev Biol. 2016;4:23.
10. Pak E, Segal RA. Hedgehog signal transduction: key players, oncogenic drivers, and cancer therapy. Dev Cell 2016;38:333–44.
11. Wu F, Zhang Y, Sun B, McMahon AP, Wang Y. Hedgehog signaling: from basic biology to cancer therapy. Cell Chem Biol. 2017;24:252–80.
12. Ding Q, Motoyama J, Gasca S, Mo R, Sasaki H, Rossant J, et al. Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. Development 1998;125:2533–43.
13. Matise MP, Epstein DJ, Park HL, Platt KA, Joyner AL. Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. Development 1998;125:2759–70.
14. Litingtung Y, Chiang C. Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. Nat Neurosci. 2000;3:979–85.
15. Persson M, Stamataki D, te Welscher P, Andersson E, Bose J, Ruther U, et al. Dorsal-ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity. Genes Dev. 2002;16:2865–78.
16. Dai P, Akimaru H, Tanaka Y, Maekawa T, Nakafuku M, Ishii S. Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. J Biol Chem. 1999;274:8143–52.
17. Amakye D, Jagani Z, Dorsch M. Unraveling the therapeutic potential of the Hedgehog pathway in cancer. Nat Med. 2013;19:1410–22.
18. Rubin LL, de Sauvage FJ. Targeting the Hedgehog pathway in cancer. Nat Rev Drug Discov. 2006;5:1026–33.
19. Dierks C, Grbic J, Zirlik K, Beigi R, Englund NP, Guo GR, et al. Essential role of stromally induced hedgehog signaling in B-cell malignancies. Nat Med. 2007;13:944–51.
20. Hegde GV, Peterson KJ, Emanuel K, Mittal AK, Joshi AD, Dickinson JD, et al. Hedgehog-induced survival of B-cell chronic lymphocytic leukemia cells in a stromal cell microenvironment: a potential new therapeutic target. Mol Cancer Res. 2008;6:1928–36.
21. Pandolfi S, Stecca B. Cooperative integration between HEDGEHOG-GLI signalling and other oncogenic pathways: implications for cancer therapy. Expert Rev Mol Med. 2015;17:e5.
22. Pietrobono S, Gagliardi S, Stecca B. Non-canonical hedgehog signaling pathway in cancer: activation of GLI transcription factors beyond smoothened. Front Genet. 2019;10:556.
23. Burns MA, Liao ZW, Yamagata N, Pouliot GP, Stevenson KE, Neuberg DS, et al. Hedgehog pathway mutations drive oncogenic transformation in high-risk T-cell acute lymphoblastic leukemia. Leukemia 2018;32:2126–37.
24. Dagklis A, Demeyer S, De Bie J, Radaelli E, Pauwels D, Degryse S, et al. Hedgehog pathway activation in T-cell acute lymphoblastic leukemia predicts response to SMO and GLI1 inhibitors. Blood 2016;128:2642–54.
25. Dagklis A, Pauwels D, Lahortiga I, Geerdens E, Bittoun E, Cauwelier B, et al. Hedgehog pathway mutations in T-cell acute lymphoblastic leukemia. Haematologica 2015;100:e102–5.

26. Hou X, Chen X, Zhang P, Fan Y, Ma A, Pang T, et al. Inhibition of hedgehog signaling by GANT58 induces apoptosis and shows synergistic antitumor activity with AKT inhibitor in acute T cell leukemia cells. Biochimie 2014;101:50–9.
27. Chiang M, Xu L, Shestova O, Histen G, L'heureux S, Romany C, et al. Leukemia-associated NOTCH1 Alleles Are Weak Tumor Initiators but Accelerate K-ras-initiated Leukemia. J Clin Investig. 2008;118:3181–94.
28. Schreck K, Taylor P, Marchionni L, Gopalakrishnan V, Bar E, Gaiano N, et al. The notch target Hes1 directly modulates Gli1 expression and hedgehog signaling: a potential mechanism of therapeutic resistance. Clin Cancer Res. 2010;16:6060–70.
29. Ingram W, McCue K, Tran T, Hallahan A, Wainwright B. Sonic Hedgehog regulates Hes1 through a novel mechanism that is independent of canonical notch pathway signalling. Oncogene 2008;27:1489–500.
30. Inaba H, Pui C-H. Glucocorticoid use in acute lymphoblastic leukemia: comparison of prednisone and dexamethasone. Lancet Oncol. 2010;11:1096–106.
31. Schrappe M, Valsecchi MG, Bartram CR, Schrauder A, Panzer-Grumayer R, Moricke A, et al. Late MRD response determines relapse risk overall and in subsets of childhood T-cell ALL: results of the AIEOP-BFM-ALL 2000 study. Blood 2011;118:2077–84.
32. Schmidt T, Meyer A. Autoregulation of Corticosteroid Receptors. How, When, Where, and Why? Receptor 1994;4:229–57.
33. Hoeck W, Rusconi S. Groner B. Down-regulation and phosphorylation of glucocorticoid receptors in cultured cells. Investigations with a monospecific antiserum against a bacterially expressed receptor fragment. J Biol Chem. 1989;264:14396–402.
34. Schwartz J, Sarvaiya P, Vedeckis W. Glucocorticoid receptor knock down reveals a similar apoptotic threshold but differing gene regulation patterns in T-cell and pre-B-cell acute lymphoblastic leukemia. Mol Cell Endocrinol. 2010;320:76–86.
35. Niewiadomski P, Niedziolka SM, Markiewicz L, Uspienski T, Baran B, Chojnowska K. Gli proteins: regulation in development and cancer. Cells 2019;8:147.
36. Canettieri G, Di Marcotullio L, Greco A, Coni S, Antonucci L, Infante P, et al. Histone deacetylase and Cullin3-REN(KCTD11) ubiquitin ligase interplay regulates Hedgehog signalling through Gli acetylation. Nat Cell Biol. 2010;12:132–42.
37. Coni S, Antonucci L, D'Amico D, Di Magno L, Infante P, De Smaele E, et al. Gli2 acetylation at lysine 757 regulates hedgehog-dependent transcriptional output by preventing its promoter occupancy. PLoS ONE 2013;8:e65718.
38. Coni S, Mancuso AB, Di Magno L, Sdruscia G, Manni S, Serrao SM, et al. Selective targeting of HDAC1/2 elicits anticancer effects through Gli1 acetylation in preclinical models of SHH Medulloblastoma. Sci Rep. 2017;7:44079.
39. Kassel O, Herrlich P. Crosstalk between the glucocorticoid receptor and other transcription factors: molecular aspects. Mol Cell Endocrinol. 2007;275:13–29.
40. Newton R, Holden NS. Separating transrepression and transactivation: a distressing divorce for the glucocorticoid receptor? Mol Pharmacol. 2007;72:799–809.
41. Malatesta M, Steinhauer C, Mohammad F, Pandey DP, Squatrito M, Helin K. Histone acetyltransferase PCAF is required for Hedgehog-Gli-dependent transcription and cancer cell proliferation. Cancer Res. 2013;73:6323–33.
42. Gulino A, Di Marcotullio L, Canettieri G, De Smaele E, Screpanti I. Hedgehog/Gli control by ubiquitination/acetylation interplay. Vitam Horm. 2012;88:211–27.
43. Wang Y, Davidow L, Arvanites AC, Blanchard J, Lam K, Xu K, et al. Glucocorticoid compounds modify smoothened localization and hedgehog pathway activity. Chem Biol. 2012;19:972–82.
44. Wang J, Lu J, Bond MC, Chen M, Ren X-R, Lyerly HK, et al. Identification of select glucocorticoids as Smoothened agonists: Potential utility for regenerative medicine. Proc Natl Acad Sci USA. 2010;107:9323–8.
45. Heine VM, Rowitch DH. Hedgehog signaling has a protective effect in glucocorticoid-induced mouse neonatal brain injury through an 11betaHSD2-dependent mechanism. J Clin Investig. 2009;119:267–77.
46. Wang J, Barak LS, Mook RA Jr, Chen W. Glucocorticoid hedgehog agonists in neurogenesis. Vitam Horm. 2011;87:207–15.
47. Heine VM, Griveau A, Chapin C, Ballard PL, Chen JK, Rowitch DH. A small-molecule smoothened agonist prevents glucocorticoid-induced neonatal cerebellar injury. Sci Transl Med. 2011;3:105ra4.
48. Gruber W, Peer E, Elmer D, Sternberg C, Tesanovic S, Del Burgo P, et al. Targeting Class I histone deacetylases by the novel small molecule inhibitor 4SC-202 blocks oncogenic hedgehog-GLI signaling and overcomes smoothened inhibitor resistance. Int J Cancer 2018;142:968–75.
49. Tosello V, Milani G, Martines A, Macri N, Van Loocke W, Matthijssens F, et al. A Novel t(8;14)(q24;q11) rearranged human cell line as a model for mechanistic and drug discovery studies of NOTCH1-Independent Human T-Cell Leukemia. Cells 2018;7:160.