The Hedgehog/GLI signaling pathway activates transcription of Slug (Snail2) in melanoma cells

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Abstract. In melanoma and other cancers, invasion, epithelial-to-mesenchymal transition, metastasis and cancer stem cell maintenance are regulated by transcription factors including the Snail family. Slug (Snail2) protein generally supports migration and apoptosis resistance. However, its role in melanoma is not completely understood. The present study investigated the transcriptional regulation of the *SLUG* gene in melanoma. It demonstrated that *SLUG* is under the control of the Hedgehog/GLI signaling pathway and is activated predominantly by the transcription factor GLI2. The *SLUG* gene promoter contains a high number of GLI-binding sites. Slug expression is activated by GLI factors in reporter assays and inhibited by GANT61 (GLI inhibitor) and cyclopamine (SMO inhibitor). *SLUG* mRNA levels are lowered by GANT61 as assessed by reverse transcription-quantitative PCR. Chromatin immunoprecipitation revealed abundant binding of factors GLI1-3 in the four subregions of the proximal SLUG promoter. Notably, melanoma-associated transcription factor (MITF) is an imperfect activator of the *SLUG* promoter in reporter assays, and downregulation of MITF had no effect on endogenous Slug protein levels. Immunohistochemical analysis confirmed the above findings and showed MITF-negative regions in metastatic melanoma that were positive for GLI2 and Slug. Taken together, the results demonstrated a previously unrecognized transcriptional activation mechanism of the *SLUG* gene, which may represent its main regulation of expression in melanoma cells.

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Abbreviations: Slug (Snai2, Snail2), snail family zinc finger 2; Snail1, snail family zinc finger 1; EMT, epithelial-to-mesenchymal transition; ALDH1A1, aldehyde dehydrogenase 1 family member A1; Klf4, Krüppel-like transcription factor 4; GLI, GLI family zinc finger; CSC, cancer stem cells; HH, Hedgehog signaling pathway; MITF, melanoma-associated transcription factor

Key words: Slug, Hedgehog signaling, GLI family zinc finger, melanoma-associated transcription factor, melanoma

Introduction

Hedgehog (HH) signaling is a developmentally conserved pathway in numerous embryonic tissues and has been shown to be dysregulated in multiple cancers (1,2). The Sonic Hedgehog cascade involves the Sonic hedgehog (Shh) ligand binding to Patched (PTCH), a 12-pass transmembrane protein. When the ligand is absent, PTCH represses the activity of the neighboring 7-pass membrane protein Smoothened (SMO). This inhibition is released upon Shh binding. The ensuing activation of SMO triggers a chain of events that lead to the release of GLI1-3 effector proteins from the Suppressor of Fused (SuFu) and their subsequent translocation to the nucleus (2). The activated HH/GLI pathway has been linked to a number of types of human cancers and causes accelerated proliferation and survival and an enhanced rate of metastasis. HH also supports the self-renewal of cancer stem cells (CSCs), a subpopulation of tumor cells with inherent resistance to therapy (3,4). HH activity can be regulated in a noncanonical manner, and does not require the initial Shh binding to the receptor. A number of pathways, such as RAS (5), MAPK (6), AKT (7) and EGFR (8), have been shown to activate GLI factors directly in tumor cells.

The HH pathway has been shown to be essential for the oncogenic properties of melanoma (6). Moreover, blunting GLI1 and GLI2 restores sensitivity to vemurafenib in vemurafenib-resistant melanoma cells harboring BRAF mutations (9). SOX2 is crucial for the self-renewal of CSCs in melanoma and is regulated by GLI1 and GLI2, thus mediating HH signaling (10). GLI1 and GLI2 also transcriptionally regulate several genes involved in positive regulation of the cell cycle, such as E2F1, cdk1 and cyclin B (11).

The transcription factor Slug, the protein product of the *SNAIL2* gene, belongs to the Snail family of zinc-finger transcription factors (12). As early as 1998, the human Slug

protein was described to contain 268 amino acids and its molecular weight is ~30 kDa (13). Slug is expressed during embryogenesis and is critical for the development of the neural crest (14). Notably, Slug and Snail significantly contribute to the maintenance of CSCs (15). Slug is an antiapoptotic protein that contributes to the transcriptional repression of E-cadherin in epithelial tumors, thus contributing to epithelial-to-mesenchymal transition (EMT) in tumor cells (16). This is achieved by upregulating the expression of Zeb1, which is then engaged in repressing E-cadherin (17). Furthermore, Twist1 upregulates Slug which mediates Twist1-induced changes during EMT (18). Cooperatively, these transcription factors repress epithelial markers, enabling cell detachment and migration during early stages of EMT, which is a key phenomenon underlying cancer progression and invasion (19). Moreover, increased Slug expression is found in patients suffering from a number of types of cancers (20). Increased Slug levels are linked to metastasis, tumor recurrence and poor prognosis and play a role in the maintenance of CSCs (21). The CSC-like properties of tumor cells promote tumor initiation, expansion, EMT, metastasis, and tumor relapse and confer resistance to chemotherapy and radiotherapy in multiple types of cancer (22). Downregulation of Slug results in inhibition of the proliferation of cancer cell lines, and its overexpression leads to accelerated proliferation (21).

In melanoma, *SLUG* has been considered to be a pro-oncogenic gene contributing to EMT (17). Slug expression in melanoma cells has been reported to be regulated by osteonectin (SPARC). PI3/Akt kinase signaling acts upstream of SPARC, as its blockade hinders induction of the *SLUG* gene by SPARC, cell migration, and EMT-like changes (23). The protein Nodal is involved in the expression of *SNAIL* and *SLUG* genes and activation of ALK/Smads and PI3K/AKT pathways (24,25). Slug silencing has also been shown to increase the radiosensitivity of melanoma cells (26).

Despite these findings, the precise mechanisms of Slug expression and its role in EMT remain to be elucidated in melanoma. Gupta *et al* (27) described the necessity of Slug for the development of melanoma metastases in a mouse model. By contrast, Slug protein expression has been observed to be diminished in human metastases (28). Slug, together with Zeb2, was notably found to be downregulated during EMT in melanoma. Slug and Zeb2 transcription factors have been reported to drive a melanocytic differentiation program and behave as oncosuppressive proteins, whereas Zeb1 and Twist1 repress differentiation and have oncogenic properties (29). Similar conclusions were reported by Gunarta *et al* (30) after ablating GLI1 function in melanoma. GLI1-knockdown cells exhibited reduced invasion ability accompanied by downregulation of the EMT factors Snail1, Zeb1 and Twist1 but not Snail2 or Zeb2. As SLUG is one of the genes contributing to CSC maintenance, a central question for understanding the acquisition of the mesenchymal state and CSC renewal is how the expression of genes involved in EMT is regulated. In brief, inconsistent results have been reported regarding *SLUG* gene function in melanoma cells, and the mechanisms of its expression have not been extensively studied. The present study investigated the transcriptional regulation of *SLUG* in human melanoma cells and observed that Slug expression is controlled by the HH/GLI pathway, particularly the GLI2 transcription factor.

Materials and methods

Cell culture. The present study used eight melanoma cell lines (listed in Table SI). Their mutational status (BRAF and NRAS mutations) is shown in Table SII. The origin of cells has been described previously (31,32). Cells were cultivated in RPMI medium (MilliporeSigma) supplemented with 10% FCS (Thermo Fisher Scientific, Inc.), glutamine and antibiotics (MilliporeSigma) at 37˚C and 5% CO_2 in 100% humidity. All cell lines were authenticated and tested for mycoplasma using a mycoplasma detection kit (MP0035; MilliporeSigma). Cells were passaged every 72-96 h using a trypsin-EDTA solution. When plated, cells ($5x10^5$) were seeded from a stable culture into 12-well plates and incubated for 24 h at 37˚C prior to inhibitor treatment or transfection, unless specified otherwise. Normal human melanocytes were purchased from Cascade Biologics Inc. and cultivated according to the manufacturer´s instructions. The generation of inducible melanoma cell lines in which melanoma-associated transcription factor (MITF) protein can be downregulated by the addition of doxycycline (Tet-on system) has been previously described (32).

Chemical inhibitors. GANT61 (stock prepared in DMSO) and cyclopamine (stock prepared by dissolving in ethanol) were purchased from Selleck Chemicals LLC. The chemicals were applied to cells as indicated in the appropriate figures for 20 h before cell harvesting if not stated otherwise. The addition of 20 μM GANT61 for 20 h was used after the optimization of both the concentration and time to follow the changes in expression of GLI-dependent genes, when no signs of apoptosis had been yet detected in cells.

Western blot analysis. To obtain whole-cell extracts for immunoblotting analysis, cells were lysed in RIPA buffer (1% NP-40, 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, 50 mM Tris-HCl pH 7.5 and 0.1% SDS) with the addition of the protease and phosphatase inhibitors aprotinin, pepstatin and leupeptin at 1 mg/ml each. cOmplete (Roche Diagnostics) was added as recommended by the supplier. Then, 1 mM phenylmethylsulfonyl fluoride (MilliporeSigma) and phosphatase inhibitor PhosSTOP (Roche Diagnostics) were added. Equal amounts of protein (30 μg; concentration determined by Bradford's assay) were loaded on 10-12% SDS-polyacrylamide gels, separated by electrophoresis and transferred onto PVDF membranes. Blots were blocked in 5% non-fat dry milk Blotto (cat. no. sc-2325, Santa Cruz Biotechnology, Inc.), and incubated with 1:1,000 diluted primary antibodies for 2 h, washed, and then incubated at room temperature for 1 h with 1:4,000 diluted secondary anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibodies (cat. no. sc-2055 or cat. no. sc-2030; Santa Cruz Biotechnology, Inc.). Chemiluminescent detection was used. For western blotting shown in Fig. 5A and B, cells were lysed in PLB buffer (Promega Corporation), used in dual luciferase measurements and these extracts were then directly utilized. Primary antibodies for western blotting were: GLI1 (cat. no. 3538; Cell Signaling Technology, Inc.), GLI2 (cat. no. TX46056; GeneTex, Inc.), and GLI3 (cat. no. AF3690; R&D Systems, Inc.). Anti-Slug (cat. no. sc-166476) was purchased from Santa Cruz Biotechnology, Inc. Klf4 (cat. no. LS-C415468) and ALDH1A1 (cat. no. LS-B10149) from LSBio. Anti-FLAG

(M2; cat. no. F1804) and anti-β-actin (A5316) antibodies were purchased from MilliporeSigma. Anti-livin antibody (cat. no. sc-30161) was from Santa Cruz Biotechnology, Inc. and MITF (D5) antibody (cat. no. NBP2451590) was from Thermo Fisher Scientific, Inc. Brn-2 (cat. no. sc-514295) was from Santa Cruz Biotechnology, Inc. E- and N-cadherins, vimentin, Zeb1, and Zeb2 antibodies were from Cell Signaling Technology (cat. no. 9782). Western blot bands were quantified using ImageJ (v. 1.52j) software (National Institutes of Health; data not shown).

Plasmids. The 12xGLI-TK-Luc plasmid was obtained from Professor R Toftgard, Karolinska Institute, Sweden. pGL3-PTCH1 was kindly donated by Professor Aberger, University of Salzburg, Austria. The *PATCHED* gene promoter contains one active GLI-binding site responsible for its activity (data not shown). The pGL3-slug-prom-full-length promoter (-5216+112) and pGL3-slug-prom-Δmiddle (-5216-4635, -2092+112) were cloned as *Xho*I-*Hind*III (New England BioLabs, Inc.) inserts in the pGL3-basic plasmid (Promega Corporation). pGL3-slug-prom-Δproximal (-5216-2092) was cloned as the *Xho*I-*Hind*III insert. The-4635+112 promoter was cloned as the *Nhe*I-*Hind*III insert. pGL3-slug-prom-middle (-4635-2092) was cloned as the *Nhe*I-*Nhe*I (New England BioLabs, Inc.) insert and the pGL3-slug-proximal promoter (-2092+112) was cloned as the *Nhe*I-*Hind*III insert. Cloning of all GLI expression vectors has been described previously (31). Briefly, original GLI1 (GL1; cat. no. 16419), GLI2 (pCS2-MT GLI2 FL; cat. no. 17648), ΔNGLI2 (pCS2-MT GLI2 delta N; cat. no. #17649) and GLI3 (GLI3 bs-2; cat. no. 16420) were acquired from the nonprofit plasmid repository Addgene, Inc. Respective coding sequences were amplified by PCR and subsequently cloned into the pcDNA3 expression vector or into the pFLAG-CMV-4 plasmid (MilliporeSigma) background to obtain FLAG-tagged GLI proteins. The types of GLI expression plasmids were similarly effective in promoter activation. The melastatin promoter plasmid with the three MITF-binding sites was constructed as previously described (33). The construction of the short hairpin plasmid directed to MITF has been previously described (32). All final plasmid inserts were verified by sequencing on both strands (GATC Biotech).

Transfection and promoter-reporter assays. Transient cell transfections of the promoter reporters were performed using 12-well plates at 37˚C, seeded and incubated for 24 h before transfections, and the transfection reagent Mirus *Trans*IT-2020 (Mirus Bio LLC) following the manufacturer's instructions and harvested 48 h after transfection. For detection, a dual luciferase system (Promega Corporation) was used according to the manufacturer's instructions. The pGL3-basic vector was used as a negative control. Expression vectors or controls (pCDNA3 or pFLAG-CMV-4) were cotransfected together with the reporter plasmids. Cell lysates were used for dual luciferase assays performed as recommended by the manufacturer's instructions. The luciferase values were acquired on a Turner Designs 20/20 luminometer (Promega Corporation). Data were normalized to *Renilla* luciferase activity (internal control) as arbitrary units. Statistical analysis of luciferase assay results was performed using a two-tailed unpaired Student's t test and SigmaPlot software V10.0 (Systat Software Inc.). Where indicated, cells were treated with GANT61 or cyclopamine 20 h at 37˚C before harvesting. Tomatidine, a compound inactive in the HH pathway but structurally similar to cyclopamine, was tested as a negative control and revealed similar results as vehicle (data not shown).

Reverse transcription-quantitative (RT-q) PCR. Total RNA was isolated using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the supplier´s instructions ($3x10^5$ cells per 30 mm well). Then 2 μg of RNA was reverse transcribed using SuperScript IV reverse transcriptase (Thermo Fisher Scientific, Inc.), and qPCR was performed using a TaqMan QuantiTect Probe PCR kit (Qiagen GmbH) on a ViiA7 Real-Time PCR system (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions (cycling: 30 sec at 94˚C and 1 min at 60˚C). Data analysis was performed by QuantStudio 6 Software (Thermo Fisher Scientific, Inc.). Concurrent results were obtained in three independent experiments with the following PCR primers and probe for *SLUG*: Forward, 5'-AGA ACTCACACGGGGGAGAAG-3'; reverse, 5'-CTCAGATTT GACCTGTCTGCAAA-3'; probe, 5'-6-FAM-TTTTTCTTG CCCTCACTGCAACAGAGC-TAMRA-3'. β-Actin was utilized as an internal standard control: Forward, 5'-ATTGCCGAC AGGATGCAGAA, reverse, 5'-GCTGATCCACATCTGCTG GAA; probe, 6-FAM-CAAGATCATTGCTCCTCCTGA GCGCA-TAMRA. Fluorescent probes were purchased from Thermo Fisher Scientific, Inc.

Chromatin immunoprecipitation. 501mel cell cultures were transfected using Mirus *Trans*IT-2020 (Mirus Bio LLC) with the pcDNA3-GLI1, pcDNA3-GLI2 or pcDNA3-GLI3 expression plasmids in 90 mm plates. After 24 h of treatment, fresh medium was applied. After another 24 h, cells were crosslinked with 1% formaldehyde for 10 min at room temperature and incubated with glycine solution, and chromatin immunoprecipitation was performed by using the ChIP-IT High Sensitivity kit (Active Motif, Inc.) in accordance with the manufacturer's protocols. Anti-acetylated histone H3 antibody (MilliporeSigma) was used as the positive control, and rabbit nonimmune IgG (MilliporeSigma) was used as a negative control. To detect GLI1 bound to the promoter, a rabbit anti-GLI1 antibody (cat. no. sc-20687; Santa Cruz Biotechnology, Inc.) was used. Rabbit anti-GLI2 (cat. no. ab26056; Abcam) was used to precipitate GLI2. For GLI3, a rabbit anti-GLI3 antibody (cat. no. 3538; Cell Signaling Technology, Inc.) was used and 2 μg of each antibody was added in each reaction. To assess the amount of ChIP-generated DNA, PCR was performed using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Inc.). The amplification of the regions of the proximal SLUG promoter was performed with four alternative primer pairs: Region A (-2108-1766): sense, 5'-GCATACGTGTTACTC GCTAGCAC-3', antisense, 5'-TCCTTGTTTCACTCTACA CAGTCTATTCAC-3'; region B (-1769-1163): sense, 5'-AGG AAATAATAGCCATGGCGATA-3', antisense, 5'-CAT CTCTGTCCATTGCAGAC-3'; region C (-1182-490): sense, 5'-GTCTGCAATGGACAGAGATGC-3', antisense, 5'-GGG AAGCGGGAAGACAAAG-3'; and region D: (-509+112): sense, 5'-CCTTTGTCTTCCCGCTTCCCCCTTCC-3', antisense, 5'-ACACGGCGGTCCCTACAGCATCG-3'. PCR products were resolved on 1% agarose gels. The intensity of

the final PCR bands was quantified using ImageJ (v. 1.52j) software (National Institutes of Health).

Immunofluorescence. For immunofluorescence, four cell lines (501mel, Hbl, SK-MEL-5 and SK-MEL-28) were seeded into 8-well Lab-Tek II Chamber Slides (Thermo Fisher Scientific, Inc.). After 48 h, 20 μM GANT61 was added for 20 h at 37˚C. Vehicle (DMSO) alone was added to the controls. The cells were fixed with 3% paraformaldehyde at room temperature for 10 min, washed, permeabilized with 0.1% Triton X-100, and blocked with 5% goat serum. Slides were then stained with Slug primary antibody (1:1,000; cat. no. sc-166476; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h followed by 1:1,000 secondary anti-mouse fluorescein-coupled antibody (cat. no. FI-2000-1.5, Vector Laboratories, Inc.) and mounted in medium with DAPI to stain nuclei. The visualization was performed using an Olympus IX70 microscope with cellSens software V2.2 (Olympus Corporation).

Immunohistochemical analysis. Formalin-fixed paraffin-embedded tissues (skin, nevus and melanoma) were retrieved from the archive of the Department of Pathology and Molecular Medicine, Second Faculty of Medicine, Charles University, University Hospital Motol, Prague. At least four samples of each tissue were processed and similar results were obtained. Deparaffinized, rehydrated in descending ethanol series, and blocked (with 3% H_2O_2) sections were stained with 1:1,000 primary antibodies. Immunohistochemistry for MITF was performed with the primary antibody MITF (cat. no. D5; cat. no. NBP2451590, Thermo Fisher Scientific, Inc.). GLI2 was stained with antibody cat. no. GTX46056 (GeneTex, Inc.) and Slug with anti-Slug (A-7) sc-166476 antibody (Santa Cruz Biotechnology, Inc.). Detection was performed using an EnVision+ avidin-biotin detection system (Dako; Agilent Technologies, Inc.). Each tissue was examined by two pathologists. Tissues were scored on a scale of 0 to 4 based on the combined extent and intensity of staining. The final score represented the predominant staining pattern of both combined parameters. Section fields were imaged using a BX51 microscope (Olympus Corporation) equipped with a PROMICAM 3-3CP 3.1 camera and QuickPHOTO CAMERA 3.2 software (Olympus Corporation).

Statistical analysis. Statistical significance (P-values) was assessed using a two-tailed Student's t test and Mann-Whitney test. Standard error of the mean values are depicted in graphs as bars within each column in the reporters and RT-qPCR. Data not significant (P>0.05) were not labeled, values of 0.01 <P<0.05 are marked by an asterisk, and values with P<0.01 are marked by two asterisks. SigmaPlot software V10.0 (Systat Software Inc.) and GraphPad Prism v.8.4.3 software (Dotmatics) were used to perform statistical analysis. Statistical analysis was verified using one-way ANOVA followed by post hoc tests as specified in figure legends. P<0.05 was considered to indicate a statistically significant difference.

Results

Slug expression in melanomas is inhibited by the Hedgehog pathway inhibitor GANT61. Snail1 and Snail2 (Slug) are among the main players in the tumorigenic program of EMT (19).

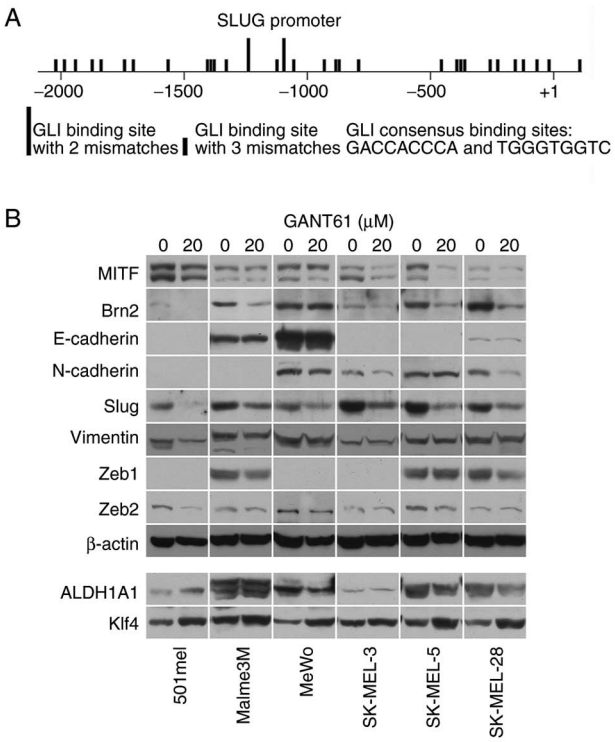

Figure 1. Distribution of GLI-binding sites in the *SLUG* proximal promoter and inhibition of Slug protein expression by GANT61 treatment. (A) Human *SLUG* promoter (proximal region -2092+112), where the +1 nucleotide denotes the start of translation, is depicted. A total of 31 GLI-binding sites are present in this promoter portion, of which two sites contain two mismatched nucleotides (longer bars) and all other sites harbor three mismatches (shorter bars). (B) Immunoblot analyses of several EMT-associated proteins after GANT61 treatment in 6 melanoma cell lines. Cells were treated with 0 μM (vehicle) or 20 μM GANT61 for 20 h and harvested for western blot analysis. The cancer stem cell markers ALDH1A1 and Klf4 were also analyzed. GLI, GLI family zinc finger.

SLUG gene deletions have been found in Waardenburg syndrome and piebaldism in humans (34,35), indicating the involvement of Slug in melanin pigmentation. The *SLUG* gene has long been thought to be under the transcriptional control of MITF (34). To further explore the regulation of Slug expression in melanoma, the present study examined the SLUG promoter and found 85 GLI-binding sites in the long SLUG promoter (-5216 +112; the start of translation is +1) and 31 sites in the proximal promoter (-2092 +112; Fig. 1A and Table SIII). Although none of the sites was a full consensus, GLI proteins, which are the final executive factors of the Hedgehog signaling pathway, bind to and are also active from sites with two or three mismatched nucleotides. This implies that SLUG gene expression could be controlled by the Hedgehog pathway.

To further investigate this hypothesis, Slug protein expression was examined in melanoma cell lines treated with GANT61, a potent and specific pan-GLI transcriptional inhibitor. It was found that GANT61 decreased Slug protein in all eight melanoma cell lines investigated as well as in normal melanocytes (Figs. 1B and SI). BRAF- or NRAS-mutated or wild-type cells for both oncogenes were present among the analyzed melanoma cell lines (Table SII). The levels of other proteins known to be involved in EMT, such as E- and N-cadherins, vimentin, Zeb1 and Zeb2, were not appreciably changed after GANT61 treatment in six melanoma cell lines.

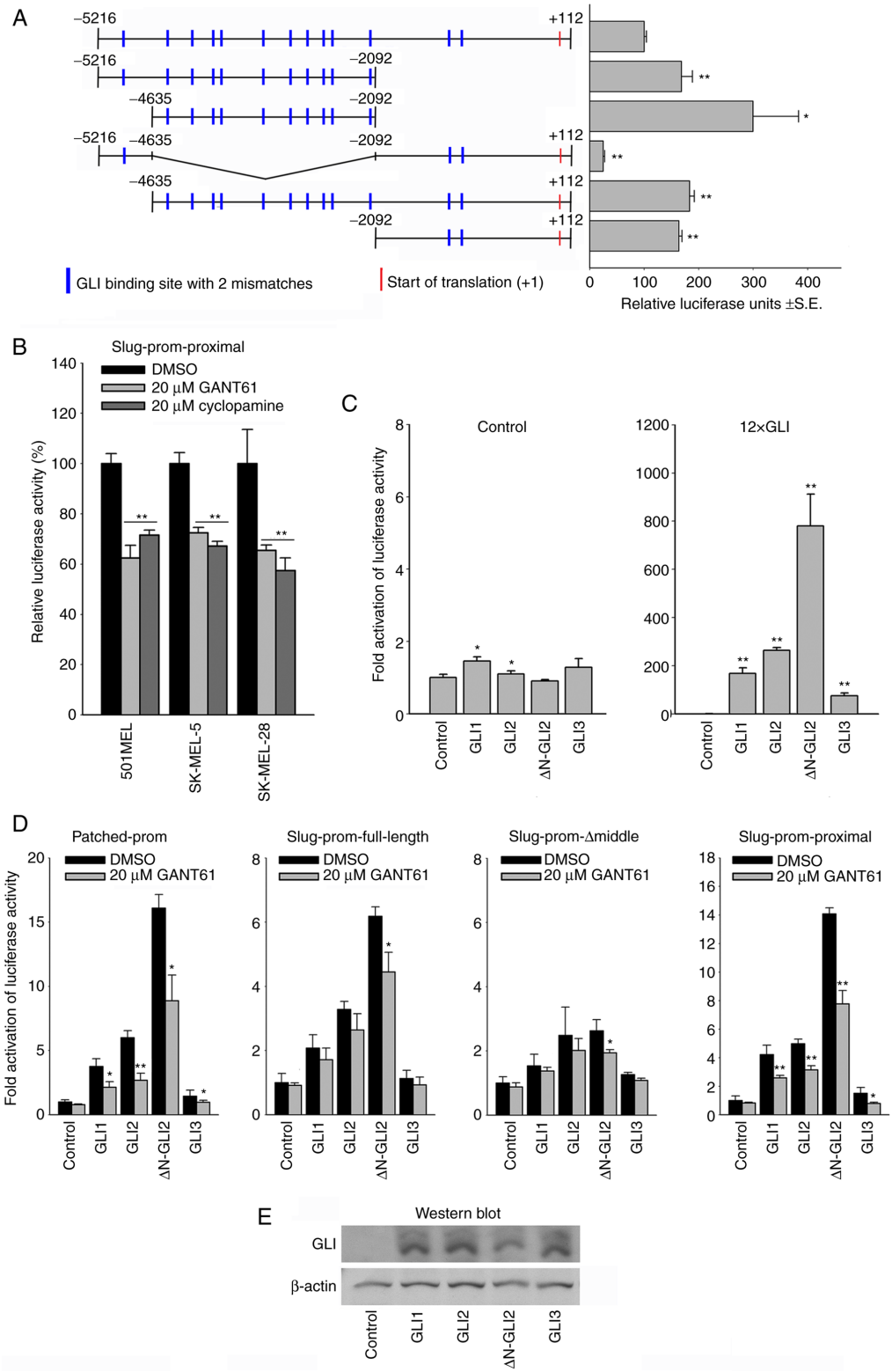

Figure 2. Promoter-reporter analysis of the *SLUG* gene promoter. (A) The long (full-length) *SLUG* promoter (-5216+112) and its truncated versions were assayed for activity in 501mel cells. Only two-mismatched consensus GLI-binding sites are shown as blue bars. pGL3-basic was used as a negative control and exhibited near zero activity (data not shown). The statistical significance of truncated promoters is related to the long promoter (100%). (B) Inhibition of the proximal *SLUG* promoter by GANT61 and cyclopamine in three cell lines. Bars are the mean ± standard error of the mean). The statistical significance is related to the activity of the untreated proximal promoter. (C) Activation of the 12xGLI-site full consensus promoter by GLI transcription factors. Left, the control plasmid (pGL3-basic) had extremely low activity and was negligibly influenced by GLI factors; right, the massive effect of GLI expression vectors on a positive control reporter plasmid with 12 full consensus GLI sites. (D) Left, inhibition of the GLI-activated *PATCHED* promoter by GANT61; middle left, middle right and right graphs, GANT61 inhibited three versions of the *SLUG* promoter. The most significant inhibition was seen with the proximal promoter (right). The GLI-mediated effect is compared with the control (pGL3-basic) in (B-left) and (D). The significance of GANT61-mediated inhibition is related to the vehicle-treated control. Statistical analyses were performed using a Student´s t test. Statistical analysis was verified using one-way ANOVA test, followed by Dunnett's post hoc test and equal results (P-values) were obtained. In all reporter assays, two or three independent experiments were performed (in triplicate) with similar results, and one representative experiment is shown. In all graphs, ± standard error of the mean bars are shown. Statistical significance: no mark, not significant, *P<0.05, **P<0.01. (E) Western blotting showing similar levels of expression of all GLI proteins in (C) and (D). GLI, GLI family zinc finger.

Likewise, the CSC markers ALDH1A1 and Klf4 were generally only slightly affected (Fig. 1B). A mild increase in Klf4 was noted in the cell lines 501mel, MeWo, SK-MEL-5 and SK-MEL-28, while ALDH1A1 levels were somewhat increased in 501mel cells and slightly diminished in SK-MEL-28 cells after GANT61 treatment (Fig. 1B). Only Brn2 (N-Oct-3, POU3F2) protein, a known repressor of MITF (36), noticeably decreased in five of six cell lines, suggesting that its transcription may also be regulated by the HH pathway in most melanomas. MITF was slightly downregulated in three cell lines (Fig. 1B). Immunofluorescence staining confirmed blunting of Slug expression after the treatment of melanoma cells with GANT61 (Fig. S2).

SLUG promoter-reporter is activated by GLI2 and inhibited by GANT61 and cyclopamine. Since the transcriptional inhibitor of GLI factors GANT61 downregulated Slug protein expression, the activity of the *SLUG* promoter was analyzed in reporter assays. Examination of luciferase expression driven by the long (full-length) promoter (-5216+112) and its truncated versions revealed that the middle part of promoter (-4635-2092) was the most active fragment (Fig. 2A). Its deletion greatly decreased the activity of the full-length promoter. The short portion most upstream (-5216-4635) probably performs an inhibitory function because its presence in the contexts of the full-length promoter, middle part and proximal promoter (-2092+112) decreased the luciferase activity (Fig. 2A). As the proximal promoter also showed appreciable activity, it was used in the following experiments.

Next, to test whether the SLUG promoter-reporter is directly activated by cotransfected expression vectors for GLI factors and inhibited by GANT61, it was first investigated whether the proximal promoter activity decreased after the addition of HH pathway inhibitors. Indeed, both GANT61 and cyclopamine lowered the activity in all three melanoma cell lines tested (Fig. 2B). As a subsequent experiment, each of the expression vectors for GLI factors (GLI1-3) were cotransfected with the GLI-responsive promoter containing 12 canonical GLI-binding sites (12xGLI). First, it was ascertained that there was only a minimal difference between the cotransfected control (pcDNA3) and GLI vectors with the pGL3-basic empty control promoter (Fig. 2C, left). By contrast, all three GLIs greatly increased 12xGLI promoter activity compared with the negative control plasmid pcDNA3 (Fig. 2C, right). GLI3 showed the weakest activation of the canonical 12xGLI promoter (but still ~80-fold), whereas the highest activation (800-fold) was achieved by ΔGLI2, a truncated version of GLI2 in which the N-terminal repression domain was removed (37). The activity of the GLI factors was then tested on a known HH-responsive promoter of the *PATCHED* gene, also a component of the HH pathway. The results were similar to those obtained with the canonical promoter, but the extent of stimulation was substantially lower, ~6-fold in the case of the most active ΔGLI2 (Fig. 2D, first graph). Similar experiments were then conducted with the three types of *SLUG* promoters, the long (full-length), Dmiddle and proximal promoters (Fig. 2D, second, third and fourth graphs). In all cases, the DGLI2 construct again showed the best activation. In accordance with the results in Fig. 2A, the Dmiddle promoter exhibited the lowest overall activity. When the

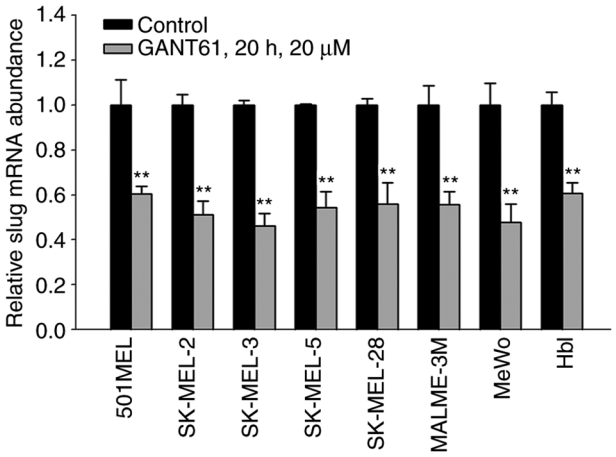

Figure 3. GANT61 decreases Slug RNA levels. mRNA levels of Slug were assessed by RT-qPCR quantification. A total of eight cell lines were treated with 20 μM GANT61 for 20 h, and total RNA was isolated. After RT, RT mixes were analyzed by qPCR. The relative mRNA levels of Slug were normalized to those of β-actin. Values are presented as the mean ± standard error of the mean. Three independent experiments were performed, each in triplicate. Similar results were obtained in all experiments, and one is depicted. The nontreated control (vehicle) was set as 1.0. All decreases in Slug RNA were statistically significant (**P<0.01) by Student's t test. RT-qPCR, reverse transcription-quantitative PCR.

PATCHED and *SLUG* promoters were tested, the addition of GANT61 more or less decreased the GLI-stimulated promoter activities (Fig. 2B and D). Western blotting verified that all GLI proteins were similarly expressed (Fig. 2E). These results together indicated that *SLUG* promoter activity is dependent mainly on GLI2 in reporter assays and that stimulation by GLI factors can be inhibited by GANT61.

Slug RNA levels are diminished after GANT61 treatment. To further evaluate the transcriptional regulation of Slug by HH/GLI, the present study examined the effect of GANT61 on Slug RNA levels using real-time PCR. RT-PCR was performed first, followed by qPCR. In all eight melanoma cell lines tested, 20 μM GANT61 significantly (P<0.01) lowered the mRNA level of Slug. This indicates that the positive effect of GLI factors on endogenous *SLUG* gene expression is mediated through the activation of transcription (Fig. 3).

ChIP assays show binding of GLI factors to the proximal SLUG promoter. The proximal *SLUG* promoter contains 31 GLI-binding sites, of which only two sites harbor two mismatches and all other sites have three mismatches (Fig. 2A and Table SIII). To investigate whether these sites are occupied by GLI proteins in cells, the 501mel cell line was used to chromatin immunoprecipitate DNA fragments bound to GLIs using specific anti-GLI1, GLI2 and GLI3 antibodies (Fig. 4). To obtain improved insight into whether the particular GLI binds to specific regions of the proximal promoter (-2092+112), the immunoprecipitated purified DNA was amplified by four primer pairs (see Materials and methods) that demarcated the four subregions A-D (Fig. 4) of the promoter. Each subregion contained several GLI-binding sequences. The most distal region A and a middle region C were bound by all three GLI factors. The middle region B was remarkably occupied only

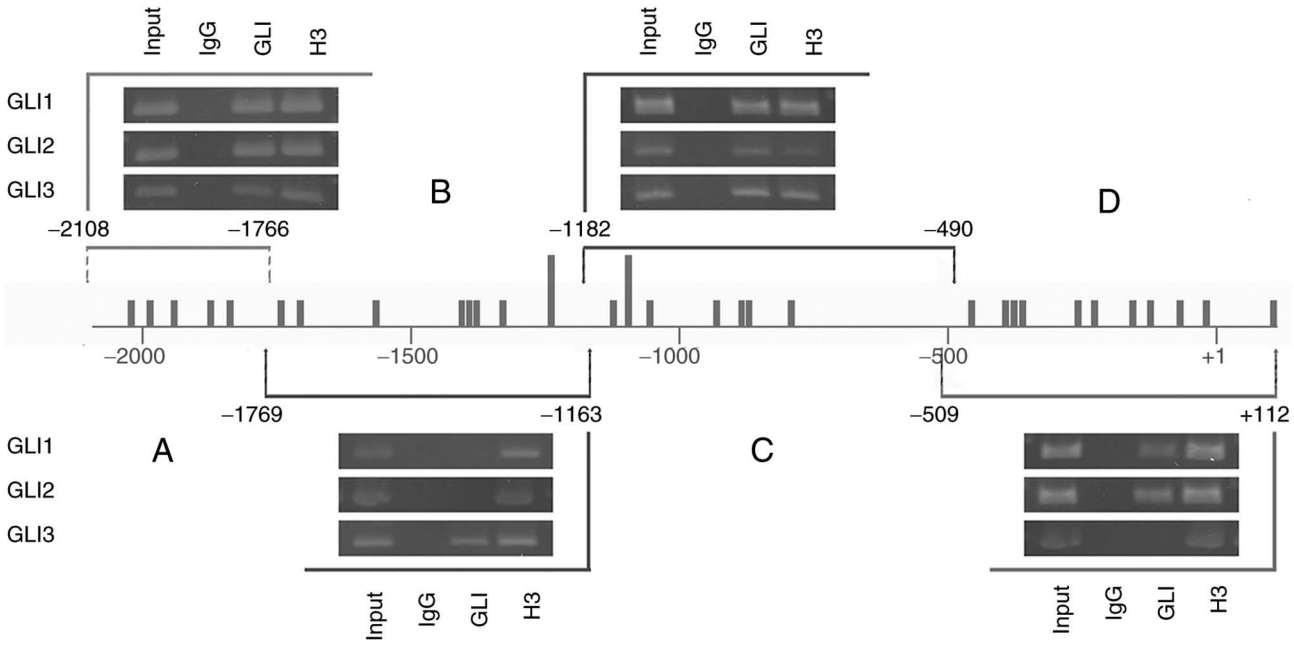

Figure 4. Binding of GLI factors to the proximal *SLUG* promoter. Chromatin immunoprecipitation was performed using the 501mel cell line. Cells were cultivated in 90 mm dishes and transfected with control vector (pCDNA3) or expression vectors for transcription factors GLI1, GL2, or GLI3. Cells were harvested for immunoprecipitation 48 h after transfection. At 24 h before harvesting, new medium was applied to the cells. After crosslinking, the cells were processed by a ChIP High Sensitivity kit as described in the Materials and methods. Immunoprecipitation was performed with the respective specific anti-GLI antibody. The final isolated coprecipitated DNA was then amplified by a set of four primer pairs (see Materials and methods). Each portion of the promoter (denoted A-D) revealed a specific pattern of GLI factor binding. Inputs and a positive control (anti-acetylated histone H3) always showed a positive band. Nonimmune IgG used as a negative control gave no bands when compared with experimental samples. All bands of PCR products were quantified using ImageJ software and the results are summarized in Table SIV. The two longer bars indicate two mismatched sites and small bars represent three mismatches of the full consensus site. GLI, GLI family zinc finger.

by the GLI3 factor. The most proximal fragment D was bound by GLI1 and GLI2 but not GLI3 (Fig. 4). Thus, regions B and D showed some preference for GLI occupancy, whereas regions A and C clearly exhibited enrichment by all three GLI factors. Acetylated histone H3, which was precipitated by anti-acetylated H3 antibody as a positive control, showed occupancy at all four subregions. Nonimmune IgG as a negative control showed no binding in any experiment. Taken together, these data indicate that GLI transcription factors are abundantly present at their recognition DNA sites within the *SLUG* proximal promoter, further confirming their role in the transcriptional regulation of Slug expression through HH signaling. The data quantifying the final DNA amount formed through PCR are in Table SIV.

Slug is an imperfect target for MITF in melanoma cells. The *SLUG* gene has been demonstrated to be transcriptionally regulated by MITF in melanocytes (34) and during *Xenopus laevis* development (38). However, data relevant to a possible regulation of Slug expression by MITF in melanoma cells are lacking. To test whether MITF overexpression influences the activity of the *SLUG* gene promoter, we performed promoter-reporter assays in which the proximal promoter was cotransfected with the MITF expression construct into 501mel cells. Additionally, we compared activation by MITF-Vp16, a hyperactive MITF derivative in which the MITF N-terminal activation domain (AD) was replaced by a stronger Vp16 AD (39), with native MITF. While MITF had no effect on promoter activity, MITF-Vp16 increased it ~2-fold (Fig. 5A left). On the other hand, the melastatin promoter, a known MITF target (33), was stimulated by both MITF and MITF-Vp16 ~4-fold and 10-fold, respectively (Fig. 5A middle). Western blotting verified the total MITF protein level (the control sample also showed the MITF protein signal because relatively high endogenous MITF protein is already present in nontransfected 501mel cells; Fig. 5A, right). Only transfected cells with ectopic MITF or MITF-Vp16 were measured for luciferase activity, which explained why promoter activity increased more compared with the overall MITF protein level. The same experiment was repeated with the FLAG-tagged vectors, and the same results were obtained. The expression of proteins expressed from transfected plasmids was verified with the anti-FLAG antibody (Fig. 5B).

To corroborate these results, doxycycline-regulatable melanoma cell lines, in which MITF could be downregulated by inducible expression of shRNA directed at MITF were used (32). Whereas a decrease in endogenous MITF protein was achieved after the addition of doxycycline in all cell lines, Slug expression remained unchanged. By contrast, the level of livin (ML-IAP), a known MITF target (40), mirrored the decrease in MITF protein (Fig. 5C). In agreement with this, overexpression of MITF did not change the endogenous level of Slug protein (data not shown). In a control experiment, inducible control nontargeting shRNA revealed no changes in the expression of all proteins tested. Thus, endogenous Slug seems to be expressed independently of MITF protein levels in human melanoma cells.

To further investigate the relationship between MITF and Slug and GLI2 vs. Slug protein expression in human samples, parallel sections of skin, nevus and melanoma metastasis were

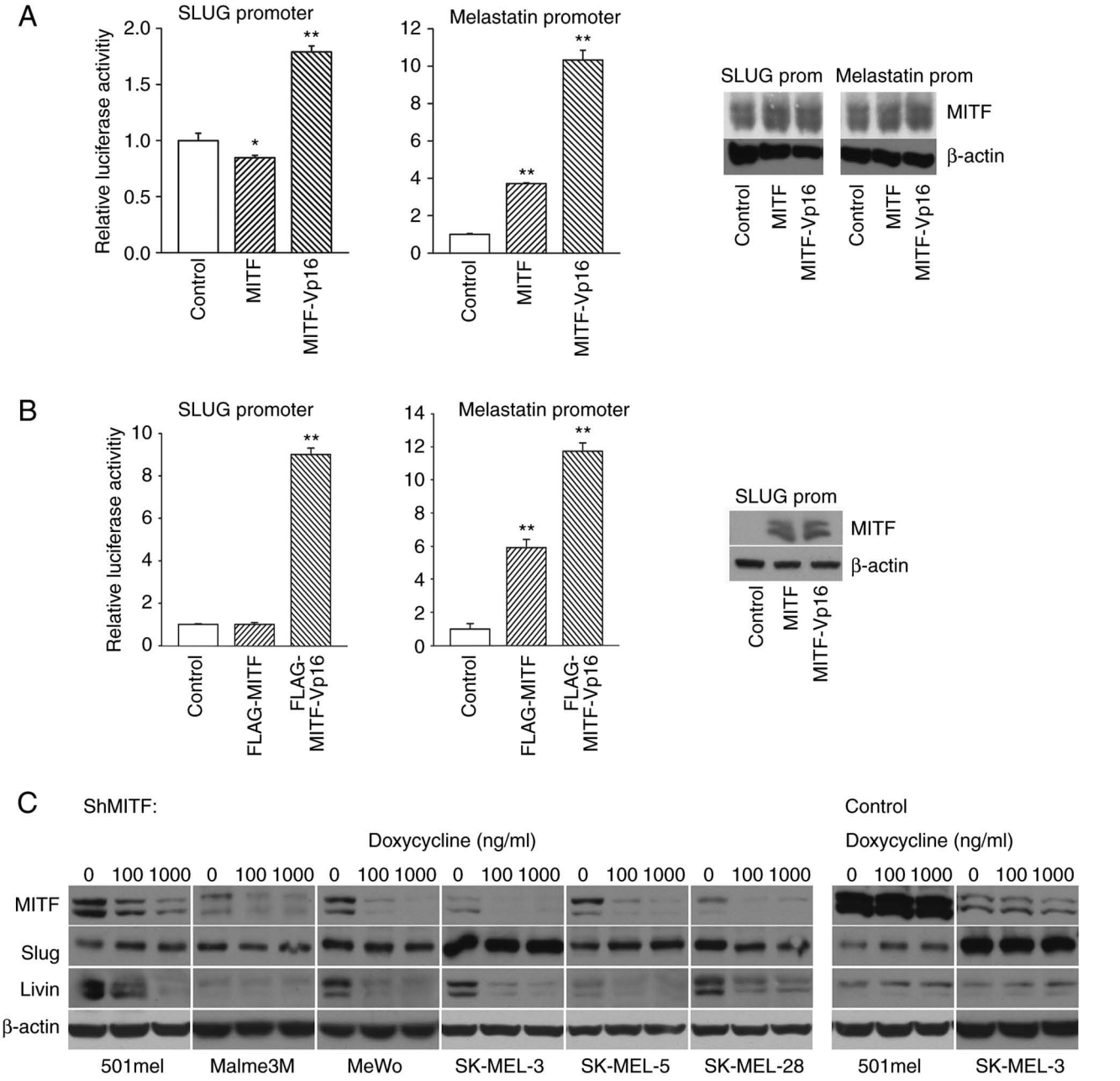

Figure 5. MITF is an imperfect activator of Slug in 501mel melanoma cells. (A) Left, *SLUG* promoter activity is slightly decreased by cotransfection of the MITF expression vector and activated ~twice by the MITF-Vp16 chimeric hyperactive MITF vector. Middle, the melastatin promoter is activated by both the MITF and MITF-Vp16 proteins. pcDNA3 plasmid served as a control. Three other melanoma cell lines likewise showed nonsignificant differences in the activity of the *SLUG* promoter compared with the control plasmid when cotransfected with the MITF vector (not shown). Right, western blotting shows MITF expression after transfection. The control sample also has a strong MITF signal because the endogenous protein level is already present in naive 501mel cells. (B) Left, *SLUG* promoter activity after cotransfection of FLAG-tagged versions of the same plasmids as shown in (A). Middle, stimulation of melastatin promoter activity. Empty pFLAG-CMV-4 plasmid served as a control. Statistical analysis was as described in Materials and methods and verified using one-way ANOVA followed by Tukey's post hoc test and equal results (P-values) were obtained. Right, Western blotting performed with the anti-FLAG antibody show expression of FLAG-tagged proteins, the same pattern of expression was seen after the transfection of melastatin promoter (not shown). (C) Western blots depicting inducible knockdown of MITF by tetracycline-regulatable shRNA repressing MITF. Concomitant expression of Slug and livin proteins was observed in the same samples, whereas no change in Slug protein levels was observed. Six melanoma cell lines (left panel) and two control lines (right panel) were analyzed. B-actin detection served as a loading control for whole cell lysates (bottom). This figure is reprinted as a part of Figure 3 from our previously published paper Vlčková K *et al*: Inducibly decreased MITF levels do not affect proliferation and phenotype switching but reduce differentiation of melanoma cells (J. Cell. Mol. Med. 22, 2018, 2240-2251) (32), with the permission of the publisher (Wiley Global Permissions, permissions@wiley.com). MITF, melanoma-associated transcription factor. *P<0.05, **P<0.01.

compared by immunohistochemical staining (Fig. 6). Positive staining by anti-GLI2 and anti-Slug antibodies was observed in the epidermis of normal skin (scored 2-3), consistent with previous observations (41,42). Only a few strongly MITF-positive (score 4) melanocytes were present in the epidermis. GLI2 and Slug were also stained in nevus cells, albeit somewhat less intensively (score 2, rare cells scored 3 in Slug staining). The nevus showed abundant MITF-positive cells (score 4). Epidermal keratinocytes were, as expected, MITF-negative in the skin and nevus sections (score 0). In metastatic

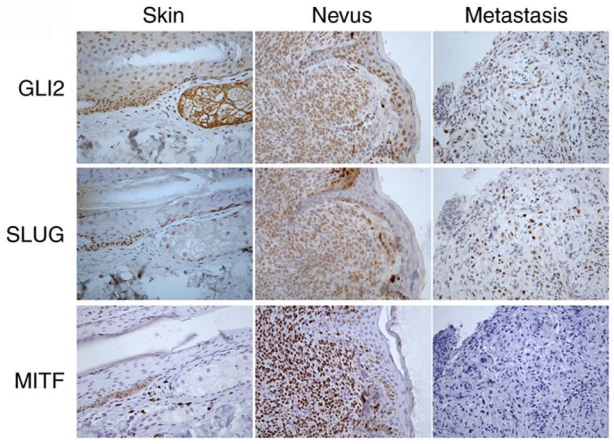

Figure 6. Slug and GLI2 expression correlate in serial sections of normal skin, nevus, and melanoma. Immunohistochemical staining was performed with antibodies against GLI2, Slug and MITF. For staining scores, see Results section (magnification, 400x). GLI, GLI family zinc finger; MITF, melanoma-associated transcription factor.

melanoma, both GLI2 and Slug staining was positive in ~half of the cell population (scored 2-4), with Slug staining slightly more positively than GLI2 staining. MITF-specific staining of metastatic melanoma was negative (score 0), with only a small number of positive cells (not shown in the picture). Sections that were negative or very slightly positive for MITF (scored 0-1) showed cells stained for both GLI2 and Slug (scores 2-3; Fig. 6). This is also consistent with the idea that invasive and metastatic cells have low or absent MITF (43). Notably, all three proteins were almost exclusively localized to the nucleus. Thus, Slug staining was associated with GLI2, but not MITF, positive staining in immunohistochemical sections of melanomas (Fig. 6). This result supports the aforementioned findings by demonstrating the positive regulation of Slug expression by GLI2, but not by MITF, in melanoma cells.

Discussion

The present study described the essential role of HH signaling and the transcription factor GLI2 in the transcription of the *SLUG* gene in human melanoma cells. The large number of GLI-binding sites present in the *SLUG* promoter led to the investigation of how GLI factors regulated *SLUG* gene expression. The presented data indicated that GLI factors activated the *SLUG* promoter in reporter assays and that the promoter was repressed by the HH signaling inhibitors GANT61 and cyclopamine. The most potent activator appeared to be GLI2. All GLI1-3 factors occupied the proximal *SLUG* promoter. Chromatin immunoprecipitation data revealed abundant and specific binding of GLI factors to four contiguous subregions of the proximal *SLUG* promoter. RT-qPCR, induced decrease of MITF and immunohistochemical experiments corroborated the foregoing data; GANT61 diminished Slug RNA abundance, decreased MITF and did not change Slug protein levels. In addition, the immunohistochemical analysis showed that MIFT-negative portions of metastatic melanomas contained Slug-positive and GLI2-positive cells. Given that an extremely high number of GLI-binding sites are present in the *SLUG* promoter, the possibility that Slug can also be upregulated by HH in other cancer cell types is worthy of further investigation. Of course, other transactivation mechanisms may also be plausible in different tumors beyond melanoma. For example, c-myb has been shown to regulate Slug expression in colon carcinoma, chronic myeloid leukemia, and neuroblastoma cells (44). C-myb-elicited EMT-like characteristics through *SLUG* gene activation in these cells. Homeobox C10 (HOXC10), which is a pro-oncogenic protein in cancer, has been shown to activate the *SLUG* promoter in melanoma through the YAP/TAZ signaling pathway (45). Das *et al* (46) reported that the oncogenic phenotype was induced by transcriptional upregulation of osteopontin through GLI1 in melanoma cells. Increased invasion, proliferation and migration was relieved by HH inhibitors. On the other hand, miR-33a-5p downregulates Slug in melanoma (47).

Previously, the essential role for HH signaling in melanoma has been demonstrated to occur mainly through the transcription factor GLI1 (6). Other signaling routes, such as RAS/MAPK and Akt/mTOR, regulate the nuclear localization of GLI1, not only in melanoma but also in other cancer cell types (6). Another report showed that GLI2 is capable of mediating the invasion and metastatic properties of melanoma (48). Furthermore, Slug is considered to be regulated by MITF, an essential transcription factor in melanoma (34). The present study showed that Slug expression depended mainly on the HH/GLI pathway. MITF probably does not serve any role in the endogenous expression of Slug because MITF downregulation had no effect on Slug protein levels in several melanoma cell lines. In the reporter assays, only the hyperactive MITF-Vp16 chimera, but not native MITF, activated the *SLUG* gene promoter (Fig. 5A).

It has been demonstrated that the oncogene *CRKL* is the downstream effector of GLI2 in lung adenocarcinoma cells (49). Crkl, which is oncogenic in several types of cancer, is activated by GLI2 through the binding of GLI2 to the site in the second intron of the *CRKL* gene. Blunting Crkl by shRNA or CRISPR-Cas9 knockout weakens the GLI2-elicited positive effect on cell viability, migration, invasion and colony formation. Thus, in lung adenocarcinoma, Crkl appears to be necessary for pro-oncogenic GLI2 effects and is itself regarded as an oncogene. For example, Crkl attenuates the therapeutic effect of several antioncogenic drugs (49) and has been found to be amplified in lung adenocarcinoma (50). It remains to be investigated whether Crkl is a general mediator of GLI factor activity in other tumor cell types. Although CRKL has been found to be amplified specifically in acral melanomas (51), no other data are available about the melanoma-specific role of the Crkl protein.

HH pathway and GLI factors are highly oncogenic and known to substantially contribute to the maintenance of CSC. In addition, GLIs are observed to be associated with EMT in various types of cancer (52-54). On the other hand, little data are available that GLI factors could be directly involved in the transcription of typical EMT-associated genes. In melanoma, GLI1 and GLI2 are reported to enhance transcription of Sox2, a stem cell marker (10). Furthermore, it was previously observed that EMT process is atypical in melanoma (55), as suggested in the present study. The present study clearly defined that GLIs, especially GLI2, are direct transcriptional regulators of Slug, a hallmark protein of EMT, in melanoma cells.

In melanoma, therapy is predominantly focused on targeting the mutated driver oncogenes BRAF and NRAS and/or kinases in the downstream MAPK signaling pathway. Unfortunately, therapies for advanced melanoma with low molecular weight inhibitors targeting these proteins result in acquired resistance. Despite advances in using a combination of drugs, the concept of targeting only the MAPK route remains questionable. As there are multiple mechanisms responsible for resistance to the inhibition of MAPK signaling in melanoma (56,57), alternative therapies should also be considered. Targeting HH and Bcl2 protein by the combination of GANT61 and obatoclax was effective in most melanoma cell lines tested previously (58). Taken together, the present study described a new mechanism of Slug transcription. It stressed the importance of the HH pathway for melanoma progression and suggested that targeting GLI2 in combination therapies could be beneficial for treatment, as GLI2 is a recognized transcriptional activator of a number of oncogenic proteins, including survivin (31). Although other mechanisms may play a role in Slug regulation in various types of cancer, the present study demonstrated that Slug is another HH/GLI target.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JV and PH conceived the project. JV and PH confirm the authenticity of all the raw data. PH, JR, KK, LO, JV and PZ performed the experiments. JB and JV Jr. performed the immunohistochemical experiments. PH and KK performed statistical analyses. PH and KK prepared the figures. PH and JV wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

Immunohistochemical analysis of paraffin sections of human tissues was approved by the Ethical Committee of the University Hospital Motol, Prague (approval no. EK-36/20). The study was carried out in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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