LETTER

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The histone methyltransferase SETDB1 is recurrently amplified in melanoma and accelerates its onset

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The most common mutation in human melanoma, *BRAF(V600E)*, activates the serine/threonine kinase BRAF and causes excessive activity in the mitogen-activated protein kinase pathway[1,2]. *BRAF(V600E)* mutations are also present in benign melanocytic naevi[3], highlighting the importance of additional genetic alterations in the genesis of malignant tumours. Such changes include recurrent copy number variations that result in the amplification of oncogenes[4,5]. For certain amplifications, the large number of genes in the interval has precluded an understanding of the cooperating oncogenic events. Here we have used a zebrafish melanoma model to test genes in a recurrently amplified region of chromosome 1 for the ability to cooperate with *BRAF(V600E)* and accelerate melanoma. SETDB1, an enzyme that methylates histone H3 on lysine 9 (H3K9), was found to accelerate melanoma formation significantly in zebrafish. Chromatin immunoprecipitation coupled with massively parallel DNA sequencing and gene expression analyses uncovered genes, including *HOX* genes, that are transcriptionally dysregulated in response to increased levels of SETDB1. Our studies establish *SETDB1* as an oncogene in melanoma and underscore the role of chromatin factors in regulating tumorigenesis.

To identify genes that promote melanoma, we focused on genomic regions that are subject to copy number amplification in human tumour samples. In a study of 101 cell lines and short-term cultures of melanoma cells, chromosome 1q21 (chr1: 147.2–149.2 megabases) was identified as a recurrently amplified interval[6] (Fig. 1a). The same region was implicated in another comprehensive analysis of copy number variation in melanoma[4]. To test candidate genes from this interval for the ability to accelerate melanoma, we developed an assay in transgenic (Tg) zebrafish in which *BRAF(V600E)* is expressed under the control of a melanocyte-specific gene (*mitfa*) promoter on a *p53* (also known as *tp53*) mutant background ($p53^{-/-}$) (Supplementary Fig. 1). Melanomas and melanocytes that develop in *Tg(mitfa:BRAF(V600E)); $p53^{-/-}$* zebrafish[7] are suppressed by a $mitfa^{-/-}$ mutation. We engineered a transposon-based vector called miniCoopR that rescues melanocytes and melanomas in a *Tg(mitfa:BRAF(V600E)); $p53^{-/-}$; $mitfa^{-/-}$* strain and drives the expression of a candidate gene in these rescued tissues. We identified genes that were present in the human 1q21 region and were overexpressed as messenger RNAs in 1q21-amplified melanomas based on Affymetrix microarrays. Candidate human genes were cloned into the miniCoopR vector and injected into one-cell stage *Tg(mitfa:BRAF(V600E)); $p53^{-/-}$; $mitfa^{-/-}$* zebrafish embryos. Tumour

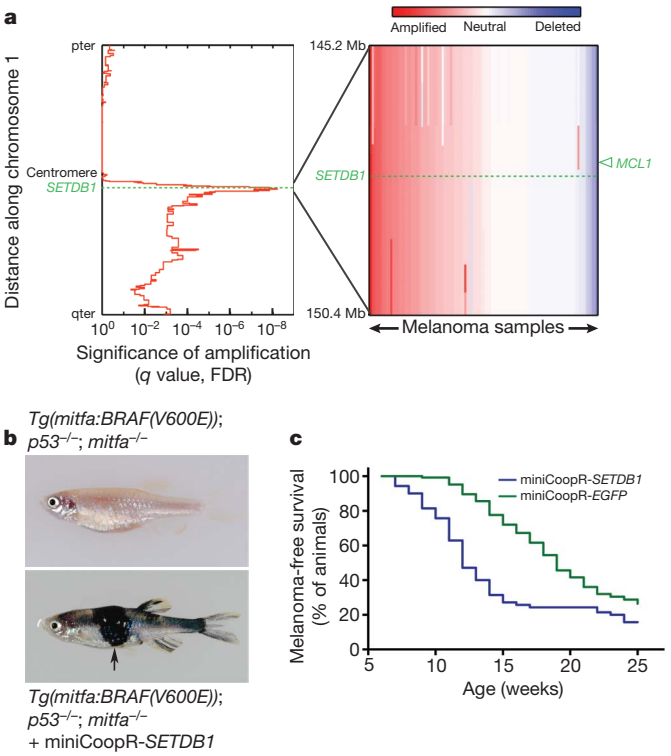

Figure 1 | *SETDB1* accelerates melanoma formation in zebrafish. **a**, Left, significance of copy number amplification along chromosome 1 in human melanoma samples, as assessed by using the algorithm GISTIC (genomic identification of significant targets in cancer)[8,20]. Significance values were determined by the false discovery rate (FDR) test. Right, copy number profiles in the human 1q21 interval in melanoma samples (vertical bars). The positions of *SETDB1* (dashed line) and *MCL1* (arrowhead) are indicated. Mb, megabase. **b**, The *Tg(mitfa:BRAF(V600E)); $p53^{-/-}$; $mitfa^{-/-}$* strain (top) was injected with miniCoopR-cloned candidate oncogenes. In animals injected with miniCoopR-*SETDB1* (bottom), the melanocytes are rescued, and melanomas (arrow) rapidly develop. **c**, Melanoma-free survival curves for *Tg(mitfa:BRAF(V600E)); $p53^{-/-}$; $mitfa^{-/-}$* zebrafish injected with miniCoopR-*SETDB1* (weighted average of 2 independent experiments, $n = 70$) or miniCoopR-*EGFP* (weighted average of 3 independent experiments, $n = 125$).

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incidence curves for the resultant adults showed that one gene in this interval, *SETDB1*, significantly accelerated melanoma onset ($P = 9.4 \times 10^{-7}$, logrank chi-squared test; Fig. 1b, c and Supplementary Fig. 2).

As in melanoma, *SETDB1* is focally amplified in non-small-cell lung cancer, small-cell lung cancer, ovarian cancer, hepatocellular carcinoma and breast cancer (Supplementary Fig. 3). The anti-apoptotic gene *MCL1* resides near *SETDB1* in the 1q21 interval, and knockdown of *MCL1* has been shown to diminish non-small-cell lung cancer proliferation and xenograft outgrowth[8]. However, *MCL1* is not overexpressed in the 1q21-amplified melanoma samples, so it was not tested in this study. No other gene accelerated the onset of melanomas, suggesting that *SETDB1* is a crucial gene that is amplified in the chromosome 1q21 interval. Using fluorescence *in situ* hybridization, we observed *SETDB1* amplification in short-term cultures of human melanoma cells (Supplementary Fig. 4), directly confirming the array-based copy number data from which our study originated.

Melanomas overexpressing *SETDB1* were more aggressive than tumours overexpressing enhanced green fluorescent protein (*EGFP*) when analysed at an equivalent stage and in the same *Tg(mitfa:BRAF(V600E)); $p53^{-/-}$; $mitfa^{-/-}$* genetic background. The melanomas expressing *SETDB1* were more locally invasive than the *EGFP* control melanomas (Fig. 2a; 94% (overexpressing *SETDB1*; $n = 18$) versus 53% (expressing *EGFP*; $n = 17$) of melanomas invaded the muscle ($P = 1.6 \times 10^{-3}$, Fisher's exact test), and 89% (*SETDB1*) versus 35% (*EGFP*) invaded the spinal column ($P = 7.2 \times 10^{-3}$, Fisher's exact test)). MiniCoopR-*SETDB1* transgenic melanomas had more extensive nuclear pleomorphism and larger nuclei than control tumours (Supplementary Fig. 5). By contrast, miniCoopR-*SETDB1* tumours showed similar levels of BRAF protein to control tumours, indicating that *SETDB1* did not accelerate melanoma formation by altering expression of the *BRAF(V600E)* transgene (Supplementary Fig. 6).

Melanocytes overexpressing *SETDB1* grew in confluent patches in zebrafish, unlike melanocytes in the *EGFP*-overexpressing control zebrafish, which grew in a wild-type stripe pattern. We analysed the genetic interactions that are responsible for these pigmentation differences. *SETDB1*-expressing melanocytes in the *Tg(mitfa:BRAF(V600E)); $mitfa^{-/-}$* strain formed confluent patches, but *SETDB1*-expressing melanocytes in the *$p53^{-/-}$; $mitfa^{-/-}$* strain grew in a striped pattern (Fig. 2b). Although *SETDB1* and *BRAF(V600E)* cooperated to override normal pigment patterning, no tumours arose in miniCoopR-*SETDB1*-injected *Tg(mitfa:BRAF(V600E)); $mitfa^{-/-}$* zebrafish, indicating that *SETDB1* overexpression does not have the same effect as loss of *p53* in tumour formation.

BRAF(V600E) induces senescence in human naevi and in cultured mammalian melanocytes[9], and we suspected that the pigmentation differences might result from a failure of senescence and excess melanocyte proliferation caused by *SETDB1*. Using senescence-associated β-Galactosidase (SA-β-Gal) staining[10,11], we confirmed that *BRAF(V600E)* induces senescence in zebrafish melanocytes *in vivo* (Supplementary Fig. 7a–c). We stained miniCoopR-rescued melanocytes and found *SETDB1*-expressing melanocytes to be less senescent than those expressing only *EGFP* (Fig. 2c). *SETDB1*-expressing melanocytes also lacked the flattened morphology of senescent cells (Supplementary Fig. 7d). These results suggest that *SETDB1* overexpression may contribute to melanoma formation by abrogating oncogene-induced senescence.

To understand the gene expression changes that occur when *SETDB1* is overexpressed, we performed microarray analyses of zebrafish melanomas. We defined a gene signature comprising 67 human orthologues of genes that are downregulated in *SETDB1*-overexpressing zebrafish melanomas (Fig. 3a) and tested the relationship between this signature and *SETDB1* expression in human melanomas. Using gene set enrichment analysis (GSEA)[12,13], we found that the gene signature was inversely correlated with *SETDB1* expression across a panel of 93 cell lines and short-term cultures of melanoma cells (Fig. 3b). *SETDB1*

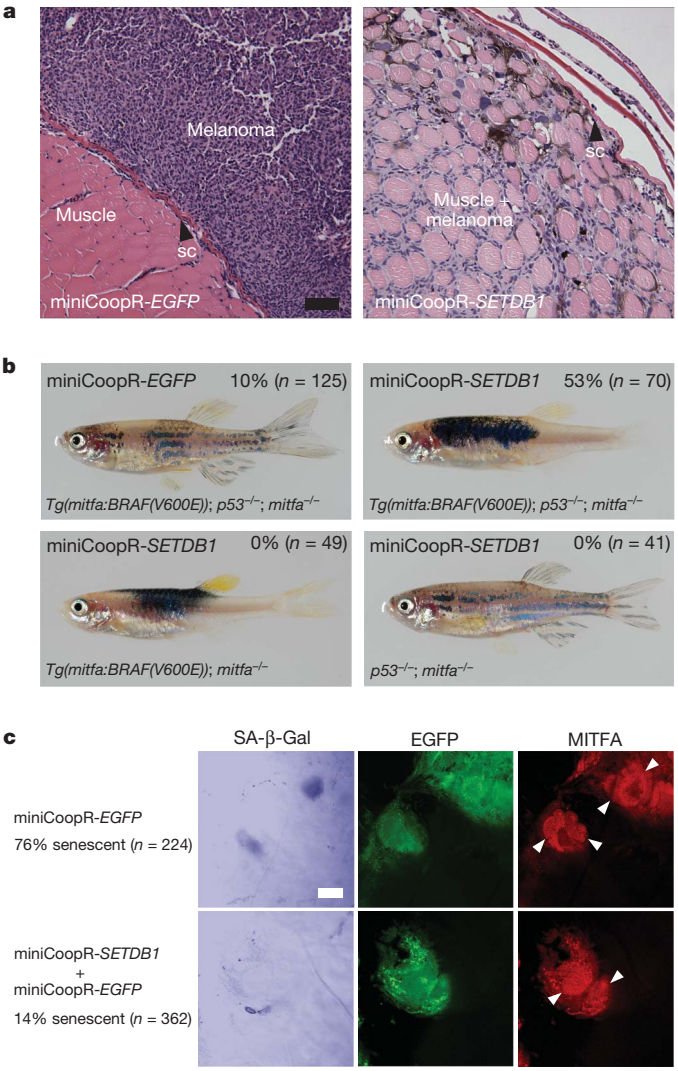

Figure 2 | Effects of *SETDB1* on melanoma cells and melanocytes. **a**, Transverse sections of zebrafish melanomas at 2 weeks post onset of melanoma, visualized by staining with haematoxylin and eosin. At this time point, dorsal miniCoopR-*EGFP* melanomas (left) have exophytic growth, whereas miniCoopR-*SETDB1* melanomas (right) have invaded from the skin, through the collagen-rich stratum compactum (sc) of the dermis, into the underlying musculature. Scale bar, 70 μm. **b**, *SETDB1* interacts with *BRAF(V600E)*, affecting the pigmentation pattern, but the *$p53^{-/-}$* mutation is required for melanoma formation. miniCoopR-*EGFP* or miniCoopR-*SETDB1* was injected into the indicated transgenic strains. The photographs indicate pigmentation pattern differences before the time point at which melanomas begin to form in the *Tg(mitfa:BRAF(V600E)); $p53^{-/-}$; $mitfa^{-/-}$* background. Percentages indicate the melanoma incidence at 12 weeks of age; n = number of fish. **c**, *SETDB1* abrogates *BRAF(V600E)*-induced senescence. Left, brightfield pseudocoloured photomicrographs of SA-β-Gal staining performed on scale-associated melanocytes. Centre and right, fluorescent photomicrographs of the same melanocytes. In this experiment, miniCoopR-rescued melanocytes express *mitfa*-promoter-driven EGFP (centre) and MITFA (right). Multiple nuclei (arrowheads) are present in *BRAF(V600E)*-expressing melanocytes. The percentage of senescent melanocytes is indicated ($P = 7.3 \times 10^{-51}$, chi-squared test); n = number of cells. Scale bar, 10 μm.

overexpression led to a broad pattern of transcriptional changes, including conserved downregulation of a group of genes that is enriched for *HOX* genes and for transcriptional regulators.

To identify the direct targets of SETDB1 across the genome in melanoma cells, we performed chromatin immunoprecipitation followed by massively parallel sequencing (ChIP-seq). We identified SETDB1 targets from WM262, a short-term culture of melanoma cells with high levels of SETDB1 expression, and from WM451Lu, a short-term

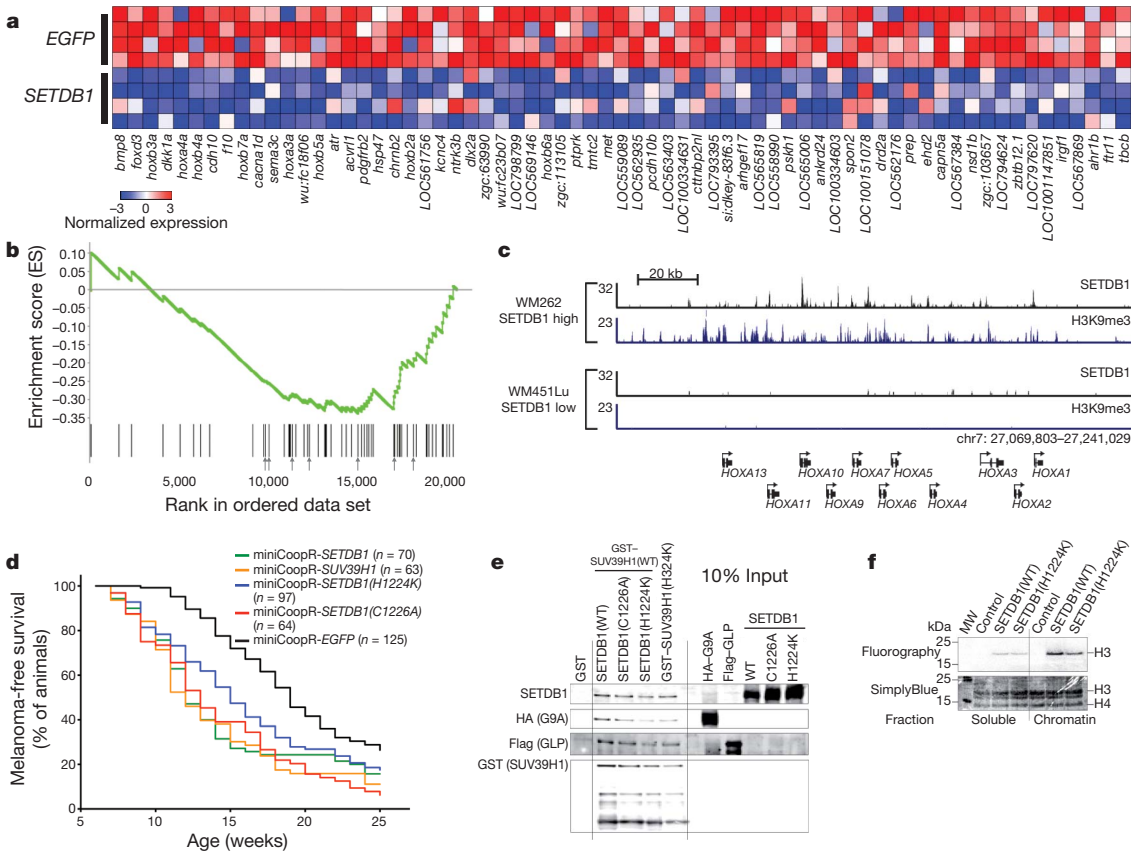

Figure 3 | SETDB1 target gene regulation and histone methyltransferase complex formation. **a**, Heat-map of genes that are downregulated in zebrafish melanomas overexpressing *SETDB1* compared with control (*EGFP*-expressing) melanomas. **b**, Graphical representation of the rank-ordered gene list derived from a panel of short-term cultures of human melanoma cells and stratified on the basis of *SETDB1* expression level. The enrichment score is calculated based on a running-sum statistic, which increases when a gene (vertical line) in the gene set is encountered and decreases when one is not. GSEA shows that human orthologues of *SETDB1*-downregulated zebrafish genes are similarly downregulated in human melanomas as the level of *SETDB1* increases (enrichment score (ES) = −0.37, normalized enrichment score (NES) = −1.47, *q* value = 0.034 (FDR test), $P = 0.034$). The arrows indicate the positions of *HOX* genes. **c**, SETDB1 and H3K9me3 ChIP-seq profiles at the *HOXA* locus in human melanoma cells. The number of sequence reads is shown on the *y* axis. SETDB1 and overlapping H3K9me3 are present at the *HOXA* locus in WM262 cells but largely absent in WM451Lu cells. kb, kilobases. **d**, Melanoma-free survival curves for *Tg(mitfa:BRAF(V600E)); p53*$^{-/-}$*; mitfa*$^{-/-}$ zebrafish expressing *SUV39H1* ($P = 6.74 \times 10^{-8}$ versus miniCoopR-*EGFP*, logrank chi-squared test) or expressing the methyltransferase-deficient SETDB1 variants *SETDB1(H1224K)* ($P = 0.24$ versus miniCoopR-*SETDB1*, and $P = 8.4 \times 10^{-5}$ versus miniCoopR-*EGFP*) or *SETDB1(C1226A)* ($P = 0.20$ versus miniCoopR-*SETDB1*, and $P = 1.3 \times 10^{-11}$ versus miniCoopR-*EGFP*). **e**, *In vitro* reconstitution of methyltransferase complexes containing SETDB1 and SUV39H1 variants. The sequential purification of glutathione *S*-transferase (GST)-tagged SUV39H1, Flag-tagged GLP and haemagglutinin (HA)-tagged G9A proteins was followed by western blotting using antibodies specific for the proteins and protein tags indicated on the left. Sequential purifications indicate that mutant SETDB1 proteins co-purify in a methyltransferase complex, as does wild-type (WT) SETDB1. **f**, Histone methylation assays of complexes purified from C2C12 myoblast cells. Complexes containing WT or mutant SETDB1 can catalyse the transfer of radiolabelled methyl groups to histone H3, as detected by fluorography.

culture of melanoma cells with low levels of SETDB1 (Supplementary Fig. 8). These short-term cultures harbour the *BRAF(V600E)* mutation (Supplementary Fig. 9), and their proliferation is sensitive to changes in SETDB1 levels (Supplementary Figs 10 and 11). In murine embryonic stem cells, SETDB1 binds to the promoters of genes encoding developmental regulators, including *Hox* genes[14]. We observed differential binding of SETDB1 to genes in the *HOXA* cluster in melanoma cell lines with high and low levels of SETDB1 expression; SETDB1 is bound to *HOXA* genes in WM262 cells, whereas there is minimal binding in WM451Lu cells (Fig. 3c and Supplementary Tables 1 and 2). SETDB1 catalyses the trimethylation of histone H3 lysine 9 (H3K9me3), thereby promoting repression of its target genes. ChIP-seq for the H3K9me3 mark showed that H3K9me3 is present at the *HOXA* locus in WM262 cells but absent in WM451Lu cells (Fig. 3c). *HOX* gene expression is inversely correlated with SETDB1 levels in short-term cultures of melanoma cells (Fig. 3b), suggesting that enhanced target gene binding and repression may have a role in the SETDB1-mediated acceleration of melanoma onset. Additional ChIP-seq, for MCAF1 (also known as AM and ATF7IP; a methyltransferase-stimulatory cofactor of SETDB1)[15] in WM262 cells, suggests that the effects of SETDB1 overexpression are mediated in part by MCAF1 (Supplementary Fig. 12).

We assayed the effects of *SETDB1* overexpression on target genes by infecting WM451Lu cells with a *SETDB1*-expressing lentivirus. Using SETDB1 ChIP-seq data from WM451Lu cells, we found that SETDB1-bound targets are significantly enriched in downregulated genes but not upregulated ones (Supplementary Fig. 13 and Supplementary Table 3), suggesting that a major consequence of *SETDB1* amplification is repression of SETDB1-bound target genes. However, many SETDB1 target genes in both WM451Lu and WM262 short-term cultures of melanoma cells are not methylated, and additional analyses show a relationship between increasing *SETDB1* levels and increasing expression of many SETDB1 target genes (Supplementary Fig. 14).

To obtain a mechanistic insight into the role of SETDB1 in regulating gene expression, we undertook genetic and biochemical studies that evaluate methyltransferase activity. Recently, a complex containing SETDB1 and the H3K9 methyltransferases SUV39H1, G9A (also known as EHMT2) and GLP (also known as EHMT1) was discovered[16]. To examine the possibility that other methyltransferases act together with *SETDB1* to modulate melanoma onset, we tested whether

SUV39H1 could accelerate melanoma formation in zebrafish. As was the case for *SETDB1*, overexpression of *SUV39H1* led to the formation of confluent melanocyte patches, and it accelerated melanoma onset (Fig. 3d). We next examined the consequences of mutations that render SETDB1 enzymatically inactive. Enzymatically deficient SETDB1 was capable of incorporating into the methyltransferase complex *in vitro* (Fig. 3e) and *in vivo* (Supplementary Fig. 15). Furthermore, in the context of enzymatically deficient SETDB1, the complex retained methyltransferase activity (Fig. 3f and Supplementary Fig. 16) and binding site localization (Supplementary Fig. 17). Last, the melanoma incidence curves for two methyltransferase-deficient SETDB1 mutants were similar to each other and to the melanoma incidence curve for zebrafish that overexpress wild-type SETDB1 (Fig. 3d). Our studies suggest a model in which activity of the methyltransferase complex containing SETDB1 and SUV39H1 alters gene expression in a way that leads to the acceleration of melanoma onset and to increased invasiveness.

To determine the extent of SETDB1 overexpression in human melanomas, and to examine potential clinical implications, we performed immunohistochemistry on melanoma tissue microarrays. After confirming antibody specificity (Supplementary Fig. 18), we observed high levels of SETDB1 expression in 5% of normal melanocytes ($n = 20$), 15% of benign naevi ($n = 20$) and 70% of malignant melanomas ($n = 91$) (Fig. 4). On the basis of our observations of pre-malignant melanocytic lesions in zebrafish, we speculate that human naevi that overexpress *SETDB1* are more likely to undergo oncogenic progression than naevi with basal levels of *SETDB1* expression. These data indicate that the majority of malignant human melanomas overexpress the SETDB1 protein.

In this study, we adapted the zebrafish as a platform for cancer gene discovery. Through the creation and analysis of more than 3,000 transgenic animals, *SETDB1* was identified as a gene capable of accelerating melanoma formation in cooperation with *BRAF(V600E)*. Amplification of the 1q21 chromosomal interval in melanoma does not preferentially occur together with the *BRAF(V600E)* mutation ($P = 0.28$, two-sample t-test). Therefore, it is probable that the tumour-promoting activity of *SETDB1* does not exclusively depend on *BRAF(V600E)*, which is common in melanomas but is found less frequently in other tumour types that have 1q21 amplification. SETDB1 forms a multimeric complex with SUV39H1 and other H3K9 methyltransferases. On the basis of our findings, we speculate that *SETDB1* overexpression can increase the activity of the H3K9 methyltransferase complex, leading to alterations in its target specificity. Inactivating mutations in histone methyltransferases and histone demethylases were recently described in renal cell carcinoma[17,18]. Our study lends functional support to the idea that perturbation of histone methylation promotes cancer. Moreover, *SETDB1* is focally amplified in a broad range of malignancies, suggesting that alterations in histone methyltransferase activity could define a biologically related subset of cancers.

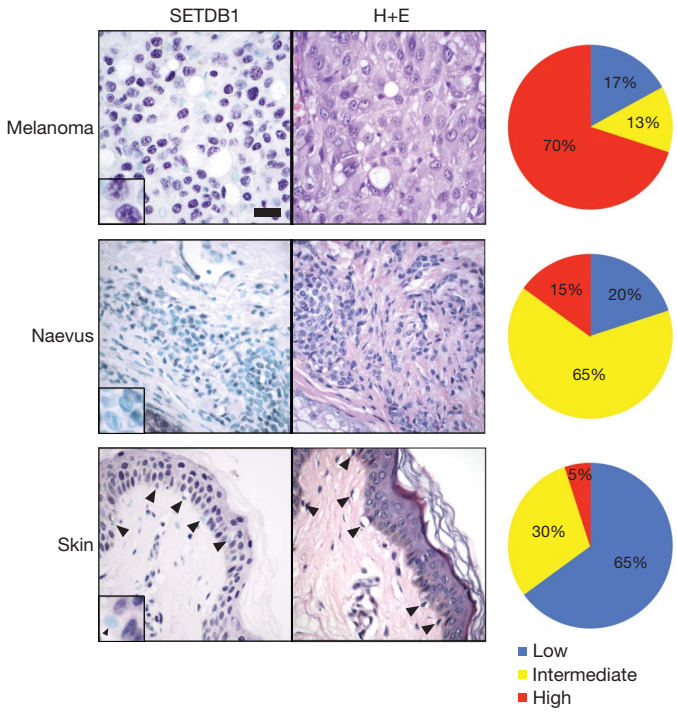

Figure 4 | High level expression of SETDB1 protein is common in human melanomas but not naevi or normal melanocytes. Immunohistochemical staining of SETDB1 (left, purple) and haematoxylin and eosin (H+E) staining (centre). Arrowheads indicate melanocytes in normal skin samples. SETDB1 expression (right, measured as described in the Methods) was scored for malignant melanomas (top, $n = 91$), naevi (centre, $n = 20$) and normal skin (bottom, $n = 20$). The percentage of samples with a low, intermediate or high level of SETDB1 staining is indicated. Summarized data and raw data from experiments with two different antibodies are described in Supplementary Tables 4 and 5, respectively. Scale bar, 30 μm; insets are magnified ×2.5.

METHODS SUMMARY

miniCoopR assay. The miniCoopR vector was constructed by inserting a zebrafish *mitfa* minigene (consisting of promoter, open reading frame and 3′-untranslated region) into the BglII restriction site of the plasmid pDestTol2pA2 (ref. 19). Individual miniCoopR clones were created by MultiSite Gateway recombination (Invitrogen) using human full-length open reading frames. Twenty-five picograms of each miniCoopR-candidate clone and 25 pg mRNA encoding the Tol2 transposase were microinjected into one-cell zebrafish embryos generated from an incross of *Tg(mitfa:BRAF(V600E)); p53*$^{-/-}$*; mitfa*$^{-/-}$ zebrafish. Rescued animals were scored weekly for the presence of visible tumours.

Senescence assay. SA-β-Gal staining was performed as described previously[10], except that scales plucked from the dorsum of melanocyte-rescued zebrafish were stained instead of tissue sections. This assay was performed on an *albino(b4)* mutant background so that melanin pigment would not obscure SA-β-Gal staining. Experimental animals were injected with 20 pg miniCoopR-*SETDB1* plus 10 pg miniCoopR-*EGFP*, and control animals were injected with 30 pg miniCoopR-*EGFP*. Rescued melanocytes were recognized as EGFP-positive cells.

Gene expression. From zebrafish, total RNA was extracted from four miniCoopR-*SETDB1* melanomas and four miniCoopR-*EGFP* melanomas. Total RNA from each was used for the synthesis of cDNA, which was hybridized to a 385K microarray (NimbleGen 071105_Zv7_EXPR). Zebrafish genes that were downregulated by *SETDB1* were selected by a fold change of >5 (when comparing the level of expression in miniCoopR-*EGFP* melanomas and the level in miniCoopR-*SETDB1* melanomas) and then filtered by a '*SETDB1* specificity score', which was defined as a fold change of >3 when comparing the level of expression in *Tg(mitfa:BRAF(V600E)); p53*$^{-/-}$ melanomas with that of miniCoopR-*SETDB1* melanomas.

Immunohistochemistry. Human melanoma tissue microarrays were independently analysed for SETDB1 protein by immunohistochemistry, using a rabbit polyclonal antibody (Sigma HPA018142, at a 1/200 dilution) and a mouse monoclonal antibody (4A3, Sigma WH0009869M7, 1/400 dilution), with a purple substrate for the secondary antibody (VIP substrate, Vector Labs). A methyl green counterstain was used.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions C.J.C. and Y.H. contributed equally to this work and are listed alphabetically. C.J.C., Y.H. and L.I.Z. conceived the project, designed and analysed the experiments, and wrote the manuscript. C.J.C. and Y.H. performed the zebrafish experiments and contributed to the other experiments. J.J.-V. performed the tissue culture experiments. S.B. performed the ChIP-seq experiments and analysed the data. V.B., L.F., S.A.-S.-A. performed the biochemistry studies on SETDB1. L.A.J. performed the fluorescence *in situ* hybridization studies. T.J.H. performed the immunohistochemistry experiments. W.M.L., R.B. and C.H.M. analysed the copy number data. D.A.O. analysed the *SETDB1*-overexpression microarray data for WM451Lu cells. F.F. designed a database to manage and analyse tumour incidence data. C.B., C.J.B., L.T. and A.U. provided technical assistance. M.L., L.A.G. and R.A.Y. provided input into the preparation of the manuscript.

Author Information The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus database under accession number GSE26372. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to L.I.Z. (zon@enders.tch.harvard.edu).

METHODS

miniCoopR assay. The miniCoopR vector was constructed by inserting a zebrafish *mitfa* minigene (consisting of promoter, open reading frame and 3′-untranslated region) into the BglII site of the plasmid pDestTol2pA2 (ref. 19). Individual miniCoopR clones were created by MultiSite Gateway recombination (Invitrogen) using human full-length open reading frames. Recombination junctions were sequence verified. Twenty-five picograms of each miniCoopR-candidate clone and 25 pg mRNA encoding the Tol2 transposase were microinjected into one-cell zebrafish embryos generated from an incross of *Tg(mitfa:BRAF(V600E)); p53*$^{-/-}$*; mitfa*$^{-/-}$ zebrafish. Transgenic animals were selected based on the presence of rescued melanocytes at 48 h post fertilization. Rescued animals were scored weekly for the presence of visible tumours.

Tumour invasion assay. Zebrafish with dorsal melanomas between the head and dorsal fin were isolated, and tumours were allowed to progress for 2 weeks, at which time the animals were killed. Tumours were formalin fixed, embedded and sectioned transversely to assess invasion.

Senescence assay. SA-β-Gal staining was performed as described previously[10], except that scales plucked from the dorsum of melanocyte-rescued zebrafish were stained instead of tissue sections. This assay was performed on an *albino(b4)* mutant background so that melanin pigment would not obscure SA-β-Gal staining. Experimental animals were injected with 20 pg miniCoopR-*SETDB1* plus 10 pg miniCoopR-*EGFP*, and control animals were injected with 30 pg miniCoopR-*EGFP*. Rescued melanocytes were recognized as EGFP-positive cells.

Gene expression and GSEA. From zebrafish, total RNA was extracted from four miniCoopR-*SETDB1* melanomas and four miniCoopR-*EGFP* melanomas. Total RNA from each was used for the synthesis of cDNA, which was hybridized to a 385K microarray (NimbleGen 071105_Zv7_EXPR). Zebrafish genes that were downregulated by *SETDB1* were selected by a fold change of >5 (when comparing the level of expression in miniCoopR-*EGFP* melanomas and the level in miniCoopR-*SETDB1* melanomas) and then filtered by a '*SETDB1* specificity score', which was defined as a fold change of >3 when comparing the level of expression in *Tg(mitfa:BRAF(V600E)); p53*$^{-/-}$ melanomas with that of miniCoopR-*SETDB1* melanomas. Human orthologues of *SETDB1*-downregulated genes were identified for GSEA (http://www.broadinstitute.org/gsea/). For GSEA of *SETDB1*-downregulated and SETDB1 'bound-bound' genes, a rank-ordered gene list was derived from the expression profiles of 93 melanoma cell lines and short-term cultures of melanoma cells[6], using *SETDB1* expression level as a continuous variable. In WM451Lu cells, the dose of *SETDB1* lentiviral infection was titrated to achieve *SETDB1* expression levels comparable to those in short-term cultures of melanoma cells with high levels of *SETDB1* expression. Total RNA was extracted and then amplified and hybridized to a Human Gene 1.0 ST Array (Affymetrix). Control gene expression values were obtained from WM451Lu cells infected with *EGFP*-expressing lentivirus.

ChIP. ChIP was performed from short-term cultures of WM262 and WM451Lu cells, and ChIP-seq data were analysed as described previously[14].

Methyltransferase complex reconstitution. *In vitro*-translated Flag–GLP, HA–G9A and untagged SETDB1 (wild type (WT) or the indicated mutant) were incubated for 4 h at 4 °C with 5 mg GST, GST–SUV39H1(WT) or GST–SUV39H1(H324K) mutant immobilized on agarose–glutathione beads. Beads were then extensively washed, as described previously[16], and protein complexes were eluted with free glutathione. The eluate was then subjected to an overnight Flag immunoprecipitation at 4 °C using Flag–agarose. After extensive washing, protein complexes were eluted with 0.1 M glycine, pH 3.0. The glycine was then neutralized with NaOH, and the eluate was renatured for 1 h at room temperature then incubated overnight at 4 °C with HA–resin. The HA–resin was then washed, and the protein complexes were eluted with SDS. Ten per cent of the input and 100% of the HA eluate were resolved by SDS–PAGE and were analysed by western blotting with the indicated antibodies. The top of the membrane was revealed with three different antibodies (anti-SETDB1, anti-HA and anti-Flag antibody) using two stripping steps.

Histone methylation assay. Purified complexes were incubated with 5 mg core histones (Upstate 13-107) and 1.5 mCi *S*-adenosyl-L-[methyl-^{3}H]methionine (PerkinElmer NET155050UC) in a buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 1% NP40, 1 mM dithiothreitol and protease inhibitors (with a reaction volume of 30 µl). The mixture was incubated for 1 h at 30 °C and was then separated by SDS–PAGE. The gel was stained with a SimplyBlue SafeStain kit (Invitrogen) and analysed by fluorography using an FLA-7000 phosphorimager (Fuji).

Immunohistochemistry. Human melanoma tissue microarrays were independently analysed for SETDB1 protein by immunohistochemistry, using a rabbit polyclonal antibody (Sigma HPA018142, at a 1/200 dilution) and a mouse monoclonal antibody (4A3, Sigma WH0009869M7, 1/400 dilution), with a purple substrate for the secondary antibody (VIP substrate, Vector Labs). A methyl green counterstain was used. Melanoma tissue microarrays were obtained from US Biomax (ME1003 and ME482). A modified visual semiquantitative method was used to score staining as described previously[21], using a two-score system for immunointensity (II) and immunopositivity (IP). II and IP were then multiplied. SETDB1 immunostaining was also performed on formalin-fixed, paraffin-embedded zebrafish melanomas.

Fluorescence *in situ* hybridization (FISH). The bacterial artificial chromosome (BAC) clone, RP11-42A12, used as the *SETDB1* probe was selected using the UCSC Genome Browser and obtained from the BACPAC Resource Center (CHORI). BAC probe preparation, labelling and hybridization were performed as described previously[22]. The SpectrumOrange-CEP1 reference probe 06J39-026 was obtained from Abbott Molecular.

Lentivirus infection. We used pLKO1-puromycin lentiviral vectors carrying short hairpin RNAs (shRNAs) specific for *SETDB1* or *GFP* sequences. The shRNA vectors were obtained from the Broad Institute RNAi Consortium (http://www.broadinstitute.org/rnai/trc), and the lentiviruses were obtained by overnight triple co-transfection of 293T cells using lipofectamine 2000 and 3 µg pLKO.1-shRNA, Δ8.9 and VSV-G vectors (in 100-mm plates). The *SETDB1* shRNA construct used was TRCN0000148112 (hairpin target sequence gctcagat gataacttctgta). Knockdown efficiency was determined by RT–PCR and by immunoblot analysis using *SETDB1*-specific primers and antibody, respectively. At days 2 and 3 post infection, supernatants were collected and filtered with a 45-µm filter to remove 293T cells. Virus was added to cells (plated to attain 30–40% confluence at the time of infection) and incubated for 16 h in the presence of polybrene at 5 µg ml^{-1}. After infection, virus was removed and fresh media added. Forty-eight hours post infection, cells were subjected to a 3-day puromycin selection. To elevate *SETDB1* expression in WM451Lu cells, we used a pLEX980 lentiviral vector into which the wild-type *SETDB1* open reading frame had been recombined. Infection, selection and monitoring of SETDB1 concentration were performed as described above.

Cell proliferation assays. Cells were plated in 12-well plates at 20,000 cells well^{-1} in 2 ml medium. At each time point, cells were trypsinized from 3 wells and counted with a cell counter (Beckman Coulter).

Western blot analyses. Western blots were performed with primary antibodies recognizing SETDB1 (Abcam ab12317), BRAF (Santa Cruz Biotechnology sc5284), α-tubulin (Cell Signaling Technology 2144), SUV39H1 (Upstate 07-550), the Flag epitope (Sigma M2), G9A (Clinisciences D141-3), and GLP (R&D Systems PP-B0422-00). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (Amersham) were used. Thirty micrograms total protein was loaded per lane. SETDB1 was recognized as a doublet at approximately 150 kDa (predicted molecular mass 143 kDa).

***p53BP2* quantitative PCR analysis.** Chromatin extracts from HeLa cells were prepared as described[16], using micrococcal nuclease digestion and mild sonication (without any formaldehyde crosslinking) to enrich them in mono-nucleosomes. Flag-HA purification was then performed. A fraction of the input or 1/3 of each Flag-HA purified complex (chromatin associated) was treated with proteinase K and RNase. Samples were then phenol–chloroform extracted, and DNA was precipitated using ethanol. *p53BP2* primers were described previously[23]. Results were normalized to input values and presented as fold enrichment compared with the control sample (Flag-HA purification from HeLa control cells).

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