Whole-transcriptomic Profile of SK-MEL-3 Melanoma Cells Treated with the Histone Deacetylase Inhibitor: Trichostatin A

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Abstract. Background: Malignant melanoma cells can rapidly acquire phenotypic properties making them resistant to radiation and mainline chemotherapies such as dacarbazine or kinase inhibitors that target RAS-proto-oncogene independent auto-activated mitogen-activated protein kinases (MAPK)/through dual specificity mitogen-activated protein kinase (MEK). Both drug resistance and inherent transition from melanocytic nevi to malignant melanoma involve the overexpression of histone deacetylases (HDACs) and a B-Raf proto-oncogene (BRAF) mutation. Materials and Methods: In this work, the effects of an HDAC class I and II inhibitor trichostatin A (TSA) on the whole transcriptome of SK-MEL-3 cells carrying a BRAF mutation was examined. Results: The data obtained show that TSA was an extremely potent HDAC inhibitor within SK-MEL-3 nuclear lysates, where TSA was then optimized for appropriate sub-lethal concentrations for in vitro testing. The whole-transcriptome profile shows a basic phenotype dominance in the SK-MEL-3 cell line for i) synthesis of melanin, ii) phagosome acidification, iii) ATP hydrolysis-coupled proton pumps and iv) iron transport systems. While TSA did not affect the aforementioned major systems, it evoked a dramatic change to the transcriptome: reflected by a down-regulation of 810 transcripts and up-regulation of 833, with fold-change from –15.27 to +31.1 FC (p<0.00001). Largest differentials were found for the following transcripts: Up-regulated: Tetraspanin 13 (TSPAN13), serpin family i member 1 (SERPINI1), ATPase Na+/K+ transporting subunit beta 2 (ATP1B2), nicotinamide nucleotide adenyl transferase 2 (NMNAT2), platelet-derived growth factor receptor-like (PDGFRL), cytochrome P450 family I subfamily A member 1 (CYP1A1), prostate androgen-regulated mucin-like protein 1 (PARM1), secretogranin II (SCG2), SYT11 (synaptotagmin II), rhophilin associated tail protein 1 like (ROPN1L); down-regulated: polypeptide N-acetylglactosaminyltransferase 3 (GALNT3), carbonic anhydrase 14 (CAXIV), BCL2-related protein A1 (BCL2A1), protein kinase C delta (PRKCD), transient receptor potential cation channel subfamily M member 1 (TRPM1), ubiquitin associated protein 1 like (UBAP1L), glutathione peroxidase 8 (GPX8), interleukin 16 (IL16), tumor protein p53 (TP53), and serpin family H member 1 (SERPINH1). There was no change to any of the HDAC transcripts (class I, II and IV), the sirtuin HDAC family (1-6) or the BRAF proto-oncogene v 599 transcripts. However, the data showed that TSA down-regulated influential transcripts that drive the BRAF–extracellular signal-regulated kinase (ERK)1/2 oncogenic pathway (namely PRKCD and MYC proto-oncogene which negatively affected the cell-cycle distribution. Mitotic inhibition was corroborated by functional pathway analysis and flow cytometry confirming halt at the G2 phase, occurring in the absence of toxicity. Conclusion: TSA does not alter HDAC transcripts nor BRAF itself, but down-regulates critical components of the MAPK/MEK/BRAF oncogenic pathway, initiating a mitotic arrest.

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Aggressive melanomas are highly resistant to radiation and chemotherapy drugs such as dacarbazine, and account for fatal metastatic disease. B-Raf proto-oncogene (BRAF) somatic missense mutations within the kinase domain from a single substitution (V599E) account for a large majority of malignant cutaneous melanoma. (1) This mutation in melanoma is often found in the absence of an NRAS or KRAS mutation, but renders autoactivation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) cascade (2-4). Despite the attempted use of combination therapies including dual-specificity mitogen-activated protein kinase (MEK) inhibitors (e.g., trametinib, and cobimetinib) or BRAF inhibitors (e.g., vemurafenib and dabrafenib), there is
a high degree of relapse (5-9). At the root of this pervasive resistance could be epigenetic changes that enable tumor cells to survive toxic insults, including those incurred from chemotherapy drugs. Some of the main epigenetic controlling elements are histone deacetylases (HDACs), which remove acetyl groups from histone tails and cores that drive nucleosome-constrictive gene silencing [reviewed in (10)]. The overexpression of HDACs in melanoma is a characteristic of acquired chemotherapeutic resistance (11) and drugs that inhibit HDACs such as panobinostat, and vorinostat prevent resistance to mainline drugs such as dacarbazine (12) and sensitize melanoma to BRAF/MEK inhibitors (13-16). HDAC inhibitors are believed to impair tumor-survival systems in part, by reducing the expression of anti-apoptotic proteins [survivin, B-cell lymphoma-extra-large (BCL-XL), BCL2, myeloid cell leukemia sequence 1 protein (MCL1), X-linked inhibitor of apoptosis (XIAP)], while at the same time elevating expression of contributors to death such as BCL2-like protein 4 (BAX), BCL2-associated X, apoptosis regulator (BAK) (17), death receptors (DR4 and DR5) (18), and the cell-cycle inhibitor (CKDN1A) (11). In this study, we evaluated the effects of trichostatin A (TSA), a potent class I and II HDAC inhibitor on the whole-transcriptome profile of SK-MEL-3 human melanoma cells, which carry the BRAF V599E mutation (1).

Materials and Methods

Cell culture media, phosphate-buffered saline, 96-well plates, pipette tips, fetal bovine serum (FBS), Alamar Blue and penicillin/streptomycin, as well as general reagents and supplies were all purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and VWR International (Radnor, PA, USA). SK-MEL-3 cells (ATCC HTB-69) and McCoy’s 5a Medium Modified were obtained from the American Type Culture Collection (Rockville, MD, USA). All microarray equipment, reagents, and materials were purchased from Affymetrix/Thermo Fisher (Waltham, MA, USA).

**HDAC activity.** Nuclear lysates from untreated SK-MEL-3 cells were extracted using the EpiQuik™ Nuclear Extraction Kit (Epigentek; Farmingdale, NY, USA) and used for kinetic HDAC activity assays (ab156064; Abcam, Cambridge, MA, USA) according to the manufacturer’s guidelines. The data were acquired using a Synergy HTX Multi-Mode Reader (BioTek, Winooski, VT, USA) with excitation at 355/40 nm, emission at 460/40 nm, optics: top, and gain: 37. Data were acquired using Gen5™ Data Analysis Software 2.06.10 (BioTek).

**Cell culture.** SK-MEL-3 (ATCC # HTB-69) cells were cultured in 175 cm2 flasks using ATCCs-formulated McCoy’s 5a Medium, supplemented with FBS (10%) and 100 U/ml penicillin G sodium/100 μg/ml streptomycin sulfate. Cells were grown at 37°C in 95% atmosphere/5% CO₂ and sub-cultured every 3-5 days.

**Cell viability assay.** Alamar Blue cell assay was used to determine both 24-h viable cell count and 7-day cell proliferation. In this assay, viable cells reduce resazurin to resorufin, a compound detectable by fluorescence. Briefly, 96-well plates were seeded with cells at a density of 5x10⁶ cells/ml (24-h) or 0.5x10⁶ cells/ml (7-day). Cells were treated with or without TSA (0.05-25 μM) and cultured at 37°C, in 5% CO₂ atmosphere. Alamar Blue (0.1 mg/ml in Hanks balanced salt solution [HBSS]) was added at 15% v/v to each well, and the plates were incubated for 6-8 h. Quantitative analysis of dye conversion was measured by Synergy™ HTX Multi-Mode microplate reader (BioTek) at 550nm/580nm (excitation/emission). The data were expressed as viable cell count as a percentage of untreated controls.

**Fluorescence microscopy.** Actin staining was conducted on untreated controls vs. cells treated with 1.56 μM TSA for 24 h. Staining was achieved using Alexa Fluor 488® phalloidin (ThermoFisher, Norcross, GA, USA) and images were obtained using a fluorescent/inverted microscope, CCD camera and data acquisition by ToupTek View (ToupTek Photonics Co, Zhejiang, PR. China).

**Whole-transcriptome human 2.1ST arrays.** After treatment (1 μM of TSA vs. untreated controls) for 24 h, cells were washed three times in HBSS, followed by rapid freezing and storage at –80°C. Using the basic trizol/chloroform method for RNA extraction led to failure at several quality control points, where it was determined that melanin appeared to be bound to the RNA and directly inhibited polymerase chain reactions. Consequently, total RNA was isolated and purified using Aurum total RNA mini kit (Bio-Rad, Hercules, CA, USA) which was effective in removal of melanin. Next, the whole-transcriptome analysis was conducted using preparation and instructions according to the GeneChipTM WT PLUS Reagent Manual for the human ST 2.1 array chips (Affymetrix/ThermoFisher Scientific, Waltham, MA, USA). Briefly, RNA was synthesized to first-strand cDNA, second-strand cDNA, followed by transcription to cRNA. cRNA was purified and assessed for yield, before synthesis of second-cycle single-stranded cDNA, hydrolysis of RNA and purification of second-cycle single-stranded cDNA. cDNA was then quantified for yield and equalized to 176 ng/ml. Subsequently, cDNA was fragmented, labeled, and hybridized onto the arrays before being subject to fluidics and imaging using the Gene Atlas (Affymetrix/ThermoFisher Scientific, Waltham, MA, USA). The array data quality control and initial processing from CEL to CHP files were conducted using expression console, before data evaluation using the Affymetrix transcriptome analysis console. The data were reported as fold change of TSA-treated cells relative to the control group.

**Flow cytometry for cell-cycle phase determination.** Cells were plated in 75 cm² flasks and cultured in low serum media (0.5% FBS) for 24 h to synchronize cells in the cell cycle. After 24 h, low serum media was removed, and high serum culture media (10% FBS) was added before treatment with TSA. After 24 h, cells were trypsinized, centrifuged and washed twice with assay buffer (Cayman Chemical, Ann Arbor, MI, USA), re-suspended to a density of 106 cells/ml in a cell suspension, fixed and stored at –20°C. After 48 h, the suspension was centrifuged at 300 x g for 2 min, the fixative was removed, and the pellet was re-suspended in 0.5 ml of staining solution containing propidium iodide (PI) and RNase A (Cayman Chemical). The distribution of DNA in all cell-cycle phases was assayed in replicates, and the proportion of cells in each stage was determined within 2 h by using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). For each sample, a total of 20,000 individual events from the gated subpopulation were analyzed separately. CellQuest Pro software
(BD Biosciences) was used for acquisition and analysis of the data, and the percentage of cells in each phase was determined using ModFit LT 3.2.1 Software (Verity Software House, Topsham, ME, USA).

Data analysis. Statistical analysis was performed using Graph Pad Prism (version 3.0; Graph Pad Software Inc. San Diego, CA, USA) with the significance of the difference between the groups assessed using a one-way ANOVA followed by Tukey post-hoc means comparison test or Student’s t-test. Microarray data were analyzed using Affymetrix expression console, transcription analysis software – incorporating analysis from the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (19).

Results

TSA-mediated reduction of HDAC enzyme activity. Nuclear HDAC activity in SK-MEL-3 cells was confirmed by monitoring de-acetylated peptide formation as relative fluorescence, in the presence and absence of TSA (2.6 nM) over time (Figure 1A). Significant signal/noise at 37°C was achieved at approximately 170 min of incubation, a time-point selected to generate a dose–response inhibition curve for TSA. The data show that the half maximal inhibitory concentration (IC₅₀) for TSA HDAC inhibition is less than 690 pM, which is extremely potent (Figure 1B). The SK-MEL3 HDAC nuclear lysate solution produced approximately 269 nM of deacetylated peptide product at 170 min under assay conditions (Figure 2A), as quantified according to the deacetylated peptide standard curve. Figure 2B represents the time course for the formation of a fluorometric product from the de-acetylated peptide product using the developer solution (lysl endopeptidase). TSA did not interfere with the product developer solution (Figure 2B), showing specificity only for the inhibition of HDAC (Figure 1).

Cytotoxicity of TSA. 24-hour toxicity of TSA in SK-MEL-3 cells was conducted where the data show a relatively high-dose sub-lethal concentration yielding no observable effects on cell viability (Figure 3A) or structural morphology (Figure 3B) at 1.56 nM of TSA. A 7-day proliferation study was also conducted at the same concentrations, where the data show TSA to exert cytostatic effects similar to the negative taxol control (1 μM) (Figure 3B). These findings clearly show a predominant effect on the cell cycle, rather than apoptosis.

Such a high concentration of melanin in SK-MEL-3 cells completely blocked all in vitro transcription and PCR amplification processes using the standard trizol/chloroform technique of RNA extraction. Therefore, after 24 h of treatment with 1 μM TSA, total RNA was extracted using steps essential to remove melanin from the nucleotide component using a spin-column technique. Basic array analysis on the control cells set showed a basic phenotype of SK-MEL-3 cells (Table I) with the inherent dominance of melanin synthesis, phagosome acidification, ATP hydrolysis-coupled proton and iron transport, where these systems remained largely unaffected by TSA.
In contrast, there were enormous changes to the whole-transcriptome evoked by TSA which is reflected by the volcano plot with highlighted gene symbols shown in Figure 4; most dominant changes are also presented in Table II, with data available for downloading at NCBI’s Gene Expression Omnibus accessible through GEO Series accession number GSE104265 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104265). In brief, the information provides...
Table I. Global transcriptomic profile by dominant processes within SK-MEL-3 (ATCC® HTB-69™) cells which were derived from a metastatic site (lymph node) from a 42-year-old Caucasian female with malignant melanoma. Data were acquired using the PANTHER (Protein ANalysis Through Evolutionary Relationships) Classification System (61, 62).

| GO biological process complete | No. of genes studied | No. found | No. expected | Fold enrichment | p-Value |
|--------------------------------|----------------------|-----------|--------------|-----------------|---------|
| Melanin biosynthetic process   | 13                   | 6         | 0.19         | 32.1            | 4.14E-04|
| Melanin metabolic process      | 14                   | 6         | 0.2          | 29.8            | 6.39E-04|
| Metabolic process              | 9916                 | 191       | 142.59       | 1.34            | 1.33E-04|
| Organic substance metabolic process | 9468               | 187       | 136.15       | 1.37            | 2.62E-05|
| Cellular metabolic process     | 8976                 | 180       | 129.07       | 1.39            | 2.43E-05|
| Cellular process               | 14970                | 271       | 215.26       | 1.26            | 5.08E-11|
| Organic hydroxy compound biosynthetic process | 153               | 13        | 2.2          | 5.91            | 4.27E-03|
| Secondary metabolite biosynthetic process | 16               | 6         | 0.23         | 26.08           | 1.39E-03|
| Phagosome acidification         | 27                   | 8         | 0.39         | 20.61           | 7.17E-05|
| Phagosome maturation            | 40                   | 8         | 0.58         | 13.91           | 1.42E-03|
| Organelle organization          | 3129                 | 82        | 44.99        | 1.82            | 2.47E-04|
| Cellular component organization | 5261                 | 127       | 75.65        | 1.68            | 6.98E-07|
| Cellular component organization or biogenesis | 5484               | 137       | 78.86        | 1.74            | 3.99E-09|
| Intracellular pH reduction      | 45                   | 9         | 0.65         | 13.91           | 2.38E-04|
| Regulation of intracellular pH  | 92                   | 12        | 1.32         | 9.07            | 1.28E-04|
| Regulation of cellular pH       | 96                   | 12        | 1.38         | 8.69            | 2.03E-04|
| Regulation of pH               | 103                  | 14        | 1.48         | 9.45            | 4.76E-06|
| Monovalent inorganic cation homeostasis | 132                | 14        | 1.9          | 7.38            | 1.06E-04|
| Cation homeostasis             | 633                  | 26        | 9.1          | 2.78            | 1.91E-02|
| Inorganic ion homeostasis       | 648                  | 26        | 9.32         | 2.79            | 2.88E-02|
| Cellular monovalent inorganic cation homeostasis | 110               | 12        | 1.58         | 7.59            | 8.71E-04|
| Cellular cation homeostasis     | 569                  | 25        | 8.18         | 3.06            | 9.28E-03|
| Cellular ion homeostasis        | 584                  | 25        | 8.4          | 2.98            | 1.47E-02|
| ATP-coupled proton transport    | 27                   | 7         | 0.39         | 18.03           | 1.52E-03|
| ATP hydrolysis-coupled cation transport | 58               | 9         | 0.83         | 10.79           | 1.98E-03|
| Ion transport                   | 1312                 | 42        | 18.87        | 2.23            | 1.00E-02|
| Transport                       | 4382                 | 163       | 63.01        | 2.59            | 1.38E-32|
| Establishment of localization   | 4491                 | 164       | 64.58        | 2.54            | 7.05E-32|
| Localization                    | 5459                 | 174       | 78.5         | 2.22            | 3.67E-27|
| Transmembrane transport         | 1220                 | 41        | 17.54        | 2.34            | 4.03E-03|
| ATP hydrolysis-coupled ion transport | 70                | 9         | 1.01         | 8.94            | 9.28E-03|
| Energy coupled proton transport, | 28                   | 8         | 0.4          | 19.87           | 9.47E-05|
| Hydrogen ion transmembrane transport | 116               | 13        | 1.67         | 7.79            | 1.87E-04|
| Proton transport                | 149                  | 13        | 2.14         | 6.07            | 3.19E-03|
| Hydrogen transport              | 151                  | 13        | 2.17         | 5.99            | 3.69E-03|
| Transferrin transport           | 36                   | 8         | 0.52         | 15.45           | 6.41E-04|
| Protein transport               | 1358                 | 71        | 19.53        | 3.64            | 1.42E-17|
| Peptide transport               | 1383                 | 71        | 19.89        | 3.57            | 3.89E-17|
| Amide transport                 | 1404                 | 72        | 20.19        | 3.57            | 2.04E-17|
| Nitrogen compound transport     | 1667                 | 83        | 23.97        | 3.46            | 4.83E-20|
| Organic substance transport     | 2030                 | 93        | 29.19        | 3.19            | 1.35E-20|
| Ferric iron transport           | 39                   | 8         | 0.56         | 14.27           | 1.17E-03|
| Iron transport                  | 58                   | 9         | 0.83         | 10.79           | 1.98E-03|
| Transition metal ion transport  | 110                  | 11        | 1.58         | 6.95            | 6.90E-03|
| Trivalent inorganic cation transport | 39               | 8         | 0.56         | 14.27           | 1.17E-03|

analysis of 48,226 transcripts, of which 1,643 genes were differentially expressed: 833 genes were up-regulated, and 810 genes were down-regulated.

There was no differential change to HDACs (class I, II and IV) 1-12 (Figure 5) nor the sirtuin HDAC class (1-6) (data not shown), and similarly no change in transcript for mutated BRAF (data not shown).

Using the Affymetrix transcriptome console, major changes were found in three areas related to tumor control: MAPK signaling (Figure 6A), cell-cycle regulation (Figure 6B), with
mitotic arrested also confirmed by flow cytometry (Figure 6C) and apoptosis (Figure 6D). In total, these findings demonstrate the global impact of inhibition of HDAC enzyme activity in SK-MEL3 malignant melanoma cells.

**Discussion**

Malignant melanoma can advance rapidly toward radiation and drug resistance, worsened by an inherent mutation of the \( \text{BRAF} \) gene which enables auto-activated RAF-independent oncogenic MAPK signaling. While there are dozens of \( \text{BRAF} \) mutations, the most common is a substitution at nucleotide 1799 where a mutation leads to valine being replaced by glutamate at codon 599 or 600. Treatment with inhibitors of \( \text{BRAF} \) (dabrafenib) or \( \text{MEK} \) (trametinib) can be initially effective, with time, there is often a high degree of relapse and development of resistance to chemotherapy agents (5-9).
The epigenetic events which contribute to chemoresistant phenotypes might offer therapeutic targets for combination drug strategies. Moreover, while there are thousands of biological epigenetic controls, HDACs function as high-capacity removers of acetyl groups from histones to sustain silencing. Epigenetic drugs that inhibit one or more HDACs can broadly alter the transcriptome by reactivating either coding or non-coding functional mRNAs which contribute to antitumor phenotypes (11). In the case of melanoma, combined therapy with HDAC inhibitors reportedly prevents resistance to dacarbazine (12) and sensitize melanoma to BRAF and MEK inhibitors (13-16), but the mechanism for this is not well understood. In addition, while there is potential for the use of HDACs of diverse types (e.g., vorinostat, tubacin, sirtinol, vorinostat, suberoyl bis-hydroxamic acid) in the treatment of melanoma [review in (20)], HDAC inhibitors are broad-based, not specific to areas of the genome, and can elicit unwanted side-effects (11), such as hematological toxicity, fatigue and nausea (21).

Given the research community interest regarding the direct effects of HDAC inhibitors on BRAF-mutant melanoma, the goal of this investigation was to examine the efficacy of a known HDAC inhibitor (TSA) and its ability to alter the transcriptome in a BRAF-mutant melanoma cell model. While a discussion of the data in its entirety is not possible given the hundreds of changes to the transcriptome, furthermore there are changes to many transcripts for which little is known, we briefly discuss changes relevant to BRAF signaling.

MAPK signaling. BRAF signaling in tumor cells is overactive corresponding to abnormally high levels of phosphorylation signaling. The data in this work confirm that TSA neither alters the transcription of several HDAC classes or the expression of BRAF itself. In contrast, TSA was found to reduce transcript levels of controlling elements of BRAF signaling, namely the upstream target protein kinase C delta (PRKCD) and downstream target MYC. PRKCD is upstream of BRAF and is highly overexpressed in a number of types of aggressive metastatic cancer (22) being an activator of phospho-ERK 1/2 signaling which can drive chemoresistant tumors (23-25), unbridled cell proliferation (22), phosphorylation of E-cadherin (which can perpetuate metastasis) (26) and in hypoxic tumors, up-regulate mRNA levels of hypoxia-inducible factor 1 alpha (HIF1α) (27) and glucose metabolism (28). The TSA-mediated reduction in MYC is one of the most compelling targets for treating chemoresistant melanoma. Down-regulation of MYC alone essentially blocks all four oncogenic pathways which lead to its up-regulation, namely NRAS proto-oncogene, GTPase (NRAS), BRAF, phosphatidylinositol-3-kinase and NOTCH (29), which are driving elements of MYC-directed tumor initiation, maintenance, and metastasis (30). Analysis of a large data pool of patient-derived BRAF-mutant melanoma showed that several pathways singly converge on activated overexpressed MYC, which manifests itself during relapse, resistance to BRAF/MEK inhibitors (29, 31, 32) and transition of normal human skin to dysplastic nevi (33). The transformative

Figure 5. Effects of trichostatin A (TSA) (1 μM) treatment for 24 h on histone deacetylase (HDAC) transcripts. The data represent the mean±SEM of the log2 bi-weighted signal from whole-transcriptome arrays. There were no significant differences between the two groups.
Table II. Differentially expressed transcripts in trichostatin A (TSA)-treated vs. control-untreated SK-MEL-3 cells. The data are expressed as fold change (FC) and level of significance represented by a p-Value and false discovery rate (FDR) adjusted p-value.

| Gene symbol | Description                                             | Ctrl mean bi-weighted. signal (log2) | TSA mean bi-weighted. signal (log2) | Fold-change | p-Value | FDR p-value |
|-------------|---------------------------------------------------------|-------------------------------------|-------------------------------------|-------------|---------|-------------|
| TSPAN13     | Tetraspanin 13                                          | 4.50                                | 9.26                                | 27.1        | 2.0E-06  | 0.020       |
| SERPIN1     | Serpin peptidase inhibitor, clade I (neuroserpin)      | 3.42                                | 7.98                                | 23.5        | 2.6E-04  | 0.046       |
| ATP1B2      | ATPase, Na+/K+ transporting, beta 2 polypeptide         | 3.85                                | 7.11                                | 11.0        | 1.3E-04  | 0.041       |
| NMNAT2      | Nicotinamide nucleotide adenyltransferase 2             | 3.04                                | 6.41                                | 10.4        | 1.8E-03  | 0.072       |
| PDGFRL      | Platelet-derived growth factor receptor-like            | 4.56                                | 7.89                                | 10.1        | 8.8E-05  | 0.038       |
| CYP1A1      | Cytochrome P450, family 1, subfamily A, polypeptide 1   | 3.60                                | 6.88                                | 9.8         | 3.3E-05  | 0.032       |
| PARM1       | Prostate androgen-regulated mucin-like protein 1        | 4.46                                | 7.73                                | 9.6         | 4.5E-04  | 0.050       |
| ROPN1L      | Rho-philin associated tail protein 1-like              | 2.80                                | 6.04                                | 9.5         | 1.3E-04  | 0.041       |
| SCG2        | Secretogranin II                                        | 3.87                                | 7.11                                | 9.5         | 2.6E-04  | 0.046       |
| SYT11       | Synaptotagmin XI                                        | 6.21                                | 9.43                                | 9.4         | 3.0E-04  | 0.047       |
| HIST1H2AG   | Histone cluster 1, H2ag                                 | 3.74                                | 6.93                                | 9.1         | 7.5E-04  | 0.057       |
| CTGF        | Connective tissue growth factor                        | 3.57                                | 6.73                                | 9.0         | 2.9E-05  | 0.030       |
| SYN1        | Synapsin I                                             | 3.85                                | 6.97                                | 8.7         | 6.7E-04  | 0.056       |
| DNER        | Delta/NOTCH like EGF repeat containing                  | 5.52                                | 8.62                                | 8.6         | 4.8E-05  | 0.034       |
| FABP4       | Fatty acid binding protein 4, adipocyte                | 1.79                                | 4.80                                | 8.1         | 5.6E-04  | 0.052       |
| STC1        | Stanniocalcin 1                                         | 2.46                                | 5.48                                | 8.0         | 2.2E-04  | 0.045       |
| EFNB2       | Ephrin-B2                                               | 3.06                                | 6.03                                | 7.8         | 8.5E-04  | 0.060       |
| SRXN1       | Sulfiredoxin 1                                          | 7.18                                | 10.10                               | 7.6         | 9.6E-05  | 0.039       |
| CYFIP2      | cytoplasmic FMR1 interacting protein 2                  | 4.43                                | 7.34                                | 7.5         | 4.9E-05  | 0.034       |
| SLCTA11     | solute carrier family 7                                 | 6.85                                | 9.75                                | 7.5         | 5.3E-04  | 0.051       |
| SEMA3D      | Semaphorin 3D                                           | 3.16                                | 6.07                                | 7.3         | 1.6E-03  | 0.072       |
| ELOVL4      | ELOVL fatty acid elongase 4                             | 3.82                                | 6.72                                | 7.5         | 3.8E-04  | 0.048       |
| GUSBP3      | Glucuronidase, beta pseudogene 3                        | 3.50                                | 6.37                                | 7.3         | 3.1E-03  | 0.090       |
| MIR2999     | MicroRNA 2999                                           | 6.36                                | 9.21                                | 7.2         | 1.1E-03  | 0.084       |
| ID1         | Inhibitor of DNA binding 1                              | 3.97                                | 6.80                                | 7.1         | 1.0E-03  | 0.083       |
| TMEM47      | Transmembrane protein 47                                | 4.65                                | 7.47                                | 7.1         | 3.0E-04  | 0.047       |
| CXADR; BTG3 | Cxosackie virus and adenovirus receptor                 | 4.59                                | 7.36                                | 6.8         | 1.6E-02  | 0.171       |
| SPTLC3      | Serine palmitoyltransferase, long chain base subunit 3  | 4.88                                | 7.61                                | 6.6         | 4.7E-04  | 0.050       |
| VGF         | VGF nerve growth factor inducible                       | 4.75                                | 7.47                                | 6.6         | 1.2E-03  | 0.068       |
| EID3        | EP300 interacting inhibitor of differentiation 3         | 4.23                                | 6.99                                | 6.6         | 2.3E-03  | 0.080       |
| BASP1       | Brain-abundant, membrane-attached signal protein 1      | 4.01                                | 6.68                                | 6.4         | 1.1E-02  | 0.142       |
| LOC730101   | uncharacterized LOC730101                               | 4.51                                | 7.14                                | 6.2         | 6.0E-06  | 0.025       |
| ATP8A1      | ATPase, aminophospholipid transporter (APLT)             | 3.60                                | 6.22                                | 6.2         | 2.1E-05  | 0.029       |
| CYR61       | Cysteine-rich, angiogenic inducer, 61                   | 4.37                                | 6.94                                | 5.9         | 8.5E-05  | 0.038       |
| PCDH9       | Protocadherin 9                                         | 3.33                                | 5.09                                | 5.9         | 1.9E-04  | 0.044       |
| PRKAR2B     | Protein kinase, cAMP-dependent, reg 2B                  | 5.08                                | 7.63                                | 5.9         | 7.0E-04  | 0.056       |
| EFN3        | Ephrin-A3                                               | 3.75                                | 6.27                                | 6.7         | 1.3E-04  | 0.041       |
| HIST2H4B, 4A| Histone cluster 2, H4b; histone cluster 2, H4a         | 5.87                                | 8.34                                | 5.5         | 2.7E-04  | 0.048       |
| ATP1B1      | ATPase, Na+/K+ transporting, beta 1 polypeptide         | 4.81                                | 7.24                                | 5.4         | 1.1E-03  | 0.083       |
| MYC1        | MYC target 1                                            | 2.68                                | 5.12                                | 5.4         | 3.4E-04  | 0.047       |
| SSBP2       | Single-stranded DNA binding protein 2                   | 4.46                                | 6.89                                | 5.4         | 7.2E-04  | 0.056       |
| STK17A      | Serine/threonine kinase 17a                             | 5.65                                | 8.07                                | 5.4         | 1.4E-03  | 0.088       |
| ANXA1       | Annexin A1                                             | 5.69                                | 8.07                                | 5.2         | 3.7E-04  | 0.048       |
| PEG10       | Paternally expressed 10                                 | 7.65                                | 10.01                               | 5.1         | 2.9E-03  | 0.087       |
| HMOX1       | Heme oxygenase 1                                        | 6.13                                | 8.45                                | 5.0         | 1.2E-03  | 0.065       |
### Table II. Continued

| Gene Symbol | Description | Fold Change | p-value |
|-------------|-------------|-------------|---------|
| SLC9A7      | Solute carrier family 9, subfamily A (NHE7) | 2.61        | 4.92    | 4.92E-03 | 0.122  |
| LIFR        | Leukemia inhibitory factor receptor alpha | 2.33        | 4.60    | 4.99E-03 | 0.108  |
| DHRS2       | Dehydrogenase/reductase (SDF family) member 2 | 2.61        | 4.89    | 4.93E-03 | 0.133  |
| NLRP1       | NLR family, pyrin domain containing 1 | 5.98        | 8.25    | 4.86E-03 | 0.066  |
| RALGAPA2    | Rap1 GTPase activating protein, as2 | 5.89        | 8.15    | 4.82E-03 | 0.079  |
| B3GALT1     | UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase 1 | 2.75        | 5.01    | 4.84E-03 | 0.116  |
| ST8SIA4     | ST8 alpha-N-acetyl-neuraminide sialyltransferase 4 | 4.90        | 7.15    | 4.85E-04 | 0.051  |
| PRDX1       | Peroxiredoxin 1 | 5.50        | 7.75    | 4.81E-03 | 0.072  |
| ACTB1       | Actin, beta-2 | 2.45        | 4.70    | 4.71E-04 | 0.045  |
| ENBP2       | Ectonucleotide pyrophosphatase/phosphodiesterase 2 | 6.91        | 9.15    | 4.79E-04 | 0.047  |
| PELI1       | Pellino E3 ubiquitin protein ligase 1 | 5.64        | 7.87    | 4.84E-03 | 0.070  |
| SMIM10L2B   | Small integral membrane protein 10 like 2B | 2.76        | 4.98    | 4.83E-03 | 0.092  |
| MAP2        | Microtubule associated protein 2 | 3.65        | 5.87    | 4.84E-03 | 0.073  |
| SLC4A12     | Solute carrier family 41 (magnesium transporter), member 2 | 3.86        | 6.07    | 4.83E-03 | 0.127  |
| PAIP2B      | Poly(A) binding protein interacting protein 2B | 5.01        | 7.22    | 4.61E-03 | 0.074  |
| UNC5D       | Unc-5 netrin receptor D | 2.90        | 5.11    | 4.61E-04 | 0.060  |
| PRKAA2      | Protein kinase, AMP-activated, alpha 2 catalytic subunit | 4.15        | 6.36    | 4.66E-03 | 0.123  |
| TTLL7       | Tubulin tyrosine ligase-like family member 7 | 5.18        | 7.37    | 4.66E-03 | 0.072  |
| USP57       | Ubiquitin specific peptidase 53 | 5.73        | 7.92    | 4.66E-03 | 0.072  |
| MGAT4A      | Mannosyl glycoprotein n-acetyl/glucomannosyltransferase | 3.13        | 5.31    | 4.51E-03 | 0.070  |
| CRIM1       | Cysteine rich transmembrane bmp regulator 1 (chordin-like) | 4.40        | 6.57    | 4.51E-03 | 0.072  |
| SEL1L3      | Sel-1 suppressor of lin-12-like 3 (C. elegans) | 3.60        | 5.77    | 4.51E-04 | 0.044  |
| SLC2A3      | Solute carrier family 2 | 3.93        | 6.09    | 4.51E-03 | 0.072  |
| MYEF2       | Myelin expression factor 2 | 4.15        | 6.27    | 4.41E-03 | 0.090  |
| HPSE2       | Heparanase | 3.62        | 5.74    | 4.41E-03 | 0.066  |
| LIPH        | Lipase, member h | 2.39        | 4.50    | 4.31E-03 | 0.034  |
| DNAJB4      | DNA (hsp40) homolog, subfamily b, member 4 | 4.56        | 6.67    | 4.31E-03 | 0.034  |
| GCML        | Glutamate-cysteine ligase, modifier subunit | 6.12        | 8.23    | 4.31E-03 | 0.072  |
| MXD1        | Max dimerization protein 1 | 5.10        | 7.20    | 4.31E-03 | 0.034  |
| BCO2        | Beta-carotene oxygenase 2 | 3.07        | 5.15    | 4.21E-03 | 0.047  |
| EYA1        | EYA transcriptional coactivator and phosphatase 1 | 4.89        | 6.96    | 4.21E-03 | 0.068  |
| CDH19       | Cadherin 19, type 2 | 5.30        | 7.37    | 4.21E-03 | 0.034  |
| ENBP1       | Ectonucleotide pyrophosphatase/phosphodiesterase 1 | 7.61        | 9.66    | 4.11E-03 | 0.048  |
| ARRD4C      | Arrestin domain containing 4 | 6.28        | 7.32    | 4.11E-03 | 0.050  |
| ABCD2       | ATP binding cassette subfamily d member 2 | 2.26        | 4.30    | 4.11E-03 | 0.051  |
| EGR1        | Early growth response 1 | 3.73        | 5.77    | 4.11E-03 | 0.065  |
| FREM2       | Fras1 related extracellular matrix protein 2 | 3.19        | 5.21    | 4.11E-03 | 0.047  |
| HIST1H2BD   | Histone cluster 1, H2bd | 3.39        | 5.41    | 4.11E-03 | 0.086  |
| ARHGAP29    | Rho GTPase activating protein 29 | 3.25        | 5.27    | 4.31E-03 | 0.053  |
| MGAM2       | Malate-glucomannose 2 (putative) | 3.78        | 5.79    | 4.21E-03 | 0.087  |
| AHR         | Aryl hydrocarbon receptor | 8.63        | 10.64   | 4.21E-03 | 0.067  |
| NPTX2       | Neuronal pentraxin II | 4.71        | 6.71    | 4.01E-03 | 0.107  |
| MGAM2       | Malate-glucomannose 2 (putative) | 3.85        | 5.84    | 4.01E-03 | 0.086  |
| TGN1        | Transcobalamin 1 | 3.20        | 5.19    | 4.01E-03 | 0.094  |
| MGAM2       | Malate-glucomannose 2 (putative) | 2.81        | 4.80    | 4.01E-03 | 0.185  |
| PRKCD       | Protein kinase C, delta | 8.08        | 9.49    | -2.64E-04 | 3.61E-04 | 0.048  |
| FAR33       | Poly (ADP-ribose) polymerase family member 3 | 7.11        | 5.11    | -4.01E-04 | 2.11E-04 | 0.045  |
| ATOH8       | Alkaline bHLH transcription factor 8 | 5.74        | 7.13    | -4.01E-04 | 7.64E-04 | 0.057  |
| ACAT2       | Acetyl-CoA acetyltransferase 2 | 7.50        | 9.49    | -4.01E-04 | 3.40E-03 | 0.092  |
| SNORD91B    | Small nucleolar RNA, C/D box 91B | 6.36        | 4.34    | -4.01E-04 | 1.21E-02 | 0.149  |
| TRIP6       | Thyroid hormone receptor interactor 6 | 7.80        | 5.77    | -4.01E-04 | 6.31E-04 | 0.055  |
| TGFB1I1     | Transforming growth factor beta 1 induced transcript 1 | 7.88        | 5.94    | -4.01E-04 | 2.11E-03 | 0.078  |
Table II. Continued

| MYC         | v-my c Avian myelocytomatosis viral oncogene homolog |
|-------------|------------------------------------------------------|
| PATZ1       | 5.63 3.60 -4.1 4.3E-04 0.049                        |
| ZC3H7B      | 6.71 4.67 -4.1 3.5E-05 0.032                        |
| KCNJ13      | 6.10 4.06 -4.1 2.2E-03 0.079                        |
| IGF2BP1     | 6.59 4.54 -4.2 3.3E-03 0.092                        |
| RFTN2       | 5.98 3.90 -4.2 2.9E-03 0.087                        |
| PRKG2       | 6.25 4.18 -4.2 6.1E-03 0.114                        |
| PHF19       | 6.29 4.19 -4.3 1.6E-02 0.145                        |
| ARHGAP31    | 6.01 3.90 -4.3 1.6E-04 0.044                        |
| MMP14       | 8.69 6.57 -4.4 2.6E-04 0.046                        |
| SLC45A2     | 9.67 7.52 -4.4 4.9E-04 0.050                        |
| MIR3142     | 6.19 4.04 -4.4 4.7E-03 0.103                        |
| MEPCE       | 7.15 4.99 -4.5 2.0E-05 0.029                        |
| NFATC2      | 8.94 6.71 -4.7 5.4E-05 0.034                        |
| CXCL1       | 6.05 3.81 -4.7 1.2E-05 0.029                        |
| TEAD2       | 5.62 3.38 -4.7 1.3E-03 0.068                        |
| SLC24A5     | 9.18 6.92 -4.8 1.1E-04 0.039                        |
| GYPC        | 6.56 4.30 -4.8 4.0E-04 0.048                        |
| PER3        | 6.22 3.93 -4.9 9.7E-04 0.063                        |
| ANGPTL2     | 7.23 4.93 -4.9 7.1E-04 0.056                        |
| OAS2        | 6.66 4.35 -5.0 5.3E-04 0.051                        |
| HAS2        | 6.72 4.41 -5.0 7.6E-04 0.057                        |
| ZBTB2       | 6.30 3.94 -5.1 6.9E-04 0.056                        |
| LGI3        | 9.08 6.71 -5.2 5.9E-04 0.053                        |
| HIST1H2BM   | 7.25 4.85 -5.3 3.3E-03 0.092                        |
| UCN2, PFKFB4| 7.58 5.17 -5.3 3.3E-04 0.047                        |
| SNORA72     | 5.30 2.87 -5.4 2.5E-02 0.209                        |
| LCP2        | 7.46 5.00 -5.5 2.0E-04 0.044                        |
| SPR         | 7.77 5.31 -5.5 2.1E-04 0.045                        |
| APOBEC3C    | 9.26 6.75 -5.7 1.8E-04 0.044                        |
| MIR4334     | 7.40 4.87 -5.8 1.1E-03 0.064                        |
| FRG2DP      | 7.72 5.18 -5.8 3.3E-04 0.047                        |
| MIR146A     | 7.81 5.27 -5.8 4.3E-04 0.049                        |
| TPCN2       | 8.86 6.31 -5.9 2.0E-03 0.077                        |
| FXYD3       | 6.98 4.38 -6.1 3.0E-05 0.030                        |
| SERPINH1    | 7.66 5.05 -6.1 6.8E-04 0.056                        |
| LOC285000   | 5.93 3.26 -6.4 3.8E-04 0.048                        |
| TP53        | 8.64 5.95 -6.5 1.2E-04 0.040                        |
| IL16        | 6.60 3.90 -6.5 2.6E-04 0.046                        |
| SNORA75     | 7.98 5.17 -7.0 9.4E-04 0.062                        |
| GPX8        | 6.24 3.43 -7.1 2.1E-03 0.078                        |
| UBAP1L      | 6.88 4.00 -7.2 4.7E-05 0.034                        |
| PYCARD      | 7.98 5.10 -7.3 3.7E-04 0.048                        |
| TRPM1       | 6.61 3.73 -7.4 2.4E-05 0.030                        |
| BCL2A1      | 8.62 5.50 -8.7 9.0E-05 0.038                        |
| CA14        | 8.19 4.80 -10.5 4.0E-06 0.022                       |
| GALNT3      | 6.85 3.44 -10.6 4.4E-04 0.050                        |
| LINCO0681   | 8.77 4.84 -15.3 1.4E-05 0.029                        |
resistance of BRAF-mutant melanoma is reversible by knockdown or drug inhibition of MYC alone (29, 34-36).

**Mitosis.** Functional pathway analysis shows that TSA reduced several controls over key cell cycle-related transcripts including: ataxia telangiectasia mutated serine/threonine kinase (ATM) that is activated by DNA damage during radiation, responsible for activation of checkpoint cell-cycle controlling kinase 2 (CHK2), which can further prevent cells from entering mitosis at the G2/M phase, all of which is believed to maintain stability of the genome (37-39). While it is uncertain what role this would have in cancer growth, a loss of function of ATM is believed to render greater genomic instability, which in theory would augment effects of radio-or chemotherapies. TSA also evoked a loss in the F-box protein S-phase kinase-associated protein 2 (SKP2) which is part of the SKP1–Cullin1–F-box protein ubiquitin ligase complex responsible for the degradation of cyclin-dependent kinase inhibitor p21 (CIP1/WAF1) which halts the cycle at the S-phase (40). A loss of SKP2 would be detrimental in several aspects such as initiating degradation of tumor suppressor p27 and KIP1 accumulation of p21, which causes S-phase arrest, cell proliferation and aggressive oncogenic potential (41-43). Loss-of-function of the SKP-Cullin, F-box containing complex (SCF)/SKP2 and degradation of the cyclin inhibitor p27KIP1 appear to be controlling factors in migration, inadequate growth arrest and invasion of diverse cancer types (42, 44). Recent work indicated that activated CDK2 and several of the polo-like kinases collaborate to phosphorylate G2 checkpoint kinase (WEE1), which could promote its ubiquitination by SCF (beta-transducing repeat containing (beta-TRCP) and in this manner perpetuate feedback signals to reinforce cycle transition (45).

TSA also reduced transcripts of the cell division cycle protein 45 (CDC45), which is required for DNA synthesis during genome duplication. CDC45 is overexpressed in several types of tumor cell, where it responsible for sustaining rapid rounds of cell division, amplification of DNA replication, chromosomal loading and unwinding and DNA synthesis at the replication fork (46). These events then activate CDKs and Dbf4-dependent kinase (DDK) to allow continued binding of CDC45 and heterotetramer of Sld5, Psp1, Psp2, and Psp3 (GINS) required for the CDC45/MCM2–7/GINS complex, and initiation/elongation of DNA for replication by DNA polymerase (47, 48). Furthermore, TSA rendered considerable loss of expression of CDK4, endothelial differentiation-related factor 1 (EDF1) and EDF3, all of which play critical roles in the cell cycle at the G1/S transition. CDK4 normally phosphorylates retinoblastoma protein (Rb) (49) causing its disassociation from E2F transcription factor, where E2F is then free to transcribe S-phase-promoting genes. Normally the Rb tumor suppressor restrains the cell cycle by binding E2F1, rendering its inability to transcribe E2F genes that encode many proteins involved with DNA replication. Down-regulation of CKD4 could leave more unphosphorylated Rb, and therefore halt the cell cycle at this point. Several studies show this where knockdown of CDK4 or use of compounds that are involved with its reduction such as asiatic acid will induce G0/G1 phase arrest of the cells (49, 50). Alterations in any key target of the CDK–Rb machinery will disrupt cell-cycle regulation, this being a valuable target for chemotherapeutic drug development.

**Apoptosis.** While TSA itself has been reported to induce apoptosis, cell death was not observed in this study at concentrations where HDAC inhibition occurred (51, 52). In contrast, we found confounding evidence of opposing forces in apoptosis, with significant losses of both tumor suppressor (TP53) and oncogenes (BCL2). While we confirm the work of others in the loss of BCL2 commonly reported with HDAC inhibitors (51, 53), we also confirm the less-reported attenuation of p53 (20). In general, it appears that HDAC inhibitors mediate anti-mitotic effects which predominate at a lower concentration over apoptotic effects.

**Carbonic anhydrase.** New findings in this work show that TSA induced a large loss in carbonic anhydrase 14 (CAXIV; 10-fold change, p<0.0001). Human carbonic anhydrases (EC 4.2.1.1) types IX and XII are overexpressed in a variety of cancer types and play a large role in pH regulation required to drive metastasis and growth, with greater importance to solid hypoxic tumors (54). Drugs such as CAIX inhibitor FC16-670A or any other compound that can down-regulate carbonic anhydrases will inevitably reduce the capacity of tumor cells to maintain acid-base equilibrium and thereby deal a vital blow to cancer survival, growth and resistance (e.g., sulfamides (acetazolamide) and coumarins (umbelliferon)) (55, 56). Combined efficacy of chemotherapy drug treatment is greater when combined with an inhibitor of carbonic acids, proton pumps (57), or HDACs (58), all of which could reduce tumor acidity.

**Tetraspanin 13.** Here we report the TSA-mediated up-regulation of tetraspanin 13 (TSP13; 27.1-fold change, p<0.0001), the ramifications of which have not been subject to much research. Sparse research on its role shows TSP13 and therefore halt the cell cycle at this point. Several studies show this where knockdown of CDK4 or use of compounds that are involved with its reduction such as asiatic acid will induce G0/G1 phase arrest of the cells (49, 50). Alterations in any key target of the CDK–Rb machinery will disrupt cell-cycle regulation, this being a valuable target for chemotherapeutic drug development.

This work provides a basic framework showing global transcriptomic changes using a pan-HDAC inhibitor in BRAF-mutant melanoma. The data provide evidence that
Figure 6. Continued
Figure 6. Pathway analysis. Effects of histone deacetylase (HDAC) enzyme inhibition with trichostatin A (TSA) (1 μM) at 24 h on A: Mitogen-activated protein kinases (MAPK) signaling, B: cell-cycle signaling, C: cell-cycle distribution, and D: apoptosis. In C, representative cytograms are shown in the upper two panels; in the lower panel, the data represent the mean percentage of cells per phase (±SEM; n=4), and significance of differences from controls was determined with a t-test. *Significantly different at p<0.05.
TSA while inhibiting HDAC enzyme activity, does not alter transcription of various classes of HDAC nor BRAF itself, but in fact down-regulates critical components of MAPK–MEK–BRAF oncogenic pathways, initiating a mitotic arrest. Functional pathway analysis showed that TSA negatively affected cell cycle progression, with flow cytometry confirming a halt at the G2 phase with no effect on apoptosis. The loss of anti-apoptotic BCL2 juxtaposed on the loss of apoptotic TP53 may account for the lack of toxicity observed in TSA-treated cells. These data provide a basis for further investigation as to the mechanisms of action for HDAC inhibition in BRAF-mutant malignant melanoma.

Data Sharing

The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE102891 LOCATED AT https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104265

Conflicts of Interest

The Authors confirm that there are no known conflicts of interest associated with this publication and there was no significant financial support for this work that could have influenced its outcome.

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