RESEARCH PAPER

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Cancer-linked satellite 2 DNA hypomethylation does not regulate *Sat2* non-coding RNA expression and is initiated by heat shock pathway activation

Gaëlle Tilman, Nausica Arnoult, Sandrine Lenglez, Amandine Van Beneden, Axelle Loriot, Charles De Smet and Anabelle Decottignies*

Genetic and Epigenetic Alterations of Genomes Research Group; de Duve Institute; Faculty of Pharmacy and Biomedical Sciences; Catholic University of Louvain; Brussels, Belgium

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Abbreviations: *Sat2*, satellite 2; HS, heat shock; ICF, immunodeficiency, centromeric region instability and facial anomalies; ncRNAs, non-coding RNAs; HSF1, heat shock factor 1; DNMT, DNA methyltransferase

Epigenetic dysfunctions, including DNA methylation alterations, play major roles in cancer initiation and progression. Although it is well established that gene promoter demethylation activates transcription, it remains unclear whether hypomethylation of repetitive heterochromatin similarly affects expression of non-coding RNA from these loci. Understanding how repetitive non-coding RNAs are transcriptionally regulated is important given that their established upregulation by the heat shock (HS) pathway suggests important functions in cellular response to stress, possibly by promoting heterochromatin reconstruction. We found that, although pericentromeric satellite 2 (*Sat2*) DNA hypomethylation is detected in a majority of cancer cell lines of various origins, DNA methylation loss does not constitutively hyperactivate *Sat2* expression, and also does not facilitate *Sat2* transcriptional induction upon heat shock. In melanoma tumor samples, our analysis revealed that the HS response, frequently upregulated in tumors, is probably the main determinant of *Sat2* RNA expression in vivo. Next, we tested whether HS pathway hyperactivation may drive *Sat2* demethylation. Strikingly, we found that both hyperthermia and hyperactivated RasV12 oncogene, another potent inducer of the HS pathway, reduced *Sat2* methylation levels by up to 27% in human fibroblasts recovering from stress. Demethylation occurred locally on *Sat2* repeats, resulting in a demethylation signature that was also detected in cancer cell lines with moderate genome-wide hypomethylation. We therefore propose that upregulation of *Sat2* transcription in response to HS pathway hyperactivation during tumorigenesis may promote localized demethylation of the locus. This, in turn, may contribute to tumorigenesis, as demethylation of *Sat2* was previously reported to favor chromosomal rearrangements.

Introduction

It has now become evident that cancer is a disease driven not only by genetic abnormalities, but also by epigenetic changes involving both losses and gains of DNA methylation as well as altered patterns of histone modifications. Repetitive DNA is normally highly methylated. Hypomethylation of these regions is therefore largely responsible for the global loss of DNA methylation in cancer and is believed to contribute to tumor formation and progression as malignancy grade is often associated with the level of global DNA hypomethylation.[1] The mechanisms underlying cancer-linked hypomethylation are still poorly understood;[2] it is also unclear whether demethylation occurs as a unique event or as successive waves. Besides, it appears that all repetitive genomic loci do not behave similarly when facing cellular demethylation processes,[3-5] suggesting the involvement of locus-specific demethylation mechanisms that may differ in intensity and/or nature, according to the cellular context.

Satellite 2 DNA (*Sat2*), a locus mapping within the 1q12 pericentromeric region, is among the best-characterized hypomethylated sequences in cancer cells.[1] Although *Sat2* hypomethylation is not sufficient to induce rearrangements in this region, it is associated with pericentromeric instability.[6] Accordingly, this locus is known to be a fragile site.[7] In addition to its involvement in chromosomal instability, hypomethylation of CpG islands within regulatory regions reportedly activates gene transcription. Recent studies unraveled that, unlike what was previously anticipated, heterochromatic regions like *Sat2* are transcriptionally

*Correspondence to: Anabelle Decottignies; Email: anabelle.decottignies@uclouvain.be
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competent and can produce non-coding RNAs (ncRNAs), the function of which is still unclear.[8,9] The observation that satellite DNA transcription is increased by a wide range of cellular stresses including heat shock, DNA damaging agents, heavy metals, UV-C, oxidative stress and hyper-osmotic stress suggests that they may play important roles in response to stress, possibly by promoting heterochromatin reconstruction.[10-14] Strong overexpression of pericentromeric satellite RNAs was also detected in experimentally-induced mouse pancreatic adenocarcinoma and in a variety of other cancer samples isolated from human patients.[15] To date, however, the impact of DNA hypomethylation on *Sat2* expression has remained elusive and conflicting data were reported. Eymery et al.[16] showed that cell treatment with 5-aza-2'-deoxycytidine demethylating agent upregulates pericentromeric transcription. On the other hand, no increased expression of satellite III RNA was observed in HCT116 colon cancer cells deficient for DNMT3B and/or DNMT1, and a poor correlation between DNA hypomethylation and satellite DNA transcription was reported in ovarian and Wilms tumor samples.[17] Similarly, cultured lymphocytes from DNMT3B-deficient patients suffering from the immunodeficiency, centromeric region instability and facial anomalies (ICF) syndrome do not display strong constitutive transcription of pericentromeric *Sat2* DNA sequences.[17]

Knowing that repetitive elements represent more than two-thirds of the human genome,[18] understanding how their transcription is regulated is undoubtedly important. Here, we performed a quantitative study evaluating the influence of DNA demethylation on the transcription level of *Sat2* in a variety of human cancer cells of distinct origins. In a second part of the study, we investigated the impact of heat shock response induction on *Sat2* transcription and DNA methylation.

Results

***Sat2* DNA hypomethylation is not associated with constitutive overexpression of *Sat2* RNA in cancer cell lines.** To assess the putative impact of cancer-linked *Sat2* hypomethylation on transcription of the locus, we first selected a series of cancer cell lines displaying varying *Sat2* methylation levels using the quantitative and rapid *Sat2* MethylLight assay described previously.[3] A total of 10 glioblastoma, 14 melanoma, 10 sarcoma and 22 carcinoma cell lines were included in the survey. To exclude any aberrant DNA demethylation linked to prolonged culture of cancer cell lines, *Sat2* methylation levels were compared with those measured in non-tumoral cell lines obtained by immortalization with h*TERT* telomerase subunit of epithelial melanocytes (HNEMhTERT), foreskin fibroblasts (HFF2hTERT), human embryonic kidney cells (HEKhTERT) or mammary epithelial cells (HMEChTERT). Human mesenchymal stem cells (HMSCs) were further included in the analysis as sarcomas were previously suggested to derive from this cell type.[19]

Strong *Sat2* hypomethylation was detected in most glioblastoma, melanoma and sarcoma cell lines, while it was less pronounced in carcinoma cell lines (**Fig. 1A**). Based on this survey, we selected eight sarcoma cell lines, four carcinoma cell lines and one melanoma cell line for further analyses. The non-tumoral HFF2hTERT fibroblast cell line was used as reference. A detailed analysis of *Sat2* methylation was then obtained by bisulfite sequencing of the previously reported 351-bp CpG-rich region (**Fig. 1B**).[20] Although the position of *Sat2* promoter is unknown, the sequenced region contains several putative heat shock factor 2 (HSF2) binding sites and one predicted HSF1 binding site (www.cbrc.jp/htbin/nph-tfsearch) (**Fig. 1B**), a transcription factor previously reported to promote satellite DNA transcription.[12-14] *Sat2* RNA levels were measured by quantitative RT-PCR, as previously described.[17] In all cases, RNA was treated with DNaseI and measurements were also performed in control samples lacking reverse transcriptase. Importantly, to avoid any bias due to amplification/deletion at the locus, *Sat2* cDNA levels were normalized first to *ACTB* cDNA levels and then to relative *Sat2* genomic DNA content obtained by normalizing *Sat2* gDNA to the total content in *Alu* repeat sequences (**Fig. 1C**). The *Alu* quantitative PCR amplifies a consensus motif conserved in ancient and more recent individual *Alu* repeats subfamilies (*Alu-J, SluSp, AluSx, AluSq, AluSc, AluY, AluSb2, AluYb8, AluYa5* and *AluYa8*).[3] Our results revealed no correlation between *Sat2* DNA methylation and normalized *Sat2* RNA levels in the 13 cancer cell lines that we tested ($R = 0.27$, $p = 0.19$) (**Fig. 1C and D**). As positive control of *Sat2* transcriptional induction, *Sat2* cDNA levels were measured in fibroblasts or HeLa cancer cells subjected to heat shock (HS), as previously reported.[10,11] This revealed that *Sat2* transcription remains at low basal level in cancer cell lines, even in the most hypomethylated ones (**Fig. 1E**). Altogether, these data show that *Sat2* hypomethylation is not associated with constitutive hyperactivation of *Sat2* expression in cancer cell lines.

***Sat2* hypomethylation in ICF fibroblasts does not exacerbate HS-induced *Sat2* RNA levels.** Although reduced methylated CpG content in cancer cell lines does not appear to impact on constitutive *Sat2* expression, we next tested whether *Sat2* hypomethylation may upregulate *Sat2* RNA induction under HS conditions. To address this issue in comparable cell lines, we derived the ICFhTERT cell line from DNMT3B-deficient ICF fibroblasts and measured both basal and HS-induced *Sat2* RNA levels in ICFhTERT and related HFF2hTERT fibroblasts displaying normal *Sat2* methylation. As expected, *Sat2* methylation levels were low in ICFhTERT, with methylated CpG content amounting to only 16.8% of total CpG, and high in HFF2hTERT, reaching 72.5% (**Fig. 2A**). In line with data obtained in cancer cell lines, we found that extensive hypomethylation of *Sat2* sequences in ICFhTERT was not associated with constitutive hyperactivation of *Sat2* transcription (**Fig. 2B**), which was instead triggered following exposure of ICFhTERT and HFF2hTERT fibroblasts to HS (**Fig. 2C**). Importantly, hypomethylation did not appear to exacerbate *Sat2* transcriptional activation in heat shocked ICFhTERT cells (**Fig. 2C**). Further support for the lack of correlation between DNA hypomethylation and constitutive *Sat2* RNA upregulation came from the demonstration that treatment of HFF2 hTERT cells with 5 μM 5-aza-2'-deoxycytidine (5-aza) demethylating agent for 1 to 5 d only transiently upregulated *Sat2* RNA (**Fig. 2D**). Consistent with a previous report,[16] we found that *Sat2* RNA levels were about 10-fold higher after 3 d of treatment with 5-aza with no concomitant increase in

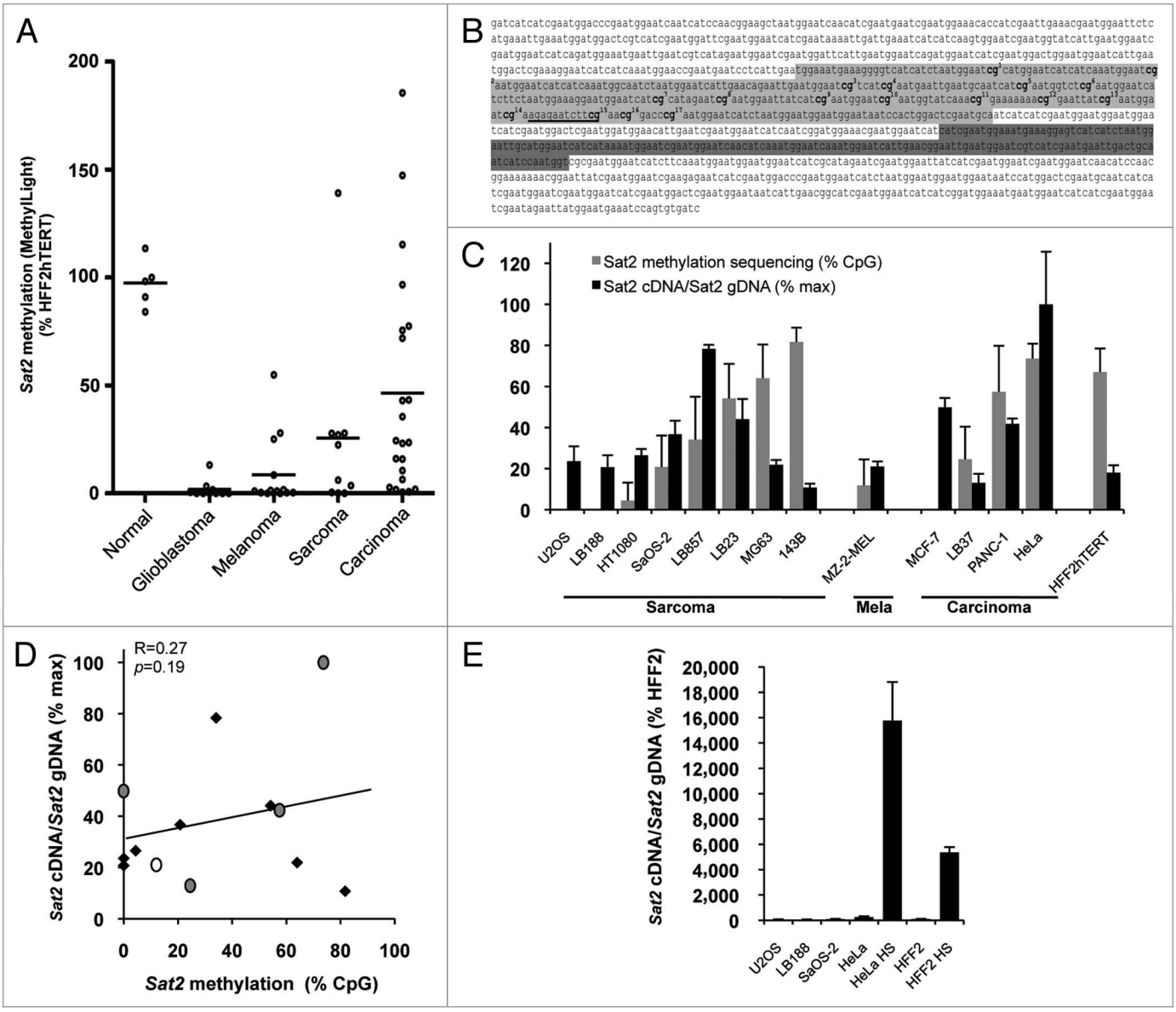


Figure 1. *Sat2* hypomethylation is not correlated with constitutive *Sat2* RNA overexpression in cancer cell lines. (A) The MethyLight assay[3] was used to quantify *Sat2* methylation on bisulfite-treated DNA isolated from glioblastoma (n = 10), melanoma (n = 14), sarcoma (n = 10) and carcinoma (n = 22) cell lines. Non-tumoral control samples included four h*TERT*-immortalized cell lines (HFF2hTERT, HEKhTERT, HNEMhTERT, HMEChTERT) and HMSC mesenchymal stem cells. Methylation levels were compared with *Sat2* methylation in HFF2hTERT. Bars indicate median values. (B) The *Sat2* sequence of chromosome 1 corresponds to GenBank accession number X72623.1. In light gray: *Sat2* fragment analyzed after bisulfite treatment of genomic DNA for methylated CpG content determination by sequencing. The 17 *CpG* sites are numbered. In dark gray: *Sat2* fragment amplified by PCR on cDNA for quantitative RT-PCR measurement of *Sat2* RNA transcripts. The same region (dark gray) was amplified in ChIP experiments. The HSF1 binding site predicted by *tfsearch* is underlined. (C) *Sat2* methylation level in cancer cell lines derived from sarcoma, melanoma and carcinoma and in HFF2hTERT non-tumoral fibroblasts determined by bisulfite sequencing (gray bars). Data are indicated as % ±SD of total *CpG*. Relative *Sat2* cDNA/*Sat2* gDNA ratio were calculated as follows: (*Sat2* cDNA/*ACTB* cDNA)/(*Sat2* gDNA/*Alu* gDNA) (black bars). Data are presented as % ±SEM of the maximal expression level measured in cell lines. (D) Linear regression of data from (C). Black diamonds: sarcoma; gray dots: carcinoma; white dot: melanoma. (E) Normalized *Sat2* RNA levels were measured in either HFF2 or HeLa cells after 1 h of heat shock (HS) at 42°C followed by 1 h of recovery at 37°C. Basal expression levels in U2OS, LB188 and SaOS-2 hypomethylated cancer cell lines are also shown. Data are presented as % ±SEM of the expression measured in HFF2.

transcription of *HSP70,* one of the main HSF1 target genes.[21] Upregulation of *Sat2* RNA coincided with the transcriptional activation of *MAGE-A1* and *MAGE-A4* cancer germline genes (**Fig. 2D**) and the expected timing of 5-aza-induced genome-wide demethylation in cultured cells.[22] While, as expected,[22] *MAGE-A1* and *MAGE-A4* remained stably induced during the time-course of 5-aza treatment, *Sat2* RNA had almost returned to basal levels after 4 d of treatment (**Fig. 2D**), unraveling a very transient induction by the demethylating agent.

As suggested before by Eymery et al.[16] the failure of *Sat2* hypomethylation to constitutively hyperactivate *Sat2* transcription may be explained by the acquisition of additional repressive

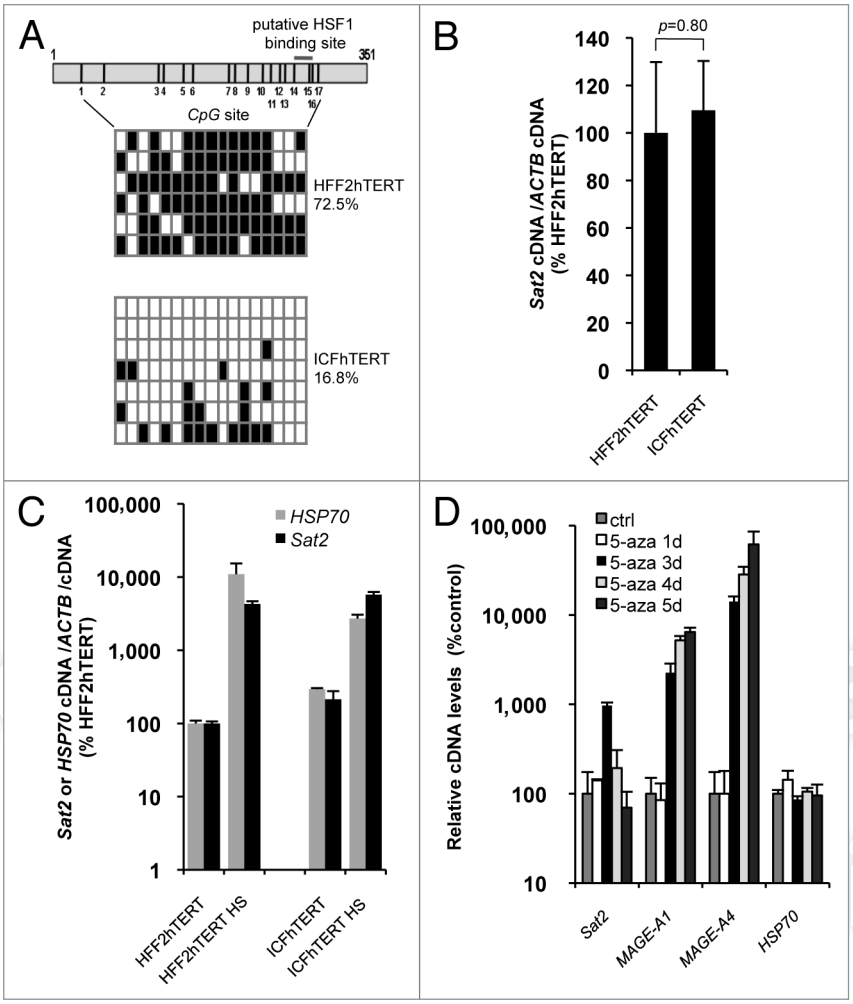


Figure 2. *Sat2* hypomethylation in DNMT3B-deficient ICFhTERT fibroblasts does not exacerbate heat shock-induced *Sat2* RNA expression. (A) Upper panel: schematic representation of the *Sat2* fragment analyzed by sequencing. Positions of the 17 CpG sites and of the putative HSF1 binding site are indicated. Lower panel: *Sat2* methylation level determined by sequencing of bisulfite-treated DNA molecules isolated from HFF2hTERT and ICFhTERT cells. White squares represent unmethylated CpG, black squares represent methylated CpG. (B) Relative *Sat2* RNA levels in HFF2hTERT and ICFhTERT cells. Data are indicated as % ±SEM of the expression measured in HFF2hTERT. (C) Basal and HS-induced *Sat2* and *HSP70* RNA levels in HFF2hTERT and ICFhTERT. For HS, cells were incubated for 1 h at 42°C followed by 1 h of recovery at 37°C. Data are presented as % ±SEM of the basal expression measured in HFF2hTERT. (D) HFF2hTERT cells were incubated in the presence or absence (ctrl) of 5 μM 5-aza-2'-deoxycytidine (5-aza) for 1 to 5 d and relative expression levels of *Sat2, MAGE-A1, MAGE-A4* and *HSP70* were measured by qRT-PCR. Data are presented as % ±SEM of the expression measured in HFF2 hTERT control cells.

histone marks that would compensate for DNA methylation loss. To address this hypothesis, we performed chromatin immunoprecipitation experiments with antibodies against H3K9me3 or H3K27me3 repressive histone marks in HFF2hTERT and ICFhTERT cell lines. As expected, *Sat2* was enriched in both H3K9me3 and H3K27me3 repressive histone marks when compared with the highly uncondensed *GAPDH* promoter (**Fig. 3A**). Strikingly, when compared with HFF2hTERT cells, we detected a 2-fold enrichment in H3K27me3 density at *Sat2* locus of ICFhTERT cells ($p = 3.5 \times 10^{-5}$) (**Fig. 3A**). To test whether this enrichment in H3K27me3 density was specific to hypomethylated *Sat2*, we analyzed a series of subtelomeric (1q, 2q, 5p, 10q, 13q, 15q, 16p and 21q) TERRA CpG-island promoters.[23] TERRA promoter methylation amounts to about 48.5% in normal telomerized fibroblasts and drops to 7.3% in ICFhTERT cells, resulting in strong upregulation of telomere transcription (data not shown). ChIP analyses revealed a similar increase in H3K27me3 density at hypomethylated TERRA promoters, showing that it is not restricted to *Sat2* DNA. To test whether increased H3K27me3 density may nevertheless repress basal transcription of *Sat2* in ICFhTERT, we transfected cells with siRNAs against *EZH2*, the histone-lysine N-methyltransferase of polycomb repressive complex 2. However, although global H3K27me3 levels were reduced by 60–65%, we did not detect any upregulation of *Sat2* RNA (**Fig. 3B and C**), suggesting that the increased density of H3K27me3 in ICFhTERT cells does not downregulate steady-state *Sat2* RNA levels. On the other hand, H3K9me3 density at *Sat2* repeats was similar in HFF2hTERT and ICFhTERT cell lines (**Fig. 3A**), in agreement with the recent observations that DNA methylation status does not impact on H3K9me3 density at several human chromosomal loci.[24]

***Sat2* RNA levels correlate with *HSP70* expression in melanoma tissues.** Having concluded that *Sat2* hypomethylation does not constitutively upregulate *Sat2* RNA levels, we next wished to get hints into *Sat2* transcriptional regulation in tumors. The previous demonstration that, when activated experimentally, the HS pathway is able to trigger *Sat2* transcription and the established role of HSF1 and heat shock proteins in the maintenance of cancer cell viability,[25-29] led us to test whether the HS pathway may similarly regulate *Sat2* expression in cancer tissues in vivo. To answer this question, we measured the expression levels of *Sat2* and *HSP70* in either primary (n = 6) or metastatic (n = 4) melanoma tumors. *Sat2* expression levels were highly variable and not statistically different between primary and metastatic melanoma tumors (p = 0.73) (**Fig. 4A**). Importantly, our results indicated a good correlation between the expression levels of *Sat2* and *HSP70* in melanoma tissues (R = 0.69, p = 0.01) (**Fig. 4B**), suggesting that, in vivo, *Sat2* RNA levels are correlated with activation of the HS pathway. We believe that, similarly to what was observed for cancer cell lines, DNA hypomethylation is not responsible for constitutive hyperactivation of *Sat2* transcription in melanoma tissues as we found no positive correlation between *Sat2* RNA and *MAGE-A1/2* gene expression, previously linked to profound *Sat2* and genome-wide hypomethylation (**Fig. 4C**).[4,30,31]

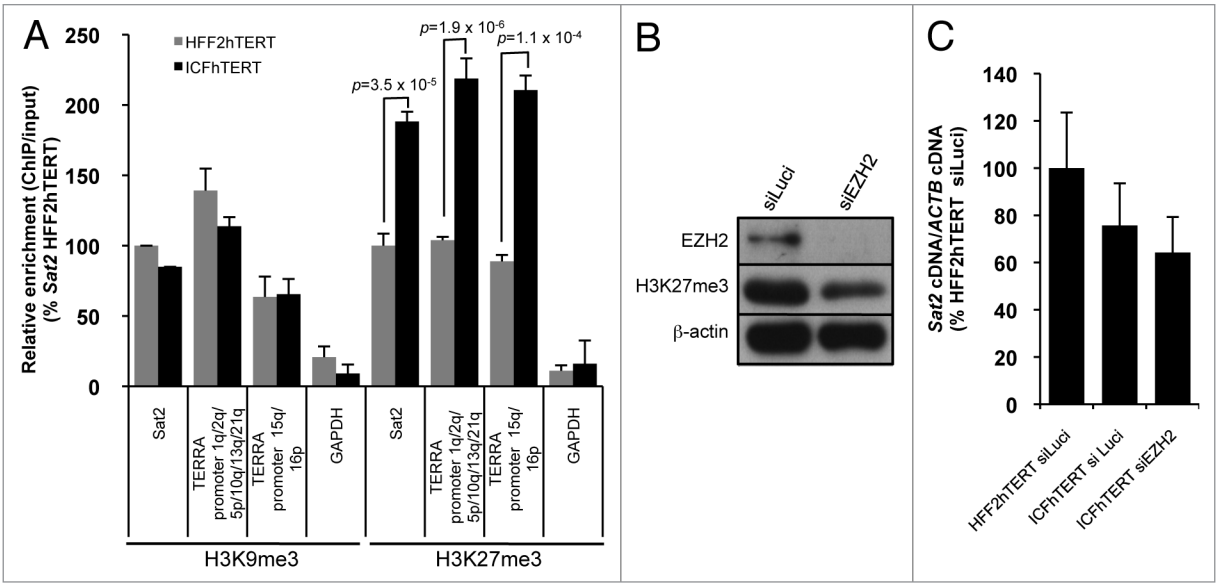


Figure 3. Hypomethylated *Sat2* in ICFhTERT is characterized by increased density of H3K27me3 marks. (A) Density of H3K9me3 or H3K27me3 at *Sat2* locus of HFF2hTERT and ICFhTERT determined by ChIP followed by qPCR. These marks were also quantified at *GAPDH* and TERRA (1q, 2q, 5p, 10q, 13q, 15q, 16p and 21q) promoters as controls. ChIP/input ratios were expressed as % ±SEM of the ratios obtained for *Sat2* DNA in HFF2hTERT with either H3K9me3 or H3K27me3 antibody. (B) Western blot analysis of EZH2 and H3K27me3 after treatment of ICFhTERT cells with siRNAs against either Luciferase (siLuci) or *EZH2* (siEZH2) for 72 h. β-actin is shown as loading control. (C) Relative *Sat2* RNA levels in ICFhTERT treated as in (B) and expressed as % ±SEM of *Sat2* RNA in HFF2 hTERT siLuci control cells.

Heat shock and RasV12 oncogene induce *Sat2* DNA demethylation. Hyperactivation of the HS pathway is likely to be a common event in the early steps of tumorigenesis, allowing cancerous cells to escape from oncogene-induced senescence.[25,26,28,29,32] Given that HS pathway hyperactivation was previously reported to be associated with chromatin remodeling events,[10,11,33,34] we next tested the possible role of the HS pathway in inducing *Sat2* DNA demethylation. To address this possibility, we exposed HFF2hTERT cells to HS for 1 h at 42°C, followed by either 1 h or 4 d of recovery at 37°C. As expected, both *HSP70* and *Sat2* transcripts were strongly induced after 1 h of recovery from HS (**Fig. 5A**), but resulting *Sat2* methylation levels (73.5%) were not statistically different from the ones measured in untreated cells (72.4%) (Yates' χ^2 test: p = 1) (**Fig. 5B**). Strikingly, however, we detected a reduction of *Sat2* methylation by about 14% in heat shocked-fibroblasts allowed to recover for four days at 37°C (p = 0.018) (**Fig. 5B**). These results indicate that the HS pathway induces *Sat2* demethylation by a process that appears to require several rounds of DNA replication (cells underwent three population doublings within the time course of recovery).

Ras oncogene was previously reported to activate HSF1.[35] Hence, we tested the impact of constitutively activated RasV12 protein, a mutant form frequently detected in tumors,[36] on *Sat2* methylation. HFF2 fibroblasts were transduced with either pBabe-puro::*RasV12* or an empty pBabe-puro vector as control. RasV12 upregulated *Sat2* RNA and the transcript was still detected 16 d after retroviral transduction (**Fig. 5C**). At this late time point, *HSP70* mRNA was not detected (data not shown) but *HSF1* transcripts were about 3-fold more abundant than in control cells (**Fig. 5C**). Analysis of RasV12-expressing cells 16 d after the transduction revealed a marked reduction of *Sat2* methylation level, which dropped from 78.7% to 51.4% (p < 0.0001) (**Fig. 5D**). Interestingly, in both HS and RasV12 conditions, *Sat2* demethylation appeared to occur mainly within the last 7 CpG dinucleotides of the analyzed sequence, in a region encompassing the putative HSF1 binding site (**Fig. 5B, D and E**). Altogether, the above results suggested that the heat shock response may be involved in *Sat2* DNA demethylation. Importantly, in our experimental conditions, we did not detect any concurrent reduction of the methylation level of either *LINE-1* interspersed repetitive element, *D4Z4* non-satellite subtelomeric repeats located at 4q35.2 and 10q26.3 or the promoter of cancer germline gene *MAGE-A1* (**Fig. 5F and G** and data not shown), suggesting a specific effect of HS on the demethylation of selected CpG dinucleotides within *Sat2* sequence.

Cancer cell lines display epigenetic scars of possible heat shock pathway activation during tumorigenesis. Our in vitro assay showed that HS pathway activation in fibroblasts is able to induce localized demethylation events within the last 7 CpG dinucleotides of *Sat2* sequence, giving rise to a HS-dependent demethylation signature. In order to provide evidence that the HS response may be involved in tumorigenesis-associated *Sat2* demethylation processes, we analyzed the methylation profile of *Sat2* in eight cancer cell lines, using HFF2hTERT and HEKhTERT as non-tumoral reference cell lines. Methylation levels within the *MAGE-A1* promoter, *D4Z4* and *LINE-1* sequences were also tested in these samples. As shown in **Figure 6A**, four cancer cell lines (LB23, LB45, PANC-1 and LB159) displayed an intermediate level of hypomethylation, as all four tested genomic regions retained a significant proportion of methylated *CpG*s.

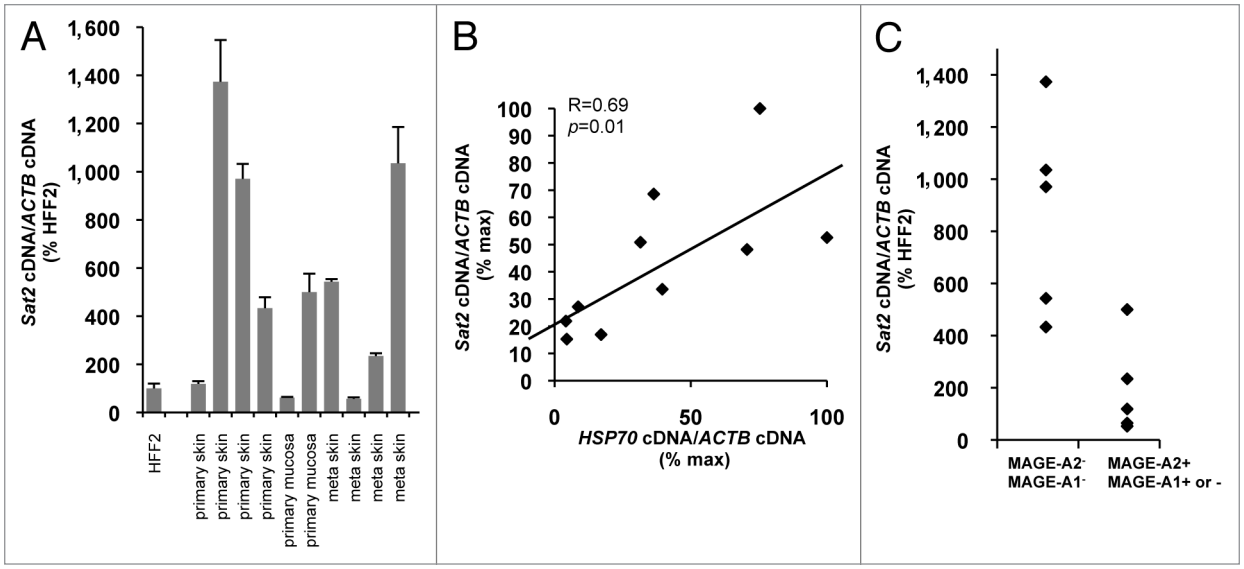


Figure 4. *Sat2* RNA levels are correlated with *HSP70* expression in melanoma tissues. (A) Relative *Sat2* RNA levels measured in six primary melanoma tumors and four melanoma metastases. Data are presented as % ±SEM of the expression measured in HFF2. (B) Comparison between *Sat2* and *HSP70* expression levels in melanoma tissues from A. Both *Sat2* and *HSP70* cDNA levels were normalized to *ACTB* cDNA and expressed as % of the maximal value measured in the samples. (C) Melanoma tissues from A were classified into two groups according to the presence (+) or the absence (-) of *MAGE-A2* or *MAGE-A1* transcripts. Relative *Sat2* RNA levels, as described in A, are indicated.

The other four cancer cell lines (U2OS, LB188, HT1080 and MZ-2-MEL), instead, were characterized by extensive genome-wide hypomethylation as evidenced by the complete or nearly complete demethylation of the tested loci (**Fig. 6A**). Closer analysis of cancer cell lines with intermediate genome-wide hypomethylation revealed preferential demethylation of the last 7 CpG dinucleotides within the *Sat2* sequence (**Fig. 6A and B**), similarly to what we observed following experimentally induced HS.

Discussion

Hypomethylation of repetitive DNA is largely responsible for the global loss of DNA methylation in cancer cells and pericentromeric *Sat2* locus is frequently hypomethylated.[1] Given the possible important role of repetitive ncRNAs in cancer cell biology, understanding how these ncRNAs are transcriptionally regulated is important. Our quantitative analysis of various cancer cell lines revealed that *Sat2* transcription is not constitutively hyperactivated by DNA hypomethylation and, in agreement with a previous study,[17] we did not detect constitutive overexpression of *Sat2* RNA in DNMT3B-deficient ICFhTERT fibroblasts either. We further showed that *Sat2* hypomethylation does not exacerbate *Sat2* RNA induction upon heat shock. Because we do not know where *Sat2* transcription starts, we cannot however exclude the possibility that, as suggested earlier,[17] dispersed chromosomal copies of *Sat2*, with undefined methylation profiles, may be transcribed and contribute to cellular *Sat2* RNA. The previous demonstration that HS induces the production of long molecules of satellite III transcripts emanating mostly—if not entirely—from chromosome 9 pericentromeric region suggests, however, that, if any, short transcripts from dispersed copies of *Sat2* are unlikely to contribute significantly to the total amount of *Sat2* RNA.[10,11]

A previous study suggested the existence of two distinct pathways for *Sat2* transcriptional induction, one relying on the HS pathway, through HSF1 activation, and the other one induced by treatment with 5-azadeoxycytidine demethylating agent independently of HS pathway activation.[16] We found that 5-aza only transiently upregulated *Sat2* RNA after three days of treatment without any detectable increase in *HSP70* transcription. Two possible explanations may account for this observation: (1) the heat shock pathway has been only transiently activated in response to genome-wide DNA damage[37] and, because *HSP70* mRNA may be much less stable than *Sat2* RNA, we did not detect it, or (2) *Sat2* transcription was hyperactivated by DNA demethylation but rapidly shut down by a still unknown mechanism, possibly linked to the immediate acquisition of additional and undefined repressive marks. Although this did not appear to counteract basal *Sat2* expression, we found an increase of H3K27me3 density at hypomethylated *Sat2* locus in ICFhTERT cells that was also detected at hypomethylated subtelomeric TERRA promoters. The reason why DNA hypomethylation is correlated with increased H3K27me3 density is unknown but fits with the observation that demethylation of the mouse imprinted *Rasgrf1* locus is similarly associated with increased H3K27me3 density, and suggests the existence of a—still unexplained—antagonism between these two repressive marks.[38] Other repressive marks, like heterochromatin protein 1 (HP1), may account for *Sat2* transcriptional repression. In support of this, mouse satellite ncRNAs were shown to promote the recruitment of HP1α to pericentric heterochromatin through direct interaction between RNA and the hinge domain of HP1α.[39] In the future, it would be interesting to test this hypothesis further.

Overexpression of satellite RNAs was previously detected in a variety of tumors.[15] Our analysis of *Sat2* expression in primary

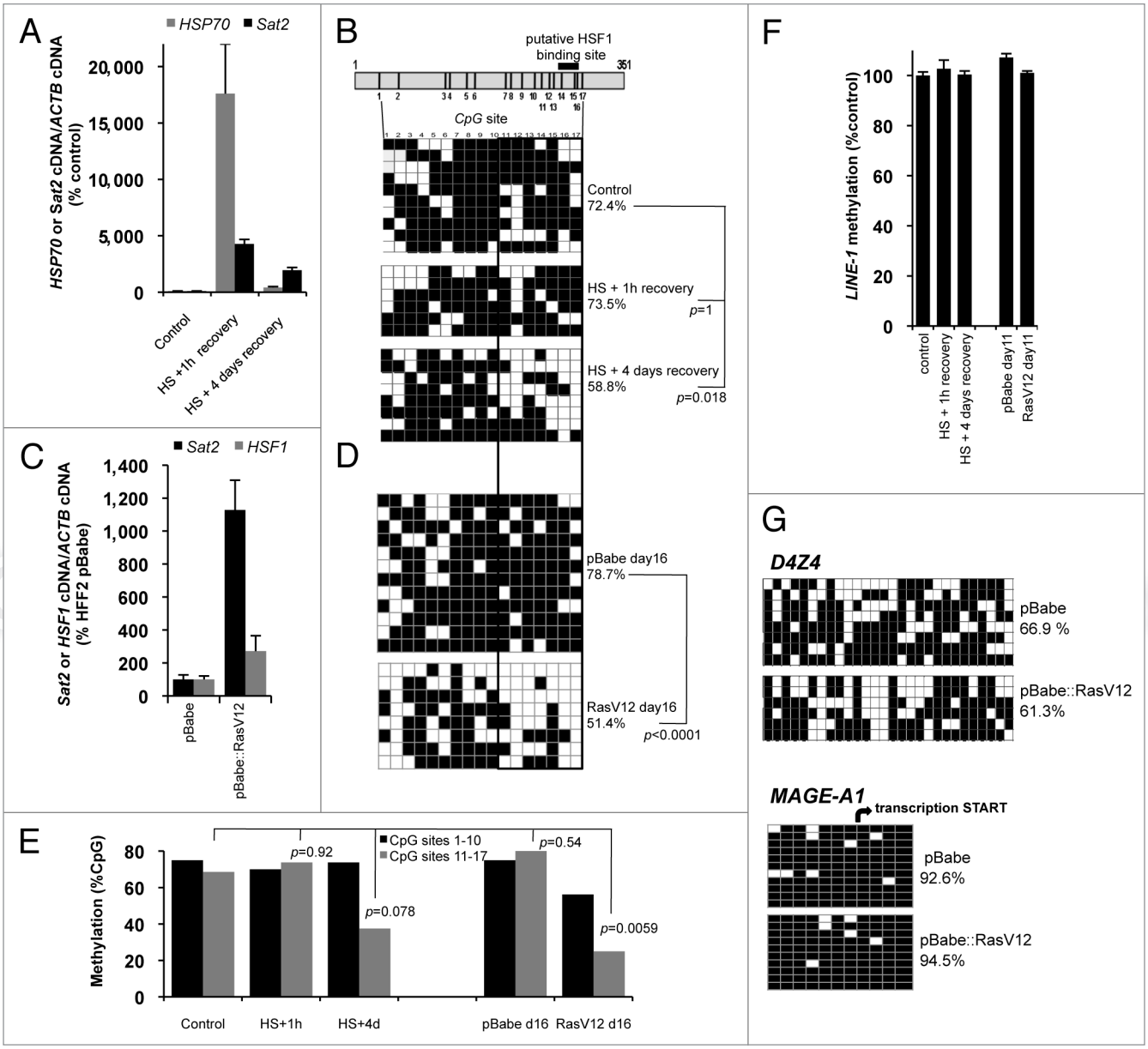
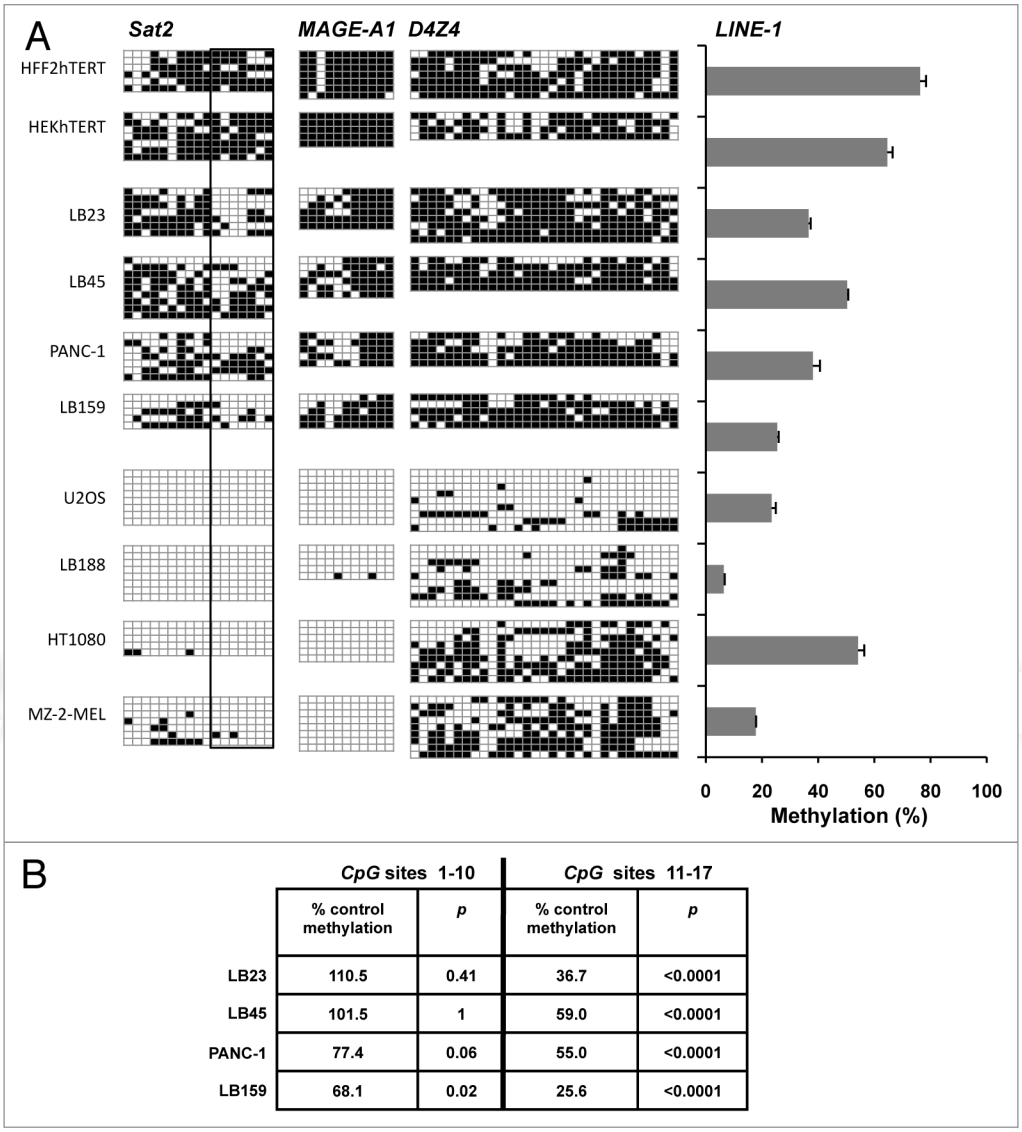


Figure 5. Heat shock and RasV12 oncogene induce *Sat2* DNA demethylation. (A) *Sat2* and *HSP70* expression levels in HFF2hTERT cells subjected or not to HS for 1 h at 42°C followed by either 1 h or 4 d of recovery at 37°C. Data are given as % ±SEM of the expression levels measured in control cells. (B) Upper panel: schematic representation of the *Sat2* fragment analyzed by sequencing. The 17 CpG dinucleotides and the putative HSF1 binding site are indicated. Lower panel: analysis of *Sat2* methylation by sequencing of individual molecules amplified from bisulfite-treated DNA isolated from cells described in (A). White squares represent unmethylated CpG, black squares represent methylated CpG. A Yates' χ^2 test was applied to assess the statistical differences in methylated CpG content with the control. (C) Relative *Sat2* and *HSF1* RNA levels in HFF2 fibroblasts 16 d after transduction with either empty pBabe or pBabe::*RasV12* plasmid and expressed as % ± SEM of the expression in HFF2pBabe. (D) *Sat2* methylation analysis of HFF2pBabe and HFF2pBabe::*RasV12* determined 16 d after transduction. See B for details. (E) Average methylation level of CpG sites 1 to 10 (black bars) or 11 to 17 (gray bars) of *Sat2* determined from (B and D). A Yates' χ^2 test was applied to assess the statistical differences in methylated CpG content within CpG sites 11 to 17 in all tested conditions. (F) *LINE-1* methylated CpG content determined by qMS-PCR on bisulfite-treated DNA prepared from samples described in (B and D). [Methylated *LINE-1*/(methylated *LINE-1* + unmethylated *LINE-1*)] ratios were expressed as % ± SEM of *LINE-1* methylation in control cells. (G) Analysis of *D4Z4* and *MAGE-A1* promoter methylation in HFF2pBabe and HFF2pBabe::RasV12 fibroblasts 16 d after transduction.

and metastatic melanoma tumor samples suggested that the level of HS pathway activation in cancer tissues may be the main determinant of *Sat2* expression in vivo, irrespective of DNA methylation status. The HS pathway is indeed frequently activated in cancerous lesions that experience oxidative stress, oncogene activation or proteotoxic damages and, not surprisingly, HSF1 is required for cancer cell survival, both in mouse and in human.[25-29,32] While this manuscript was in preparation, a study similarly reported that, although frequently detected in prostate cancer tissues, rRNA (rRNA) overexpression is not



B

	CpG sites 1-10		*CpG* sites 11-17	
	% control methylation	*p*	% control methylation	*p*
LB23	110.5	0.41	36.7	<0.0001
LB45	101.5	1	59.0	<0.0001
PANC-1	77.4	0.06	55.0	<0.0001
LB159	68.1	0.02	25.6	<0.0001

Figure 6. Heat shock pathway-dependent signature of *Sat2* demethylation is also observed in cancer cell lines. (A) Left panel: methylation profile of *Sat2*, *D4Z4* and *MAGE-A1* promoter determined by sequencing of bisulfite-treated DNA molecules isolated from sarcoma (LB23, U2OS, LB188, HT1080), melanoma (LB45, MZ-2-MEL) and carcinoma (PANC-1, LB159) cell lines and from HFF2hTERT and HEKhTERT non-tumoral cell lines; right panel: *LINE-1* methylation level determined by qMS-PCR on bisulfite-treated DNA with [methylated *LINE-1*/(methylated *LINE-1* + unmethylated *LINE-1*)] ratios expressed as % ±SEM (B) Relative methylation levels of CpG sites 1–10 or 11–17 of *Sat2* in LB23, LB45, PANC-1 and LB159 cancer cell lines compared with the methylated CpG content of *Sat2* DNA from HFF2hTERT and HEKhTERT control cells together. Yates' χ^2 p values are indicated.

linked to rDNA promoter hypomethylation, and authors provided evidences that rRNA levels are instead correlated with *MYC* mRNA levels, a transcriptional activator of rDNA.[40] In addition to being required for survival of fully transformed cells, HSF1 also acts as a powerful driver of oncogenic transformation, notably by ensuring escape from oncogene-induced senescence.[28,29,32] Hence, we can reasonably assume that the HS pathway is strongly hyperactivated during the first steps of tumorigenesis. We found that both HS treatment and RasV12 oncogene overexpression reduced *Sat2* methylation level by, respectively, 13 and 27%, suggesting that hyperactivation of HS pathway may be causally involved in tumorigenesis-driven *Sat2* demethylation. In support of this, we observed similar patterns of *Sat2* demethylation in cancer cell lines with moderate genome-wide hypomethylation. Interestingly, we found that HS pathway-induced demethylation of *Sat2* mainly occurred within a region encompassing the putative HSF1 binding site. These observations are reminiscent to the demonstration that recruitment of DNA-binding factors to chromatin is sufficient to locally induce DNA methylation loss,[41] and fits with a more recent base-pair-resolution methylome analysis in mouse stem cells, which unraveled an overlap between low-methylated regions and DNA-binding factor occupancy.[42] It is likely that, in light of the role of ncRNA in heterochromatin formation, HS-induced transcriptional upregulation, although leading to localized DNA demethylation, may be associated with the acquisition of other repressive marks to maintain pericentromeric heterochromatin.

Whether HS response hyperactivation may induce demethylation of other repetitive DNA sequences remains to be established as transcription of a variety of other centromeric and pericentric sequences was reported to occur upon HS.[16] However, the observation that, instead of being localized within the last seven CpG sites, demethylated CpG sites spread over the entire *Sat2* sequence in cancer cell lines displaying strong genome-wide hypomethylation, suggests the existence of additional and still unexplained demethylation processes that may also be responsible for *MAGE-A1* and *D4Z4* demethylation. Cancer-testis gene promoter demethylation was reported to be a late event in prostate cancer progression[43] and it is assumed that, while cancer-linked DNA hypomethylation is generally found early during carcinogenesis, more extensive DNA hypomethylation can appear later during tumor progression.[1] Hence, we propose that HS pathway activation may contribute to initial demethylation events of *Sat2,* which may, in some instances, be followed by additional undefined genome-wide demethylation events. We do not exclude the possibility that the latter events may be facilitated by—and spread from—early-induced localized demethylation events.

In summary, our results indicate that cancer-linked *Sat2* hypomethylation is not associated with constitutive hyperactivation of *Sat2* but that HS pathway activation in cancer tissues is likely to be the main determinant of *Sat2* expression. Our study further suggests that strong HS response activation during tumorigenesis may play a role in localized demethylation of pericentromeric *Sat2*. In the future, it would be interesting to address further the role of the HS pathway and its downstream effectors in cancer-linked DNA demethylation.

Materials and Methods

Non-tumoral or cancer cell lines and melanoma tissues. HFF2 normal human foreskin fibroblasts were purchased from ATCC (SCRC-1042). HMEC human mammary epithelial cells and ICF fibroblasts were purchased from Coriell. HNEM normal human epidermal melanocytes and HMSC human mesenchymal stem cells were kindly provided by E. De Plaen (Ludwig Institute for Cancer Research) and E. Sokal (UCL) respectively. HFF2hTERT,[44] ICFhTERT, HMEChTERT and HNEMhTERT cell lines were obtained by retroviral transduction with pBMN::*hTERT* plasmid (kindly provided by C. Heirman, VUB). Cancer cell lines used in this study are listed in **Table S1**. Cell culture media were from GIBCO (Life Technologies) and FBS was from HyClone (Thermo Fisher Scientific, SV30160.03). HEKhTERT cDNA and gDNA samples were provided by A. Londoño-Vallejo (Institut Curie). Melanoma tumor samples were obtained from patients undergoing surgery or tumor resection and immediately frozen in liquid nitrogen. Experimental procedures involving the use of biological material were approved by the Ludwig Institute for Cancer Research Institutional Review Board. All patients gave informed consent mentioning that part of the tumor samples could be used for research purposes. Most tumor biopsies were obtained as part of screening procedures for participation in clinical immunotherapy trials.

Cell treatment with 5-aza-2'-deoxycytidine. HFF2hTERT cells were incubated for 1 to 5 d in culture medium containing 5 μM 5-aza-2'-deoxycytidine (Sigma-Aldrich).

Retroviral transductions with RasV12 oncogene. 150,000 HFF2 cells were incubated with retroviral supernatant containing either pBABE-puro or pBABE::*Ha-RasV12*-puro plasmid (kindly provided by F d'Adda di Fagagna, IFOM foundation, Milan, Italy) and 4 μM polybrene (Sigma-Aldrich, H9268) for 24 h. Transduction procedure was repeated once and cells were then selected with 0.8 μg/ml puromycin (Sigma-Aldrich, P7255). The day following the second transduction was designated as day 1.

siRNA transfections. Cells were transfected with Lipofectamine 2000 (Life Technologies, 11668019) and siRNAs (Eurogentec) against either Luciferase (5'-CUU ACG CUG AGU ACU UCG A) or EZH2 (5'-GAC UCU GAA UGC AGU UGC U).[45] Six hundred and fifty thousand cells were transfected with 0.7 nmole siRNA and collected for RNA or protein extraction 72 h post-transfection.

Genomic DNA extraction and sodium bisulfite treatment. Genomic DNA was extracted with the Wizard Genomic DNA Purification Kit following manufacturer's instructions (Promega, A1125). Sodium bisulfite treatment of 10 μg gDNA was performed as described earlier and resuspended in a final volume of 100 μl.[46]

***Sat2* and *LINE-1* methylation measurement by qPCR.** When indicated, quantification of *Sat2* methylation was achieved using the MethylLight assay on bisulfite-treated gDNA and values were normalized by the amount of CpG-depleted Alu sequences as described previously.[3] *LINE-1* methylation was quantified as follows. First, a semi-quantitative methylation-independent PCR was performed on a 1:100 dilution of bisulfite-modified gDNA (30 sec at 95°C, 30 sec at 58°C and 30 sec at 72°C for 25 cycles). Next, two independent qPCR were performed on a 1:100 dilution of the first PCR with primers specific for either the methylated or the unmethylated *LINE-1* sequence. Annealing temperature was of 60°C for both *Sat2* MethylLight and *LINE-1* qMS-PCR. Primer and probe sequences are listed in **Table S2**.

Methylation analysis of *Sat2, D4Z4* and *MAGE-A1* promoter by sequencing. Methylation CpG content determination of *Sat2*,[20] *D4Z4*,[31] and *MAGE-A1* promoter,[47] was performed by sequencing of bisulfite-treated DNA using primers listed in **Table S2**. In all cases, the final PCR product was cloned into pJET 1.2/blunt vector using CloneJET PCR Cloning kit (Fermentas, Thermo Fischer Scientific, K1232) before sequencing (Macrogen Inc.).

Quantitative RT-PCR. Total RNA was extracted from cell lines using TriPure Isolation Reagent (Roche Applied Science) and treated with DNaseI (Ambion, Life Technologies, AM2238) according to manufacturer's instructions. Reverse transcription was performed as described previously.[31] *Sat2/ACTB* cDNA ratios were normalized to *Sat2/Alu* gDNA ratios determined by qPCR on a 1:1,000 dilution of gDNA. The *Alu* sequences that were amplified correspond to an *Alu* consensus motif determined from the sequences of ancient and more recent individual *Alu* repeats subfamilies (*Alu-J, SluSp, AluSx, AluSq, AluSc, AluY,*

AluSb2, AluYb8, AluYa5 and *AluYa8*).[3] Annealing temperature was of 60°C for all qPCR except for *ACTB* (β-actin) (62°C). qPCR reactions were performed using either *Taq*Man or SYBR Green technology. Primers and probes (listed in **Table S2**), qPCR Core kit and SYBR Green were purchased from Eurogentec.

Western blot. Cell lysates were obtained by resuspending cell pellets into 2× Laemmli sample buffer (25% glycerol, 2% SDS, 0.01% bromophenol blue, 0.0625 M TRIS-HCl, pH 6.8) with Complete mini protease inhibitor cocktail (Roche Applied Science, 11836153001). The following antibodies were used: EZH2 (Millipore, 17-662, 1:2,000), H3K27me3 (Active Motif, 39156-39158, 1:10,000) and β-Actin (Sigma-Aldrich, A5441, 1:50,000).

CHIP assay. About 20 × 10^6 cells were cross-linked with 1% formaldehyde (Sigma-Aldrich, 344198) at RT for 10 min before addition of 125 mM glycine (Sigma-Aldrich, G7126) followed by resuspension into lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1, 1× Complete mini complete protease inhibitor cocktail (Roche, 11836153001)] and sonication with Bioruptor (Diagenode) for 9 × 30 s. Immunoprecipitations were performed with OneDay ChIP kit (Diagenode, Kch-oneDIP-180) following manufacturer's instructions and with antibodies purchased from Millipore (H3K9me3, 07-442; H3K27me3, 17-622). Immunoprecipitated *Sat2* DNA was quantified by qPCR, together with *GAPDH* and subtelomeric TERRA (1q, 2q, 5p, 10q, 13q, 15q, 16p and 21q) promoter DNA as controls. Primers are described in **Table S2**.

Statistical analyses. Results obtained from experiments relying on either qRT-PCR or qPCR on bisulfite-treated gDNA are shown as means ± standard error of the mean (SEM). SEM was obtained by dividing the standard deviation by the square root of the number of measurements performed (SD/√n). Results obtained after sequencing of bisulfite-treated gDNA molecules are given as means ± standard deviations. p values were calculated with Student's t-tests. Linear regression analyses and Yates' chi-square (χ^2) were performed on http://faculty.vassar.edu/lowry/VassarStats.html.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/21107

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