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ORIGINAL ARTICLE

Inhibition of GLI, but not Smoothened, induces apoptosis in chronic lymphocytic leukemia cells

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The Hedgehog (Hh) pathway regulates cell proliferation and survival and contributes to tumorigenesis. We investigated the expression and function of this pathway in B-cell chronic lymphocytic leukemia (CLL) cells and in healthy B lymphocytes. Profiling of cognate Hh pathway members revealed reduced expression of two key Hh signaling effectors, Smoothened (SMOH) and GLI, in CLL cells, whereas transcription levels of other investigated members resembled normal B-lymphocyte levels. Examining the functional role of SMOH and GLI in cell survival, we found that CLL cells were hardly sensitive toward specific SMOH inhibition, but showed an unspecific decline in cell viability in response to high concentrations of the SMOH antagonist cyclopamine. In contrast, treatment with the novel GLI antagonist GANT61 reduced expression of the target gene *Patched* and preferentially decreased the viability of malignant cells. Specific RNA interference knockdown experiments in a CLL-derived cell line confirmed the autonomous role of GLI in malignant cell survival. GANT61-induced apoptosis in primary leukemic cells was partly attenuated by protective stromal cells, but not soluble sonic hedgehog ligand. In summary, our data show a downregulation of the classical Hh pathway in CLL and suggest an intrinsic SMOH-independent role of GLI in the *ex vivo* survival of CLL cells.
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Introduction

The Hedgehog (Hh) signaling pathway regulates crucial steps during early embryonic development and pattern formation and influences cell cycle progression, survival, differentiation and self-renewal in multiple tissue types and species (Nusslein-Volhard and Wieschaus 1980; Hammerschmidt *et al*., 1997; Ingham and McMahon, 2001). In the classical view of canonical Hh signaling, the Hh receptor Patched (PTCH1, PTCH2) functions as a pathway repressor by inhibiting functional signal transduction using the transmembrane protein, Smoothened (SMOH), in the absence of the three known Hh ligands, Sonic Hedgehog (SHH), Indian Hedgehog and Desert Hedgehog. On ligand binding to PTCH, this repression is abolished and signals are transduced to the nucleus, where GLI transcription factors, GLI1, GLI2 and, to a lower extent, GLI3, activate transcription of downstream targets. Direct target genes of Hh signaling include Hh pathway regulators, such as GLI1 and PTCH1, as well as regulators of cell proliferation, survival and metastasis (Marigo and Tabin 1996; Lee *et al*., 1997; Duman-Scheel *et al*., 2002; Regl *et al*., 2002; Ikram *et al*., 2004).

Sustained Hh pathway activation contributes to the development of various malignancies including cancers of the skin, brain, lung, pancreas, prostate, gastrointestinal tract and hematopoietic system (Oro *et al*., 1997; Berman *et al*., 2003; Thayer *et al*., 2003; Watkins *et al*., 2003; Karhadkar *et al*., 2004; Sheng *et al*., 2004; Marino, 2005; Peacock *et al*., 2007). Canonical Hh signaling pathway activation by stromal cell-derived paracrine or autocrine production of Hh ligands was reported to influence proliferation and survival of hematopoietic cells during normal homeostasis and malignancy (Bhardwaj *et al*., 2001; Lowrey *et al*., 2002; Sacedon *et al*., 2005; Dierks *et al*., 2007; Hegde *et al*., 2008). However, this view was recently challenged by the unexpected finding that canonical Hh signaling is dispensable for normal adult hematopoiesis (Gao *et al*., 2009; Hofmann *et al*., 2009). In addition, inconsistent data exist on the role of SMOH for the onset and progression of leukemias. Although Hh signaling by SMOH was reported to be required in a chronic myeloid leukemia-like disease (Dierks *et al*., 2008; Zhao *et al*., 2009), it was found to be dispensable for the development of acute leukemias induced by either the

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MLL-AF9 fusion gene or an activated form of Notch (Gao *et al*., 2009; Hofmann *et al*., 2009).

B-cell chronic lymphocytic leukemia (CLL) is the most prevalent adult leukemia in the Western world and characterized by a massive accumulation of functionally incompetent B lymphocytes in the peripheral blood (PB), bone marrow (BM) and secondary lymphoid tissues (Chiorazzi *et al*., 2005). As proliferation and survival of CLL cells are dependent on specific combinations of cell types and factors in specific niches, microenvironmental interactions have a pivotal role in CLL pathogenesis. Stromal cells in close contact with the accumulating leukemic pool are thought to provide long-term support and survival benefits to malignant cells (Lagneaux *et al*., 1998; Burger *et al*., 2005; Pleyer *et al*., 2009). As stromal cell-derived Hh ligands were reported to elicit PTCH/SMOH-mediated antiapoptotic signaling in mouse B-cell lymphomas (Dierks *et al*., 2007), we were interested in a potential role of Hh signaling in CLL survival and investigated the expression and function of this pathway in CLL cells compared with normal B lymphocytes.

Results

Profiling of Hh pathway members in CLL and normal B cells

We first comprehensively determined the RNA expression profile of molecules associated with Hh signaling in CLL cells compared with normal B lymphocytes. We found Hh ligands Indian Hedgehog and Desert Hedgehog, but not SHH, expressed in both cell types (Figure 1a). Furthermore, CLL and normal B cells expressed similar levels of the two known Hh receptors, PTCH1 and PTCH2 (Figure 1b). In contrast, the intracellular signal transducer, SMOH, was expressed significantly lower (9.3-fold, $P = 0.001$) in CLL cells than in normal B lymphocytes. Suppressor of Fused, a negative regulator of GLI proteins, was similarly detected in both cell types (Figure 1c). Among the group of GLI transcription factors, GLI1 was the only factor broadly expressed in malignant and normal B lymphocytes, but was 2.6-fold decreased ($P = 0.002$) in CLL cells (Figure 1c). In contrast to GLI1, we hardly detected GLI2 and GLI3 transcripts in CLL cells and in normal B lymphocytes using specific Taqman assays. To confirm our data, we used an additional PCR approach, recently reported to show high sensitivity, but lower quantitative specificity, than Taqman-based PCR (Matsenko *et al*., 2008). Performing this SYBR Green-based technique, we detected low transcript levels of GLI2, whereas GLI3 expression remained undetectable. As observed for GLI1, levels of GLI2 were also decreased in CLL (Supplementary Figure 1).

High-risk CLL patients express increased transcript levels of key Hh effectors

We next aimed to evaluate the relevance of the Hh signaling pathway in CLL progression. Therefore, we compared Hh member expression within prognostically relevant risk groups on the basis of Rai staging (Rai *et al*., 1975), immunoglobulin heavy chain variable gene mutational status, CD38 expression (Damle *et al*., 1999) and cytogenetic abnormalities (Dohner *et al*., 2000; Wiestner *et al*., 2003; Zanotti *et al*., 2007). We detected significantly higher SMOH expression in groups with unfavorable cytogenetic abnormalities compared with normal karyotypes ($P = 0.035$), as increased SMOH expression was observed in patients with trisomy 12 (Tri12; Figure 2a). Tri12 is present in about 20–40% of CLL cases with cytogenetic abnormalities and correlates with atypical histology (Knuutila *et al*., 1986; Dohner *et al*., 1999). Although SMOH ($P = 0.051$) and other critical components upstream of GLI, such as Suppressor of Fused ($P = 0.046$) and PTCH2 ($P = 0.218$), were significantly, or by trend, increased in the prognostically unfavorable group of patients with unmutated immunoglobulin heavy chain variable (Figure 2b), GLI1 levels were not influenced by any tested risk parameter.

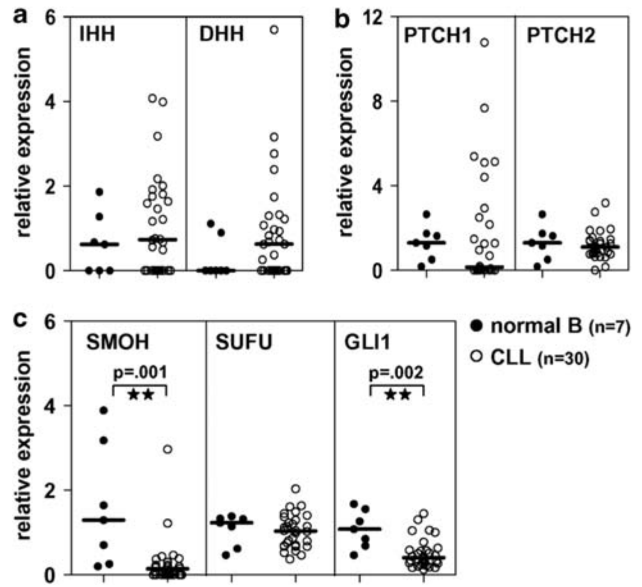

Figure 1 RNA expression profile of Hh pathway members of peripheral blood (PB)-derived CLL cells compared with normal B lymphocytes. Peripheral blood mononuclear cells (PBMCs) of CLL patients ($n = 30$) or of healthy donors ($n = 7$) were isolated using Ficoll density gradient centrifugation and subsequently CD19+ MACS sorted. RNA isolation and cDNA preparation were performed immediately after lymphocyte preparation. Real-time PCR was performed using TaqMan assays and expression levels of CLL cells were compared with those of healthy donor-derived B cells as described in Materials and methods. The figure shows relative RNA expression levels of cognate (**a**) Hh ligands, (**b**) receptors and (**c**) intracellular members of PB-derived CLL cells. Data are presented in a scatter diagram showing the median marked as a line. Abbreviations: IHH, Indian Hedgehog; DHH, Desert Hedgehog; SUFU, suppressor of fused.

Cyclopamine has only minor specific effects on CLL survival

Next, we determined the function of Hh signaling in CLL, normal B- and T-cell survival using the popular SMOH antagonist cyclopamine. Treatment with 5 μM

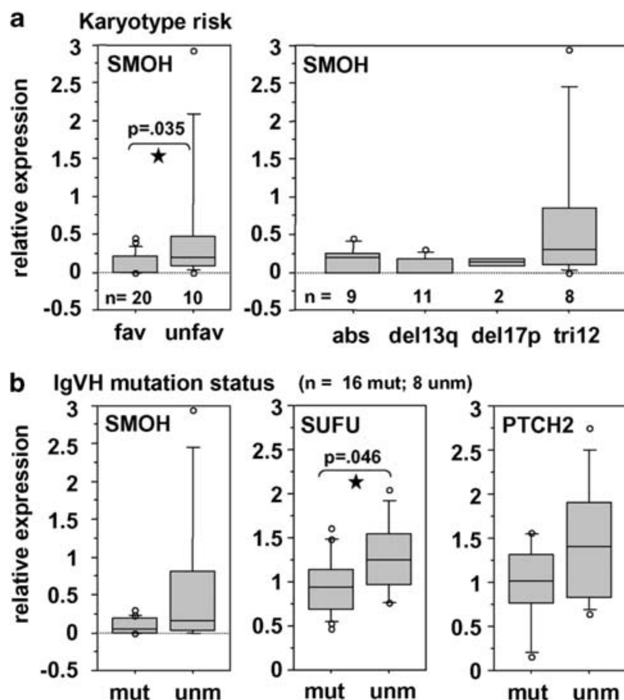

Figure 2 Expression levels of Hh members in correlation with prognostic CLL risk parameters. Relative expression levels of Hh members as determined by real-time PCR were analyzed according to prognostic CLL risk factors. Fluorescence *in situ* hybridization and immunoglobulin heavy chain variable (IgVH) mutation analyses were routinely performed to determine the (**a**) karyotype risk and (**b**) IgVH mutation status of CLL patients ($n=30$). Standard definition of the risk is as follows: favorable (fav): absent aberration (abs) or deletion in band 13q14 (del13q), unfavorable (unfav): deletion in band 17p13 (del17p) or trisomy of chromosome 12 (tri12); IgVH mutational status (>2% as mutated (mut) versus ⩽2% unmutated (unm)). Data are presented in box-and-whisker format: the 25th and 75th percentiles form the box, with the median marked as a line. The 10th and 90th percentiles form the whiskers and all observations <10th percentile or >90th percentile are presented as dots.

cyclopamine for 48 h significantly reduced CLL median cell viability by about 15% ($P \leqslant 0.0001$) and 10 μM cyclopamine by about 30% ($P \leqslant 0.0001$, Figure 3a). Tri12 CLL samples hereby showed higher spontaneous apoptosis rates than those without Tri12 karyotypes, but comparable sensitivity toward cyclopamine (Supplementary Figure 2). Normal B-cell survival was also significantly decreased by cyclopamine treatment, whereas T-cell viability was not affected (Figure 3a). However, reported IC50 concentrations for cyclopamine are about 1 μM (Romer *et al.*, 2004) and high concentrations were recently shown to cause off-target effects (Yauch *et al.*, 2008), recommending a careful interpretation of cyclopamine-based results. Therefore, we incubated CLL cells with lower cyclopamine concentrations and found that 1 or 2.5 μM cyclopamine did not significantly affect their viability (Figure 3b), in contrast to the observed dramatic effects using higher concentrations. Additional stimulation of the canonical Hh signaling with recombinant SHH ligand before cyclopamine treatment did not increase specific sensitivity either (Figure 3c). Furthermore, as a negative control for cyclopamine activity, we incubated CLL cells with tomatidine (Figure 3e), a steroidal alkaloid structurally similar to cyclopamine, without inhibitory effects on Hh signaling (Watkins *et al.*, 2003). It is noteworthy that tomatidine treatment reduced CLL cell viability in a manner similar to that of cyclopamine treatment (Figure 3d). Therefore, we next decided for additional use of the SMOH antagonist SANT-1 (IC50 ≈ 20 nM; Chen *et al.*, 2002) in a paired experimental setting. This antagonist reduced CLL cell viability to a much lower extent than observed for cyclopamine treatment performed in parallel (Figures 3d and f). In addition, we analyzed the involvement of SMOH in survival of two CLL-derived prolymphocytic cell lines, MEC-1 and MEC-2 (Stacchini *et al.*, 1999), which expressed highly increased levels of SMOH compared with primary normal B and CLL cells (Supplementary Figures 3a and b). Similar to primary CLL cells, high-dose cyclopamine treatment (that is, 5 and 10 μM) significantly decreased MEC-1 and MEC-2 cell viability, whereas lower concentrations (1 and 2.5 μM) did not significantly affect viability (Supplementary Figures 3c and d). Specific SMOH downregulation using three different small interfering RNAs (Supplementary Figure 4a) did not cause any reduction in cell viability, (Supplementary Figure 4b) suggesting off-target effects of the high dose being responsible for apoptosis induction rather than specific SMOH inhibition, although incompleteness of the knockdown may also contribute to the lack of effect. In addition, to determine whether induction of the Hh signaling pathway by SMOH activation reveals a protective effect on CLL cells, we also treated primary CLL cells with the SMOH agonist SAG, but did not observe any effect (Supplementary Figure 5). In summary, although primary CLL cells, CLL-derived cell lines and normal B lymphocytes were sensitive toward high-dose cyclopamine, specific SMOH targeting did not influence CLL viability. Our data suggest that observed CLL sensitivity toward cyclopamine, which was recently reported and discussed relating to therapeutical exploitation (Hegde *et al.*, 2008), may at least in part be due to unspecific off-target effects.

CLL cells, but not normal B cells, are highly sensitive toward the GLI antagonist GANT61

Having shown that canonical autocrine Hh signaling by SMOH has an inferior role in CLL survival, we next addressed the putative involvement of GLI transcription factors, which can be regulated by SMOH-independent signals. To this end, we used the novel GLI antagonist GANT61, a small molecule interfering with GLI DNA binding and effecting Hh downstream signaling with an IC50 of ~5 μM (Lauth *et al.*, 2007a). We first confirmed the specificity of GANT61 by the Hh-responsive murine cell line C3H/10T1/2 (Figure 4a). In this cell line, SHH treatment led to a 180-fold upregulation of endogenous Gli1 transcription that could be dose-dependently inhibited by GANT61, in line with the reported IC50

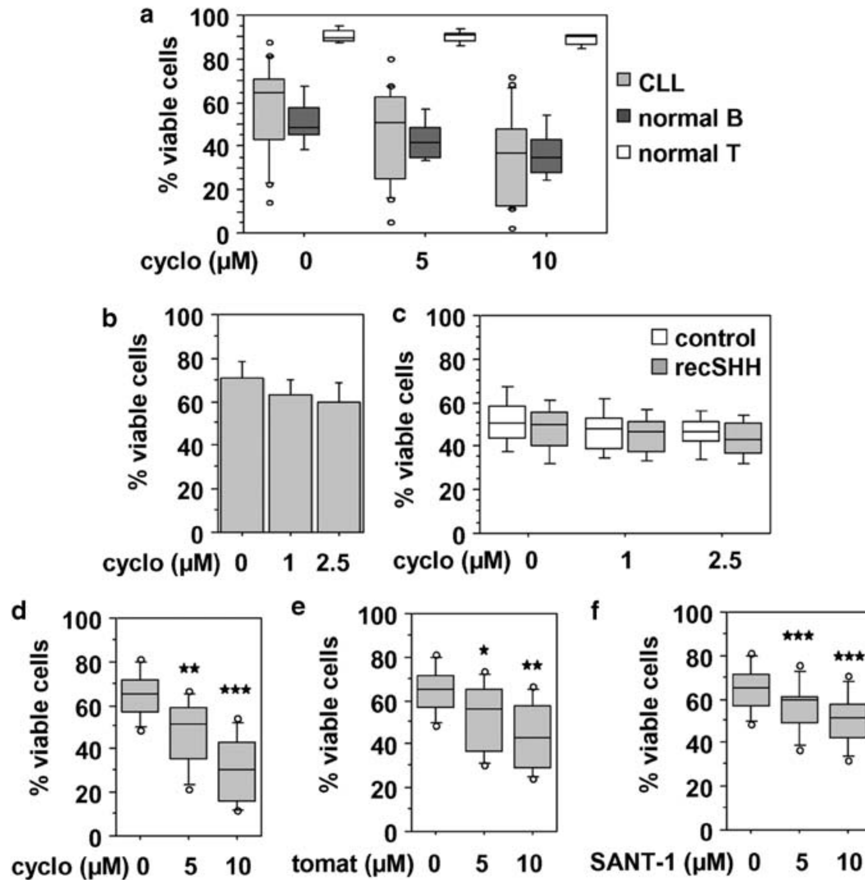

Figure 3 Influence of SMOH antagonists on viability of CLL cells and normal lymphocytes. (**a**) CLL patient ($n = 16$)- and healthy donor ($n = 5$)-derived PBMCs were treated with 5 and 10 μM cyclopamine for 48 h. Cell viability was analyzed by multicolor flow cytometry using AnnexinV/7-Aminoactinomycin D and anti-CD19 and anti-CD5 monoclonal antibodies to distinguish B from T lymphocytes. CLL cells were defined as CD19+/CD5+, normal B cells as CD19+/CD5− and normal T cells as CD19−/CD5+ cells. (**b**) CLL cells ($n = 5$) treated with lower concentrations of cyclopamine (1, 2.5 μM) showed no significant decrease in viability. (**c**) Biologically active human recombinant Shh-N (recShh) was produced in an Sf9 insect cell-based baculovirus expression system and applied on the CLL cells 7 h before cyclopamine addition. Viability of CLL cells ($n = 7$) treated with 5 and 10 μM (**d**) cyclopamine, (**e**) tomatidine or (**f**) SANT-1. Viable cells were considered AnnexinV and 7-AAD negative. Data are presented in box-and-whisker format with the median marked as a line or as bar diagram showing mean values ± 1 s.d.

value. It is noteworthy that primary CLL cells were highly sensitive toward GLI inhibition with GANT61 (Figure 4b). A significant reduction in cell viability could already be observed 24 h after treatment. After 48 h of treatment with 5 μM GANT61, we observed a reduction in CLL viability of about 20% ($P = 0.003$) and of about 75% on 10 μM GANT61 ($P = 0.001$). By contrast, normal B lymphocytes showed less sensitivity toward GANT61 treatment than CLL cells, which was very obvious after 48 h of treatment (Figure 4b, $P = 0.0001$). We ensured the specificity of GANT61-mediated GLI blockage by measuring the direct GLI target gene *PTCH1*. GLI inhibition by GANT61 treatment reduced expression of PTCH1 in all primary CLL samples tested ($n = 4$, Figure 4c), whereas GLI expression itself remained unaffected (data not shown). We confirmed the specific role of GLI in CLL survival by using two different GLI1 small hairpin RNAs. Strikingly, transduction of MEC-1 cells targeting GLI1 led to highly decreased cell survival (Figure 4d), accompanied by reduction in GLI1 mRNA levels (Figure 4e).

Next, we tested the influence of paracrine factors of the BM microenvironment on Hh signaling in CLL. We first determined potential differences in the expression of Hh-associated molecules in PB CLL samples compared with BM aspirates. It was recently suggested that SMOH and GLI1 on CLL cells are activated by Hh ligand derived from the stromal tumor microenvironment (Hegde *et al*., 2008). Therefore, we tested protein expression of these molecules by immunohistochemistry (Figures 5a–c). We used purified CLL cells from PB and BM aspirates (Figures 5a and b) embedded in paraffin blocks and whole BM sections for *in situ* examination (Figure 5c) and found similar SMOH and GLI1 expression in PB and BM. RNA profiling supported the immunohistochemical findings (Figure 5d). Transcript levels of pathway ligands (Indian Hedgehog, Desert Hedgehog) and receptors (PTCH1, PTCH2) were comparable in PB and BM samples (data not shown).

As CLL cells gain *in vitro* survival benefits by the presence of accessory cells, we further evaluated the effect of GLI antagonism on CLL cell survival in

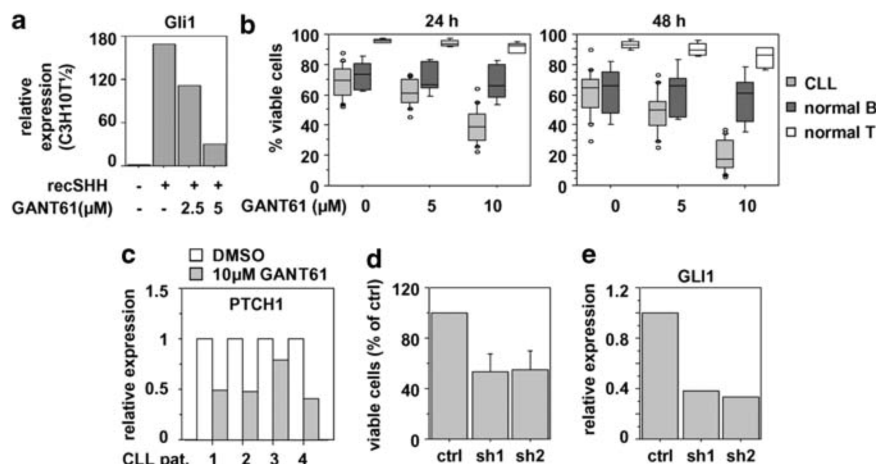

Figure 4 Influence of GANT61 on viability or target gene expression of CLL cells, normal lymphocytes and the Hh-responsive murine cell line C3H/10T1/2 (**a**) Relative RNA expression level of the specific Hh target Gli1 in the Hh-responsive murine cell line C3H/10T1/2 in the presence or absence of GANT61. (**b**) CLL patient ($n = 16$)- and healthy donor ($n = 5$)-derived PBMCs were isolated using density gradient centrifugation and treated with 5 and 10 μM of GANT61 for up to 48 h. Viability of cells was analyzed by multicolor flow cytometry using AnnexinV/7-AAD and lineage-specific antibodies (anti-CD19 and anti-CD5). CLL cells were defined as CD19+/CD5+, normal B cells as CD19+/CD5− and normal T cells as CD19−/CD5+ cells. Data are presented in box-and-whisker format, with the median marked as a line. (**c**) Relative RNA expression levels of specific Hh target gene *PTCH1* in primary CLL after GANT61 treatment. (**d**) Viability of MEC-1 cells after specific knockdown of GLI1 using two different small hairpin RNAs (shRNAs) and control shRNA. (**e**) Relative expression of GLI1 after shRNA-mediated knockdown of GLI1 in MEC-1 cells.

coculture with HS-5 stromal cells (Burger *et al*., 2005; Seiffert *et al*., 2007; Hegde *et al*., 2008). We first analyzed transcript levels of Hh ligands in HS-5 cells using real-time PCR and found all three cognate ligands, Indian Hedgehog, Desert Hedgehog and SHH, being expressed (Figure 6a). Furthermore, we observed a significant increase in CLL cell viability when cultured in the presence of HS-5 cells (Figure 6b, $P = 0.003$). Addition of GANT61 declined this viability in the presence of these stromal cells (Figure 6b). We hereby excluded any influence of GANT61 treatment on survival and Hh ligand expression of stromal cells (Supplementary Figure 6b). Next, we reexamined these effects with primary patient-derived BM stromal cells. In complete analogy to the HS-5-based data, these cells moderately protected CLL cells from spontaneous and partially from GANT61-induced apoptosis (Figure 6c). Hereby, autologous CLL cells were protected to the same extent as heterologous CLL samples (data not shown).

We also used the murine stromal cell line M2-10B4, which had previously been shown to produce the highly conserved Hh ligands and to support human leukemic cell growth (Dierks *et al*., 2007). In contrast to the moderate protective effects by human primary BM stromal and HS-5 cells, the presence of M2-10B4 cells restored full viability of CLL cells and addition of GANT61 did not decrease CLL viability (Supplementary Figure 7). In addition, M2-10B4 cells more strongly protected against fludarabine-induced apoptosis. However, combinatorial treatment of GANT61 and fludarabine resulted in higher apoptosis onset than single-agent treatment (Supplementary Figure 7). Moreover, GANT61 treatment also induced apotosis in *in vitro* fludarabine-resistant CLL samples (Figure 6d). Furthermore, as coincubation of CLL cells with SHH ligand was reported to increase CLL viability (Hegde *et al*., 2008), we tested SHH-induced effects. Functionality of the SHH ligand was assured by the use of an SHH responsive murine cell line that highly upregulated Gli expression on SHH addition (Figure 4a). However, SHH treatment did not increase CLL cell viability nor did it attenuate GANT61-induced onset of apoptosis (Figure 6e).

In summary, GLI inhibition by the novel compound GANT61 and GLI small hairpin RNA resulted in a significant reduction of CLL viability, downregulation of specific target genes and was efficient in fludarabine-resistent samples, indicating an indispensable cell-autonomous function of GLI in CLL survival.

Discussion

Emerging evidence indicates a key role of supportive microenvironmental niches, composed of specific combinations of cell types and molecular factors, in CLL pathogenesis (Panayiotidis *et al*., 1996; Caligaris-Cappio, 2003). Previous reports showed that Hh ligands secreted by stromal cells can serve as survival signals for B- and plasma-cell malignancies (Dierks *et al*., 2007; Hegde *et al*., 2008). These Hh ligands, especially SHH, were thereby described to induce a classical/canonical activation of the PTCH/SMOH-GLI cascade in malignant cells that could be antagonized by SMOH inhibition. Here, we report a SMOH-independent role of GLI in the *ex vivo* survival of CLL cells (Figure 7).

To study the role of the Hh signaling pathway in CLL, we first profiled the expression of Hh pathway

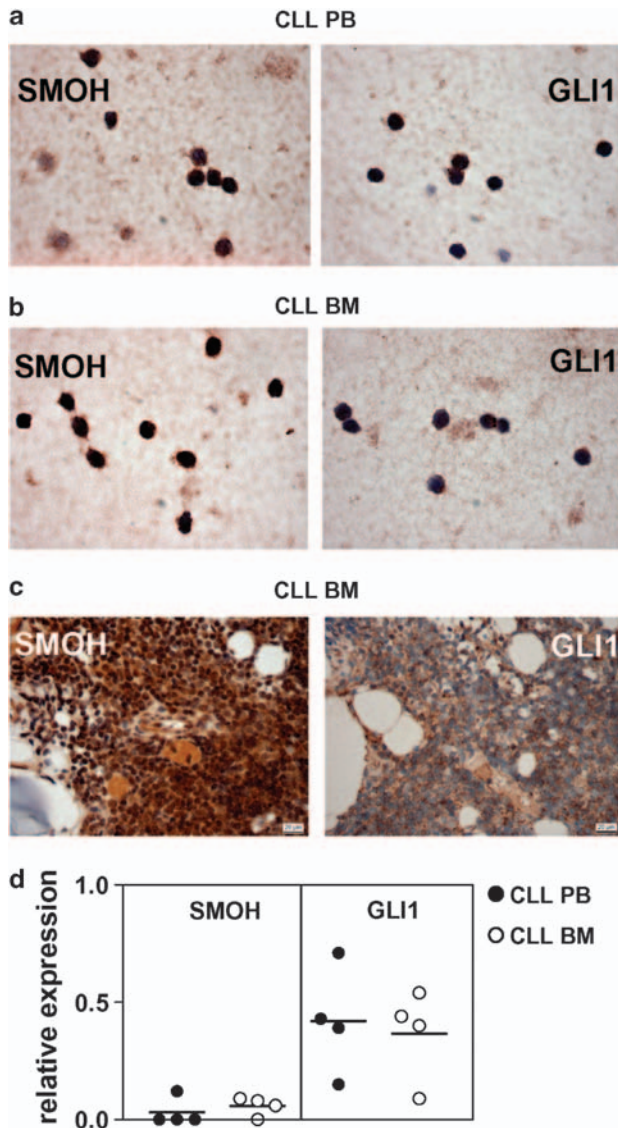

Figure 5 Immunohistochemical staining and RNA transcript expression of SMOH and GLI1 in PB and BM aspiration-derived primary CLL samples. SMOH and GLI1 expression in purified and subsequently in paraffin-embedded cells of (**a**) peripheral blood and (**b**) bone marrow-derived CLL cells. (**c**) SMOH and GLI1 expression in paraffin sections of CLL bone marrow. To characterize and quantify the infiltrate of the CLL further, immunohistochemistry was carried out using monoclonal antibodies against CD3, CD5, CD10, CD20, CD23 and CD79. (**d**) Relative expression levels of SMOH and GLI1 transcripts of paired PB and BM samples.

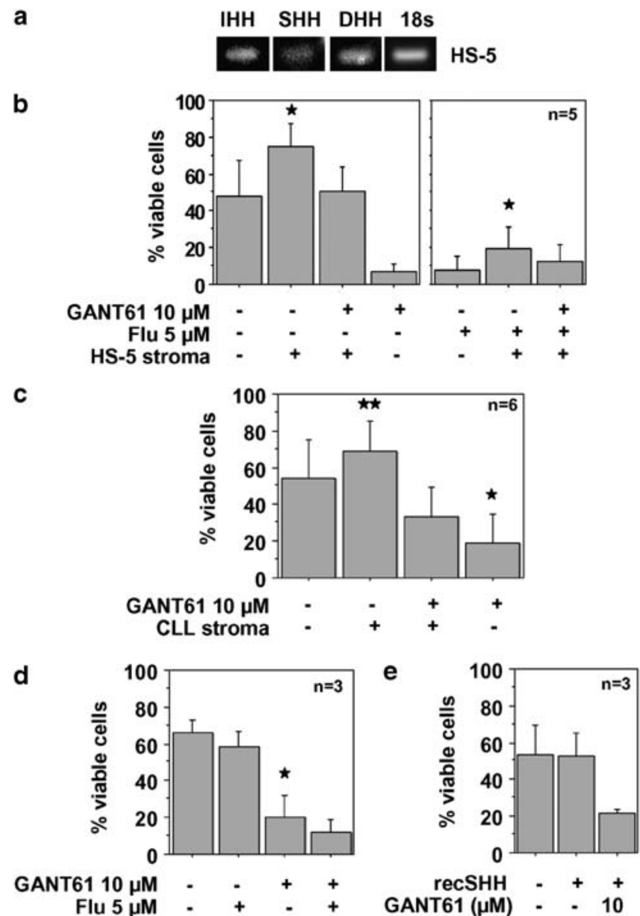

Figure 6 Influence of GANT61, recSHH and fludarabine treatment on viability of CLL cells cultured in the presence of the human stromal cell line HS-5. (**a**) Expression of Hh ligands in HS-5 stromal cells was measured using real-time PCR. (**b**) CLL patient-derived PBMCs ($n=5$) were added on HS-5 stromal cells seeded in a 24-well plate in a ratio of 5:1 and incubated with 10 μM of the Hh antagonist GANT61 and 5 μM fludarabine. (**c**) CLL cells ($n=6$) were added to cultures of CLL patient-derived primary stromal cells and incubated with 10 μM of the Hh antagonist GANT61. (**d**) *In vitro* fludarabine-resistant CLL cells are highly sensitive toward GANT61. (**e**) No significant influence of recSHH treatment on spontaneous and GANT61-induced apoptosis of primary CLL cells. The influence of single compounds or combined treatment on CLL viability in the presence or absence of HS-5 stromal cells was measured using multicolor flow cytometry using AnnexinV/7-AAD and lineage-specific antibodies (anti-CD19 and anti-CD5). Viable cells were considered AnnexinV and 7-AAD negative. Data are shown in a bar diagram presenting the mean values ± 1 s.d. Abbreviations: DHH, Desert Hedgehog; IHH, Indian Hedgehog.

members. We detected transcripts of key effectors, such as the signal transducer SMOH, PTCH receptors and GLI1 and GLI2 transcription factors, in CLL cells and normal B lymphocytes. However, we found a prominent downregulation of SMOH and GLI1 transcripts in CLL. In addition, we did not observe *in vitro* sensitivity of CLL cells toward specific SMOH inhibition. Thus, we do not confirm previously reported active canonical Hh signaling by SMOH in CLL (Hegde *et al.*, 2008). However, this previous interpretation was based on experiments using high doses of the popular and commercially available SMOH antagonist cyclopamine, which was also recently reported to cause unspecific *in vitro* growth repression due to off-target effects when used at these high concentrations (Yauch *et al.*, 2008). Indeed, while using cyclopamine above a certain concentration ($\geqslant 5$ μM), we observed a strong decline in CLL and normal B-cell viability, consistent with the previous observation (Hegde *et al.*, 2008). However, at such concentrations, tomatidine, a negative control compound for cyclopamine activity, also induced significant CLL apoptosis. Lower cyclopamine concentrations did not

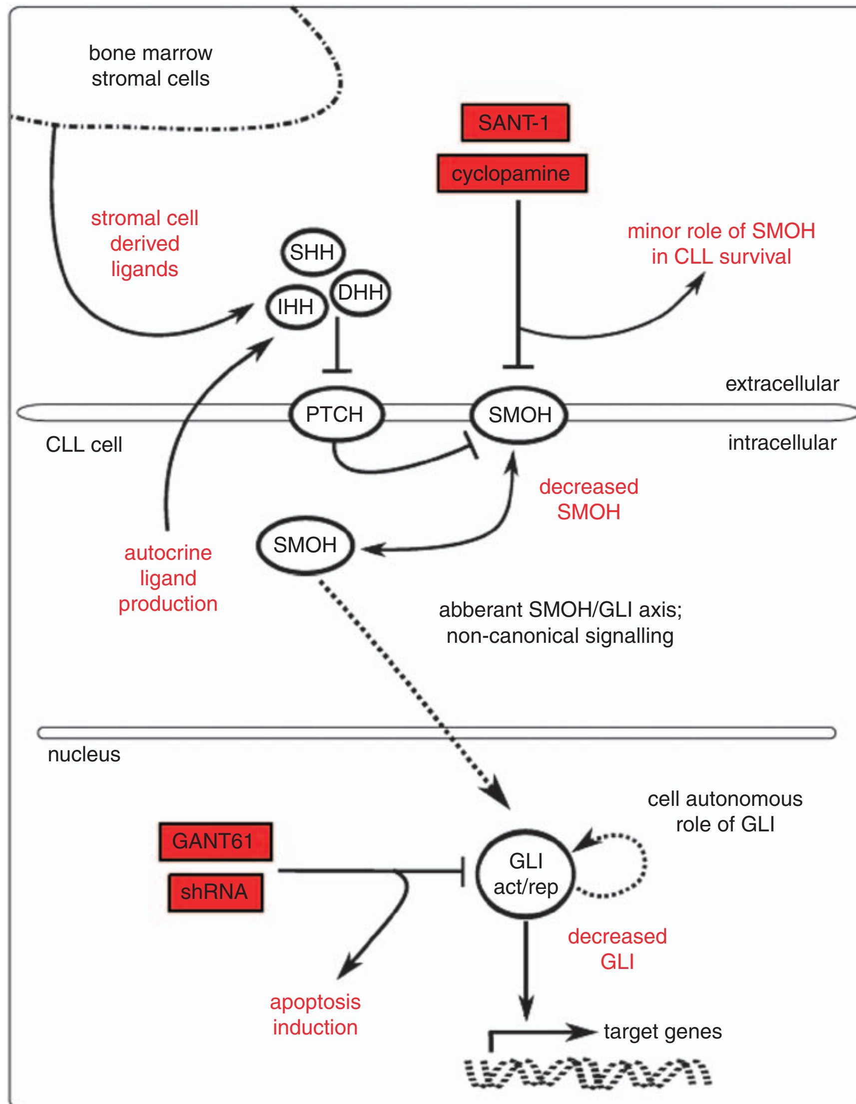

Figure 7 A hypothetical model of the Hh pathway in CLL. Although marrow stromal cells and CLL cells secrete Hh ligands potentially influencing SMOH activation, direct and specific SMOH blockage did not interfere with CLL survival. In contrast, direct GLI inhibition or knockdown resulted in CLL apoptosis, suggesting a cell autonomous role of GLI and/or noncanonical Hh pathway activation influencing GLI effects on CLL survival.

result in significant CLL apoptosis. In addition, we observed only a minor onset of apoptosis by a second SMOH antagonist, SANT-1. Moreover, small interfering RNA-mediated knockdown of SMOH did not reduce viability of a CLL-derived cell line nor did the coincubation of CLL cells with a described SMOH agonist provide any survival benefit, arguing against a prominent role of SMOH in CLL survival *in vitro*.

Transcription factors GLI1 and GLI2 are described to be activating elements during developmental patterning, whereas GLI3 functions rather repressively (Wang *et al*., 2000; Wang and Li, 2006). We confirm recent data on GLI1 expression in CLL (Hegde *et al*., 2008), although we also observe significantly lower GLI1 expression in CLL cells than in normal B lymphocytes, which was not examined in the previous report. GLI1 expression can be regulated by microRNAs (Ferretti *et al*., 2008), and databank searches revealed that the GLI1 3′ untranslated region shows a putative microRNA150 binding site. As we observe higher microRNA150 expression in CLL than in normal B lymphocytes (data not shown), this microRNA might be one of the responsible factors for the intrinsic GLI1 downregulation. Interestingly, we did not find any expression of GLI3 in both, normal B lymphocytes and CLL cells. GANT61 is a novel small molecule interfering with GLI binding to target gene promoters (Lauth *et al*., 2007a). In contrast to SMOH antagonism, GLI inhibition by GANT61 resulted in a massive onset of apoptosis in CLL cells, but not in normal lymphocytes. The specificity of this compound was confirmed by showing transcriptional reduction of the cognate Hh-GLI target gene *PTCH1* and small hairpin RNA approaches targeting GLI1. As GLI1 transcription

and CLL cell response to GLI inhibition were not influenced by the presence of any tested CLL risk parameter (data not shown), GLI targeting might be an option for CLL patients independently of their risk profile. Interestingly, we observed a clear response of *in vitro* fludarabine-insensitive CLL samples toward GANT61 treatment. This observation suggests a divergence of the GLI pathway and the p53 axis in CLL, which is known to be involved in fludarabine resistance (Dohner *et al*., 1995). It also commends a further evaluation of GLI targeting as potential treatment strategy for high-risk patients.

GLI activation by SMOH-dependent canonical Hh signaling induced by stromal cell-derived Hh ligands has previously been reported to support the survival of malignant hematopoietic cells (Dierks *et al*., 2007; Lindemann, 2008). Using two stromal cell lines, as well as primary, CLL-derived stromal cells producing Hh ligands (Dierks *et al*., 2007), we confirm the previous observations of stroma-mediated survival benefits and show stromal cell-dependent attenuation of GANT61-induced CLL apoptosis. We did not observe any effect of GANT61 on the viability or Hh ligand expression of stromal cells. Addition of soluble SHH did not provide any survival benefit to CLL cells and was unable to compensate for GANT61-induced apoptosis, suggesting the contributions of other stromal cell-derived secreted/paracrine/soluble factors or adhesive events relevant in CLL–stroma interactions, rather than soluble stromal cell-derived Hh ligands. In addition, despite the high conservation of this pathway, different effects of human- and murine-derived stromal cells were observed on CLL cell survival under GANT61 treatment. This might be based on either ‘dose-dependent’ effects of the same protecting components or on the presence of different protecting components against GANT61-induced apoptosis in the murine compared with the human system. Coculture with stromal cells in long-term culture experiments did not influence GLI1 levels of CLL cells, further supporting the above-mentioned SMOH-independent role of GLI1 in CLL cell survival (data not shown). Our data point toward additional, noncanonical mechanisms of GLI activation in line with recent publications, suggesting that GLI transcription factors are not solely regulated by Hh/SMOH signaling but are also influenced by crosstalk with other pathways, such as RAS, PI3K/Akt, transforming growth factor-β, PKCδ, or extracellular signal-regulated kinase pathways, which are downstream and independent of SMOH (Riobo *et al*., 2006; Dennler *et al*., 2007; Stecca *et al*., 2007; Ji *et al*., 2007, Lauth *et al*., 2007b). All these pathways and supposedly additional pathways as well are able to modulate GLI1 and GLI2 activity independent of the presence of Hh ligands. The finding of noncanonical GLI activation is likely to influence the design of future drug development strategies based on targeting the Hh pathway downstream of SMOH rather than direct SMOH antagonism. In CLL, GLI inhibition could thus be a therapeutic option for the circulating pool or for CLL cells within BM and lymphoid organs in combination with agents targeting the niche-malignant cell interaction and potentially mobilizing CLL cells into the periphery.

In constrast to Hegde *et al*. (Hegde *et al*., 2008), our findings show only a minor role of SMOH and stromal cell-derived Hh ligands in the *ex vivo* survival of PB CLL cells. However, we do not discount the possibility that SMOH or canonical Hh signaling is important for CLL leukemogenesis *in vivo*. In chronic myeloid leukemia and multiple myeloma, SMOH is thought to be responsible for the maintenance of the cancer stem cell sub-population propagating the leukemia (Peacock *et al*., 2007; Dierks *et al*., 2008; Zhao *et al*., 2009). Although in CLL, such a tumor-initiating sub-population has not yet been identified, Hh pathway activity may indeed be present in a very small sub-population driving the disease. Recent data confirm the presence of a relevant population of proliferative CLL cells, supposedly localized within pseudofollicles in secondary lymphoid tissues (Messmer *et al*., 2005; Patten *et al*., 2008). As we observe high SMOH expression in the proliferating MEC CLL-derived cell line, but very low expression in cell-cycle-arrested primary PB CLL cells, it is tempting to speculate that SMOH expression may mark this proliferative pool. However, optimized techniques for isolation of this sub-population or *in vivo* animal CLL models with conditional ablation of SMOH function will be necessary to expand our knowledge on CLL origin and progression. Increased understanding of the role of the Hh pathway in leukemic bulk cells versus leukemia-initiating cells may also lead to the development of more effective and targeted therapies for a broad range of tumors, including CLL.

Materials and methods

Patient material and cell culture

At the University Hospital Salzburg, a cohort of 65 CLL patients was enrolled after obtaining informed consent. Clinical, genetic and prognostic data were routinely collected (Supplementary Table 1). Peripheral blood mononuclear cells were isolated from PB and BM aspirates using density gradient centrifugation and maintained in complete RPMI-1640 medium (PAA Laboratories, Pasching, Austria). MEC cell lines (DSMZ ACC 497, MEC-1; ACC 500 and MEC-2) were cultured in complete Iscove’s modified Dulbecco’s medium (Invitrogen, Carlsbad; CA, USA). Treatment with GANT61 (Alexis Corporation, Lausen, Switzerland), cyclopamine, tomatidine and SANT-1 (Sigma-Aldrich, St Louis, MO, USA) was performed in concentrations of 1, 2.5, 5 and 10 μM, and cells were incubated for up to 72 h at 37 °C, 5% CO_2. Fludarabine (Sigma-Aldrich) was used at 5 μM. SAG (Sigma-Aldrich) was used at 200 nM. For coculture of CLL cells with human BM-derived stromal cells, or with HS-5 cells (ATCC; CRL-11882) or M210-B4 (ATCC; CRL-1972), a ratio of 5:1 was used. Biologically active human recombinant Shh-N was produced in an Sf9 insect cell-based baculovirus expression system and applied on the Hh-responsive murine cell line C3H/10T1/2 (ATCC; CCL-226) as described (Ecke *et al*., 2008). Primary stromal cells were generated by a modified protocol from the study by Jaganathan *et al*., (2007). Briefly, peripheral blood mononuclear cells-derived adherent cells were cultured in Dulbecco’s modied Eagle’s medium with low

glucose, supplemented with 20% FCS, glutamine and 25 ng/ml basic fibroblast growth factor (Biolegend, San Diego, CA, USA). After about 2 weeks, a layer of spindle-shaped stromal cells had formed. Cells were passaged 1:3 at 80% confluence.

RNA isolation and real-time PCR
To obtain high purity (>95%) of CLL cells or normal B lymphocytes, peripheral blood mononuclear cells were CD19+ sorted by MACS separation (Miltenyi Biotec, Auburn, CA, USA) before RNA isolation. Total RNA was isolated with the QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany). Reverse transcription of RNA was performed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR using Taqman Gene Expression Assays was performed using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Results were quantified according to the 'delta-delta-CT method' based on the relative expression of a target gene versus a reference gene (18 s rRNA) and normalized to the expression levels of normal B lymphocytes.

Apoptosis assay
Cells were stained with Annexin-V-FITC, 7-Aminoactinomycin D and anti-CD19-PE and anti-CD5-PC7 antibodies (BD Bioscience, Bedford, MA, USA). CLL cells were defined as CD19+/CD5+, normal B cells as CD19+/CD5− and normal T cells as CD19−/CD5+ cells. Viable cells were considered as AnnexinV and 7-Aminoactinomycin D negative. Cell were analyzed on the FC-500 Cytomics, and data were analyzed using Cytomics RXP Software (Beckman Coulter, Fullerton, CA, USA).

Immunohistochemistry
For cell block preparation, MEC cells and PB and BM-derived CLL cells were harvested and resuspended in 200 µl of citrate plasma (supplied by the Institute of Transfusion Medicine, PMU, Salzburg, Austria) and 200 µl of Thromborel S (Siemens Healthcare Diagnostics, Deerfield, IL, USA). After coagulation, cells were fixed in neutral-buffered saline containing 7% formalin and paraffin embedded. Cell blocks were cut into 5 µM sections and deparaffinized using graded alcohol.

BM specimens were routinely fixed with 5% (v/v) phosphate-buffered saline-buffered formalin immediately after removal, decalcified in 0.3M EDTA, dehydrated and embedded in paraffin. Thick paraffin sections of 5 µm were cut and stored at room temperature until use. Routine histology (hematoxylin and eosin staining) was performed to evaluate basic histomorphology of the BM and to characterize the cellularity of specimens. To characterize and quantify the infiltrate of the CLL, further immunohistochemistry was carried out using monoclonal antibodies listed in Supplementary Table 2.

Knockdown experiments
Knockdown experiments in MEC cells were performed with a total cell number of 5×10^5 cells. Three different SMOH small interfering RNAs (SMO Stealth Select 3 RNAi Set, Invitrogen, Paisley, UK; Oligo IDs: HSS110025, HSS185994 and HSS185995) and negative control oligonucleotides (Stealth RNAi siRNA Negative Control Hi GC; 12935-400) were purchased from Invitrogen (Stealth Select 3 RNAi Set). Hiperfect (Qiagen) was used according to the manufacturer's instructions. Viral transduction of MEC cells was performed with virus supernatants of GLI1 sh88 (TRCN0000020488), GLI1 sh86 (TRCN0000020486) and control shc002 (Sigma-Aldrich) lentiviral particles. Production was performed as previously described (Kasper *et al.*, 2007). Stably transduced cells were selected with 2 µg/ml of puromycin. At 8 days after transduction, viability was assayed by flow cytometry analysis of 7-Aminoactinomycin D/AnnexinV-stained cells.

Statistics
Statistical analyses (analysis of variance *post hoc* with Fisher's paired or unpaired *t*-test, Mann–Whitney test) were carried out using StatView 5.0.1. Software (SAS Institute Inc., Cary, NC, USA) and effects were considered significant at $P \leqslant 0.05$ with $*P \leqslant 0.05$; $**P \leqslant 0.001$; $***P \leqslant 0.0001$.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)