Melanoma cells rely on developmental programs during tumor initiation and progression. Here we show that the embryonic stem cell (ESC) factor Sall4 is re-expressed in the Tyr::NrasQ61K; Cdkn2a−/− melanoma model and that its expression is necessary for primary melanoma formation. Surprisingly, while Sall4 loss prevents tumor formation, it promotes micrometastases to distant organs in this melanoma-prone mouse model. Transcriptional profiling and in vitro assays using human melanoma cells demonstrate that SALL4 loss induces a phenotype switch and the acquisition of an invasive phenotype. We show that SALL4 negatively regulates invasiveness through interaction with the histone deacetylase (HDAC) 2 and direct co-binding to a set of invasiveness genes. Consequently, SALL4 knockdown, as well as HDAC inhibition, promote the expression of an invasive signature, while inhibition of histone acetylation partially reverts the invasiveness program induced by SALL4 loss. Thus, SALL4 appears to regulate phenotype switching in melanoma through an HDAC2-mediated mechanism.
General hallmarks of cancer include genetic mutations and chromosomal rearrangements. To sustain growth and eventually progress to metastatic disease, tumors undergo several additional molecular changes including epigenetic rewiring and altered transcription of specific genes. The aberrant re-expression of genes reminiscent of the embryonic cell-of-origin and the hijacking of its transcriptional programs have been identified as possible drivers of cancer progression. In different cancer types, stem-cell-like cancer cells have been associated with tumor initiation, sustained growth, and metastasis formation. Furthermore, the reacquisition of features reminiscent of stem cells has been associated with reduced anticancer immunity, resistance to different therapies and disease relapse.

Cutaneous melanoma is the most aggressive skin cancer due to its high metastatic potential. The embryonic cell-of-origin of melanocytes, from which melanoma arises, is the neural crest stem cell (NCSC). Melanoma cells are known to hijack neural crest (NC)-related migratory programs during tumor initiation, invasion, and metastasis formation. For instance, the transcription factors PAX3, FOXD3, and SOX10 are part of a gene regulatory network in early NC development that also supports melanoma cell growth, migration, and resistance to targeted therapy, respectively. Furthermore, the transcription factor YY1 has recently been shown to control comparable metabolic pathways in NC and melanoma cells and to be equally required for both development and melanoma formation. Likewise, the neurotrophin receptor CD271/NGFR/p75NTR, which marks migratory NCSCs, is re-expressed in melanoma cells and renders them more invasive, metastatic, and therapy resistant and of note, transient ectopic expression of NGFR was shown to promote phenotype switching—the dynamic transition of melanoma cells from a proliferative to a highly invasive state.

Here we identify the stem cell factor SALL4 as a regulator of melanoma phenotype switching. We find that SALL4 is the strongest upregulated transcription factor in hyperplastic, melanoma-prone murine melanocytes when compared to normal wild-type melanocytes and that SALL4 is crucial for primary melanoma growth. SALL4 is a known embryonic stem cell (ESC) regulator and its aberrant re-expression has been reported for an increasing number of cancer types, such as germ cell tumors, hepatocellular carcinoma, gastric cancer, leukemia, and others. Due to its re-expression in cancers, SALL4 has been dubbed an ‘oncofetal’ gene. Since such genes are not expressed in adult tissues except for malignant lesions, factors such as SALL4 represent ideal targets for cancer diagnosis and disease treatment. In the present study, we show that while upregulation of SALL4 is crucial to sustain melanoma tumor growth, its depletion or downregulation increases invasiveness and metastasis formation in melanoma. Intriguingly, SALL4 appears to regulate a melanoma-specific invasion program through HDAC2-mediated epigenetic silencing of invasiveness genes.

Results

SALL4 is re-expressed in hyperplastic murine melanocytes. To identify factors essentially involved in melanoma tumorigenesis, we analyzed RNA sequencing data previously obtained from wild-type melanocytes and hyperplastic melanocytes isolated from the skin of 3-months-old Tyr::Nras^Q61K; Cdkn2a^−/− mice (Fig. 1a). Loss of the tumor suppressor Cdkn2a together with gain of Nras function in the melanocytic lineage of these mice leads to hyperplastic melanocytes already at birth and primary melanoma arising around the age of 6 months. When comparing hyperplastic to wild-type melanocytes by RNA sequencing (RNA seq) (Supplementary Data 1), we found among the top 20 upregulated genes one transcription factor, SALL4 (logFC 9.59, p value < 0.0001) (Fig. 1b). Upregulation of SALL4 in hyperplastic versus wild-type melanocytes was confirmed by immunohistochemistry on mouse skin sections (Fig. 1c). Moreover, murine primary melanoma displayed prominent SALL4 expression (Supplementary Fig. 1). Likewise, we could not detect SALL4 expression in melanocytes of healthy human skin, in agreement with a previous study, while SALL4 was strongly expressed in human melanoma tissue (Supplementary Fig. 2a, b).

Conditional knockout of SALL4 in the melanocytic lineage leads to reduced primary melanoma formation. Given the re-expression of SALL4 in hyperplastic melanocytic lesions, we next addressed whether SALL4 is essential for melanoma formation. We therefore crossed the Tyr::Nras^Q61K; Cdkn2a^−/− melanoma mouse model with inducible Tyr::CreERT2 Sall4^lox/lox and R26R-LSL-GFP mice (Fig. 2a). By doing so, we obtained a mouse model that spontaneously developed melanoma, in which SALL4 could be deleted in melanocytes upon tamoxifen (TM) injections (Fig. 2b) and Cre activity could be traced by GFP expression (Supplementary Fig. 3a). We assessed SALL4 expression levels in this model by mRNA analysis of isolated melanocytes from recombined postnatal day 8 mice of either wild-type (Tyr::CreERT2 LSL-R26R-GFP or -tdTomato), Sall4^+/− conditional knockout (cko) (Tyr::Nras^Q61K Cdkn2a^−/−; Tyr::CreERT2 Sall4^cko) mice (Fig. 2e; Supplementary Fig. 3c). Melanoma identity of the micrometastases was confirmed by immunohistochemical staining for the melanocyte marker MITF (Fig. 2g). Strikingly, we found a significant increase in micrometastases counts in Sall4^+−/− cko mice as well as in Sall4^+/−/− cko mice compared to the control animals (Fig. 2h).

SALL4 loss results in an increased metastatic burden in vivo. In addition to proliferation, SALL4 has been associated with increased migration and invasion of solid tumors other than melanoma. Therefore, we assessed whether loss of SALL4 affects melanoma metastasis formation in our melanoma-susceptible mouse model. For this purpose, we quantified the formation of lung micrometastases traced by GFP expression in Sall4^−/− cko and Sall4^+/− cko animals (Fig. 2f, g, h; Supplementary Fig. 4a, b). Melanoma identity of the micrometastases was confirmed by immunohistochemical staining for the melanocyte marker MITF (Fig. 2g). Strikingly, we found a significant increase in micrometastases counts in Sall4^−/− cko as well as in Sall4^+/− cko mice compared to the control animals (Fig. 2h). These data suggest that in melanoma—similar to other cancer types—SALL4 is essential for tumor growth, while its depletion or downregulation leads to increased micrometastases formation, which is in contrast to its function observed in other cancer types.

SALL4 promotes proliferation and suppresses invasion in human melanoma cells. To validate the findings from our mouse model in human melanoma cells, we carried out siRNA-mediated SALL4 knockdown experiments in various human melanoma cell lines with different mutational backgrounds. Namely, M010817
SALL4 negatively regulates a set of melanoma-specific invasiveness genes. To determine the molecular mechanism by which SALL4 loss leads to increased invasion, we performed RNA seq on siControl and siSALL4-treated M010817 cells and found 1004 genes significantly upregulated and 1140 genes significantly downregulated in siSALL4 over siControl samples (Fig. 4a; Supplementary Data 2). Gene ontology (GO) analysis with MetaCore™ revealed that the upregulated genes were strongly enriched in Process Networks related to cell adhesion/cytoskeleton, developmental processes related to epithelial-to-mesenchymal transition, angiogenesis, and others (Fig. 4b; Supplementary Data 2). Oppositely, the downregulated genes were enriched in Process Networks related to cell cycle regulation, inflammation, and developmental processes such as hedgehog signaling or melanocyte differentiation (Fig. 4c; Supplementary Data 2). The differential gene expression obtained upon SALL4 knockdown pointed towards an upregulation of EMT-related genes and a downregulation of differentiation genes, overall suggesting acquisition of a transcriptional signature typical of melanoma cell phenotype switching. To assess whether there was a significant
enrichment in genes related to phenotype switching among our differentially expressed genes upon siSALL4, we carried out Gene Set Enrichment Analysis (GSEA)39, where the ranked siSALL4 signature was compared to published melanoma phenotype switching signatures. We found that SALL4-regulated genes showed a significant enrichment and positive correlation with published invasiveness signatures40–42, while they significantly anti-correlated with their corresponding proliferation signatures40–42 (Fig. 4d). Next, siSALL4-mediated upregulation of a set of invasiveness genes representing the top enriched MetaCore™ Process Networks and also the published melanoma phenotype switching signatures (bold genes in Fig. 4b, c) was
Fig. 2 Sall4 is essential for primary tumor formation, but its depletion leads to increased micrometastasis. a Genetics scheme of the Tyr::NrasQ61K; Cdkn2a−/− transgenic mouse model spontaneously developing melanoma, which was crossed with inducible Tyr-CreERT2; Sall4lox/lox; R26R-LSL-GFP mice, allowing ablation of Sall4 from the melanocytic lineage upon tamoxifen administration. b Experimental scheme depicting how at 1 month of age the experimental mice from a undergo Cre-mediated recombination due to 5 consecutive i.p. tamoxifen injections. Hyperplasia gradually develops from birth of the pups. Primary tumors and metastasis were assessed at around 6 months of age. c Photographs of control and heterozygous (Sall4fl/fl; termed Sall4+/−/cko) and homozygous (Sall4fl/fl; termed Sall4−/−/cko) Sall4cko animals (left panel). Hematoxylin and eosin staining of back skin from respective control, Sall4+/−/cko or Sall4−/−/cko animals (right panel), which has been repeated in independent experiments with similar results 7 times. Scale bars 500 µm. d Quantification of primary tumor numbers of control (Sall4+/+ and non-tamoxifen-injected animals), Sall4+/−/cko and Sall4−/−/cko animals. e Quantification of proliferation rate, assessed by immunohistochemistry (see Supplementary Fig. 3c), in primary tumors of control and Sall4+/−/cko animals, f Binocular images of mouse lungs. The endogenous fluorescent GFP signal was imaged under a fluorescent binocular and for visualization inverted and set to black/white (B/W). Dark spots therefore represent inverted GFP spots set to B/W. Scale bars 500 µm. g Immunohistochemical stainings of mouse lung sections to verify melanoma identity of GFP+ spots by means of MITF expression. Scale bars top panel 100 µm, second and third panel 25 µm. h Quantification of GFP lung metastases of tamoxifen-injected control (Sall4+/−/h), Sall4+/−/cko and Sall4−/−/cko animals. Metastasis score 0 indicates <5 GFP+ lesions, 1>5 lesions, 2>20 lesions, 3>50 lesions, 4>100 lesions. In d, e, h, error bars represent mean ± SEM with N indicated in the respective figures. Two-sided t-tests between groups were performed for significance with p values ≥0.05 = n.s.; <0.05 = *; <0.01 = **; with Sall4−/−/cko in d P = 0.0049; Sall4+/−/cko in e P = 0.0456; Sall4+/−/cko in h P = 0.0011; Sall4−/−/cko in h P = 0.0129. Source data for d, e, h are provided as a Source Data file.

SALL4 and HDAC2 interact and directly regulate a set of target genes. SALL4 has been shown to regulate transcription by a variety of different mechanisms.26 In mouse ESCs for example, Sall4 has been shown to exert stemness regulatory function via direct binding and activation of a distal enhancer of the gene Pou5f1, which encodes the pluripotency regulator Oct4.25 Furthermore, in adult human CD34+ hematopoietic stem cells, a whole set of direct targets of SALL4 has been identified by Chromatin Immuno precipitation (ChIP)-chip and additional ChIP-qPCR validation.43 Interestingly, neither POU5F1 nor the 16 validated genes (except for HNF1) from Gao et al., (2013b) were significantly changed in our RNA sequencing upon SALL4 knockdown in melanoma cells. Similarly, MYC has previously been shown to be a direct target of SALL4 in endometrial cancer,38 but we could not detect any altered MYC expression upon SALL4 knockdown. We therefore hypothesized that in melanoma SALL4 might exert its regulatory function in an alternative manner and have a different array of targets.

It has previously been reported that SALL4 can interact with epigenetic co-factors both in stem cells as well as in cancerous cells.26 One type of epigenetic enzymes that has been shown to interact with SALL4 are histone deacetylases (HDACs), specifically HDAC1 and HDAC2, which are part of the Nucleosome Remodeling Complex (NuRD).46 Since HDAC1, HDAC2, and HDAC3 have been reported to be overexpressed in melanoma cells compared to primary melanocytes,47 we addressed whether in human melanoma cells, SALL4 can interact with one of the HDACs. By Co-immunoprecipitation (Co-IP) experiments we detected protein interaction between SALL4 and HDAC2 in the human melanoma cell line M010817 (Fig. 5a; Supplementary Fig. 7), which led to the hypothesis that SALL4 might repress invasiveness genes via recruitment of histone deacetylases, leading to epigenetic silencing of target genes such as invasiveness genes. To test this idea and address which genes are directly bound by both SALL4 and HDAC2, we carried out a cleavage under targets and release using nuclease (CUT&RUN) sequencing experiment for SALL4 as well as for HDAC2. Of note, we chose to perform CUT&RUN with two different antibodies per factor, each set consisting of one antibody that had previously been published for CUT&RUN or ChIP sequencing plus one additional one (Supplementary Data 3). To determine target genes of both SALL4 and HDAC2, we performed peak calling with SEACR for loci that contained significant peaks with at least 3 of the 4 antibodies used (Fig. 5b, c, d; Supplementary Data 4). The ’sof4’ antibody approach allowed us on one hand to strengthen the specificity of the SALL4-HDAC2 targets and on the other hand to identify novel peaks that might only be recognized by one or another antibody due to epitope masking in protein complexes at specific locations. Interestingly, several of the identified SALL4-HDAC2 peaks were associated with putative invasiveness genes, with peaks either at the transcription start site (TSS) (such as for VEGFR-1) (Fig. 5c), downstream of the TSS (such as for TGFBR2) (Fig. 5f), or within annotated putative regulatory elements (such as for PDGFC, CDH2 (N-cadherin), and FNI) (Fig. 5g–i). The fact that these genes were upregulated upon SALL4 depletion (Fig. 4b, c) and direct targets of both SALL4 and HDAC2 is consistent with the idea that SALL4 recruits HDAC2 to these specific loci, resulting in histone deacetylation and hence repression of these invasiveness-related target genes.

Next, we analyzed with HOMER the DNA binding motifs of the CUT&RUN peaks that are unique for SALL4 (Supplementary Figs. 8, 9, 10), unique for HDAC2 (Supplementary Figs. 11, 12, 13), and most importantly, the DNA binding motifs that are shared between SALL4 and HDAC2 (Supplementary Fig. 14). Interestingly, we found amongst our top SALL4-HDAC2 shared de novo DNA binding motifs matches for known transcriptional regulators of the NC that are re-expressed and have functional implication in melanoma, such as SOX10 and SOX9,58 or RUNX1,49,50 and also key regulators of melanocyte differentiation and hence melanoma, such as MITF or TFAP2C,52,53 (Fig. 5j). This further strengthens the hypothesis that SALL4 and HDAC2 together regulate melanocyte and melanoma-specific cellular processes.

Differentially expressed SALL4-HDAC2 targets enrich in cell adhesion-related processes. Since we had found that SALL4 and HDAC2 bind to a large set of common loci, we wanted to correlate their direct targets with differential expression upon SALL4 knockdown. As we hypothesized that SALL4 recruits HDAC2 to specific loci to repress gene activity, we assessed which SALL4-HDAC2 targets (peaks with at least 3 of 4 SALL4-HDAC2 antibodies) were significantly upregulated after SALL4 knockdown validated by qRT-PCR in the five previously used human cell lines (Fig. 4e) and, for selected gene products, by immunocytochemistry and western blot in M010817 cells (Supplementary Fig. 6a, b). Together, our data suggest that the reduced expression of SALL4 induces human melanoma cell invasion via upregulation of known melanoma invasiveness genes, which could explain the increased metastasis burden seen upon SALL4 loss in the Tyr::NrasQ61K; Cdkn2a−/− melanoma mouse model.
(RNA Seq) and found 184 direct SALL4-HDAC2 targets with increased expression upon SALL4 KD (Fig. 6a; Supplementary Data 4). MetaCore™ Process Network enrichment analysis revealed that these targets were associated with cell adhesion, TGFβ signaling, angiogenesis, and EMT (Fig. 6b; Supplementary Data 4). For instance, among the SALL4-HDAC2 targets upregulated upon SALL4 knockdown, we found integrins (ITGB1, ITGA6, ITGA4), N-cadherin (CDH2), TGFB2, VEGFR-1, PDGF, LOXL2, MAPK8, and many others which are associated with invasive melanoma phenotypes40–42 (Fig. 6b, Supplementary
Data 4). These results strongly support our hypothesis that SALL4 and HDAC2 co-repress invasiveness genes in melanoma and that either SALL4 or HDAC inhibition induces their expression.

We next analyzed the SALL4-HDAC2 target genes that were downregulated upon SALL4 loss (Supplementary Fig. 15a; Supplementary Data 4). Of note, MetaCore® analysis showed that in general the Process Network enrichment of the siSALL4 downregulated direct SALL4-HDAC2 targets was less significant and processes were represented by fewer genes (Supplementary Fig. 15b) than was the case for the siSALL4 upregulated direct SALL4-HDAC2 targets (Fig. 6b). Nevertheless, of interest, the top enriched process of siSALL4 downregulated direct SALL4-HDAC2 targets was related to melanocyte differentiation, represented by downregulated direct targets such as MITF, DCT, β-catenin (CTNNB1), and tyrosinase (TYR), among others (Supplementary Fig. 15b). These data suggest that melanocyte differentiation genes, although they can be bound by SALL4-HDAC2, are subject to positive regulation by SALL4 by a mechanism that remains to be elucidated. Possibly, transcriptional activation in these cases might involve tertiary co-factors recruited to SALL4-HDAC2 target loci. To address this, we reanalyzed the SALL4-HDAC2 peaks within melanocyte differentiation genes (Supplementary Data 5) using CIIDER to predict transcription factors (TFs) significantly enriched at these peaks (Supplementary Data 5). The resulting list of TFs (Supplementary Data 5) putatively bound to the same loci as SALL4 and HDAC2 was filtered for high stringency and further analyzed with STRING to predict protein–protein interactions with SALL4 (Supplementary Fig. 15c, red cluster). Interestingly, this in silico approach revealed TFs regulating NC development and melanoma as putative SALL4 binding partners at melanocyte differentiation gene loci with joint SALL4 and HDAC2 peaks such as TFAP2A (Supplementary Fig. 15c, red cluster), a well-known transcriptional activator in NC cells54,55 and a regulator of melanocyte differentiation genes53. Hence, our data raise the possibility that amongst others, TFAP2A can be recruited to SALL4-HDAC2-target elements of melanocyte differentiation genes and that SALL4 (and possibly HDAC2) loss leads to a loss in TFAP2A-mediated transcriptional activation of these genes even if HDAC2-mediated repression is attenuated after SALL4 loss.

We also elaborated on genes that might putatively be regulated exclusively by either SALL4 or HDAC2 by screening for protein-coding genes that only show CUT&RUN peaks with the two SALL4 antibodies the two HDAC2 antibodies, respectively, but no 3 of 4 shared peaks (Supplementary Data 6). By doing so, we found that genes exclusively bound by SALL4 enrich in biological processes related to neurogenesis, neurophysiological processes, and others, while genes exclusively bound by HDAC2 enriched in various different process classes such as immune cell adhesion, cell cycle, apoptosis, and developmental processes (Supplementary Data 6). These data suggest that SALL4 largely relies on the interaction with HDAC2 to negatively regulate invasiveness genes in melanoma.

To functionally strengthen this hypothesis, we inhibited HDACs in human melanoma cell lines with HDAC inhibitors (HDACi) for 48 h and measured gene expression by qRT-PCR (Fig. 6c). Specifically, treatment with the class 1 HDACi inhibitor Mocetinostat, which inhibits HDACs 1, 2, and 3, resulted in a differential gene expression pattern similar to SALL4 knockdown, in that invasiveness genes were upregulated, while melanocyte differentiation genes were downregulated (Fig. 6c). A similar, although less striking effect was detected after treatment with the pan-HDAC inhibitor Panobinostat (Fig. 6c). Importantly, phenotypically HDACi treatment led to increased invasion in vitro for the majority of tested human melanoma cell lines (Fig. 6d, e).

These findings are in accordance with our results that showed increased invasiveness of melanoma cells upon SALL4 loss (Fig. 3i–k) and with previous studies that have reported HDAC inhibitor-induced invasiveness in melanoma cells and other cancer cells56,57. We therefore further addressed the expression of invasiveness genes in HDACi-treated melanoma cells in vivo. Human melanoma xenografts in athymic nude mice were treated with Mocetinostat and Pанобиностат (Supplementary Fig. 16a) and tumor lysates were analyzed for expression of invasiveness genes at the experimental endpoint. While mice treated with either Panobinostat or Mocetinostat showed reduced xenograft tumor growth (Supplementary Fig. 16b), the expression of established invasiveness genes, such as AXL or NGFR was upregulated (Supplementary Fig. 16c). Moreover, we detected a trend of reduced expression of melanocyte differentiation genes, such as MITF, MLANA, or DCT, in the lysates of HDACi-treated tumors (Supplementary Fig. 16c).

SALL4 knockdown leads to differential histone acetylation in invasiveness genes. Given that SALL4-HDAC2 target genes belonging to invasiveness processes are upregulated upon SALL4 loss or HDAC inhibition, we wanted to assess whether SALL4 regulates epigenetic activation of invasiveness genes in general. Since HDACs catalyze deacetylation of histones and, consequently, transcriptional repression of target genes58,59, we
Fig. 4 RNA sequencing of SALL4 knockdown reveals an invasiveness gene expression signature. a RNA seq row z-score heatmap of differentially expressed (DE) genes after 48 h SALL4 knockdown in the human cell line M010817. Cutoffs were set at Log2 ratio ≥0.27 or ≤−0.27, p value < 0.05 and FDR < 0.05 and resulted in 1004 genes significantly upregulated and 1140 genes significantly downregulated. b Top 12 MetaCore™ process networks of upregulated genes. Top ten most upregulated genes per process are listed and genes validated in additional cell lines are highlighted in bold. c Top 12 MetaCore™ process networks of downregulated genes. Top ten most downregulated genes per process are listed and genes validated in additional cell lines are highlighted in bold. d Gene Set Enrichment Analysis (GSEA) of DE genes after SALL4 knockdown (log2 ratio-ranked) as in a with published genesets characterizing invasive versus proliferative melanoma cells. e Log2 expression ratio heatmap of qRT-PCR-based gene expression analysis of specific genes of interest (normalized to PPIA and set relative to siCtrl-treated cells) in human melanoma cell lines (M010817: NRASQ61K, M070302: unknown mutational status; M150548: BRAFV600E, M121224: NRASV60K and BRAFV600E, M150536: BRAFV600E) after 48 h treatment with two different siRNAs targeting SALL4. Two-sided t-tests were performed with N = 3 and p values ≥0.05 is n.s. Source data for e are provided as a Source Data file.
hypothesized that SALL4 knockdown would lead to derepression and increased acetylation of target genes associated with invasiveness. To address this hypothesis, we performed an H3K27ac Chromatin Immunoprecipitation sequencing (ChIP-seq) experiment of cells treated with siCtrl or siSALL4 (Supplementary Data 7) to identify genes with differential histone acetylation upon SALL4 knockdown (Fig. 7a, Supplementary Data 8). Overall, we found more gained H3K27ac peaks than lost ones upon SALL4 knockdown (Fig. 7a, b). Most of the peaks were annotated to intron and intergenic regions or promoter/
genes with lost H3K27ac levels, 137 genes showed decreased (Fig. 7i, Supplementary Data 8), while within the more than 1000 H3K27ac marks, 261 genes also showed increased mRNA levels seq, respectively. Within the more than 2000 genes with gained H3K27ac peaks (Fig.7c)

to proximity to TSS by exclusively analyzing changed peaks (Fig. 17a). To do so, we restricted our H3K27ac ChIP-seq analysis

to SALL4 and HDAC2 interact and have a set of common target genes in melanoma cells. a Western blot for SALL4 and HDAC2 after Co-Immunoprecipitation (Co-IP) with a SALL4 antibody in the human melanoma cells M010817. Experiment has been repeated independently with similar results two times. b CUT&RUN (C&R) in M010817 cells with two antibodies against SALL4 (SALL4_Ab#1 (S_Ab#1) and SALL4_Ab#2 (S_Ab#2)) and two antibodies against HDAC2 (HDAC2_Ab#1 (H_Ab#1) and HDAC2_Ab#2 (H_Ab#2)) and Ctrl (anti-FLAG) visualized as read density heatmaps of the centered peaks (within 10 kb) for all loci showing peaks with at least 3 of the 4 (2x SALL4, 2x HDAC2) antibodies. c C&R peak numbers called with SEACR for single antibodies, shared between antibodies and shared between at least 3 of the 4 SALL4-HDAC2 antibodies. In total 3319 loci contained peaks for at least 3 of 4 antibodies tested and were found in total 2301 different genes. Those peaks were used for further analyses correlating the direct targets with either expression (Fig. 6a, b) or acetylation status (Fig. 7h). d Annotation of C&R peaks to genetic regions. Green: the 5019 SALL4_Ab#1 and SALL4_Ab#2 shared peaks; yellow: the 1925 HDAC2_Ab#1 and HDAC2_Ab#2 shared peaks; grey: the 3319 peaks shared between at least 3 of the 4 SALL4_Ab#1, SALL4_Ab#2, HDAC2_Ab#1, and HDAC2_Ab#2 antibodies. TTS: transcription termination site, TSS: transcription start site, UTR: untranslated region. e-i Specific gene tracts visualized with IGV. Green: SALL4_Ab#1 and SALL4_Ab#2; yellow: HDAC2_Ab#1, and HDAC2_Ab#2. Red dashed lines highlight significantly called peaks (with at least 3 of 4 antibodies.) Selected de novo DNA binding motifs of SALL4-HDAC2 (at least 3 of 4 antibodies) shared peaks analyzed by HOMER. Source data for a and d are provided as a Source Data file.

transcription start sites (TSS) (Fig. 7b), such as for the invasive- ness genes VEGFR-1, FN1, PDGFC, and NGFR (promoter) or AXL (upstream), which showed significantly gained H3K27ac peaks (Fig. 7c-g, red bars and red, dashed highlighting) mediated by SALL4 knockdown, while no significantly lost H3K27ac peaks were found at the present tracks. Since we had hypothesized that increased histone acetylation in siSALL4 cells can partially be linked to reduced SALL4-mediated HDAC2 recruitment to specific target genes (i.e., invasiveness genes), we next wanted to address whether differential acetylation patterns can indeed be detected upon SALL4 knockdown at loci bound by both SALL4 and HDAC2. In line with our hypothesis, differential acetylation ChIP-seq peaks were present within 10 Kb intervals of the SALL4-HDAC2 peaks, as determined by means of a read density heatmap (Fig. 7h). Thus, H3K27ac is differentially regulated within a fraction of the SALL4-HDAC2-bound loci. Moreover, when we correlated the loci bound by SALL4-HDAC2 and showing increased H3K27ac ChIP-seq peaks in siSALL4 (Fig. 7h) with increased RNA expression after SALL4 knockdown (Fig. 4b, Supplementary Data 2), we again found, among others, invasiveness genes such as TGFBR2, ITGA6, or VEGFR-1 (Supplementary Data 9), that were enriched in biological processes related to cell adhesion, TGFβ signaling, and others (Supplementary Data 9).

Of importance, the heatmap correlating SALL4-HDAC2 targets with differential acetylation (Fig. 7h) also revealed that a large fraction of SALL4-HDAC2 peaks likely regulate more distant regulatory elements and not the exact same loci, to which the two proteins directly bind. This is to be expected due to the fact that any genomic locus that becomes functionally proximal to the SALL4-HDAC2 protein duet—which could also happen via genomic looping of far distant regulatory regions—could be differentially acetylated by HDAC2. Therefore, as it is difficult to define the exact genomic loci that the SALL4/HDAC2 complex regulates, we decided to investigate the functional impact of SALL4-recruited HDAC2 on a genome-wide level by correlating the regions of differential acetylation with the genes differentially expressed after SALL4 knockdown (Fig. 7i, Supplementary Fig. 17a). To do so, we restricted our H3K27ac ChIP-seq analysis to proximity to TSS by exclusively analyzing changed peaks within 15+/10 kb of the TSS. We found significantly gained H3K27ac peaks in 2566 genes and significantly lost H3K27ac peaks in 1131 genes (Supplementary Data 8). Next, we did an overlay of the genes with significantly gained or lost H3K27ac marks and increased or decreased transcription based on RNA seq, respectively. Within the more than 2000 genes with gained H3K27ac marks, 261 genes also showed increased mRNA levels (Fig. 7i, Supplementary Data 8), while within the more than 1000 genes with lost H3K27ac levels, 137 genes showed decreased mRNA expression (Supplementary Fig. 17a, Supplementary Data 8). Interestingly, among the 261 genes with gained H3K27ac and increased transcription, we could again identify invasiveness genes such as VEGFR-1, PDGFC, ITGA6, NGFR, AXL, FN1, SERPINE1, among others, and MetaCore “process network enrichment again resulted in pathways related to cell adhesion, EMT, angiogenesis, and others (Fig. 7i).

GSEA on the 398 genes with gained or lost histone acetylation and increased or decreased expression, respectively, (combined from Fig. 7i and Supplementary Fig. 17a) confirmed a positive correlation of the siSALL4 signature with the invasiveness signature of Verfaillie and colleagues (2015), while it showed a negative correlation with the corresponding proliferation signature (Fig. 7k), further suggesting that downmodulation of SALL4 changes the activating chromatin mark H3K27ac of genes related to phenotype switching.

Of note, while we hypothesize that SALL4 knockdown leads to derepression of invasiveness genes through attenuated HDAC2-mediated target gene repression, epigenetic activation of target genes after SALL4 loss could be boosted by direct targets of SALL4 and HDAC2 that function as epigenetic activators. For instance, we found the lysine demethylase 4 C (KDM4C) or the lysine acetyltransferase 2B (KAT2B) as direct targets of SALL4 and HDAC2 (Supplementary Fig. 18a, b), which have been identified as SALL4 targets in other systems as well60,61. Hence, upregulation of invasiveness genes after SALL4 depletion might be induced by a joint epigenetic mechanism of lost repression and gained activation of target genes.

To further test whether siSALL4-mediated upregulation of invasiveness genes was indeed dependent on differential acetylation, we combined SALL4 knockdown with an inhibitor of histone acetyl transferases (HATs), CTK7A, and quantified the expression of invasiveness genes. Of note, upregulation of invasiveness genes, such as ITGA6, FLT1 (VEGFR-1), PDGFC, FN1, NGFR, AXL, ETS1, and others, could be significantly rescued by addition of the HAT inhibitor CTK7A (Supplementary Fig. 19a-c), supporting our hypothesis that SALL4 regulates melanoma invasiveness genes via chromatin modulating mechanisms involving histone deacetylation.

Discussion

SALL4 is a member of the spalt-like (SALL) gene family, which are the vertebrate homologues of the developmental spalt (sal) genes in Drosophila62. Sall4 is a zinc finger transcription factor that plays an essential role in maintaining pluripotency and self-renewal of ESCs by regulating Pou5f1 and by direct binding to Nanog and Oct425,63. Sall4 is vastly expressed during murine embryonic development and of note, in a previous study we have detected its expression also in murine neural crest stem cells19. In the adult, however, Sall4 is mostly absent and detectable only in the germ cells of the ovaries or testis84. Similarly to mice, SALL4

ARTICLE

Fig. 5 SALL4 and HDAC2 interact and have a set of common target genes in melanoma cells. a Western blot for SALL4 and HDAC2 after Co-Immunoprecipitation (Co-IP) with a SALL4 antibody in the human melanoma cells M010817. Experiment has been repeated independently with similar results two times. b CUT&RUN (C&R) in M010817 cells with two antibodies against SALL4 (SALL4_Ab#1 (S_Ab#1) and SALL4_Ab#2 (S_Ab#2)) and two antibodies against HDAC2 (HDAC2_Ab#1 (H_Ab#1) and HDAC2_Ab#2 (H_Ab#2)) and Ctrl (anti-FLAG) visualized as read density heatmaps of the centered peaks (within 10 kb) for all loci showing peaks with at least 3 of the 4 (2x SALL4, 2x HDAC2) antibodies. c C&R peak numbers called with SEACR for single antibodies, shared between antibodies and shared between at least 3 of the 4 SALL4-HDAC2 antibodies. In total 3319 loci contained peaks for at least 3 of 4 antibodies tested and were found in total 2301 different genes. Those peaks were used for further analyses correlating the direct targets with either expression (Fig. 6a, b) or acetylation status (Fig. 7h). d Annotation of C&R peaks to genetic regions. Green: the 5019 SALL4_Ab#1 and SALL4_Ab#2 shared peaks; yellow: the 1925 HDAC2_Ab#1 and HDAC2_Ab#2 shared peaks; grey: the 3319 peaks shared between at least 3 of the 4 SALL4_Ab#1, SALL4_Ab#2, HDAC2_Ab#1, and HDAC2_Ab#2 antibodies. TTS: transcription termination site, TSS: transcription start site, UTR: untranslated region. e-i Specific gene tracts visualized with IGV. Green: SALL4_Ab#1 and SALL4_Ab#2; yellow: HDAC2_Ab#1, and HDAC2_Ab#2. Red dashed lines highlight significantly called peaks (with at least 3 of 4 antibodies.) Selected de novo DNA binding motifs of SALL4-HDAC2 (at least 3 of 4 antibodies) shared peaks analyzed by HOMER. Source data for a and d are provided as a Source Data file.
expression in adult human tissue is thought to be restricted to testes and ovaries\(^6\) and hematopoietic stem/progenitor cells (HSPCs)\(^4\).

Several studies have reported the re-expression of the stem cell factor SALL4 in the adult in different cancer types\(^2\). Mostly, the re-expression of SALL4 was associated with increased tumor cell proliferation and decreased patient survival. Here, we show that SALL4 is upregulated in hyperplastic murine melanoma-prone melanocytes and that its expression is essential for melanoma primary tumor growth. However, in contrast to previous studies on other cancer types, our results reveal that depletion of SALL4 in a murine melanoma model leads to increased micrometastasis formation while preventing sustained tumor growth. In line with these in vivo results, knockdown of SALL4 in human melanoma cells leads to reduced tumor cell proliferation and to the upregulation of a set of well-known melanoma invasiveness genes, inducing an invasive cell phenotype. Hence, our findings identify SALL4 as a negative regulator of melanoma cell...
phenotype switching, i.e. the reversible change from a high proliferative/low invasive to a low proliferative/high invasive cell state\textsuperscript{23,24,40}. Notably, SALL4 negatively controls melanoma invasiveness-related genes, as for instance NGFR, a potent regulator of phenotype switching\textsuperscript{22}, FN1, VEGFR-1, CDH2 (N-cadherin), and other genes implicated in melanoma invasiveness.

The binding of SALL4 to chromatin and epigenetic modifier enzymes and the resulting epigenetic rewiring of target genes has previously been shown in ESCs as well as in cancer. Amongst the published epigenetic co-factors of SALL4 are DNA methyltransferases (DNMT-1, DNMT-3A, DNMT-3B, and DNMT-3L), histone demethylase LSD1/KDM1A, and others\textsuperscript{26}. Additionally,
Fig. 7 SALL4 can regulate invasiveness genes through an epigenetic mechanism. a Read density heatmap of gained and lost H3K27ac ChIP sequencing peaks (±2 kb) upon SALL4 knockdown (left panels) and average read distribution of gained and lost H3K27ac peaks within ±5 kb from peak center (right panels). b Distribution of the gained and lost H3K27ac peaks in different genetic regions. TTS: transcription termination site, TSS: transcription start site, UTR: untranslated region. c-q Representative tracks of genes with significantly gained (red bars; highlighted by red, dashed lines) H3K27ac peaks in siSALL4 over siCtrl. (no significantly lost H3K27ac peaks were detected for the visualized gene tracks). b) Read density heatmap of differential H3K27ac ChIP-seq peaks (within 10 kb) in siSALL4 over siCtrl at direct target genes of SALL4-HDAC2 (CUT&RUN peaks with at least 3 of 4 SALL4/HDAC2 antibodies). i Significantly upregulated genes after SALL4 knockdown (Fig. 4a, b) were overlaid with those genes that have significantly gained H3K27ac marks (×15–×10 kb of TSS (a, ocher panel). This resulted in 261 genes with activating chromatin marks that were at the same time upregulated after SALL4 knockdown. j MetaCore™ Process Network enrichment of the 261 genes from i. The top 12 most significant processes are indicated with the specific genes of each process listed in red. k Gene Set Enrichment Analysis (GSEA) of the combined 261 upregulated (K) and 137 downregulated genes (Supplementary Fig. 17a) with differential acetylation after SALL4 knockdown (ranked according to log2 expression ratio of RNA seq results) with the previously published melanoma programs of Verfaillie and colleagues (2015). Source data for b are provided as a Source Data file.

Based on tandem mass spectrometry studies on ESCs and 293 T cells overexpressing SALL4, it has been shown that SALL4 can also bind to the NuRD complex members HDAC1 and HDAC2. Here we show that SALL4 can build a protein complex together with HDAC2 also in human melanoma cells and that SALL4 and HDAC2 together directly bind to genes involved in melanocyte and melanoma biology, such as VEGFR-1, CDH2 (N-cadherin), FN1, TGFBR2, MITF, and others.

Strengthening the hypothesis that SALL4-HDAC2 epigenetically repress transcriptional activity of invasiveness genes, knockdown of SALL4 leads to increased H3K27 acetylation and increased transcription of melanoma invasiveness-related genes, such as FN1, VEGFR-1, PDGFC, and NGFR, which could be partially rescued by administration of a histone acetyltransferase (HAT) inhibitor. Likewise, HDAC inhibition with two different HDAC inhibitors, leads to upregulation of a similar set of invasiveness-related genes and to increased invasiveness in vitro. This supports the hypothesis that SALL4 inhibits invasiveness-related genes in melanoma via interaction with HDACs. HDAC inhibitors have been considered for use in combinatorial therapies for several cancers including melanoma. Our data suggest caution in the use of these inhibitors because of potential adverse, metastasis-promoting effects of HDAC inhibitors in melanoma patients.

Others have shown different sets of target genes epigenetically silenced by SALL4. Lu et al (2009), for instance, identified by ChIP the SALL4 targets SALL1 and PTEN. Since the binding sites of those target genes were co-occupied by NuRD components with HDAC activity, the authors argued that SALL4 silences PTEN and SALL1 by interacting with NuRD. Phenotypically, the decreased expression of the tumor suppressor PTEN in SALL4 transgenic mice was associated with myeloid leukemia and cystic kidneys. Indeed, in our experiments, binding of SALL4 and HDAC2 to PTEN (but not SALL1) was confirmed by CUT&RUN (Supplementary Data 3 and 4). However, we did neither observe differential acetylation patterns in ChIP-seq nor transcriptional changes in RNA seq of PTEN or SALL1 upon SALL4 knockdown. In addition, other previously identified direct targets of SALL4 have not come up in our analyses either, which suggests that SALL4 might regulate a set of specific targets in melanoma cells.

Regarding melanoma disease progression, there is increasing evidence that the re-expression of NCSC-related factors can regulate melanoma initiation and later stages of the disease, such as resistance to therapies or invasion and metastatic spread. However, unlike melanoma cells undergoing phenotype switching, NCSCs during embryonic development continue to proliferate when they migrate and invade distant tissues. Intriguingly, while some NCSC factors, such as SOX10 and YY1, are activated upon melanoma formation and are required for melanoma growth, other NCSC-associated factors, such as CD271/NGFR/p75NTR, PAX3, and FOXD3, promote melanoma cell invasiveness and metastasis formation. This suggests that the embryonic program active in NCSCs segregates in melanoma to regulate distinct aspects of phenotype switching, namely proliferation vs. invasion. Our study adds SALL4 to the growing list of stem cell factors known to control melanoma cell biology. Similar to SOX10 and YY1, SALL4 is upregulated in melanoma cells and is essential for primary tumor formation and proliferation. Importantly, however, SALL4 depletion in melanoma leads to increased invasiveness and micrometastasis formation. This is reminiscent of melanoma cells displaying reduced SOX10 levels, which is associated with increased expression of SOX9, another factor involved in NC development, and increased invasiveness. It will be important to elucidate to what extent the gene regulatory program of NCSCs is functionally implicated in mediating cellular plasticity during melanoma disease progression. Knowledge of this program might allow defining strategies targeting both tumor growth and metastasis formation.

Methods

Transgenic mice and in vivo TM application. Tyr::NrasQ61K animals and Cdkn2a-deficient mice have been described previously. Also the Tyr::CreERT2 murine line, Sall4lox mice, and R26r-LSL-GFP mice have been analyzed previously. Mice were bred and crossed in-house to generate the Tyr::NrasQ61K, Tyr::CreERT2; Sall4lox/lox, R26r-LSL-GFP genotype and resulted in a mixed genetic background. All animal breeding, housing and experimentation was conducted according to the guidelines of the veterinary office of the Canton of Zurich, Switzerland. Specifically, animals were housed in a controlled environment with a 12 h light/dark cycle, with free access to water and food and at temperatures of 21–25 °C and humidity of 40–60%. Genotyping was performed on toe or ear biopsies, followed by PCR on isolated DNA using the Tap PCR Core Kit (201225, Qigene) and primers as listed in Supplementary Table 2. For conditional ablation of Sall4, 8-week-old transgenic mice of both genders were injected intraperitoneally (i.p.) with 100 µl tamoxifen (TM) (T5648, Sigma-Aldrich) diluted in ethanol and sunflower oil (19 ratio) at a concentration of 1 mg d−1 for 5 days according to an established protocol. Melanoma-developing mice were monitored and euthanized at an endpoint defined by adverse clinical symptoms, such as multiple skin tumors Ø >5 mm, weight loss (Δ> 15%) or a hunched back. All animal experiments have been approved by the veterinary authorities of Canton of Zurich, Switzerland and were performed in accordance with Swiss law.

Quantification of skin melanomas and metastases. At sacrifice of the animals, skin melanomas and metastases were assessed. Above a diameter of 2 mm (Ø > 2 mm), developing trunk skin lesions were considered as melanomas. The tumors of the heterozygouscko group were verified to have been recombined and lost Sall4 by either GFP or Sall4 immunohistochemical stainings of tumor sections. Animals with non-recombined tumors were excluded from the analysis. The Control group consisted of noninjected Tyr::NrasH129Cdnk2a−/−/Tyr::CreERT2 Sall4lox/lox R26r-GFP and TM-injected Tyr::NrasH129Cdnk2a−/−/Tyr::CreERT2 Sall4lox/lox R26r-GFP animals for analysis of primary tumor numbers and of TM-injected Tyr::NrasH129Cdnk2a−/−/Tyr::CreERT2 Sall4lox/lox R26r-GFP animals for analysis of lung metastases. To assess whether GFP spots in the mouse lungs were of melanoma identity, MITF and GFP co-stainings were performed on histological sections of the mouse lungs. For quantification of micrometastasis, the number of GFP spots in the lungs were categorized as follows: <5 lesions = score 0; >5 lesions = score 1; >20 lesions = score 2; >50 lesions = score 3; >100 lesions = score 4. Group sizes are indicated for each experiment in the respective figures.
Malignant melanoma is known to be a recalcitrant disease due to its strong resistance against conventional therapies. The development of effective therapies is urgent. Therefore, it is desirable to develop a comprehensive system that integrates imaging and functional assessment of the therapeutic effects in vivo. Based on these considerations, we have developed an immunocompromised nude mouse xenograft model to study the effects of HDAC inhibitors on human malignant melanoma. The xenografts were generated by subcutaneous injections of melanoma cell lines, followed by treatment with HDAC inhibitors. The results showed that the HDAC inhibitors could significantly inhibit the growth of the xenografts, and the inhibition effect was dose-dependent. The study provides a valuable tool for the evaluation of HDAC inhibitors in the treatment of malignant melanoma.

The xenograft model was established by subcutaneous injection of melanoma cell lines into immunocompromised nude mice. The mice were treated with HDAC inhibitors at different doses, and the growth of the xenografts was monitored over time. The results showed that the HDAC inhibitors could significantly inhibit the growth of the xenografts, and the inhibition effect was dose-dependent. The study provides a valuable tool for the evaluation of HDAC inhibitors in the treatment of malignant melanoma.

RNA isolation and real-time PCR. RNA extraction and DNase treatment of samples were performed using the ReliaPrep® RNA Miniprep Kit (Promega, Z6010) according to the manufacturer's guidelines. Purified RNA was quantified using Nanodrop and subjected to reverse transcription using the SuperScript II RNase H-Reverse Transcriptase (Life Technologies), and a mix of penicillin–streptomycin antibiotics (150,000 units/ml) and 10% FCS, 4 mM L-Glutamine, Pen-Strep) with a volume of 100 µl was injected i.p. for each treatment. Volumes of the drugs dissolved in DMSO were diluted with 90 µl sun

Protein isolation and western blotting. Cytoskeletons were lysed and protein extracted as described previously22. Protein concentrations were determined with the BCA Protein Assay Kit (23227, Thermo Fisher Scientific) using a DTX 880 Multimode Detector at 562 nm. Thirty micrograms of total protein were run through Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad) and transferred onto nitrocellulose membranes (Bio-Rad, 2895), which were stained with primary antibodies in Odyssey blocking buffer (LI-COR Biosciences) at 4 °C and visualized using secondary antibodies in Odyssey blocking buffer for 45 min at RT. Blots were scanned and quantified with an Odyssey imaging system (LI-COR Biosciences).

xCELLigence real-time cell proliferation analysis. For assessment of cell proliferation, we made use of the xCELLigence® Real-Time Cell Analysis (RTCA) DP Instrument (ACEA Biosciences). This system allows impedance-based real-time growth/proliferation measurements of adherent cells by measuring the net adhesion/confluent (Cell Index) of cells to high-density gold electrodes on custom-designed plates. The assay was performed according to the manufacturer's guide-

Human xenografts in immunocompromised mice. Nude mice (Harlan) were purchased from Harlan and housed under standard conditions with free access to water and food at temperatures of 21–23 °C and humidity of 40–60 %. Experiments were carried out with female mice of 6–10 weeks of age. Xenografts of human melanoma cells were generated by dissociating cultured cells with PBS + 2 mM EDTA to generate single-cell suspensions, which were resuspended in 100 µl of RPMI-1640 medium and mixed 1:1 with Matrigel matrix (356234, BD Biosciences). A total volume of 200 µl of the cell/matri
gel mix was injected subcutaneously per injection site with a 1 ml syringe with a 25-gauge hypodermic needle. For xenografts of siRNA-treated M010817 and M150548 cells, 1,000,000 tumor cells were subcutaneously injected per injection site and left for growth for 6 days. For the generation of xenografts that were treated with HDAC inhibitors, 300,000 M010817 cells were grafted subcutaneously per injection site (two injections per mouse). For each xenograft injection site, mice were sacrificed and dissected. The tumors were formalin-fixed and embedded in paraffin, and then cut into 5 µm sections. The sections were stained with H
epathy and eosin according to standard procedures or stained for immuno
histochemical analyses.

siRNA transfection. Cells were cultured in starvation medium (0.5% FCS) and after 24 h transfected at 60% confluency in complete growth medium (RPMI 1640 with 10% FCS, 4 mM L-Glutamine, Pen-Strep) with a final siRNA concentration as indicated in Supplementary Table 4. For transfection, the JetPrime transfection kit (114, Polypus transfection) was used according to the manufacturer’s guidelines. Further information on siSALL1#1 and R can be found in Supplementary Table 4.

RNA isolation and real-time PCR. RNA extraction and DNase treatment of samples was performed using the ReliaPrep® RNA Miniprep Kit (Promega, Z6010) according to the manufacturer’s guidelines. Purified RNA was quantified using Nanodrop and subjected to reverse transcription using the SuperScript II RNase H-Reverse Transcriptase (Life Technologies), and a mix of penicillin–streptomycin antibiotics (150,000 units/ml) and 10% FCS, 4 mM L-Glutamine, Pen-Strep) with a volume of 100 µl was injected i.p. for each treatment. Volumes of the drugs dissolved in DMSO were diluted with 90 µl sun
Corning® Transwell® migration assay. The migration assay was carried out similarly to the manufacturer’s guidelines. Specifically, prior to seeding the cells into the transwell migration chamber they were incubated in starvation medium (RPMI + 1% FBS, P/S, L-Glut) for 24 h. Next, 50,000 cells in 500 µl FBS-free starvation medium were seeded per well onto the porous membrane of Clear Transwell Inserts (Corning, 3464) and placed within a multi-well plate with 800 µl of normal growth medium (RPMI + 10% FBS + P/S + L-Glut) on the bottom of the well. Cells in the transwell chambers were then incubated at 37 °C for 16 h. At the experimental endpoint, inserts were taken out and remaining starvation medium in the upper chamber was carefully pipetted up and down and then collected with the nonmigratory cells in suspension, centrifuged, and cell pellets further processed for RNA extraction. The cell pellets from 3 wells were used to make one sample. Next, membranes now free of nonmigratory cells were cut out of the flipped transwells and three membranes were collected in one Eppendorf to make one sample and were further processed with lysis buffer to extract RNA of migratory cells. RNA extraction and qRT-PCR were performed as described above.

Corning® Matrigel® invasion assay. The invasion assay was carried out similarly to the manufacturer’s guidelines. Specifically, prior to seeding of the cells into the invasion assay, the invasion chamber was incubated in starvation medium (P/S, L-Glut) for 48 h. On the day of cell seeding, invasion plates with inserts (Corning, 354480) were equilibrated by adding 800 µl blunt medium into the wells and 500 µl into each insert and incubated for 2 h at 37 °C. Cells were then collected, washed once in PBS, reseeded in starvation medium and seeded into each insert in a final volume of 500 µl. Of the cell line M010817 cells 200,000 cells were seeded into M070302 160,000 and of M150548 200,000 cells, respectively. Inserts were then placed into wells where blunt medium was aspirated and replaced by 800 µl of growth medium (RPMI + 10% FBS + P/S + L-Glut) and seeded cells were incubated for 24 h at 37 °C. For analysis, the medium in the inserts was aspirated and the membranes were washed slowly but vigorously with cotton swabs and washed once with PBS. Cells were then fixed with 4% Roti Histofix (Carl Roth, P087.3) for 15 min at room temperature and washed once with PBS. Inserts were then carefully cut out with a scalpel and placed downside up onto microscopy glass slides, covered with some drops of Fluorescent Mounting Medium (Dako, S3023) with 1:1000 Hoechst 33342 (Sigma, H5540) and formaldehyde (Sigma, F8528). The slides were then coupled overnight at 37 °C. The next day, the beads were washed with HB and LB solution (always +0.1% Tween-20) respectively, and finally washed and with resuspended in SB solution (as in manufacturer instructions).10 M100817 cells were harvested, weighted and resuspended in Extraction buffer in a ratio 1:9 (+Complete, Mini, EDTA-free Protease Inhibitor, Sigma #11836170001). Cells were lysed for 15 min on ice, centrifuged at 2600 × g for 5 min at 4 °C and the supernatant was transferred to a new tube. The antibody coupled beads were then washed with 900 µl of Extraction buffer, collected with a magnet and gently resuspended in the cell lysate. Coupled beads and cell lysate were incubated for 30 min at 4 °C on a rotator. The beads were then washed for six times with the Extraction buffer, incubated 5 min at RT in WB buffer (+0.01% Tween-20) and finally 5 min at RT in elution buffer (EB). The supernatant was then used to perform western blots and either 1% input or 0.01% input were used as positive control for the pulverids of SALL1 (1%) or of HDACs (0.01%).

Clavage under targets and release using nuclease (CUT&RUN) sequencing. M100817 cells were harvested by adding EDTA to the cell medium to a final concentration of 2 mM and by pelleting 300 × g for 5 min and resuspending in 2 ml wash buffer (20 mM HEPEs pH 7.4, 150 mM NaCl, 2 mM Spermidine, EDTA-free protease inhibitor) each time. Cells were counted, and 500,000 cells per sample were bound to 40 µl of ConcanaValin A beads prepared according to a previously established protocol.17 Beads were divided into 2 ml tubes (150 mM NaCl, 2 mM CaCl2) and pelleted. Cells were then washed three times by pelleting at 300 × g for 5 min and resuspending in 2 ml wash buffer (20 mM HEPEs pH 7.4, 150 mM NaCl, 2 mM Spermidine, EDTA-free protease inhibitor, 0.025% digitonin, 2 mM EDTA). Beads were then washed twice to wash off the beads. Beads were resuspended in binding wash buffer (20 µl of 0.5× CUTANA pG-MNase (EpiCypher) solution (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM Spermidine, EDTA-free protease inhibitor, 0.025% Digitonin, 2 mM EDTA)) and used for the row z-score heatmap and the MetaCoreTM analysis (also included in Supplementary Data 2) in Fig. 4 a, b.

Immunocytochemistry. For staining of adherent cells, culture medium was aspirated from culture wells, cells washed with PBS (Thermo Fisher, 10010) and fixed in 4 % Roti Histofix (Carl Roth, P087.3) for 15 min at room temperature. Histofix was removed and cells washed three times for 5 min with PBS. Sections were then fixed for 50 min at room temperature in blocking buffer containing 1% BSA (Sigma-Aldrich, 65470) and 0.2% Triton X-100 (Sigma-Aldrich, 9002-93-1) in PBS. After aspirating the blocking buffer, primary antibodies were applied in blocking buffer at the concentrations indicated in Supplementary Table 3 and incubated for 1.5 h at room temperature. After washing the sections again 2x with PBS, they were incubated with secondary antibodies in blocking buffer for 45 min at room temperature. Cells were again washed 2x with PBS and finally, nuclei were stained using 33342 (Sigma, 14533) and slides were mounted with Fluorescent Mounting Medium (Dako, S3023). Sections were imaged with a DMI 6000B microscope (Leica).

Gene Set Enrichment Analysis (GSEA). GSEA39 version 4.1.0 was run if there was a minimum of 15 shared genes between the datasets to compare and on default settings with 1000 permutations.

Co-immunoprecipitation. Co-immunoprecipitation experiments were prepared using the Dynabeads co-Immunoprecipitation Kit (Thermo Fisher, 11167D) and followed the manufacturer’s instructions. In brief, Dynabeads M-270 Epoxy per sample were initially washed with 1 ml of C1 solution. The beads were collected using a magnet and then resuspended in 55 µl C1 solution + 20 µg anti-body (either SALL4, abcam ab29112, or IgG, abcam #ab7009 or Cell Signaling # 2729 S) and incubated on ice, centrifuged at 2600 × g for 5 min at 4 °C and the supernatant was transferred to a new tube. The antibody coupled beads were then washed with 900 µl of Extraction buffer, collected with a magnet and gently resuspended in the cell lysate. Coupled beads and cell lysate were incubated for 30 min at 4 °C on a rotator. The beads were then washed for six times with the Extraction buffer, incubated 5 min at RT in LWB buffer (+0.01% Tween-20) and finally 5 min at RT in elution buffer (EB). The supernatant was then used to perform western blots and either 1% input or 0.01% input were used as positive control for the pulldowns of SALL1 (1%) or of HDACs (0.01%).
A, 50 µg/mL. Glycogen, 100 µg/mL yeast spike-in DNA) was added to each tube. Then, the tubes were placed in a 37°C heat block for 30 min to release soluble chromatin from cells. Biotin was again bound to the magnetic rack and allowed to clear completely, and the supernatant was transferred carefully into a DNA-for bind tube.

DNA was extracted using Phenol:Chloroform extraction and libraries were prepared using KAPA HyperPrep Kit (Roche) using KAPA UU adapters (Roche). Libraries were pooled, and libraries were sequenced on a NextSeq 500 using a NextSeq 500/550 High Output Kit v2.5 (75 Cycles), generating 36 bp paired-end FASTQ files. Reads were trimmed using BBduk, removing overrepresented repeat sequences (i.e., [TA]_{18}, [G]_{36}), artifact, and adapter sequences. Reads were aligned to the human genome (hg38) using bowtie2 with settings -X 700 -m1 -v 3. Duplicate reads were removed, were trimmed using BBDuk, removing overrepresented repeat sequences (i.e., [TA]_{18}, [G]_{36}), artifact, and adapter sequences. Reads were aligned to the human genome (hg38) using bowtie2 with settings -X 700 -m1 -v 3. Duplicate reads were removed, and files were sorted using samtools. Mapped reads were filtered for size, keeping only reads with a fragment size at or below 120 base pairs. Bedgraph files were generated using bedtools genocov, and peaks were called using SEACR version 1.3 ([https://seacr.fredhutch.org/](https://seacr.fredhutch.org/)), in relaxed mode, normalized to the negative control. Single-gene tracks were visualized with the Integrative Genome Viewer (IGV) version 2.8.13 ([https://software.broadinstitute.org/software/igv/download](https://software.broadinstitute.org/software/igv/download)).

**HDAC inhibitor treatment in vitro.** With Mocetinostat (MCGD1013; Selleckchem, S1122) and Panobinostat (LBH589; Selleckchem, S1030) stocks of 10 mM drug in DMSO (Sigma–Aldrich, D2650) were generated and stored at −20°C. For working concentrations of 600 nM Mocetinostat and 7 nM Panobinostat, respectively, stock solutions were further diluted in complete growth medium and added to cells in culture for 48 h.

**Chromatin immunoprecipitation of H3K27ac and sequencing.** ChIP analysis was performed as previously described. Briefly, 1% formaldehyde was added to cultured cells to crosslink proteins to DNA. Isolated nuclei were then lysed and sonicated using a Bioruptor ultrasonic cell disruptor (Diagenode) to shear genomic DNA to an average fragment size of 200 bp. Twenty microliters of chromatin was diluted to a total volume of 50 µl with ChIP buffer (16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 0.1% SDS, 1.1% Triton X-100) and precleared with 10 µl packed Sepharose beads for 2 h at 4°C. Precleared chromatin was incubated overnight with the indicated antibodies. The next day, Dynabeads protein-A were added and incubated for 4 h at 4°C. After washing, bound chromatin was eluted with the elution buffer (1% SDS, 100 mM NaHCO₃). Upon proteinase K digestion (50°C for 3 h) and reversion of crosslinking (65°C, overnight), DNA was purified with phenol/chloroform, ethanol precipitated and quantified.

**Supplementary methods.** The data deposition of SALL4 and HDAC2 in M010817 cells generated for this study are available on ENA under the accession code PRJEB39209. The ChIP-seq data of H3K27ac in siControl and siSALL4-treated M010817 cells generated for this study are available on ENA under accession code PRJEB39209. The CUT&RUN seq data of SALL4 and HDAC2 in M010817 cells generated for this study are available on ArrayExpress under accession code E-MTAB-10163. The list of differentially expressed genes from RNA seq experiments or lists of called peaks from ChIP-seq and C&R seq tests) were done using GraphPad Prism 5.0 and Excel with values > 0.05 deemed significant.

**References**

1. Tianzhan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* 100, 57–70 (2000).
2. Nebbioso, A., Tambaro, F. P., Dell’Aversana, C. & Altucci, L. Cancer epigenetics: moving forward. *PloS Genet.* 14, e1007362 (2018).
3. Suvá, M. L., Riggi, N. & Bernstein, B. E. Epigenetic reprogramming in cancer. *Science* 339, 1567–1570 (2013).
4. Bernardi, M. et al. SOX2-expressing neural progenitor cells differentiate into neural crest mesenchymal cells. *Nature* 540, 185–190 (2016).
31. Ackermann, J. et al. Metastasizing melanoma formation caused by expression.
32. Shakhova, O. et al. Sox10 promotes the formation and maintenance of giant
33. Bosenberg, M. et al. Characterization of melanocyte-specific inducible Cre
34. Elling, U., Klassen, C., Eisenberger, T., Anlag, K. & Treier, M. Murine inner cell
35. Simon, C., Lickert, H., Götz, M. & Dimou, L. Sox10-iCreER T2: a mouse line
36. Zhang, Y. et al. Analysis of the NuRD subunits reveals a histone deacetylase
37. He, J. et al. Inhibition of SALL4 reduces tumorigenicity involving epithelial-
38. Liu, L. et al. SALL4 as an epithelial-mesenchymal transition and drug
39. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based
40. Hoek, K. S. et al. In vivo switching of human melanoma cells between
41. Verfaillie, A. et al. Decoding the regulatory landscape of melanoma reveals
42. TIROS, I. et al. Dissecting the multicellular ecosystem of metastatic melanoma
43. Gao, C. et al. SALL4 is a key transcription regulator in normal human
44. Kloet, S. L. et al. Towards elucidating the stability, dynamics and architecture
45. 54. Mitchell, P. J., Timmons, P. M., Hébert, J. M., Rigby, P. W. J. & Tjian, R.
46. Zhang, Y. et al. Analysis of the NuRD subunits reveals a histone deacetylase
47. Kim, T. et al. The transcriptional repressor SOX10 interacts with an epigenetic
48. Shakhova, O. et al. Antagonistic cross-regulation between Sox9 and Sox10
49. Giricz, O. et al. The RUNX1/IL-34/CSF-1R axis is an autocrinally regulated
50. Hoek, K. S. et al. In vivo switching of human melanoma cells between
51. Ackermann, J. et al. Metastasizing melanoma formation caused by expression
52. Serrano, M. et al. Role of the INK4a locus in tumor suppression and cell
53. Seberg, H. E. et al. TFAP2 paralogs regulate melanocyte differentiation in
54. Mitchell, P. J., Timmons, P. M., Hébert, J. M., Rigby, P. W. J. & Tjian, R.
55. 58. Grunstein, M. Histone acetylation in chromatin structure and transcription.
56. Kong, N. & Y. et al. Zinc finger protein SALL4 functions through an AT-rich
57. Jiang, G. M. et al. Histone deacetylase inhibitor induction of epithelial-
58. 51. Eckschlager, T., Plch, J., Stiborova, M. & Hrabeta, J. Histone deacetylase
Author contributions
J.D., A.B., and L.S. designed the study. J.D., A.B., M.P., D.D., J.H., and S.S. performed experiments and analyzed data. J.D., A.B., M.P., P.C., L.L., S.V., L.St., K.B., R.S., and C.C. analyzed data and provided intellectual guidance in experimental designs and interpretation of results. M.T. provided the Sall4lox/lox mouse. R.D. and M.L. provided melanoma patient-derived cell cultures. J.D. and L.S. wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-25326-8.

Correspondence and requests for materials should be addressed to L.S.

Peer review information Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021