Hedgehog controls neural stem cells through p53-independent regulation of *Nanog*

**Agnese Po^{1,5}, Elisabetta Ferretti^{1,5},
Evelina Miele¹, Enrico De Smaele¹,
Arianna Paganelli¹, Gianluca Canetti¹,
Sonia Coni¹, Lucia Di Marcotullio¹,
Mauro Biffoni², Luca Massimi³,
Concezio Di Rocco³, Isabella Screpanti¹
and Alberto Gulino^{1,4,*}**

¹Department of Experimental Medicine, Sapienza University, Rome, Italy, ²Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy, ³Institute of Neurosurgery, Division of Pediatric Neurosurgery, Catholic University of Rome, Rome, Italy and ⁴Department of Neuroscience, Istituto Neurologico Mediterraneo Neuromed, Pozzilli, Italy

Hedgehog (Hh) pathway has a pivotal function in development and tumorigenesis, processes sustained by stem cells (SCs). The transcription factor *Nanog* controls stemness acting as a key determinant of both embryonic SC self-renewal and differentiated somatic cells reprogramming to pluripotency, in concert with the loss of the oncosuppressor p53. How *Nanog* is regulated by micro-environmental signals in postnatal SC niches has been poorly investigated. Here, we show that *Nanog* is highly expressed in SCs from postnatal cerebellum and medulloblastoma, and acts as a critical mediator of Hh-driven self-renewal. Indeed, the downstream effectors of Hh activity, Gli1 and Gli2, bind to *Nanog*-specific cis-regulatory sequences both in mouse and human SCs. Loss of p53, a key event promoting cell stemness, activates Hh signalling, thereby contributing to *Nanog* upregulation. Conversely, Hh downregulates p53 but does not require p53 to control *Nanog*. Our data reveal a mechanism for the function of Hh in the control of stemness that represents a crucial component of an integrated circuitry determining cell fate decision and involved in the maintenance of cancer SCs.

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Introduction

Nanog is a core intrinsic determinant of self-renewal and pluripotent cellular state of embryonic stem cells (ESCs) of

the blastocyst inner cell mass, and its expression is down-regulated on differentiation (Chambers *et al*, 2003; Mitsui *et al*, 2003; Boiani and Scholer, 2005; Pan and Thomson, 2007). *Nanog* is also required for early embryo development, as *Nanog*-deficient mice fail to develop beyond the blastocyst stage (Mitsui *et al*, 2003). Reprogramming experiments of differentiated somatic cells by expression of Oct4, Sox2, c-Myc and Klf4, reactivate the production of *Nanog*⁺ pluripotent stem cells (SCs) (Takahashi and Yamanaka, 2006; Brambrink *et al*, 2008), indicating that *Nanog* has an important function in determining stemness features. Consistently, overexpression of *Nanog* gives rise to pluripotent SCs (Yu *et al*, 2007; Hanna *et al*, 2009). Recently, *Nanog* has been described to be reactivated in cancer cells (e.g. glioma, pancreas, colorectal, endometrial) possibly as a reminiscence of stemness properties of cancer SCs (Clement *et al*, 2007; Hubbard *et al*, 2009; Jeter *et al*, 2009; Ji *et al*, 2009; Meng *et al*, 2010; Wen *et al*, 2010).

These observations have emphasized the notion that cancer cells and SCs might be governed by common mechanisms. Such conclusions are also supported by recent works demonstrating that loss of the tumor suppressor p53 is another key determinant of stemness and of transcriptional activation of *Nanog* (Lin *et al*, 2005; Hong *et al*, 2009; Kawamura *et al*, 2009). Thus, the elucidation of the poorly understood events that regulate *Nanog* in postnatal SCs represents a fundamental aspect to elucidate both SC and tumor biology.

Hedgehog (Hh) pathway has a pivotal function in cell development and tumorigenesis in a wide variety of tissues, both processes sustained by SCs. More specifically, Hh signalling sustains embryonic and postnatal NSCs of forebrain subventricular zone and hippocampus (Ahn and Joyner, 2005; Lai *et al*, 2003; Machold *et al*, 2003; Palma *et al*, 2005; Palma and Ruiz i Altaba, 2004), as well as cerebellar NSCs and glioma SCs overexpressing a stemness gene signature (e.g. *Nanog*, Oct4, Sox2, CD133) (Clement *et al*, 2007; Stecca and Ruiz i Altaba, 2009). A paradigmatic Hh-target organ is cerebellum, where Hh is critically required to keep transit-amplifying granule cell progenitors (GCPs) undifferentiated, to promote their proliferation (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999) and to cause medulloblastoma (Mb) (Ruiz i Altaba, 2006). Aberrant Hh signalling activation occurs in Mb as a consequence of genetic or epigenetic changes affecting several components of the pathway (recently reviewed in Teglund and Toftgard, 2010). For instance, loss-of-function mutation of Patched1 (Ptc1) (the receptor of the Hh ligands) mimics the ligand-induced relief of the inhibition on the transducer Smoothened (Smo), leading to constitutive pathway activation. Downstream effectors of Smo activity are Gli transcription factors, which act on a set of target genes promoting cell proliferation and reducing cell differentiation. These target genes include *Gli1* itself, thus autoreinforcing the signalling strength and representing a sensitive read out of the pathway.

*Corresponding author. Department of Experimental Medicine, Sapienza University, 324 Viale Regina Elena, Rome 00161, Italy. Tel.: +39 6 446 4021; Fax: +39 6 446 1974; E-mail: alberto.gulino@uniroma1.it

⁵These authors contributed equally to this work

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The identity of Hh/Gli-target genes involved in the control of stemness in NSCs and cancer SCs is poorly understood. Here, we show that both *Nanog* and Gli1 are highly expressed in postnatal cerebellar NSCs and in Hh-dependent mouse and human Mb SCs. *Nanog* is required as a critical mediator of Hh-driven self-renewal of NSCs, as Hh acts through transcriptional activation of both mouse and human *Nanog*. Our data also suggest *Nanog* involvement in the Hh-dependent stemness properties of brain tumors.

Results

Cerebellar neurospheres coexpress *Nanog* and *Gli1*

Earlier studies have shown the existence of a subpopulation of NSCs in mouse postnatal cerebellum (Klein *et al*, 2005; Lee *et al*, 2005). Thus, to investigate a functional connection between Hh and intrinsic stemness determinants, we sorted Proliferin1⁺ cells, (Supplementary Figure S1A) from cerebella of 4-day-old mice. We found that these cells displayed enhanced expression of stemness markers (*Nanog*, Oct4, Sox2, Bmi1, Musashi1, Proliferin1, Nestin) together with Hh pathway target genes (*Ptc1*, *Hip1*, *Cyclin D1-2* and *Gli1*), compared with Proliferin1-negative cells (Figure 1A). Conversely, differentiation markers (e.g. the granule cell lineage markers *Math1* and *Zic1*) were significantly reduced (Figure 1A; Supplementary Figure S1B), suggesting that Hh activity preferentially associates to stemness features.

Cerebellar SCs formed primary neurospheres and were further propagated as secondary neurospheres from single-cell suspensions indicating that they were able to self-renew.

To characterize SCs, we analysed mRNA and protein expression of stemness markers, differentiation markers and Hh activity (*Gli1* expression) in neurospheres with respect to pre-neurosphere cells. Although we found loss of differentiation markers *Math1* and *Zic1* (Figure 1B), we observed enhanced expression of stemness markers together with Hh pathway activation in neurospheres (Figure 1B and C).

Notably, we also found, by immunofluorescence, co-expression of *Gli1* and *Nanog* in about 40% of cells within neurospheres (Figure 1D), whereas *Nanog* did not stain rare cells positive for neuronal differentiation marker MEF2D (Supplementary Figure S1C, upper panels). These results suggest us a possible functional connection between Hh signalling and *Nanog*.

To analyse this relationship, we studied *Nanog* expression *ex vivo* in conditions of Hh pathway constitutive activation using the *Ptc1*^{-/-} mouse embryo fibroblasts (MEFs) model, in which the activation of Gli is a consequence of *Ptc1* deletion (Goodrich *et al*, 1997). In this context, we observed increased *Nanog* mRNA and protein levels (Figure 1E and F). Accordingly, in neurospheres derived from murine *Ptc1*^{+/-} Mbs, both *Nanog* and *Gli1* levels were higher than in pre-neurosphere cells and mostly displayed cellular coexpression (Figure 1G and H). Collectively, these observations further support the notion that *Nanog* and Hh signalling are functionally connected.

Function of Hh-Gli and *Nanog* in cerebellar neurospheres

Cerebellar neurosphere multipotency is indicated by the ability to differentiate into distinct lineages (Figure 2A) when cultured under appropriate conditions (Lee *et al*,

2005), whereas the reduction of *Gli1*, *Gli2*, *Nanog* and Sox2 expression suggests that Hh activity and stemness features were coregulated during differentiation (Figure 2B). To understand the function of Hh in the control of cerebellar NSCs, we tested the effects of the activation of the pathway by the Smo-agonist SAG (Chen *et al*, 2002; Ruiz i Altaba, 2006). SAG enhanced *Gli1*, *Nanog* and Proliferin1 mRNA and protein levels (Figure 2C and D; Supplementary Figure S2A) as well as the percentage of neurosphere-forming cells (Figure 2E). Conversely, treatment with the Hh inhibitor KAAD cyclopamine had a suppressive effect on NSCs self-renewal and on *Gli1* and *Nanog* expression (Figure 2E; Supplementary Figure S2B). Importantly, SAG-induced *Gli1* is coexpressed with *Nanog* (Supplementary Figure S2C), indicating that both are targets of Hh activity. Consistently, inactivation of Hh signalling by siRNA-mediated depletion of Smo significantly reduced the levels of Smo, *Gli1* and *Nanog* (Figure 2F) as well as the self-renewal ability, both after SAG treatment and under basal conditions (Figure 2G).

As Hh enhances the proliferation of GCPs (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999), we hypothesized that it could favour NSCs towards the expansion of the granule cell lineage. Treatment of neurospheres with SAG for 4 or 10 days followed by shift to differentiation medium did not influence the multipotency of cerebellar NSCs, as no significant change in the generation of the various cerebellar cell lineages was observed compared with untreated neurospheres (Supplementary Figure S2D). Overall, these findings show that Hh signalling is required to maintain both self-renewal and *Nanog* expression in cerebellar NSCs.

***Nanog* is required for Hh-induced NSCs self-renewal**

To understand the function of *Nanog* in postnatal NSCs and its connection with Hh signalling, we studied the self-renewal potential of *Nanog*-positive cells. To this purpose, we infected NSCs with a *Nanog*-GFP lentiviral vector expressing GFP protein under the control of *Nanog* promoter (*Nanog*-GFP). We observed a significant expression of GFP in NSCs, whereas we did not detect any expression in GCPs obtained from primary cerebellar cell cultures (as described in Di Marcotullio *et al*, 2006) (Figure 3A). GCPs are 'already committed' precursor cells, which do not express stem markers but are characterized by neuronal markers (MEF2D) as shown in Supplementary Figure S1C.

As control, a CMV-driven-GFP lentivector (Zeo-GFP) was similarly expressed in both cell types. Of relevance, *Nanog*-GFP expression paralleled the one of endogenous *Nanog* in NSCs, whereas both were not expressed in differentiated GCPs (Figure 3B and C).

To show the function of *Nanog* in NSC self-renewal, we sorted *Nanog*^{GFP+} cells and we found that they were able to form secondary neurospheres to a significantly higher extent compared with Zeo-GFP-positive cells (Figure 3D). Accordingly, silencing of *Nanog* reduced the expression of Proliferin1 (Figure 3E) and significantly impaired the ability to form neurospheres compared with control (Figure 3F and G), elucidating its function in self-renewal. Moreover, *Nanog* depletion abrogated the enhancing activity of SAG on neurosphere-forming assay (Figure 3F), indicating that *Nanog* is required for Hh effects on NSCs.

Hh regulates SC self-renewal through a p53-independent pathway

As p53 suppresses *Nanog* transcription and self-renewal ability in ESCs (Lin *et al*, 2005) and can be repressed by Hh

signalling through Mdm2 (Abe *et al*, 2008; Stecca and Ruiz i Altaba, 2009), we addressed the function of p53 in Hh-induced regulation of *Nanog* and NSC self-renewal. To this end, cerebellar NSCs have been isolated from p53-deficient

Figure 2 Function of Hh-Gli and Nanog in cerebellar neurospheres. (A, B) Percentage of positive cells detected with immunofluorescence staining (A) or western blot (B) with the indicated antibodies in neurospheres cultured in D-poly-ornithine-coated chamber slides with N2 medium plus PDGF (10 ng/ml) for 7 days (A, Gran, Granule cells; Purk, Purkinje cells; Astr, astrocytes; Olig, oligodendrocytes) (B, differentiated (Diff)). (C, D) mRNA (C) and protein levels (D) of Gli1 and Nanog evaluated by RT-qPCR or western blot relative to housekeeping controls, in neurospheres before (Ctrl) and after 48-h SAG treatment. (E) Neurosphere-forming assay of single cells derived from secondary neurospheres cultured in basal stem medium (–) or in the presence of SAG or KAAD cyclopamine for 10 days. (F, G) Western blot (F) or neurosphere-forming assay (G) after transfection with control (siCtrl) or Smo siRNA (siSmo) in the absence or in the presence of SAG for 10 days. * $P < 0.05$ versus untreated cells.

Figure 1 Cerebellar neurospheres coexpress Nanog and Gli1. (A, B) Heat map of RT-qPCR gene expression (stemness, Hh pathway and differentiation (Diff) markers) of FACS-sorted Prominin1⁺ versus Prominin1[–] cells from eight cerebella of 4-day-old mice (three representative experiments) (A) or 24 h cultured cerebellar cells (T0) versus derived neurospheres (B). A red-green colour scale (–10 to +30) depicts markers expression normalized to three housekeeping genes. (C) Western blot analysis of endogenous Gli1 and stemness markers levels in cerebellar cells (T0) and derived neurospheres (NS). (D) Immunofluorescence or nuclear Hoechst (blue) staining with Nanog (green), Gli1 (red) or both antibodies in disaggregated neurospheres from murine cerebellum. The relative percentage of either Nanog or Gli1 or double-positive (marked) cells is indicated on the right. (E) mRNA levels evaluated by RT-qPCR of Hh (Gli1, Gli2) or stemness (Nanog, Sox2) markers in mouse embryonic fibroblasts (MEFs) Ptc1^{–/–} respect to wild-type MEFs (wt). * $P < 0.05$ versus wild-type MEFs. (F) Western blot analysis of Gli1 and Nanog levels in Ptc1 wt versus Ptc1^{–/–} MEFs. (G) Western blot analysis of endogenous Gli1 and Nanog in Mb cells (T0) and derived neurospheres (NS). (H) Immunofluorescence or nuclear Hoechst (blue) staining with Nanog (green) and Gli1 (red) antibodies in disaggregated neurospheres from mouse Ptc1^{+/–} Mb.

Figure 3 Nanog is required for Hh-induced NSC self-renewal. (A) Histograms showing the percentage of GFP⁺ cells in postnatal cerebellar secondary neurospheres (NS) and GCPs 48 h after infection with *Nanog*-GFP or Zeo-GFP control lentiviral vector. * $P < 0.05$ versus Zeo-GFP-positive cells. (B) mRNA and protein levels of Nanog and Gli1 in neurospheres (NS) or GCPs before (T0) or after 2 days culture (DIV2). (C) Western blots of Gli1 and Nanog in *Nanog*-GFP versus Zeo-GFP-sorted neurosphere-derived cells. (D) Secondary neurosphere-forming assay of GFP-sorted cells as in panel (A). (E) mRNA (upper panel) and protein levels (bottom panel) of Nanog and Prominin1 in neurospheres after siRNA-mediated silencing of Nanog (siNanog +) versus control siRNA (–). (F) Secondary neurosphere-forming assay after control siRNA (siCtrl) or Nanog siRNA (siNanog), treated (+) or not (–) with SAG. * $P < 0.05$ versus untreated or Zeo-GFP cells. (G) Representative bright field images of cerebellar neurosphere after transfection with control siRNA (siCtrl) or Nanog siRNA (siNanog).

mice (Jacks *et al*, 1994). The derived neurospheres displayed a significantly enhanced expression of stemness markers including *Nanog* (Figure 4A and B) and, consistently, a higher clonogenic activity with respect to wild-type cells (Figure 4C). Interestingly, p53-deficient neurospheres also displayed higher levels of Gli1 (Figure 4A and B), suggesting that p53 downregulates Hh signalling and confirming recent observations (Stecca and Ruiz i Altaba, 2009). Importantly, abrogation of Hh signalling in p53-deficient neurospheres by means of siRNA-mediated depletion of Smo, still reduced *Nanog* levels and neurosphere-forming ability (Figure 4D–F), suggesting that p53 is at least in part dispensable in this context. Therefore, in addition to p53-mediated control, Hh regulates *Nanog* and NSCs self-renewal also in a p53-independent manner.

Hh/Gli activate *Nanog* transcription

To address how Hh signalling affects *Nanog* expression, we performed a time course of SAG treatment. SAG increased *Nanog* mRNA (as early as 3–6 h) and protein levels (9 h) mimicking the induction of a direct Hh target, Gli1 (Figure 5A and B). Of note, other stemness markers, such as Sox2 (Figure 5A and B), were not affected, suggesting that Hh-mediated enhancement of *Nanog* could depend not only on expansion of NSC population, but also on specific transcriptional regulation. Indeed, a number of potential Gli-binding sequences were observed in a region spanning from –1060 to –200 bp of *Nanog* (Figure 5C; Supplementary Figure S3A). As Gli2 is an early transducer of SAG-dependent Smo activation leading to its accumulation into the nucleus (Supplementary Figure S3B) (Park *et al*, 2000; Ikram *et al*, 2004; Lipinski *et al*, 2006), we performed chromatin immunoprecipitation (ChIP) experiments to determine the *in vivo* occupancy of endogenous Gli2 onto the *Nanog* promoter in mouse NSCs. Gli2 was recruited to the S1 (–200 bp) and S2 to S5 (–775, –935, –1000 and –1060 bp) sites (but not to –2100 bp site devoid of Gli consensus sequence) following 2.5 and 5 h SAG treatment (Figure 5D and F). Gli2 recruitment was accompanied by an increase of acetylated histone H3, indicating that this promoter was transcriptionally activated by SAG (Figure 5D and F). As SAG-activated Gli2 enhances Gli1 transcription, representing the second wave of Hh activity, we determined Gli1 recruitment to *Nanog* promoter after SAG treatment. At later time, Gli1 substituted Gli2 on the S1 and S2–S5 sites, together with hyperacetylation of histone H3 (Figure 5E and F). According to p53-mediated regulation of Hh, a similar enhanced recruitment of Gli1 was observed in p53-deficient NSCs in the absence of SAG treatment (Figure 5E). A basal recruitment of Gli2 and Gli1 onto *Nanog* promoter was also detected, although lower than that found after SAG treatment with respect to an unrelated control gene (actin) or to non-specific –2100 bp *Nanog* site devoid of Gli consensus sequence. Consistently, the activation of *Nanog* and Gli1 expression observed in neurospheres derived from *Ptc1*^{+/–} Mbs was associated with an enhanced Gli1 occupancy of *Nanog* promoter (Figure 5G).

To verify whether these Gli-binding sites were responsible for the transcriptional activation of *Nanog*, we determined the effect of Gli transcription factors overexpression on mouse *Nanog* promoter-luciferase reporter. The luciferase activity was strongly induced by Gli1 and Gli2, whereas

Figure 4 Hh regulates stem cell self-renewal through a p53-independent pathway. (A, B) RT-qPCR (A) and western blot (B) analysis of stemness markers and Gli1 in neurospheres from p53-deficient mouse cerebella (p53^{-/-}). (A) Results are expressed as mean values of five different neurosphere cultures from p53^{-/-} mice with respect to p53^{+/+} wild type (dashed line). (C) Secondary neurosphere-forming assay of p53^{-/-} cells with respect to cells from p53 wild-type mice. (D) Western blot analysis of Smo, Gli1 and Nanog in p53^{-/-} neurospheres after transfection with siRNA against Smo (siSmo) compared with control siRNA (Ctrl). (E, F) Secondary neurosphere-forming assay (E) and representative bright field images (F) of cerebellar p53^{-/-} cells after transfection with siRNA against Smo (siSmo) or control siRNA (siCtrl). **P* < 0.05 versus untreated p53^{+/+} cells (A, C) or siCtrl (E).

this induction was significantly reduced by deletion of the (–1060 to –935) Gli-binding sites or mutations of critical nucleotides in S1 to S5 sites (Figure 6A; Supplementary Figure S3A).

Moreover, to further strengthen the p53-independent and direct regulation of *Nanog* by Gli factors, we investigated the effect of the modulation of Hh signalling on *Nanog* promoter-luciferase reporter in a p53^{-/-} neurospheres. We first observed that in this context, *Nanog* promoter has a basal higher activity when compared to wild type. The modulation

Figure 5 Hh/Gli activate *Nanog* transcription. (A, B) RT-qPCR (A) and western blot (B) analysis of *Nanog*, *Sox2* and *Gli1* levels in neurosphere cultures after SAG treatment up to 24 h (means $\pm$ s.d. from four different experiments). $*P < 0.05$ versus untreated cells. (C) Representation of *Nanog* promoter showing putative Gli-responsive elements (GliRE). (D–F) ChIP (D, E) and real-time qPCR-ChIP (F) assays from untreated (nt), 2.5 h (F), 5 h (D, F) and 24 h (E, F) SAG-treated neurospheres or *p53*^{−/−} neurospheres (E), using anti-Gli2 (D, F) or anti-Gli1 (two different antibodies, see Materials and methods and panels (E, F)) and anti-acetyl-H3 antibodies. Eluted DNA was PCR amplified with primers shown in Supplementary Figure S4B. Real-time qPCR-ChIP results are expressed as fold induction versus endogenous β -actin-amplified ChIP controls. Bars represent the mean of three independent experiments $\pm$ s.d. ($*P < 0.05$ versus nt). (G) RT-qPCR ChIP assay from untreated murine cerebellar neurospheres (mNSC) and murine medulloblastoma-derived neurospheres (mMbSC) using anti-Gli1 antibody. Eluted DNA was PCR amplified with primers shown in Supplementary Figure S4B. Bars represent the mean of three independent experiments $\pm$ s.d. ($*P < 0.05$ versus mNSC). A full-colour version of this figure is available at *The EMBO Journal* Online.

of Hh signalling by Gli1 or Gli2 overexpression or by Smo siRNA increased or reduced, respectively, the luciferase activity, indicating that p53 is dispensable for Hh regulation (Figure 6B). Overall, these findings indicate the presence of discrete Gli-binding sites in the *cis*-regulatory sequences of *Nanog* that are responsible for its Hh-mediated transcriptional activation.

Conservation of human *Nanog* regulation by Hh

To investigate whether Hh-induced transcriptional regulation is also active on human *Nanog*, we treated human

neurospheres (human neuronal precursor cells, HNPCs) with SAG. This resulted in enhanced transcription of *Gli1* as well as of *Nanog* mRNA and protein (Figure 7A and B), suggesting that Hh signalling may enhance also human *Nanog* transcription. Interestingly, the −256 to −118 bp region of *Nanog*, including the S1 Gli site, is conserved between mouse and human (Figure 7C; Pan and Thomson, 2007). Accordingly, the activity of a human *Nanog* reporter (−2586/+1 bp), spanning from −2586 bp to the transcription start site, was upregulated by Gli1 and Gli2 overexpression in human cells (Figure 7D; Supplementary Figure S3C).

Figure 6 Transcriptional activity of Gli-responsive sites of *Nanog* promoter. **(A)** Relative luciferase activity driven by either mouse *Nanog* (–2500/+1 bp) or (–877/+1 bp) region or (–2500/+1 bp) reporter constructs carrying either mutagenized S1 to S5 Gli consensus sequences (indicated in Supplementary Figure S3A) or *Ptc1* promoter/reporter or PgL4 empty vector, transfected into mouse cerebellar neurospheres together with mock (continuous line), Gli1, Gli2 or CREB (as a negative control). Data are indicated as mean ratios with respect to pRL-CMV-Renilla Luciferase control (ctrl). * $P < 0.05$ versus Ctrl. ** $P < 0.05$ versus wild-type constructs (dashed lines). **(B)** Relative luciferase activity driven by *Nanog* promoter in p53^{-/-} compared with p53 wt neurospheres (continuous line). The day before Gli1 and Gli2 overexpression, cells were transfected with siRNA against *Smo* (siSmo) or control. * $P < 0.05$ versus Mock-transfected cells; ** $P < 0.05$ versus siCtrl.

Hh-dependent activation of *Nanog* in human Mb SCs

As Hh hyperactivation is responsible for cerebellar cell transformation into Mb and cancer SCs have a critical function in tumorigenesis (Singh *et al*, 2004; Morrison and Kimble, 2006; Ruiz i Altaba, 2006; Fan and Eberhart, 2008; Jiang and Hui, 2008), we investigated whether the possible connection between *Nanog* and Hh could also occur in cancer SCs. ChIP experiments show that Gli1 was recruited to the S1 site of the *Nanog* promoter in human Mb neurospheres, together with an increase of acetylated histone H3 (Figure 8A), indicating that this Gli1-bound promoter was transcriptionally active. Moreover, human Mb neurospheres display enhanced expression of both *Nanog* and Gli1 with respect to pre-neurosphere cells (Figure 8B), which were also coexpressed (Figure 8C), confirming the mouse Mb data (Figure 1G and H).

Furthermore, we analysed *Nanog* levels in human primary tumor samples (hMb) compared with normal cerebellum (controls, hCb). Notably, significantly higher levels of *Nanog* were detected in hMb with hyperactive Hh signalling (high Gli1 levels, HG) compared both to low Gli1 Mb (LG) and controls (Figure 8D).

In addition, we analysed the distribution of *Nanog* values as a function of Gli1 in all tumors. This revealed that *Nanog* positively correlated with Gli1 ($P < 0.01$; Figure 8D). These observations suggest that Hh signalling is responsible for enhanced *Nanog* expression in Mb SCs. Therefore, to test whether Hh was required to maintain high *Nanog* expression in human Mb neurospheres, we abrogated Hh signalling by *Smo* siRNA and observed significantly reduced levels of *Nanog* (Figure 8E). Consistently, the *Smo*-antagonist KAAD cyclopamine also reduced the self-renewal of hMb neurospheres (Figure 8F). Overall, these findings support that enhanced expression of *Nanog* in Mb SCs is sustained by Hh signalling.

Discussion

We suggest here a model in which *Nanog* is a critical mediator of Hh effect on NSCs (Figure 8G). Indeed, both *Nanog* and Hh activity are specifically coexpressed in post-natal cerebellar NSCs and are essential to drive their self-renewal. We also show that *Nanog* is downstream to Hh, as its expression is required for Hh-induced self-renewal. Such

Figure 7 Conservation of human *Nanog* regulation by Hh. (A, B) RT-qPCR (A) of *Nanog*, *Sox2* and *Gli1* levels and western blot analysis (B) of *Nanog* and *Gli1* levels in HNPC after SAG treatment up to 24 h (means $\pm$ s.d. from four different experiments). * $P < 0.05$ versus untreated cells. (C) Alignment of mouse and human *Nanog* upstream *cis*-regulatory sequences, showing a good homology in the -256 to -118 bp region, encompassing a conserved *Gli* consensus site at -200 bp. (D) Relative luciferase activity driven by human *Nanog* (-2586/+1 bp) reporter transfected into human HNPC together with Mock, *Gli1* and *Gli2*. Data are indicated as mean ratios with respect to pRL-CMV-Renilla Luciferase control (ctrl). * $P < 0.05$ versus ctrl. A full-colour version of this figure is available at *The EMBO Journal* Online.

stemness promoting activity is sustained by transcriptional activation of *Nanog*, which is conserved in mouse and human cells, through the *in vivo* binding of *Gli1* and *Gli2* to specific promoter *cis*-regulatory elements. These findings provide the key evidence that the Hh/*Gli* cascade activates a critical pluripotency gene in postnatal cerebellar NSCs.

Hh signalling is active in cerebellar NSCs

We show that the window of Hh activity also extends to cerebellar NSCs, besides the lineage committed GCPs, which are known to be a specific target of Hh (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). This is consistent with the increased expression of stemness genes, including *Nanog*, by *Gli1* overexpressing prominin1-positive cells in mouse cerebellar tissue (Stecca and Ruiz i Altaba, 2009) and with the *in vivo* expansion of cerebellar NSCs observed in mice where the Hh pathway has been artificially activated (i.e. nestin-*Gli1* transgenic mice or animals with hyperactive Hh signalling through *Ptc1* deletion or *Smo* activation in GFAP-expressing NSCs) (Schuller *et al*, 2008; Yang *et al*, 2008; Stecca and Ruiz i Altaba, 2009). This raises the question of the possible relationships between the functions of Hh activity in either NSCs and committed GCPs. Indeed, although NSC and GCP lineage may be unrelated, at least some NSCs are able to generate differentiated GCPs (Klein *et al*, 2005; Lee *et al*, 2005 and this study). However, our data suggest that Hh is

not involved in such a lineage commitment, as the GCP lineage choice or the expansion of GCP population are not influenced by Hh treatment of NSCs. These findings may be explained by the previously described ability of bFGF, which is required to maintain NSCs cultures, to inhibit the mitogenic effect of Shh on GCPs (Fogarty *et al*, 2007). Overall, these findings provide evidence that Hh signalling has a double activity: it maintains stemness properties of cerebellar NSCs and sustains proliferation of lineage committed GCPs. This could be explained by differential cooperation of the Hh pathway with other microenvironment-dependent signals involved in the control of NSCs or GCPs specific gene expression patterns.

Hh sustains NSCs through transcriptional activation of *Nanog*

To this regard, we identify here *Nanog* as one of the intrinsic determinants of the activity of Hh in NSCs, whereas Shh is not able to increase *Nanog* expression in GCPs (not shown). Although *Nanog* is known to be critical for the maintenance of pluripotency and self-renewal of ESC (Chambers *et al*, 2003; Mitsui *et al*, 2003), we describe here that it is also active in postnatal cerebellar NSCs where it acts as a stemness gene. Our findings are in keeping with the suggested function of *Nanog* to control somatic cell pluripotency *in vivo* in Huntington's disease *Hdh*-Q111 knock-in mice that display aberrant neurogenesis as well as NSCs characterized

by impaired lineage restriction together with enhanced self-renewal and *Nanog* expression (Molero *et al*, 2009). In apparent contrast with this suggestion, genetic loss of *Nanog* in ESCs leads to the *in vitro* emergence of *Nanog*-negative ESCs that keep self-renewal and multipotency and do not display impaired integration into neuro-epithelia of *Nanog*-positive/*Nanog*-negative chimeric mouse

embryos (Chambers *et al*, 2007). The latter observations do not rule out a function of *Nanog* in the control of somatic NSCs *in vivo*, as it might be sustained by non-cell-autonomous paracrine *Nanog*-induced factors produced by *Nanog*-positive NSCs in the chimerical tissue, as recently described in primitive endoderm, possibly through FGF/Erk signalling (Messerschmidt and Kemler, 2010). To fully elucidate

this issue, further studies using mice in which *Nanog* is conditionally targeted into NSCs need to be carried out.

Hh-dependent *Nanog* activation in Mb SCs

Consistent with the notion that *Nanog* may confer stemness features to somatic cells, we also show that *Nanog* is upregulated in Hh-promoted neuronal tumors, specifically featuring the cancer SC population. Notably, preferential upregulation of *Nanog* is observed in human Mbs with hyperactive Hh signalling. Additionally in Mb samples, Gli1 and *Nanog* levels positively correlate, suggesting their functional connection also *in vivo* in human tumors. Importantly, *Nanog* has been reported to be reactivated also in other tumors and suggested to have a function in tumor development (Clement *et al*, 2007; Jeter *et al*, 2009; Meng *et al*, 2010; Wen *et al*, 2010), possibly by featuring and controlling cancer SCs. The critical function of Hh pathway in promoting tumorigenesis has been recently highlighted by its ability to maintain cancer SCs in a number of tumor types (glioblastoma, breast cancer, myeloma, colon cancer and Mb) (Liu *et al*, 2006; Clement *et al*, 2007; Peacock *et al*, 2007; Varnat *et al*, 2009 and this study). Therefore, the Hh-dependent regulation of *Nanog* that we have described here in Mb SCs provides an explanation to the Hh-enhanced expression of *Nanog* in glioma (Clement *et al*, 2007) and Mb SCs (this study), as well as to the essential role of *Nanog* as a mediator of Hh-regulated glioma stemness properties and tumorigenicity (see accompanying paper by Zbinden *et al*, 2010). Overall, these observations suggest that targeting *Nanog* might be an approach to improve therapy of cancer SCs, as indicated by the ability of histone deacetylase inhibitors to suppress both the ESC signature (including *Nanog*) and growth inhibition of human embryonic carcinoma cells (You *et al*, 2009). To this regard, histone deacetylase inhibitors have been recently described to suppress Gli transcription function in Mb cells (Canettieri *et al*, 2010).

Hh and p53 cross-regulate *Nanog*

The Hh-dependent regulation of *Nanog* allows us to depict a model (Figure 8G) where a circuitry of Hh-intersecting signals converge on *Nanog* controlling stemness. To this regard, the oncosuppressor p53 has been recently described to have a critical function in reprogramming pluripotent and self-renewing SCs and to repress both *Nanog* (Lin *et al*, 2005; Hong *et al*, 2009; Kawamura *et al*, 2009) and neural cell stemness (Meletis

et al, 2006). p53 also suppresses Hh pathway (Stecca and Ruiz i Altaba, 2009 and this study) that in turn represses p53 (Abe *et al*, 2008; Stecca and Ruiz i Altaba, 2009).

We have shown the presence of a p53-independent regulation of *Nanog* and stemness through Gli-mediated direct transcriptional control, which cross-talks with previously described p53- and Gli-mediated mechanisms.

Interestingly, p53 regulates polarity of self-renewing divisions of mammary SCs, where p53 loss causes enhanced symmetric cell divisions and clonogenic activity (Cicalese *et al*, 2009), similarly to the effects induced by Hh activation in NSCs (this study). Therefore, we speculate that p53 may restrain self-renewing NSC divisions by suppressing *Nanog* expression both directly (Xu, 2005) and through cross-inhibition of Hh signalling (Figure 4B). This model may also have implications for cancer SCs where *Nanog* is reactivated (Jeter *et al*, 2009; Li *et al*, 2009 and this study) and p53 cooperates with Hh pathway for Mb tumorigenesis (Romer *et al*, 2004).

In conclusion, Hh controls cell stemness through regulation of a pluripotency gene (*Nanog*), providing a crucial component of an integrated Hh- and p53-mediated circuitry determining cell fate decisions and involved in the maintenance of cancer SCs.

Materials and methods

Unless otherwise indicated, media and supplements for cell culture were purchased from Gibco-Invitrogen (Carlsbad, CA) and chemicals were purchased from Sigma-Aldrich (St Louis, MO).

Cell culture

Mouse cerebella were obtained from postnatal 4-day-old wild-type mice and p53^{-/-} mice (The Jackson Laboratory, ME). Murine Mbs were isolated from Ptc1^{-/+} mice (Di Marcotullio *et al*, 2006) (EMMA, Monterotondo, Italy). Human Mb samples were collected during surgical resection with the approval of institutional review board as described earlier (Di Marcotullio *et al*, 2006).

Tissues were collected in HBSS supplemented with 0.5% glucose and penicillin-streptomycin, grossly triturated with serological pipette and treated with DNase I to a final concentration of 0.04% for 20 min. Finally, cells aggregates were mechanically dissociated using pipettes of decreasing bore size to obtain a single-cell suspension. SCs were cultured as neurospheres in selective medium after centrifugation, DMEM/F12 supplemented with 0.6% glucose, 25 µg/ml insulin, 60 µg/ml N-acetyl-L-cystein, 2 µg/ml heparin, 20 ng/ml EGF, 20 ng/ml bFGF (Peprotech, Rocky Hill, NJ), 1X penicillin-streptomycin and B27 supplement without vitamin A. Cell isolation procedures did not influence *Nanog* or stress-induced proteins (i.e. p53), as their levels in pre-neurosphere

Figure 8 Hh-dependent activation of *Nanog* in human Mb stem cells. (A) RT-qPCR ChIP assay from untreated HNPC and human Mb-derived neurospheres (hMbSCs) derived neurospheres with anti-Gli1 and anti-acetyl-H3 antibodies. Eluted DNA was PCR amplified with primers shown in Supplementary Figure S4B. Results are expressed as fold induction versus endogenous GAPDH-amplified ChIP controls. Bars represent the mean of three independent experiments $\pm$ s.d. (* $P < 0.05$ versus HNPC). (B) Protein levels of endogenous *Nanog* and Gli1 evaluated by western blot in cells derived from human Mb (T0) versus derived neurospheres (NS). (C) Immunofluorescence or nuclear Hoechst (blue) staining with *Nanog* (green) and Gli1 (red) antibodies in disaggregated neurospheres from human Mb. (D) mRNA levels of *Nanog* and Gli1, evaluated by RT-qPCR relative to housekeeping controls, in human hMb samples (subsets expressing high (HG) or low levels (LG) of Gli1) and cerebellar controls (hCb). * $P < 0.05$ in HG versus LG versus hCb. Inset: Regression analysis of *Nanog* versus Gli1 levels in 28 human Mb samples ($P < 0.01$ by simple regression analysis). (E) Western blot analysis of endogenous Gli1, Smo and *Nanog* levels in human Mb neurospheres after siRNA-mediated silencing of Smo (siSmo) versus control siRNA (ctrl). (F) Neurosphere-forming assay of single cells derived from secondary human Mb neurospheres in basal stem medium (—), in the presence of Hh antagonist KAAD cyclopamine for 15 days. * $P < 0.05$ versus untreated. (G) Schematic model of the regulatory loop between Hh and *Nanog* in cerebellar NSCs. Shh binds to Ptc1, releasing the inhibition on Smo, which in turn enhances Gli2 nuclear translocation and recruitment to promoters of target genes *Gli1* and *Nanog*. Gli1 and p53 form a reciprocal inhibitory loop. Hh signal through Gli1 activates the E3 Ligase Mdm2, which degrades p53 protein, thus relieving its inhibitory activities on both *Nanog* promoter and Gli1 (see text). In turn, Gli1 and Gli2 bind to specific consensus *cis*-regulatory responsive elements on *Nanog* promoter enhancing its transcription. In this way, they mediate the Hh- and p53-dependent control of *Nanog* and downstream stemness genes, which promote self-renewal of NSCs and cancer SCs of Hh-induced tumors (e.g. Mb).

cells both immediately after *ex vivo* isolation or 24 h in culture were not detectable (Supplementary Figure S4A).

Neurospheres were treated with the Smo-agonist SAG (200 nM, Alexis) and with the steroidal alkaloid Smo antagonist, cyclopamine KAAD (0.5 μ M Calbiochem, Nottingham, UK). For the neurosphere-forming assay, cells were plated at clonal density (1–2 cells/mm²) into 96-well plates and cultured in selective medium as described above. For differentiation studies, neurospheres were mechanically dissociated, and the resulting cells were plated onto D-polyornithine-coated dishes in differentiation medium (DMEM/F12 with N2 supplement and 2 μ g/ml heparin, 0.6% glucose, 60 μ g/ml N-acetyl-L-cysteine, containing 1% Calf Serum and PDGF 10 ng/ml (P3076), for 7 days. Cerebellar GCPs (from 4-day-old mice) were isolated and cultured as described earlier (Di Marcotullio *et al* 2006).

Human fetal neural stem/precursor cells (HNPC) (Lonza, Milan, Italy) were derived from whole brains of human fetus of 18 gestational weeks and were grown as neurospheres in Neural Progenitor basal medium containing bFGF and EGF (Lonza). Neurospheres were passaged every 2 weeks and were used at passage lower than 8.

Knockdown of mouse *Nanog* and Smo was performed with specific smart pool siRNAs (20 nM) (Dharmacon, Lafayette, CO) using INTERFERin siRNA Transfection Reagent (Polyplus Transfection, NY).

Neurospheres and GCPs were also transduced with pGreenZeo Lentiviral Reporter Vectors containing specific promoters for NANOG (*Nanog*-GFP) or CMV (*Zeo*-GFP) (Biotac, Heidelberg, Germany).

Fluorescence-activated cell sorting

Mechanically dissociated cerebellar cells were stained with anti-Prominin1 (Ab16518; Abcam) and with secondary antibody (Alexa Fluor 488; Molecular Probes, Invitrogen) or cells transduced with *Nanog*-GFP or *Zeo*-GFP were sorted by fluorescence-activated cell sorting (FACS) Aria (Becton Dickinson, NJ).

RNA isolation and real-time q-PCR

RNA isolation from cells and tissue samples was performed as described earlier (Di Marcotullio *et al* 2006). cDNA synthesis was performed using High Capacity cDNA reverse transcription kit from Applied Biosystems (Foster City, CA). Quantitative reverse transcription (RT-PCR) analysis of Prominin1, Musashi1, Musashi2, BMI1, GFAP, Nestin, PCNA, *Nanog*, Oct-4, Sox2, KLF4, c-Myc, Math1, Zic1, Gli1, Gli2, Gli3, CyD1, CyD2, Ptch1, Hip1, Smo, β -actin, GAPDH and HPRT mRNA expression was analysed on cDNAs using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using TaqMan gene expression assay according to the manufacturer's instructions (Applied Biosystems). Each amplification reaction was performed in triplicate, and the average of the three threshold cycles was used to calculate the amount of transcripts in the sample (SDS software, AB). mRNA quantification was expressed, in arbitrary units, as the ratio of the sample quantity to the calibrator or to the mean values of control samples. All values were normalized to three endogenous controls, GAPDH, β -actin and HPRT.

Western blot assay

Cells were lysed in Tris-HCl pH 7.6, 50 mM, deoxycholic acid sodium salt 0.5%, NaCl 140 mM, NP40 1%, EDTA 5 mM, NaF 100 mM, Na pyrophosphate 2 mM and protease inhibitors. Lysates were separated on 8 or 6% acrylamide gel and immunoblotted using standard procedures. Anti-Mouse *Nanog* (Cosmo Bio Co, Japan), anti-*Nanog* (ab80892-Abcam, Cambridge, UK), anti-Gli1 H-300 (sc-20687; Santa Cruz Biotechnology, CA), anti-Smo N-19 (sc-6366; Santa Cruz Biotechnology), anti-Sox2 (MAB4343 Millipore Billerica, MA), anti-mouse β III Tubulin (MAB 1637 Millipore), anti-Gli2 sc-28674 H-300 Santa Cruz Biotechnology, CA), anti-CD133 (prominin1) Ab16518-Abcam, Cambridge, UK anti-actin I19 (sc-1616; Santa Cruz Biotechnology) and HRP-conjugated secondary antisera (Santa Cruz Biotechnology) were used followed by enhanced chemiluminescence (ECL Amersham, Amersham, UK).

Immunofluorescence

To detect *Nanog* and Gli1, cells were plated on poly-lysine-coated Lab-Tek chamber slides (cover slips) and allowed to adhere for 3 h. To detect differentiation markers, cells were plated on polyornithine-coated Lab-Tek chamber slides (cover slips) and cultured

in N2 medium for 7 days. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature, incubated in blocking solution (5% normal goat serum (NGS), 1% BSA, 0.1% Triton X-100) and stained overnight with primary antibodies diluted in blocking solution and for 2 h with secondary antibodies. Primary antibodies were rabbit anti-*Nanog* polyclonal, ab80892 (Abcam, Cambridge, UK) and mouse anti-Gli1, #2643 (Cell Signaling Technology Inc); mouse-anti-Oligodendrocyte marker O4 monoclonal antibody (Millipore) MAB 345; mouse anti-GFAP monoclonal antibody MAB360 (Millipore); mouse anti-MEF2D, 610774 (BD Bioscience); mouse anti-Parvalbumin P3088 (Sigma); rabbit anti-S100 S2644 (Sigma); mouse anti- β -III tubulin (MAB1637) (Millipore); rabbit anti-Zic1 polyclonal ab72694 (Abcam, Cambridge, UK); rabbit anti-GABA-R G4416 (Sigma); mouse anti-NG2 MAB5384 (Millipore). In all, 594- or 488-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Molecular Probes (Invitrogen, Eugene, OR). Nuclei were counterstained with Hoechst reagent. Cover slips were mounted with fluorescence mounting medium (S3023) (Dako, Carpinteria, CA). Images were acquired with Carl Zeiss microscope (Axio Observer Z1) using Apotome technology and AxioVision Digital Image Processing Software.

Chromatin immunoprecipitation

ChIP was performed as described earlier (Canetti *et al*, 2010). More in detail, cells were cross-linked 10 min with 1% formaldehyde, and the reaction was stopped with 0.125 M glycine for 5 min. Cells were washed and harvested, and cytoplasmic membranes were lysed with lysis buffer (5 mM Pipes, 85 mM KCl, and 0.5% Nonidet P-40). After centrifugation, nuclei were lysed with sonication buffer (1% SDS, 10 mM EDTA, and 50 mM Tris (pH 8) supplemented with protease inhibitors) and sonicated to obtain chromatin fragments of about 400–600 nucleotides. After sonication, the lysates were precleared for 1 h, diluted with nine volumes of dilution buffer (0.01% SDS, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8), 1.1% Triton X-100 and 167 mM NaCl) and incubated with the specific antibodies overnight. The next day, salmon sperm-saturated protein A beads (Upstate) were added for 40 min, after which the lysates were washed five times with Buffer A (0.1% SDS, 2 mM EDTA, 20 mM Tris-HCl (pH 8), 1% Triton X-100 and 150 mM NaCl), four times with Buffer B (0.1% SDS, 2 mM EDTA, 20 mM Tris-HCl (pH 8), 1% Triton X-100 and 500 mM NaCl), and once with Buffer TE (10 mM Tris-HCl (pH 8) and 1 mM EDTA). After the final washing, the immunocomplexes were eluted with elution buffer (1% SDS and 100 mM NaHCO₃) 30 min at room temperature, and after the addition of 200 mM NaCl, the cross-linking was reversed with an overnight incubation at 65°C. Subsequently, the samples were digested with proteinase K and RNase A for 2 h at 42°C, and the DNA was purified and precipitated. Eluted DNA was PCR amplified with primers encompassing the Gli-responsive sites (Supplementary Figure S4B) of murine and human *Nanog* promoter, the *actin* or GAPDH gene as controls. The following antibodies were used: rabbit polyclonal anti-Gli1 H-300X (Santa Cruz), rabbit polyclonal anti-Gli1 2553 (Cell Signaling), rabbit polyclonal anti-Gli2-H-300X (Santa Cruz), rabbit polyclonal anti-acetyl-histone3 (Cell Signaling). Eluted DNA has been analysed with standard PCR techniques or Q-PCR. Primers were designed with Primer Express software (Applied Biosystems).

Luciferase assays

Mouse *Nanog* promoter_luciferase vector (–877 bp) was PCR amplified from genomic DNA of SC using the following primers:

M_P_Nanog_Luc_F2: ACTGGTACCGCCAGTCTCGGTACGTGA
Pro m_nanog com_Rv: ACTCTCGAGGCAGCTTCCACAGAAAG
and cloned in pGL4-vector (Promega, Madison, WI).

Mouse *Nanog* promoter_luciferase vector –2500 bp (*Nanog*5P reporter) was obtained from Addgene (Cambridge, MA). This construct was used to generate the mutant derivatives in at least two nucleotides of canonical Gli-binding sites (Pavletich and Pabo, 1993; Mizugishi *et al*, 2001).

Human *Nanog* promoter_luciferase vector –2586 bp (Human *Nanog*_Luciferase-T2A-ZeoR) was obtained from Biotac (Germany). NSCs were transfected in 24-well plates with 80 ng of *Nanog* promoter_luciferase vector (–2500 bp and –935 bp), 400 ng of Gli1, Gli2 or mock and 5 ng of or pRL-CMV-Renilla Luciferase control vector with Fugene6 Transfection Reagent (Roche). Cells were harvested and tested 24 h posttransfection with the dual-luciferase assay (Promega). Empty pGL4 vector was tested as negative ctrl

with Gli1, Gli2 or mock. PtchP1A luciferase reporter (kindly donated by R Toftgård) was used as positive control of response to Gli family elements, whereas myc-Gli2 plasmid was from A Dlugosz. Results are expressed as luciferase/renilla ratios and represent the average $\pm$ s.d. of at least three experiments, each performed in triplicate.

Site-directed mutagenesis

Mutation of critical nucleotides in Gli-binding sites was designed according to Mizugishi *et al* (2001) and Pavletich and Pabo (1993). To mutagenize the promoter constructs, the QuickChange XL Site-Directed Mutagenesis Kit and the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) were used. The reaction was carried out according to the manufacturers' protocol using the following primers:

S1 MUTfw: CCTCCCTCCAGTCTGTGCCACCTTACAGCTTCTT
S1 MUTrv: AAGAAGCTGTAAGGTGGCAGACTGGGAGGGAGG
S2 MUTfw: TTAGACGCTGAGGCACCTTAAGATTGCAGTAAGTCT
GAAG
S2 MUTrv: CTTCAGACTTACTGCAATCTTAAGTGCCCTCAGCCGTC
TAA
And for multisite mutagenesis the following ones:
S3MUT: GATCGGCAAACCTTTGAACCTGTGATTGGAAATAGGAA
GATCAGGA
S4MUT: CAGAAGCCGACTTAAGCTGTGCTAGAGTGCTTTCACTCACT
S5MUT: TCACTGTGGTAGAGTCTTCACTTGGATATGTTACGAGAGG
The mutations introduced in the Nanog sequence are underlined.

Statistical analysis

Statistical analysis was performed using StatView 4.1 software (Abacus Concepts, Berkeley, CA). Statistical differences were analysed by the Mann-Whitney *U* test for non-parametric values; MB markers were analysed by simple regression using the StatView 4.1 software (Abacus Concepts). The results are expressed as mean $\pm$ s.d. from an appropriate number of experiments as indicated in the figure legends.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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