HEDGEHOG/GLI-E2F1 axis modulates iASPP expression and function and regulates melanoma cell growth

S Pandolfi1,4, V Montagnani1,4, A Lapucci2 and B Stecca1,3

HEDGEHOG (HH) signaling is a key regulator of tissue development and its aberrant activation is involved in several cancer types, including melanoma. We and others have shown a reciprocal cross talk between HH signaling and p53, whose function is often impaired in melanoma. Here we present evidence that both GLI1 and GLI2, the final effectors of HH signaling, regulate the transcription factor E2F1 in melanoma cells, by binding to a functional non-canonical GLI consensus sequence. Consistently, we find a significant correlation between E2F1 and PATCHED1 (PTCH1), GLI1 and GLI2 expression in human melanomas. Functionally, we find that E2F1 is a crucial mediator of HH signaling and it is required for melanoma cell proliferation and xenograft growth induced by activation of the HH pathway. Interestingly, we present evidence that the HH/GLI-E2F1 axis positively modulates the inhibitor of apoptosis-stimulating protein of p53 (iASPP) at multiple levels. HH activation induces iASPP expression through E2F1, which directly binds to IASPP promoter. HH pathway also contributes to iASPP function, by the induction of Cyclin B1 and by the E2F1-dependent regulation of CDK1, which are both involved in iASPP activation. Our data show that activation of HH signaling enhances proliferation in presence of E2F1 and promotes apoptosis in its absence or upon CDK1 inhibition, suggesting that E2F1/iASPP dictates the outcome of HH signaling in melanoma. Together, these findings identify a novel HH/GLI-E2F1-iASPP axis that regulates melanoma cell growth and survival, providing an additional mechanism through which HH signaling restrains p53 proapoptotic function.

Cell Death and Differentiation (2015) 22, 2006–2019; doi:10.1038/cdd.2015.56; published online 29 May 2015

Hedgehog (HH) signaling is a conserved pathway that directs embryonic patterning through the temporal and spatial regulation of cellular proliferation and differentiation.1,2 During development, the loss of HH signaling results in severe abnormalities in mice and humans.3-5 In the adult it is mostly active in stem/progenitor cells, where it regulates tissue homeostasis, repair and regeneration.6 Conversely, unrestricted HH pathway activation is implicated in a variety of tumors, including those of the skin.7,8 Secreted HH ligands trigger downstream signaling by binding to the transmembrane receptor Patched (PTCH1). PTCH1 relieves its inhibition on the G protein-coupled receptor Smoothened (SMO), which triggers an intracellular signaling cascade regulating the formation of the zinc finger transcription factors GLI2 and GLI3 and their translocation into the nucleus.9,10 Both GLI1 and GLI2 act as main mediators of HH signaling in cancer by directly controlling the transcription of target genes, several of which are involved in proliferation.11,12

Cutaneous melanoma arises from malignant transformation of melanocytes and is the most aggressive form of skin cancer, with poor prognosis in late stages.13 In contrast to other tumors, >80% of melanomas retain wild-type (wt) p53.14,15 Nevertheless, p53 tumor-suppressor activity is impaired by various mechanisms, including the deletion of the CDKN2A locus16,17 or MDM2 and MDMX overexpression.18-21 Recently, the inhibitor of apoptosis-stimulating protein of p53 (iASPP),22,23 which is frequently upregulated in human cancers,24-29 has been proposed to hamper p53 function in melanoma.21

HH pathway is often activated in human melanoma, where it is required for proliferation and survival both in vitro and in vivo30-32 and it drives self-renewal and tumorigenicity of melanoma cancer stem cells (CSCs).33 Previous data indicate a reciprocal cross talk between HH signaling and p53. Activation of the HH pathway impairs p53 function by increasing MDM2 levels.34 In turn, p53 inhibits GLI1 levels, nuclear localization and transcriptional activity35 and, upon DNA damage, promotes GLI1 degradation through PCAF induction.36

Restoration of wt p53 function is an attractive therapeutic approach for melanoma, and recent evidence support E2F1 as a biomarker to predict the outcome of the treatment with inhibitors of MDM2/p53 interaction.37 E2F1 is a key transcriptional regulator of proliferation38-40 whose aberrant activation

1Laboratory of Tumor Cell Biology, Core Research Laboratory–Istituto Toscana Tumori (CRL-ITT), Florence, Italy; 2Department of Health Sciences, Section of Clinical Pharmacology and Oncology, University of Florence, Florence, Italy and 3Department of Oncology, Azienda Ospedaliero-Universitaria Careggi, Florence, Italy
4These authors contributed equally to this work.

Abbreviations: HH, Hedgehog; PTCH1, Patched1; SMO, Smoothened; iASPP, inhibitor of apoptosis-stimulating protein of p53; CDK1, cyclin-dependent kinase 1; PI3K, p53-inducible gene 3; p53AIP1, p53-regulated apoptosis-inducing protein 1; BCL2, B-cell lymphoma 2; BCL-XL, B-cell lymphoma extra-large; CSC, cancer stem cells; GLI-BS, GLI-binding site; shRNA, short hairpin RNA; EMSA, electrophoretic mobility shift assay; JNJ, JNJ-7706621.

Received 08.10.14; revised 26.3.15; accepted 08.4.15; Edited by B Zhivotovsky; published online 29.5.15
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Results
HH signaling modulates E2F1 expression in melanoma cells. To investigate whether HH pathway modulates E2F1 expression in melanoma, we inhibited HH signaling by SMO silencing, transducing patient-derived SSM2c and M26c, and commercial A375 melanoma cells with a replication-incompetent lentivirus expressing a short interference RNA targeting SMO (LV-shSMO). Quantitative real-time PCR (qPCR) analysis showed strong reduction of mRNA levels of SMO and of the two HH targets GLI1 and PTCH1, confirming the inhibition of HH signaling (Figure 1a). Notably, SMO silencing decreased E2F1 mRNA and protein levels compared with the control (Figures 1a and b and Supplementary Figure 1a) in all three melanoma cells. In addition, GLI2 silencing (LV-shGLI2) reduced E2F1 mRNA levels in A375 cells, which express high levels of GLI2 (Supplementary Figures 1b and c and Supplementary Figure 2a). Conversely, activation of the HH pathway by silencing the negative regulator PTCH1 (LV-shPTCH1; ref. 35) increased GLI1 and E2F1 mRNA levels (Figure 1c). Transfection of Myc-tagged GLI1 or GLI2 increased the endogenous E2F1 protein in SSM2c and M26c cells. To test whether E2F1 transactivated all the three GLI promoters, we performed chromatin immunoprecipitation (ChIP) assay in HH signaling competent murine NIH3T3 cells (Figures 1d and e). As expected, E2F1 transactivated the reporter by GLI1 and GLI2 in both SSM2c and M26c cells, whereas the mutation of the site2 (Mut2) did not have any effect (Figure 2e). Electrophoretic mobility shift assay (EMSA) in HH signaling competent murine NIH3T3 cells showed the direct binding of Gli1/2 to site1. The activation of the HH pathway by stimulation with SMO agonist SAG led to the formation of a DNA-binding complex, which was competed away by unlabeled probe. Preincubation of cell extracts with anti-GLI1 or anti-GLI2 antibodies decreased the formation of this DNA complex, suggesting the presence of GLI1 and GLI2 proteins (Figure 2f). Western blot analysis confirmed the activation of the HH signaling upon SAG treatment (Figure 2g). Together these results indicate that GLI1 and GLI2 directly interact with the E2F1 promoter by binding to a non-canonical GLI consensus sequence (CGCCTCCAG).

GLI1 and GLI2 bind to a non-canonical GLI consensus sequence at E2F1 promoter. To test whether E2F1 expression was directly regulated by GLI1 or GLI2, we performed chromatin immunoprecipitation (ChIP) assay in M26c, SSM2c and A375 cells in which HH pathway was activated by PTCH1 silencing. qPCR analysis on the immunoprecipitated DNA showed that both endogenous GLI1 and GLI2 bound to E2F1 promoter (Figure 2a and Supplementary Figure 3). The activation of HH pathway resulted in increased GLI1 binding to PTCH1 promoter (used as positive control) in M26c and SSM2c cells, and increased GLI2 binding in A375 cells. The binding of GLI1 and GLI2 to E2F1 promoter was not further increased by the HH pathway activation, and it was stronger than that to PTCH1 promoter. Nevertheless, ChIP experiment showed that the activation of HH signaling increased E2F1 binding to its promoter in the proximity of a conserved E2F-binding site (Supplementary Figure 4). This suggests that the activation of the HH pathway increases E2F1 levels, which could then contribute to sustain the transactivation of its own promoter, as already shown. Bioinformatic analysis did not identify any canonical GLI consensus sequence (GACCACCCA) in the E2F1 promoter. However, GLI transcription factors are able to bind to variant GLI-binding sites (GLI-BSs) with relatively low affinity, still leading to strong transcriptional activation. We thus speculated that the E2F1 promoter might contain a degenerate GLI-BS. To identify the region responsible for the modulation by GLI transcription factors, we cloned three different fragments of the E2F1 promoter upstream of the luciferase gene (−132, −269 and −656 bp; Figure 2c). We transfected SSM2c and M26c cells with the reporter vector along with GLI1, GLI2 or E2F1, the latter used as positive control because it binds to the E2F1 promoter close to the transcription start site. The assay showed that both GLI1 and GLI2 transactivated the reporter driven by the −269 and −656 bp fragments, but not by the −132 bp fragment (Figure 2c). As expected, E2F1 transactivated all the three regions (Figure 2c). These results indicate the presence of a functional GLI-BS between −132 and −269 bp upstream the E2F1 transcription start site.

To precisely map the GLI-BS, we identified two putative degenerated GLI consensus sequences (Figure 2d; site1 and site2) and we mutated each of them in two positions crucial for the efficiency of GLI-binding. Reporter assay showed that the disruption of the site1 (Mut1) prevented the transactivation of the reporter by GLI1 and GLI2 in both SSM2c and M26c cells, whereas the mutation of the site2 (Mut2) did not have any effect (Figure 2e). Electrophoretic mobility shift assay (EMSA) in HH signaling competent murine NIH3T3 cells showed the direct binding of Gli1/2 to site1. The activation of the HH pathway by stimulation with SMO agonist SAG led to the formation of a DNA-binding complex, which was competed away by unlabeled probe. Preincubation of cell extracts with anti-GLI1 or anti-GLI2 antibodies decreased the formation of this DNA complex, suggesting the presence of GLI1 and GLI2 proteins (Figure 2f). Western blot analysis confirmed the activation of the HH signaling upon SAG treatment (Figure 2g). Altogether these results indicate that GLI1 and GLI2 directly interact with the E2F1 promoter by binding to a non-canonical GLI consensus sequence (CGCCTCCAG).

E2F1 dictates the outcome of HH pathway activation in melanoma cells. Our data indicate that in melanoma cells HH signaling directly controls the expression of E2F1, a key transcriptional regulator of proliferation. We and others have previously shown that HH signaling is required for melanoma growth and stemness. To investigate the effects of the HH pathway that are mediated by E2F1 in melanoma, we knocked down E2F1 after the activation of the
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HH signaling. We silenced E2F1 by transducing cells with LV-shE2F1-1 and LV-shE2F1-2 lentiviruses, which strongly reduced E2F1 mRNA and protein levels (Supplementary Figures 5a and b). To investigate the presence of possible compensatory effects by other E2F members upon E2F1 silencing, we measured the expression of the activator E2F members E2F2, E2F3A and the repressor member E2F4. No significant differences were detected in E2F2, E2F3A and E2F4 expression (Supplementary Figures 5c and d) in two melanoma cells. To activate HH signaling, we silenced PTCH1, thus mimicking the canonical pathway activation. M26c, SSM2c and A375 cells were transduced with LV-shPTCH1 or LV-shE2F1, alone or in combination. qPCR and western blot analysis showed an increase of GLI1, GLI2 and E2F1 expression upon PTCH1 silencing, confirming the activation of the HH pathway and a strong decrease of E2F1 upon E2F1 silencing (Figure 3a and Supplementary Figure 6). To investigate the effect of E2F1 in mediating HH-induced cell growth, cells transduced as described above were seeded and allowed to grow for 7 days. In all melanoma cell lines, LV-shPTCH1 increased the cell number compared with LV-c, whereas LV-shE2F1 reduced it. Interestingly, in cells transfected with both LV-shPTCH1 and LV-shE2F1 the reduction of cell growth was greater than in cells transduced with LV-shE2F1 alone (Figure 3b).

Cytometric analysis of proliferation index (PI) showed that HH pathway activation increased cell proliferation, as expected. E2F1 silencing by itself did not significantly affect PI, but it markedly decreased the effect of LV-shPTCH1 (Supplementary Figures 7a and c). Cell cycle distribution did not vary, although LV-shPTCH1/LV-shE2F1-transduced cells presented a significant increase in the population with subG0 DNA content, corresponding to the apoptotic fraction (Supplementary Figures 7b, d and e). Annexin V-PE/7-AAD labeling showed a consistent increase in the percentage of cells in late apoptosis when E2F1 was silenced in combination with HH signaling activation (Figure 3c). The apoptotic response observed in LV-shPTCH1/LV-shE2F1-transduced cells was consistent with the increased expression of the proapoptotic p53-target genes PIG3 and p53AIP1, and decreased levels of the antiapoptotic factors BCL- XL and BCL2 (Figures 3d and e and Supplementary Figure 8), suggesting the involvement of p53 in controlling cell death. Altogether these data indicate that E2F1 is critical to sustain melanoma cell growth induced by the activation of the HH signaling and suggest that E2F1 controls the balance between cell proliferation and apoptosis upon HH pathway activation.

**HH signaling regulates iASPP expression and function through E2F1.** Recent evidence indicate that melanomas harboring wt p53 express high levels of phosphorylated iASPP which functionally inactivates p53. Little is known about the regulation of iASPP expression, therefore we sought to investigate whether it might be affected by the HH signaling. qPCR analysis showed that in M26c, SSM2c and A375 cells inhibition of HH pathway by SMO silencing produced a decrease of iASPP mRNA (Figure 4a). Conversely, activation of the HH signaling by PTCH1 silencing resulted in iASPP induction (Figure 4b). Two different antibodies targeting the N- or C-terminal regions of iASPP, both validated by iASPP silencing (Supplementary Figure 9), were used to assess the impact of HH pathway activation on iASPP protein. Although we were not able to clearly detect a slow-migrating iASPP that could be compatible with the Cycin B1/CDK1-phosphorylated form, western blot analysis showed that PTCH1 depletion increased iASPP protein level, confirming qPCR results (Figures 4c and 5c). Because iASPP inhibits p53 function by interacting with it,21 we assessed whether activation of the HH pathway might affect iASPP-p53 interaction. Indeed, in M26c and SSM2c cells transduced with LV-shPTCH1, iASPP co-immunoprecipitated p53 more efficiently than in control cells (Figure 4c), suggesting that HH signaling activation increases iASPP-p53 binding, contributing to restrain p53 function through iASPP.

Because PTCH1 silencing, combined with E2F1 depletion, produced a marked apoptotic response with the upregulation of p53-target genes (Figures 3c, d and e and Supplementary Figure 8), we speculated that E2F1 might mediate iASPP induction upon HH signaling activation. Interrogation of publicly available databases revealed the presence of a conserved E2F-binding site within iASPP promoter (Supplementary Figure 10a). Indeed, ChIP analysis showed that E2F1 bound to iASPP promoter and that its binding was greatly increased by HH pathway activation (Figure 5a and Supplementary Figure 10b). To assess whether E2F1 mediates iASPP induction upon HH signaling activation, we performed western blot and qPCR analyses in melanoma cells transduced with LV-shPTCH1 and/or LV-shE2F1. PTCH1 silencing induced iASPP levels in an E2F1-dependent manner, because E2F1 depletion abolished the effect of HH pathway activation (Figures 5b and c and Supplementary Figure 11). Altogether these data indicate that HH signaling modulates iASPP expression through E2F1.

The ability of iASPP to interfere with p53 function depends mainly on its phosphorylation by CDK1/Cyclin B1 complex.21 We thus investigated whether HH pathway might affect also CDK1 or Cyclin B1 levels. Western blot analysis on melanoma cells transduced with LV-shPTCH1 and/or LV-shE2F1 showed that CDK1 expression was completely abolished upon E2F1 silencing (Figure 5c), consistently with a previous report...
indicating that E2F1 regulates CDK1 expression.\textsuperscript{53} ChIP analysis revealed a strong enrichment of E2F1 on the CDK1 promoter (Figure 5d and Supplementary Figure 12), supporting a direct regulation,\textsuperscript{54} and the binding was further increased upon HH pathway activation. PTCH1 depletion also increased Cyclin B1 levels, as previously reported\textsuperscript{55,56} and this increase was independent of E2F1, because it occurred also in cells transduced with LV-shPTCH1/LV-shE2F1 (Figure 5c). These data indicate that HH signaling contributes to both iASPP induction, through E2F1, and to iASPP function, via Cyclin B1 and E2F1-dependent regulation of CDK1.

HH signaling contributes to iASPP function through E2F1-dependent regulation of CDK1 (Figure 5). Therefore we speculated that the inhibition of CDK1 activity with the CDK1 inhibitor JNJ-7706621 (JNJ, 500 nM),\textsuperscript{57} which was recently shown to block iASPP phosphorylation,\textsuperscript{21} would mimic the effect of E2F1 depletion. JNJ treatment markedly reduced the ability of iASPP to co-immunoprecipitate p53 (Figure 6a), confirming the efficacy of CDK1 inhibition. Growth assay showed that JNJ treatment produced a stronger reduction in the cell number of melanoma cells with activated HH pathway compared with the control (Figure 6b), paralleling the results obtained by the E2F1 silencing (Figure 3b). Cytometric analysis revealed that cells with activated HH signaling were more sensitive to CDK1 inhibition than the control cells, undergoing increased apoptosis and cell death upon JNJ treatment (Figures 6c and d). Altogether these data show that the inhibition of CDK1 activity produces the similar increase of apoptosis as E2F1 depletion in cells with activated HH pathway, suggesting that E2F1 determines the outcome of HH signaling activation by controlling not only iASPP expression but also its function through CDK1.

Depletion of E2F1 suppresses HH-mediated melanoma xenograft growth. To investigate whether E2F1 regulates HH-induced growth of melanoma xenografts in vivo, M26c cells stably transduced with LV-c or LV-shPTCH1 and/or LV-shE2F1 were engrafted subcutaneously into athymic-nude mice. Activation of HH signaling resulted in almost two-fold larger xenografts than the control cells (Figures 7a, b and c), confirming that the activation of HH pathway increases melanoma cell growth \textit{in vivo}.\textsuperscript{33,58} E2F1 silencing reduced of about 50% the size of melanoma xenografts compared with LV-c and strongly reduced the increase of tumor growth induced by PTCH1 silencing (Figures 7a and c). Western blot analysis in tumors dissected 42 days after injection confirmed the increase of E2F1 levels induced by HH pathway activation and its reduction upon E2F1 silencing (Figure 7d), consistently with the tumor growth curve. The difference in xenograft growth between LV-shE2F1 and LV-shPTCH1/LV-shE2F1 was not significant as we documented \textit{in vitro}. To explain this result, we investigated whether E2F1 depletion in a context of HH activation might affect the self-renewal of melanoma CSC grown as spheres.\textsuperscript{33,59} We found that E2F1 silencing markedly reduced the ability of melanoma CSCs to form secondary spheres, suggesting that it might be involved in controlling stemness. The effect of HH pathway activation was reduced by E2F1 silencing to the level of LV-c. However, even in absence of E2F1, PTCH1 silencing was still able to increase CSC self-renewal, suggesting that the regulation of melanoma stemness by HH signaling is only partially dependent on E2F1 (Supplementary Figure 13). Nevertheless, western blot analysis on xenografts showed the same iASPP, Cyclin B1 and CDK1 pattern obtained \textit{in vitro} (Figures 4d and 5c), suggesting that the HH/GLI-E2F1-iASPP axis contributes to regulate the melanoma cell growth \textit{in vivo}.

**Discussion**

Here we define a novel HH/GLI-E2F1-iASPP axis that regulates melanoma cell growth and identify E2F1 as a central player in determining the outcome of HH signaling, by controlling the levels and function of the p53 inhibitor iASPP (Figure 8).

Previous data indicated that E2F1 is induced in response to SHH stimulation\textsuperscript{44,45} and by GLI2 overexpression.\textsuperscript{46} Nevertheless, there are no evidence indicating a direct regulation. Here we show that the HH pathway directly regulates E2F1 expression in melanoma cells and that GLI1 and GLI2 bind to and transactivate the E2F1 promoter. Although bioinformatic analysis did not find any canonical GLI consensus sequence in E2F1 promoter, we identified a functional non-canonical GLI-Bs (CGCCTCCAG) by site-directed mutagenesis. This

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**Figure 2** GLI1 and GLI2 bind to E2F1 promoter. (a) Chromatin immunoprecipitation (ChIP) assay showing that both endogenous GLI1 and GLI2 bind to PTCH1 and E2F1 promoters in M26c cells transduced with LV-c or LV-shPTCH1. The y-axis represents the relative promoter enrichment, normalized on the input material. ACTIN promoter was used as negative control and set to 1. (b) Consensus GLI DNA-binding motif calculated from experimentally validated GLI-binding sites\textsuperscript{56} using WebLogo3.\textsuperscript{65} Positions 4C and 6C are essential for DNA binding, whereas all the other positions allow a certain degree of sequence variation. (c) Quantification of dual-luciferase reporter assay in SSM2c and M26c cells showing that GLI1 and GLI2 transactivate E2F1 promoter in a region between −132 bp and −269 bp from the transcription start site. Three fragments of different length (−132, −269 and −656 bp) of E2F1 promoter were assayed for their ability to be transactivated by GLI1 or GLI2; E2F1 was used as a positive control. The red and green arrows mark the positions of the primers used in ChIP, described in Supplementary Figure 4. Relative luciferase activities were firefly/Renilla ratios, with the level induced by control equated to 1. The data represent mean ± S.E.M. of at least five independent experiments. \( P \) values are as follows: −656 bp: \( P = 0.0214 \), SSM2c; \( P = 0.0071 \), M26c; CTR versus GLI1, \( P = 0.0011 \) in SSM2c; \( P = 0.0008 \) in M26c; CTR versus GLI2, \( P = 0.0013 \) in SSM2c; −269 bp: CTR versus GLI1, \( P < 0.001 \) in SSM2c; \( P = 0.0183 \) in M26c; CTR versus GLI2, \( P < 0.001 \) SSM2c; \( P = 0.0006 \) M26c; CTR versus E2F1, \( P < 0.004 \) in −132, −269 and −656 bp, in both the cells. (d) Putative GLI-binding sites (site1 and site2) in the −269 bp E2F2 promoter that were mutagenized (Mut1 and Mut2). Note that both the positions essential for DNA binding (4C and 6C) were mutagenized. (e) Quantification of dual-luciferase reporter assay in SSM2c and M26c cells showing that mutation of Mut1, but not Mut2 site, prevented GLI1 and GLI2 from transactivating the −269 bp fragment of the E2F1 promoter. Relative luciferase activities were firefly/Renilla ratios, with the level induced by the control equal to 1. Data represent mean ± S.E.M. of at least five independent experiments. (f) Electrophoretic mobility shift assays of site1 in E2F1 promoter. In the right panel the arrow indicates the specific DNA complex that appears upon SAG treatment in NIH3T3 cells. Competition was carried out using a 100-fold excess of unlabeled site1 oligonucleotide. Disruption of the complex is achieved with preincubation of the WCE with anti-Gli1 and anti-Gli2 antibodies. WCE, whole-cell extract; 100x comp, unlabeled site1 competitor. (g) Western blot analysis showing the increase of endogenous GlI1 and GlI2 proteins upon SAG treatment (100 nM, 48 h) in NIH3T3 cells. Hsp90 served as loading control.
site matches the minimal GLI-BS requirements and indeed mutation of two essential cytosines in the 4th and 6th position completely abolishes the ability of GLI1 and GLI2 to transactivate the E2F1 promoter. Despite the divergence from the canonical GLI-BS, EMSA analysis confirmed the binding of GLI1/2 to this site, in agreement with the reporter assay. The biological relevance of this modulation is supported by a significant positive correlation between the expression of E2F1 and of the HH pathway components (GLI1, GLI2 and PTCH1) in human melanomas (Figure 1f) and in a set of

**Figure 1:**

- **a:** ChIP analysis showing relative promoter enrichment for GLI1 and GLI2 in LV-c and LV-shPTCH1 conditions.
- **b:** EMSA analysis with the GACCACCGGGCA binding site sequence.
- **c:** Luciferase reporter assay for E2F1 promoter sites -656/-1, -269/-1, and -132/-1 with GLI1 and GLI2 expression.
- **d:** Schematic representation of E2F1 promoter sites and luciferase activity with WT and Mut1/2 conditions.
- **e:** Further luciferase activity analysis with SSM2c and M26c constructs.
- **f:** Western blot analysis for GLI1 and GLI2 in NIH3T3 cells with SAG treatment.
- **g:** Cellular expression of GLI1 and Hsp90 in NIH3T3 cells with and without SAG treatment.
Figure 3  E2F1 is required for the increase in melanoma cell growth induced by activation of the HH signaling. (a) Western blot analysis of GLI1, GLI2 and E2F1 in M26c, SSM2c and A375 cells transduced with the indicated lentiviruses. HSP90 served as loading control. (b) Growth curve in M26c, SSM2c and A375 cells transduced with the indicated lentiviruses, showing that E2F1 silencing abolishes melanoma cell growth induced by activation of the HH pathway. The data represent mean ± S.E.M. of three independent experiments. *P < 0.05. (c) Annexin V-PE/7-AAD labeling of M26c, SSM2c and A375 cells transduced with the indicated lentiviruses, showing a marked increase of the late-apoptotic fraction in cells transduced with LV-shE2F1 and with LV-shPTCH1/LV-shE2F1 lentiviruses compared with the control (LV-c). No differences in the early apoptosis are observed. The data represent mean ± S.E.M. of three independent experiments. *P < 0.05. (d) Quantitative PCR analysis of BCL-XL, PIG3 and p53AIP1 expression in M26c cells transduced with the indicated lentiviruses. The y-axis represents expression ratio of gene/(EIF2α+HPRT average). Data represent mean ± S.E.M. of three independent experiments. *P < 0.05. (e) Western blot analysis of BCL2 and p53 in M26c, SSM2c and A375 cells transduced with the indicated lentiviruses. HSP90 served as loading control.
commercial and primary melanoma cells (Supplementary Figure 2).

We previously showed that HH signaling regulates proliferation and survival of human melanoma and melanoma CSCs.30,33 E2F1 is a key regulator of cell cycle and survival and it is often upregulated or hyperactivated in a variety of tumors, including melanoma.41,42 Our data confirm the role of HH pathway and E2F1 in regulating melanoma cell growth. Interestingly, the activation of HH signaling in the absence of E2F1 in vitro produces a marked apoptotic response and reduces melanoma growth more than E2F1 depletion alone. Multiple evidence indicate that activation of HH signaling leads to the inhibition of the tumor-suppressor p53,34,35 which is itself a negative modulator of GLI1.35 Although HH activation promotes cell proliferation, enhanced GLI1 levels are associated with markers of DNA replication stress.35 As a consequence, the inhibition of p53 function contributes both to fuel the HH activation and to blunt the stress response.35 Here we identify the HH/GLI-E2F1-iASPP axis as a novel mechanism of p53 inhibition in response to HH signaling. In this context, E2F1 plays a crucial role in determining the balance between proliferation and apoptosis in response to HH pathway activation through the regulation of iASPP.

Although iASPP is overexpressed in a variety of human cancers,24–29 little is known about the regulation of its expression. Here we find that the activation of HH signaling increases iASPP expression in melanoma cells. The induction is mediated by E2F1, which binds to the iASPP promoter in a region closed to a conserved E2F-binding site; notably, the activation of HH signaling increases E2F1 binding to iASPP promoter, consistently with the presence of a HH-E2F1-iASPP axis. A previous report showed that the ability of iASPP to inhibit p53 depends on its phosphorylation by Cyclin B1/CDK1 complex.35 Our data indicate that HH signaling contributes to...
the regulation of iASPP function also at post-translational level, by controlling both the Cyclin B1 and CDK1 levels and favouring its interaction with p53. Activation of HH pathway increases Cyclin B1 expression, and this modulation is independent from E2F1. In addition, HH signaling regulates CDK1 expression through E2F1, which increases its binding to CDK1 promoter upon HH signaling activation (Figure 5).

We show that E2F1 is an important mediator of HH signaling in vivo, because its depletion abolishes the increase in tumor growth induced by the activation of the HH pathway. We do not find significant difference between LS-hE2F1 and LS-shPTCH1 xenografts. This is likely due to other downstream effectors of HH pathway that may favor CSC self-renewal independently from E2F1, thus contributing to tumor growth, as suggested by the self-renewal experiments. Nevertheless, as a confirmation of the relevance of our findings, western blot analysis on xenografts reproduces a similar pattern of iASPP, Cyclin B1 and CDK1 expression observed in vitro.

Reactivation of the endogenous wt p53 has been proposed as a parallel strategy along with targeted therapy to suppress melanoma growth more efficiently. The HH/GLI-E2F1-iASPP axis (Figure 8) described in this study provides novel targets for the therapeutic intervention for melanomas and possibly other tumors harboring wt p53 and with activated HH pathway. Our data show that E2F1 depletion in the context of activated HH pathway restores p53 apoptotic function. The fact that treatment with JNJ-7706621, a CDK1 inhibitor that prevents iASPP activation, produces the same effect in terms of cell growth and apoptosis as E2F1 silencing, confirms the relevance of the HH-E2F1-iASPP axis we show in this study. Therefore, blockade of iASPP activity through interference with E2F1 function or by CDK1 inhibition, is predicted to restore the tumor-suppressive effects of p53, turning the

Figure 5 E2F1 mediates the modulation of iASPP by HH pathway in melanoma cells. (a) Chromatin immunoprecipitation (ChIP) assay showing that endogenous E2F1 binds to iASPP promoter in M26c and SSM2c cells transduced with LV-c or LV-shPTCH1. The y-axis represents the relative promoter enrichment, normalized on input material. ACTIN promoter was used as negative control and set to 1. (b) Quantitative PCR analysis of iASPP expression in M26c cells transduced with the indicated lentiviruses. The y-axis represents the expression ratio of gene/(Elo2a+HPRT average). Data represent mean ± S.E.M. of three independent experiments. *P < 0.05. (c) Western blot analysis of iASPP, CDK1 and Cyclin B1 in M26c, SSM2c and A375 cells transduced with indicated lentiviruses. iASPP was detected using an N-terminal Ab in M26c cells and a C-terminal Ab in SSM2c and A375 cells. HSP90 served as loading control. (d) ChIP assay showing that endogenous E2F1 binds to CDK1 promoter in M26c and SSM2c cells transduced with LV-c or LV-shPTCH1 lentiviruses. The y-axis represents the relative promoter enrichment, normalized on input material. ACTIN promoter was used as negative control and set to 1.
proliferative activity of HH signaling into an apoptotic response.

Materials and methods

Patient samples and cell cultures. HEK-293T (CRL-11268), NIH3T3 (CRL-1658) and A375 (CRL-1619) cell lines were obtained from ATCC (Manassas, VA, USA). SSM2c and M26c patient-derived melanoma cells were previously described.33,58 Human melanoma specimens (Supplementary Table 1) were obtained after approved protocols by the local Ethics Committee. In brief, fresh tissue samples were digested enzymatically using 1 mg/ml collagenase A and 20 μg/ml DNase I (Roche Diagnostic, Mannheim, Germany) and grown in DMEM supplemented with 10% fetal bovine serum, 1% Penicillin-streptomycin, 2 mM L-Glutamine (Lonza, Basel, Switzerland). The identity of melanoma cells was verified by immunocytochemistry using anti-Melan A, anti-S100 and anti-Vimentin antibodies, as previously described.33 Mycoplasma was periodically tested by 4',6-diamidino-2-phenylindole inspection and PCR. Direct sequencing revealed that both SSM2c and M26c cells harbor wt p53 with codon 72 Proline polymorphism.58 A375
melanoma cells harbor wt p53. For growth curve 3000 cells/well were plated in 12-well plates and counted on days 3–5–7. For growth assay after JNJ treatment 15 000 cells/well were plated in 12-well plates and treated with the CDK1 inhibitor JNJ-7706621 (500 nM, JNJ; Merck, Damstadt, Germany) for 72 h. Puromycin was used at 2 μg/ml to select for transduced cells.

Plasmids, cloning, mutagenesis and lentiviral vectors. Vectors used for overexpression were: pCS2+MT (Addgene, Cambridge, MA, USA), pCMV-E2F1 (kind gift from M. Chiariello), 63 Myc-tagged human GLI1 (pCS2+MT-GLI1, kind gift from A. Ruiz i Altaba) 35 and GLI2 (pCS2+MT-GLI2, Addgene). 5 Three fragments of E2F1 promoter (−132, −269 and −656 bp) were PCR amplified with Platinum Pfx DNA polymerase (Life Technologies, Carlsbad, CA, USA) and cloned in pGL3Basic vector (Promega, Madison, WI, USA) using NheI-XhoI sites, to generate −132/–269/–656 bp-E2F1 prom-luc reporters. Primers used were: E2F1 prom, FW, 5′-ACGCT AGCGCGTT AAAGCCAA T AGG-3′; E2F1 prom, RV, 5′-ACCTCGAGA TC CTTTTGCCGCGAAA-3′. Mutations of E2F1 prom−269-bp reporter were introduced using QuickChange II (Agilent Technologies, Santa Clara, CA, USA) with the following oligos: Mut1, FW, 5′-AGTGTCACGGCCGCTAGCATGAGGGAA-3′; Mut1, RV, 5′-GTCGTCAGCTGCGTAGATGAGGGAA-3′; Mut2, FW, 5′-GCCCTTGCAGCAGCCGGCTGCGTAGAGGGAA-3′; Mut2, RV, 5′-GTCGTCAGCTGCGTAGATGAGGGAA-3′; Plasmid identity was verified by direct sequencing. All transfections were performed in OptiMEM (Life Technologies) using X-tremeGENE transfection reagent (Roche Diagnostic) according to the manufacturer’s protocol. Lentiviruses were produced in HEK-293T cells. Lentiviral vectors pLV-CTH (LV-c), pLV-CTH-shPTCH1 (LV-shPTCH1) 35 and pLV-CTH-shE2F1 (LV-shE2F1) lentiviruses were injected subcutaneously in athymic-nude mice. Quantification of tumor volume (n=12 per group), showing that E2F1 silencing prevented the increase of tumor growth induced by the activation of the HH signaling. *P<0.05. (b) Tumor weight of xenografts, as indicated. *P<0.05. (c) Representative images of M26c xenografts, as indicated. (d) Western blot analysis of GLI1, E2F1, iASPP, CDK1 and Cyclin B1 in tumors derived from M26c xenografts. iASPP was detected using an N-terminal Ab. HSP90 served as loading control.
shSMO (LV-shSMO) were previously described; pLKO.1-puro (LV-c), pLKO.1-puro-shE2F1-L (LV-shE2F1-1); targeting sequence 5′-GACCTCTCGACTGTGACCC-3′, exon 7) were from Open Biosoys (Lafayette, CO, USA). Most experiments were performed with LV-shE2F1-1.

 Luciferase reporter assay. E2F1 prom-luc reporters (−132 to −268/−656 bp) were used in combination with Renilla luciferase pRL-TK reporter vector (Promega) to normalize luciferase activities; pGL3Basic vector (Promega) was used to equal DNA amounts. Luminescence was measured using the Dual-Glo Luciferase Assay System (Promega) and the GloMax 20/20 Luminoimeter (Promega).

 Electrophoretic mobility shift assay. NIH3T3 cells were treated with 100 nM SAG for 4 h to activate the HH pathway, harvested and lysed in ice buffer (20 mM HEPES pH 7.9, 250 mM NaCl, 0.5% Nonidet P-40, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT) to obtain whole-cell extracts (WCE). To analyze the binding of Gli proteins to Site1 at E2F1 promoter we used the following oligonucleotide and its complementary strand: site1F, 5′-CTTAAGAGACAGGACGG-3′ and site1R, 5′-TGGAAACGACCTGACGAGGT-3′. The DNA-binding reaction was performed by incubating 20 fmol of double-stranded 32P-labeled oligo with 40 μg of WCE in a total volume of 20 μl containing 1 μg poly-dC. 100 mM KCl in 40 mM HEPES (pH 7.9), 10 mM MgCl2, 0.4 mM EDTA, 4 mM DTT and 40% glycerol for 20 min at room temperature. In indicated competition experiments, a 100-fold excess of unlabeled oligo was used. When using antibodies, proteins were preincubated with anti-GLI1 (N-16; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-GLI2 (AFA3635, R&D Systems, Minneapolis, MN, USA) antibodies for 20 min at room temperature before adding the radiolabeled probe. The samples were separated on native polyacrylamide gel (6% polyacrylamide/bisacrylamide, 29:1) at 4 °C for 3 h and the signal was detected by Cyclone Storage Phosphor System (Perkin Elmer, Waltham, MA, USA).

 Western blot and co-immunoprecipitation. Samples were lysed in ice in RIPA buffer (1% NP-40, 150 mM NaCl, 5 mM EDTA, 0.25% NaDeoxycholate, 50 mM Tris- HCl pH 7.5, 7.5% SDS 0.1%) added with protease and phosphatase inhibitors, sonicated to shear DNA and centrifuged at 14000 rpm for 20 min at 4 °C; supernatant was collected as WCE. For co-immunoprecipitation experiments 700 μg WCE were diluted with IP buffer (0.5% NP-40, 100 mM NaCl, 5 mM EDTA, 10% glycerol, 50 mM Tris- HCl pH 7.5) added with protease and phosphatase inhibitors to a final volume of 450μl and incubated overnight at 4 °C with Dynabeads Protein G (Life Technologies) pre-conjugated with anti-iASPP antibody (40.3, Santa Cruz Biotechnology) or irrelevant IgG (Life Technologies). Beads were washed three times with IP buffer, proteins were eluted with Laemmli buffer and visualized on SDS polyacrylamide gel electrophoresis. The following antibodies were used for western blot: rabbit anti-iASPP (ab3498), (Abcam, Cambridge, United Kingdom), rabbit anti-E2F1 (#3742), rabbit anti-BCL2 (#2976), mouse anti-GLI1 (L42B10) (Cell Signaling Technology, Danvers, MA, USA), goat anti-GLI2 (AFA3635; R&D Systems), mouse anti-Myc (BE10), mouse anti-HSP90 (F-8), mouse anti-p53 (DO-1), mouse anti-iASPP (2808csa), rabbit anti-CDK1 (C-19), rabbit anti-Cyclin B1 (H-433; Santa Cruz Biotechnology), mouse anti-β-Actin (AC-15, Sigma-Aldrich, St. Louis, MO, USA). Chemiluminescent detection was used.

 Quantitative real-time PCR. Total RNA from adherent cells was isolated with TriPure Isolation Reagent (Roche Diagnostics). After DNase I treatment (Roche Diagnostics), RNA was subjected to reverse transcription with High Capacity cDNA Reverse Transcription Kit (Life Technologies). qPCRs were carried out at 60 °C using FastStart SYBR Green Master (Roche Diagnostic) in a Rotorgene-Q (Qiagen). Primers used were: E2F1prom-F, 5′-GACGAGCGGATGCTGACGAGG-3′ and E2F1prom-R, 5′-TGGACGACCGTGCCTGACGG-3′; PTCH1-F, 5′-GTCGAGCGACCCTGACCTGG-3′ and PTCH1-R, 5′-TCTCGTATCAGGTCCTGACG-3′; BCL2A1-F, 5′-GCGACATCCACACTGCTGACG-3′ and BCL2A1-R, 5′-CTGACGACCGCTGACGAGGT-3′; GLI1-F, 5′-GACCTCTCGACTGTGACCC-3′ and GLI1-R, 5′-GCTTTACTGCAGCCCTCGT-3′; PTCH1-F, 5′-GACACCTGGTGTCGCTGTG-3′ and PTCH1-R, 5′-GAGGGACGACCTCTGCTCATC-3′; and SMR-O, 5′-GGCGACCTGCTAGGATGAACGG-3′ and E2F1-R, 5′-GTCAGGACCCCTCGTGAG-3′; E2F1-F, 5′-GTCAGGACCCCTCGTGAG-3′ and E2F1-R, 5′-ACCTCGTATGCTGACGAGGT-3′; iASPP-F, 5′-AGGCTAAAGAGACACAGGACG-3′ and iASPP-R, 5′-TGTATGACTGACGGCAGACT-3′; ACTINprom-F, 5′-GTCGAGCGACCCTGACCTGG-3′ and ACTINprom-R, 5′-CAGTATTTTGAGGCAACTCTG-3′. The data represent mean ± standard error of the mean (SEM). Statistical analysis. The data represent mean ± S.E.M. values and are calculated on at least 3–5 independent experiments. P-values were calculated using Student’s t-test. A two-tailed value of P < 0.05 was considered statistically significant.

 Flow cytometry analysis. Apoptosis was assessed by cytometric analysis of Annexin V/7-AAD labeled cells using Annexin V/7-AAD apoptosis kit (Becton Dickinson, Franklin Lakes, NJ, USA), according to manufacturer’s protocol. Cell death was measured by propidium iodide staining; cells were incubated with 100ng/ml PI for 5 min before analysis. Cytometric analysis was performed with FACS-Count II (Becton Dickinson).

 Cell sorting, nude mice and xenografts. Melanoma cells were transduced with either P2-CYCTH (LV-c) or P2-CYCTHshPCHT1 (LV-shPCHT1). Cells transduced with LV-c or LV-shPCHT1 also express green fluorescent protein (GFP) and were FACs (fluorescent-activated cell sorter)-sorted with the BD FACs-Aria cell sorter (Becton Dickinson). GFP-positive cells were then transduced with either pLKO.1-puro (LV-c) or pLKO.1-puro-shE2F1-I (LV-shE2F1-I) lentiviruses and selected for puromycin resistance. For xenotransplantation M26c cells were resuspended in Matrigel (Becton Dickinson) and injected s.c. into the flanks of female athymic-nude mice (Foxn1nu, Harlan Laboratories, Udine, Italy; 40-000 cells/injection). Animals were housed in SPF conditions and monitored daily. Subcutaneous tumor size was measured twice a week with a caliber. Tumor volumes were calculated using the formula: V = W2 x L x 0.5, where W and L are, respectively, tumor width and length. The experiment was approved by the Italian Ministry of Health and was in accordance with the Italian guidelines and regulations.

 Statistical analysis. The data represent mean ± S.E.M. values and are calculated on at least 3–5 independent experiments. P-values were calculated using Student’s t-test. A two-tailed value of P < 0.05 was considered statistically significant.

 Bioinformatic analysis. Publicly available gene expression data for a series of 31 primary and 73 metastatic melanomas were profiled on Affymetrix U133 platform (Gene Expression Omnibus GEO-46517). To assess the relationship between E2F1, PTCH1, GLI1 and GLI2 expression we performed Pearson’s correlation and simple regression analysis using StatGraphics Centurion XVI software (Statpoint Technologies).
Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements.

We thank Gianni Del Sal (ICGEB, Trieste, Italy), Silvia Petrobon, Roberta Santini, Maria Riveros and Alessandro Apollo (Tumor Cell Biology Unit, ITT, Florence) for helpful comments on the paper and discussion. We are grateful to Lorenzo Borgognoni, Gianni Gerlini (S. Maria Annunziata Hospital, Florence, Italy), Nicola Pimpinelli (Department of Dermatology, University of Florence, Florence, Italy) and Riccardo Gatta (Department of Medical-Surgical Critical Area, General and Oncological Surgery, University of Florence, Florence, Italy) for providing samples and Laura Pollesio (Istituto Toscano Tumori, Pisa, Italy) for providing SK-Mel-2, SK-Mel-5, SK-Mel-28 and 501MI melanoma cell lines. This work was supported by grants from AIRC (Associazione Italiana per la Ricerca sul Cancro; IG-9566 and IG-14184), Regional Health Research Program 2009 and Fondazione Cassa di Risparmio di Firenze (2011.1027, FiorGen Foundation) to BS.

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