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ORIGINAL ARTICLE

Single-cell profiling of MC1R-inhibited melanocytes

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Abstract

The human red hair color (RHC) trait is caused by increased pheomelanin (red-yellow) and reduced eumelanin (black-brown) pigment in skin and hair due to diminished melanocortin 1 receptor (MC1R) function. In addition, individuals harboring the RHC trait are predisposed to melanoma development. While *MC1R* variants have been established as causative of RHC and are a well-defined risk factor for melanoma, it remains unclear mechanistically why decreased MC1R signaling alters pigmentation and increases melanoma susceptibility. Here, we use single-cell RNA sequencing (scRNA-seq) of melanocytes isolated from RHC mouse models to define a MC1R-inhibited Gene Signature (MiGS) comprising a large set of previously unidentified genes which may be implicated in melanogenesis and oncogenic transformation. We show that one of the candidate MiGS genes, *TBX3*, a well-known anti-senescence transcription factor implicated in melanoma progression, binds both E-box and T-box elements to regulate genes associated with melanogenesis and senescence bypass. Our results provide key insights into further mechanisms by which melanocytes with reduced MC1R signaling may regulate pigmentation and offer new candidates of study toward understanding how individuals with the RHC phenotype are predisposed to melanoma.

KEYWORDS

melanocortin 1 receptor, melanocytes, melanoma, red hair color, *TBX3*

1 | INTRODUCTION

Cancer predisposition is usually defined by inherited genetic mutations which increase an individual's risk of developing the disease. Identification of the genes and molecular pathways underlying predisposition is essential for both cancer treatment and prevention. Melanoma, the most lethal form of cutaneous cancer,

arises from melanin-producing melanocytes and represents an excellent model to study cancer risk factors. Melanoma is an increasingly common skin cancer caused by high levels of sun exposure and subsequent damage by ultraviolet (UV) light. All stages of the disease are amenable to study, and the genetic basis of disease initiation is well-defined. Specifically, cutaneous melanoma usually arises from acquisition of activated pro-oncogenes (e.g., *BRAF*

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and NRAS) combined with senescence bypass through inactivation of cell cycle regulators (e.g., CDKN2A/INK4A) (Bennett & Medrano, 2002; Ha et al., 2008; Vredeveld et al., 2012; Wagstaff et al., 2022). However, in addition to genes falling into these two categories, additional genetic loci have been implicated in melanoma predisposition. Understanding the molecular basis for melanoma predisposition is a key issue which has the potential to inform melanoma prevention strategies.

RHC is a well-described melanoma predisposition phenotype. Individuals with the RHC phenotype, possessing red hair, pale skin, freckles, and an inability to tan, show an increased risk of developing melanoma compared to those with a non-RHC disposition (Rayner et al., 2019; Rhodes et al., 1987; Schiöth et al., 1999). The RHC trait is mostly explained by coding variants of reduced function in the *melanocortin 1 receptor* (*MC1R*) gene which encodes a G-protein-coupled receptor (GPCR) located on the cell surface of melanocytes. The MC1R signaling axis (MC1R-cAMP-CREB-MITF) is a major regulator of pigment/melanin production where active signaling promotes eumelanin synthesis, in contrast, inhibition of this pathway favors an environment for pheomelanin production; therefore, diminished MC1R function alters the ratio of cellular eumelanin and pheomelanin that largely determines skin, hair, and eye color. Importantly, this ratio creates an environment lacking in photoprotective eumelanin and in turn allows for almost exclusive production of cytotoxic pheomelanin (Mort et al., 2015; Pavan & Sturm, 2019; Schiöth et al., 1999). High levels of pheomelanin in RHC individuals exacerbate DNA damage through the amplification of free radicals which is worsened when cells are exposed to UV, and pheomelanin itself can contribute to melanomagenesis in the absence of UV radiation (Mitra et al., 2012; Napolitano et al., 2014). These observations may partly explain why individuals with genetic RHC variants in *MC1R* exhibit an increased somatic mutational burden in melanocytes (Robles-Espinoza et al., 2016).

Although the influence of *MC1R* RHC variants on melanin composition may play a role in melanoma predisposition, *MC1R* mutation status may also have an impact on melanoma initiation unrelated to its role in pigmentation. For example, individuals with *MC1R* variants fail to activate PTEN upon UV exposure, resulting in increased PI3K/AKT signaling and oncogenic transformation (Cao et al., 2013). However, whether *MC1R* signaling plays additional roles in the prevention of melanoma initiation remains unknown. In part, this may be because, by definition, a molecular mechanism underlying melanoma predisposition must already be operating prior to the acquisition of the ultimate trigger for melanoma initiation, such as acquiring activating BRAF or NRAS mutations.

MC1R signaling shows high evolutionary conservation among mammals; thus, murine species provide an excellent model to explore this pathway. This includes mice with active *MC1R* signaling (nonagouti; *a/a*) and suppressed *MC1R* signaling (recessive yellow, *Mc1r^{re/e}*, and lethal yellow, *A^y/a*). Nonagouti mice have a dark coat color that results from a loss-of-function (LOF) allele of the nonagouti gene (*a*), which encodes the mouse homolog of agouti signaling protein (ASIP). In the absence of this important *MC1R* antagonist,

Significance

Human migration toward extreme latitudes precipitated selection of skin color variation to maximize the absorption of ultraviolet radiation (UVR) needed for Vitamin D3 synthesis. A prime example is loss-of-function coding variants in the melanocortin 1 receptor (*MC1R*), associated with red hair color (RHC) trait characterized by red hair, fair skin, and poor tanning ability. It is also associated with increased skin cancer risk. The mechanisms surrounding the impact of RHC on skin color and cancer remain unclear. This study uses transcriptomic profiling of individual skin cells to elucidate the physiological landscape of RHC melanocytes. We identified a set of genes associated with RHC and highlight a transcriptional regulator, *TBX3*, as a new candidate in the regulation of melanogenesis and melanoma predisposition.

nonagouti melanocytes predominantly synthesize eumelanin. In contrast, recessive yellow (*Mc1r^{re/e}*) and lethal yellow (*A^y/a*) mice provide two models which mimic known causes of RHC (Kempf et al., 2022; Rayner et al., 2019; Schiöth et al., 1999), each with melanocytes that synthesize primarily pheomelanin due to distinct molecular mechanisms which cause reduced *MC1R* signaling. Recessive yellow mice carry a LOF allele in the *MC1R* receptor while the similar yellow coat color of lethal yellow mice is caused by constitutive overexpression of the *MC1R* antagonist, agouti signaling protein (Hauschka et al., 1968; Michaud et al., 1993; Michaud et al., 1994). While demonstrated subtle differences in phenotype between these two models suggest agouti may have agonist functions in addition to its well-characterized *MC1R* antagonist function (Ollmann et al., 1998), the overlap in constitutive pheomelanin expression suggests they could both be used to identify common candidate regulators responsible for RHC.

Given the role of the RHC trait in melanoma risk, a better understanding of the gene regulatory network (GRN) underlying in vivo melanocytes with inhibited *MC1R* signaling is needed. Previously, April et al. identified several novel genes linked to pheomelanogenesis in mouse models using bulk RNA sequencing (April & Barsh, 2006). Since this study used whole skin layers, of which melanocytes comprise only a small fraction of the total cell population, gene expression from other cell types such as keratinocytes, basal cells, and dendritic cells potentially masked the detection of melanocyte-intrinsic changes. Haltaufderhyde and Oancea performed bulk RNA sequencing on human epidermal melanocytes to assess global gene expression changes between light and dark colored cells (Haltaufderhyde & Oancea, 2014). However, the genotypes were unknown, and melanocytes were not exclusively producing one form of melanin over the other. While these previous studies established an important foundation for our understanding of pigmentation, additional, high-resolution genomic

studies are needed to fully evaluate the intricate molecular networks regulating eumelanogenesis, pheomelanogenesis, and melanoma development.

To better understand the GRN associated with the RHC trait, we utilized single-cell RNA sequencing (scRNA-seq) to compare melanocytes with both active and suppressed MC1R signaling. We defined a set of gene candidates, the MC1R-inhibited Gene Signature (MiGS), as potential new or previously overlooked factors in the regulation of melanogenesis and melanomagenesis. We further explored the chromatin occupancy of TBX3, a MiGS- and melanoma-associated T-box family member. Our results provide key insights into how RHC trait variants may increase melanoma risk via altered regulation of MiGS candidate genes in melanocytes which have inhibited MC1R signaling.

2 | MATERIALS AND METHODS

2.1 | Animals, tissue harvest, and fluorescent-activated cell sorting

Nonagouti (*a/a*) (C57BL/6J; JAX Mice #000664), lethal yellow (*B6.Cg-A^y/a*; JAX Mice #000021), and recessive yellow (*C57BL/6J-Mc1r^{e/e}*; kindly provided by Jonathan Zippin, equivalent to JAX Mice #000060) mice were used in this study with each of the mutant alleles on a C57BL/6J background. Breeding age nonagouti and lethal yellow mice were crossed (*a/a* × *A^y/a*) to generate two male littermate samples for comparison of nonagouti (*a/a*) or lethal yellow (*A^y/a*) melanocytes. Similarly, nonagouti (*a/a*) and recessive yellow (*Mc1r^{e/e}*) melanocytes were isolated for comparison from male pups harvested in parallel from two breeding pairs (*a/a*; *Mc1r^{+/+}* × *a/a*; *Mc1r^{+/+}* and *a/a*; *Mc1r^{e/e}* × *Mc1r^{e/e}*). Pups were phenotypically distinguishable due to their different black or yellow fur color and genotype was confirmed in the sequencing results.

Single-cell solutions for each sample were derived from the dorsal skin of postnatal day 3 (P3) animals following a protocol modified from Harris et al. (2018). Dorsal skin was removed along anatomically defined margins consisting of four incisions: two transverse cuts; one between each axilla, and the other between the anterior pelvises, and bilateral incisions midline from the axilla to the anterior pelvis. Following a brief incubation in ice cold 1xPBS, the hypodermis was removed manually with forceps. Skins were then incubated in 3mLs of 0.25% Trypsin-EDTA (ThermoFisher #25200056) at 37°C and 10% CO₂ for 30–35 min to facilitate separation of the epidermis and dermis. The epidermis was manually removed, and the remaining dermal tissue underwent further digestion in 0.3mg/mL of the enzyme Liberase (Roche #05401119001) on a rotating platform at 37°C for 45 min. The resulting dermal suspension was mechanically digested with repeated passage through a 70µm filtered (Corning #352350) syringe (Covidien #8881560125). This single-cell suspension was immunolabeled with CD117-PEcy7 (c-KIT, BD Pharmingen #553772), CD45.2-FITC (BD Pharmingen #558163), and LIVE/DEAD™ Fixable Aqua Dead Cell Stain (Invitrogen #L34965).

Using FACS Aria (BD Biosciences), melanocyte cells were positively selected by gating on CD117+, CD45.2-, and Aqua+.

2.2 | Single-cell RNA sequencing and differential expression analysis

FACS-isolated melanocytes were captured in a solution of 1xPBS+0.04% FBS. Cell viability and concentration were analyzed using a Luna-FX7 cell counter (Logos Biosystems). Gel Bead-in-Emulsions were prepared by loading 5000 cells/sample onto a Chromium Chip G (10x Genomics #1000127) and run using the Chromium controller (10x Genomics #1000204). cDNA libraries were generated with Chromium Single Cell 3' GEM, Library and Gel Bead Kit V3.1 (10x Genomics #1000128). Samples were indexed, and pairs for each experiment were pooled for sequencing using the NextSeq 500/550 Hi Output KT v2.5 (Illumina #20024907) on an Illumina NextSeq550. Cell Ranger (10x Genomics) was used for alignment to mm39 genome, barcode counting, and UMI counting. Downstream processing of the data was performed with Partek Flow™ Genomic Analysis Software. Downstream processing of the data was performed with Partek Flow™ Genomic Analysis Software.

Single-cell QA/QC included steps to filter out cells which did not meet the following parameters defined in the two pairwise experiments (lethal yellow / recessive yellow) as barcode count 600–32,000/3000–45,000, number of genes detected 500–6000/500–7000, and % ribosomal counts 12%–43%/12%–39%. Of the 31,017 annotated murine genes, 16,793 (54.14%)/20,060 (64.7%) passed an exclusion cutoff where genes were excluded if they had zero counts in at least 99.9% of the cells. The remaining read counts were normalized using a log₂-based method on Counts Per Million (CPM). Normalized counts underwent dimensional reduction via a principal components analysis (PCA) on the first 100 principal components and unsupervised, non-biased, graph-based clustering through a Louvain algorithm on the first 20 principal components. Graph-based clusters underwent further dimensional reduction for visualization on a Uniform Manifold Approximation and Projection diagram. Postnatal mouse skin cell types including dermal fibroblasts, dermal papilla, transit amplifying cells, matrix cells, and epidermal cells were identified by utilizing published signature gene lists (Rezza et al., 2016). The most highly expressed genes (top 10%) from the signature gene lists were overlaid on the UMAP, and the graph-based clusters which coincided with expression overlay were assigned to each cell type. All non-melanocyte cell types were subsequently filtered out, leaving only clusters classified as melanocytes for downstream analysis. The melanocyte only population underwent a further iteration of PCA, graph-based clustering, and UMAP display. To eliminate the variable of cellular heterogeneity as influenced by the mitotic state, we selected for cells within the G₀ phase using a gene expression overlay established by Hsiao et al (Hsiao et al., 2020). The melanocytes within the G₀ phase underwent a further iteration of PCA, graph-based clustering, and UMAP analysis. Differential

gene expression lists of melanocytes were generated using the Partek ANOVA differential expression analysis. Genes considered statistically significant had a false-discovery rate of <0.05 and a fold-change of ± 2 .

2.3 | Gene ontology and pathway analysis

Genes comprising the MiGS were uploaded to the Gene Ontology Resource for GO Enrichment Analysis via PANTHER for Overrepresentation Tests (GO Ontology database [10.5281/zenodo.6399963](https://www.ebi.ac.uk/GO); Released 2022-03-22). The MiGS was run against a reference list comprising all *Mus musculus* genes in the database. Fisher's exact test was performed with a false-discovery rate correction for GO Biological Process Complete, GO Cell Component Complete, and GO Molecular Function Complete with significant results being those with an FDR <0.05 . To identify predicted upstream regulators, we utilized QIAGEN's Ingenuity® Pathway Analysis (73,620,684 (Release Date: 2022-03-12). Testing was done via Expression Core Analysis on the observed experimental fold-change of the MiGS for both pairwise experiments.

2.4 | Gene set enrichment analysis

Gene lists comprising the significant DE+FC and -FC genes regulated by TBX3 were uploaded to the Molecular Signatures Database (MSigDB) for gene set enrichment analysis utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG). For the +FC list, the top 500 genes by fold-change for TBX3_13.8 were used as the maximum input value is 500 genes. Each list was tested independently with significant terms being those with an FDR q -value <0.05 .

2.5 | Cell lines

501mel-iTBX3-3xHA-PuroR for ChIP-seq studies were maintained in RPMI-1640+10% FBS and the Ludwig Institute for Cancer Research. 501mel cells at the National Human Genome Research Institute were grown in DMEM +10% FBS, 1 mM sodium pyruvate, and 4 mM L-Glutamine for TBX3 knockdown experiments.

2.6 | Chromatin immunoprecipitation sequencing (ChIP-seq)

Fifteen 15-cm dishes of 501mel-iTBX3-3xHA-PuroR cells were cultured for each replicate of ChIP-seq, and three were used for ChIP-qPCR. Cells were cross-linked for 10 min by adding formaldehyde (Sigma #F8775) to a final concentration of 0.8%, and then quenched for 10 min by adding glycine to a final concentration of 200 mM. Cells were then washed, scraped, and collected into a

50-mL falcon tube (Corning #430828), and centrifuged at 1500g for 10 min. The cell pellet was lysed in 1 mL of ChIP lysis buffer (50 mM Tris-HCl at pH 8.0, 10 mM EDTA, 10 mM sodium butyrate, 1% SDS, 4× PIC [Roche #05056489001]) and passed through a 100- μ m cell strainer before being sonicated for 15 min in a Covaris S220 (peak incident = 145 W, duty factor = 5%, cycle/burst = 200) until 200- to 400-bp fragments were obtained (assessed by 1% agarose gel). The sonicated chromatin was cleared by centrifugation at 13,000g for 10 min and the supernatant was diluted eightfold in ChIP dilution buffer (16.7 mM Tris at pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 1% Triton X-100, 0.01% SDS) before 12 μ g of anti-HA antibody (Roche #11666606001) was added, and chromatin was rotated in a 50-mL falcon tube overnight. In parallel, 60 μ L of Dynabeads protein G (Invitrogen #10004D) was washed, resuspended in ChIP dilution buffer, and blocked in 0.5 mg/mL BSA overnight at 4°C. Immunoprecipitation was carried out using blocked Dynabeads for 1 h at 4°C. The beads were washed three times each in low-salt wash buffer (20 mM Tris-HCl at pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), high-salt wash buffer (20 mM Tris-HCl at pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), and LiCl wash buffer (10 mM Tris-HCl at pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% sodium deoxycholate, 1% NP-40) with beads transferred to a new DNA LoBind tube (Eppendorf #Z666548) with each wash. The beads were eluted in 200 μ L of elution buffer (100 mM NaHCO₃, 1% SDS). Reversal of cross-linking of chromatin-immunoprecipitated DNA was done overnight at 55°C with the addition of NaCl (final concentration 300 mM), 20 μ g of RNase A, and 20 μ g of Proteinase. Chromatin-immunoprecipitated DNA was recovered using a QIAquick PCR purification kit (Qiagen #28106). The concentration of chromatin-immunoprecipitated DNA used in sequencing was assessed using Qubit dsDNA HS assay kit (Invitrogen #Q32851). The two biological replicate samples were subjected to 150-bp paired-end sequencing using a NovaSeq6000 (Illumina) at the Wellcome Trust Genomic Service, Oxford.

2.7 | TBX3 ChIP analysis

Prior to mapping to the human reference genome (hg38) with Bowtie2 (v.2.3.5) (Langmead & Salzberg, 2012), quality of the raw sequencing data was evaluated using FastQC (v.0.11.7), with adapter contamination removed using CutAdapt (v.2.8). Peak calling was performed using MACS2 (v.2.1.2) (Y. Zhang et al., 2008) with a 0.01 q -value threshold. Bowtie2-generated SAM files were compressed to BAM files, indexed using SAMtools (v.1.9), and sequentially converted to bigWig files using UCSC tools (v.373). The BED files of both replicates were intersected using the UCSC Table Browser to pull out common peaks from replicate 2 (40,974) that had $\geq 80\%$ overlap with replicate 1 (32,479), which yielded a group of 20,449 common peaks. For simplicity of downstream analysis, the 20,449 peak coordinates used were derived from the BED position parameters of replicate 1.

The Genomic Regions Enrichment of Annotations Tool (GREAT v4.0.4) in the “Basal plus extension” mode was used to analyze the functional significance of cis-regulatory regions of the TBX3 bound ChIP peaks. Each annotated gene was assigned a basal regulatory domain of 5kb upstream, and 1kb downstream, of the transcriptional start site. The regulatory domain of that gene was then extended in both directions to the nearest gene's basal domain but with no more than a 100kb extension in one direction (McLean et al., 2010). Discovery of enriched binding motifs was done using the MEME-ChIP DNA binding motif analysis tool (Bailey et al., 2015). 250bps were added up and downstream of the chromosomal coordinates corresponding to the absolute summit of all overlapping peaks. A FASTA file was generated using the usegalaxy.org bedtools application “GetFastaBed” (Quinlan & Hall, 2010). This was run against the default settings in MEME-ChIP, except for maximum width for “What width motifs should MEME and STREME find?” which was increased to 20 bases. Peak distributions were obtained from GREAT and ChIPseeker (Yu et al., 2015), via usegalaxy.org. The histogram was produced by GREAT, while the Venn diagram was created with ChIPseeker.

2.8 | Bulk RNAseq of in vitro 501mel human melanoma cells

501mel cells (140,000/well) were plated in a 12-well plate (1.2mL/well). dsRNAs (NC1, TBX3 13.6, TBX 13.8; Integrated DNA Technologies) were diluted in 300uL of Opti-MEM (final concentration of 20nM). Lipofectamine RNAiMAX (6uL/100uL; Invitrogen #13778075) was then diluted in 300uL of Opti-MEM. Diluted dsRNA and Lipofectamine were mixed and set to incubate at RT for 5min. 200uL of dsRNA-Lipofectamine mix was added to each well. At 24h, cells were lysed for RNA collection via (Zymo Research #R2053). RNA purity was assessed via tapestation with all samples having a RIN purity of ≥ 9.0 . RNA samples were submitted to Omega Bioservices for bulk RNAseq. RNA purity was again assessed via an Agilent tapestation with all samples having a RIN ≥ 9.0 . Library construction was completed using NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®. Samples were sequenced using NovaSeq 6000 (Illumina) PE150 with a ~40M (20M in each direction) read throughput for each sample. Phred Mean Quality scores for all samples were ≥ 38.86 . Data were analyzed by ROSALIND® (<https://rosalind.bio/>), with a HyperScale architecture developed by ROSALIND, Inc.

2.9 | Figure construction

Figures were assembled and created with BioRender.com. UMAPs were generated with Partek Flow. Venn diagrams were produced using DeepVenn. Binding distribution, Figure 3a, was modified from original GREAT output, while Figure 3b was modified from the ChIP

seeker output from usegalaxy.org. Motif distribution for Figure 5d is modified from the MEME CentriMo tool.

3 | RESULTS

3.1 | Single-cell RNA sequencing of primary melanocytes identifies a MC1R-inhibited gene signature (MiGS)

To better understand global gene expression changes in cells with inhibited-MC1R signaling, we used single-cell RNA sequencing (scRNA-seq) to define the transcriptomic profiles of primary mouse melanocytes which predominantly synthesize either pheomelanin (lethal yellow, A^Y , $n=1$; or recessive yellow, $Mc1r^e$, $n=1$) or eumelanin (nonagouti, a , $n=2$). The dorsal skin was removed from postnatal Day 3 (P3) animals along anatomically defined margins consisting of four incisions (Figure 1a): two transverse cuts, with one between each axilla, and the other between the anterior pelvises, and bilateral incisions at the midline from the axilla to the anterior pelvis. This tissue was then enzymatically digested into a single-cell solution. Melanocytes comprise <5% of cells within the skin; therefore, after dissection and digestion of tissue, a fluorescent-activated cell sorting (FACS) approach was used to enrich the pool of melanocytes for sequencing (Harris et al., 2018). Positively selected cells were defined as c-KIT⁺ and CD45⁻ (Figure 1a,b). To eliminate selection bias among potential subpopulations of melanocytes that may vary in c-KIT levels, a lower intensity threshold for c-KIT positivity was used, yielding a population enriched for melanocytes but expected to also contain other cell types. Isolated cells were processed for library construction and RNA sequencing via the 10x Genomics Chromium pipeline. After the implementation of quality control measures (see Methods), sequencing data were obtained from a total of 11,058 cells from all samples. The corresponding data underwent dimensional reduction via non-biased principal component (PCA) and graph-based clustering analyses for graphical display by Uniform Manifold Approximation and Projection (UMAP) (Figure 1c,d). We classified and labeled captured cell types in an unbiased manner by overlaying published murine skin cell gene signatures (Rezza et al., 2016) (Figure 1c,d; Table S1, Figure S1 and S2). Cells classified as melanocytes included 4 of the 16 graph-based clusters (GBC) identified in cells isolated from a nonagouti and lethal yellow pair of samples, and 1 of 12 GBCs identified in cells isolated from a nonagouti and recessive yellow pair of samples. The classification of selected GBCs as melanocytes is supported by visualization of the expression of bona fide melanocyte markers including *Mitf* and *Dct* (Figure S1d and S2d). Subsequently, for each of the two pairwise experiments, all non-melanocyte cells were filtered out and only gene expression from the cells classified as melanocytes were used for further analysis.

Because the mitotic stage has a significant and influential effect on gene expression, all melanocytes outside the G_0/G_1

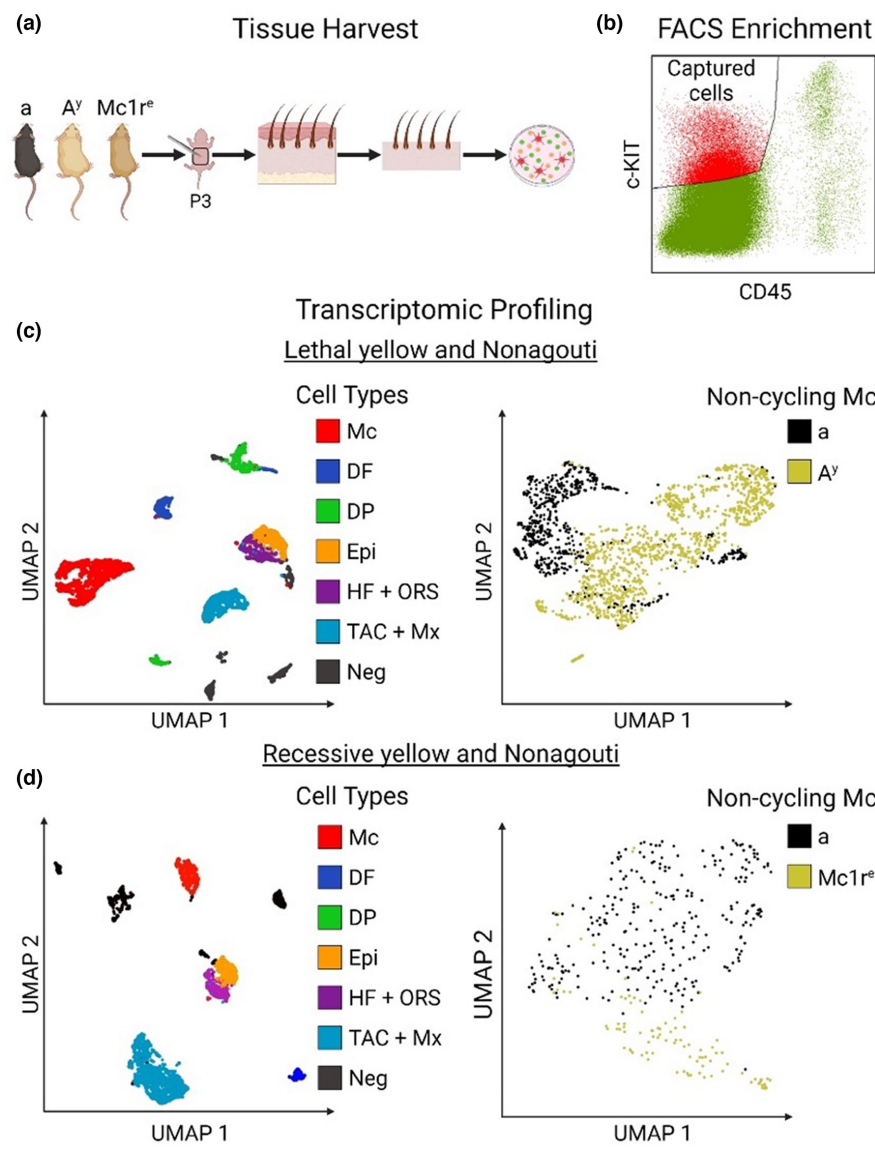


FIGURE 1 Overview of single-cell sequencing of primary murine melanocytes. (a) Schematic of tissue harvest and digestion for melanocyte isolation from dorsal skin (a = nonagouti; A^y = lethal yellow, Mc1r^e = recessive yellow). (b) Representative FACS enrichment of c-KIT⁺/CD45⁻ cells isolated from the lethal yellow sample (red). (c, d) Uniform Manifold Approximation and Projection (UMAP) plots of the sequenced cells that passed quality control for each pairwise experiment. Among the cells isolated from nonagouti and lethal yellow paired samples (6413 cells in c) and nonagouti and recessive yellow paired samples (4645 cells in d), skin cell types were identified and labeled as follows: Melanocytes (Mc), Dermal fibroblasts (DF), Dermal papilla (DP), Epidermis (Epi), Hair follicle and Outer root sheath (HF + ORS), Transit amplifying and Matrix cells (TAC + Mx), and Negative (Neg) cells which could not be classified. Right panels show UMAP plots of the 2256 cells classified as non-cycling melanocytes. The nonagouti melanocytes (black) segregate from the recessive yellow and lethal yellow (gold) melanocytes consistent with a eumelanin and pheomelanin-producing melanocytes having distinct transcriptional profiles.

TABLE 1 Pigmentation phenotype associated genes found within the MC1R-inhibited Gene Signature (MiGS) with FDR and FC values for each pairwise experiment.

Gene	Lethal yellow			Recessive yellow		
	FDR step up	Fold change	Normalized total counts	FDR step up	Fold change	Normalized total counts
<i>Tyrp1</i>	0.00E+00	-19.31	775984.48	1.12E-67	-83.9	286332.60
<i>Pmel</i>	4.82E-46	-2.33	8214929.50	5.82E-16	-2.55	2144016.69
<i>Mef2c</i>	8.98E-40	2.69	453242.33	1.93E-07	2.33	87371.49
<i>Oca2</i>	1.77E-25	-3.16	1308.27	9.28E-05	-2.47	75510.26
<i>Slc7a11</i>	5.38E-23	7.72	92466.92	3.79E-03	3.92	7263.31
<i>Irf4</i>	1.32E-19	-3.02	36655.02	7.19E-03	-2.61	17344.66
<i>Mc1r</i>	2.58E-11	2.94	553621.06	3.64E-03	3.63	62970.90
<i>Gas7</i>	1.37E-07	-2.28	14496.09	1.05E-02	-8.57	3650.95
<i>Sdc2</i>	1.70E-07	2.39	56501.17	8.94E-13	4.29	12197.97
<i>Pcbd1</i>	1.25E-03	2.12	39324.37	2.37E-02	2.34	4546.95
<i>Masp1</i>	9.76E-03	3.11	8721.57	1.73E-02	2.69	3468.97

Note: In each pairwise experiment, a negative FC indicates reduced expression in the pheomelanin-producing melanocytes (lethal yellow, recessive yellow) compared to the eumelanin producing melanocytes isolated from nonagouti littermates.

phase were removed using the same gene expression overlay approach with previously published cell cycle marker genes (Belote et al., 2021; Hsiao et al., 2020) (Figure S1e and S2e). This defined the non-cycling melanocytes that were used for differential gene expression analysis comparison. Subsequent graph-based clustering analysis on the melanocyte population showed that both RHC mouse models displayed a bifurcation between the pheomelanin melanocytes and the eumelanin melanocytes captured in parallel, as displayed by UMAP (Figure 1c,d). Consistent with this, clustering of melanocytes by genotype was unique to melanocytes and not observed in the other cell types, suggesting that the effects of MC1R signaling loss are indeed restricted to melanocytes (Guida et al., 2022) (Figure S1f and S2f).

Using this melanocyte population, differentially expressed genes (DEGs) (ANOVA; FDR < 0.05 and fold-change (FC) ≥ 2) were identified in pairwise experiments to compare lethal yellow to nonagouti melanocytes (Figure 1c) as well as recessive yellow to nonagouti melanocytes (Figure 1d). Because each pairwise experiment had small sample numbers ($n = 1$ of each genotype), the differential lists (Table S2 and S3) from each experiment were intersected to focus on identifying shared genes which have consistent FC direction and likely possess most biological significance when comparing the nonagouti (a/a) melanocytes to the lethal yellow (A^y/a) or recessive yellow ($Mc1r^{e/e}$) melanocytes. These comparisons resulted in a set of 216 genes replicated between the two RHC models which we have identified as the MiGS (Table S4, Figure S4). We next asked to what extent these genes are associated with pigmentation by querying

the MiGS against a compiled list of genes annotated as causative for pigmentation phenotypes (Baxter et al., 2019) to assess the degree of overlap. Interestingly, only 5% of the MiGS genes appeared in the previously annotated pigmentation gene list (Table 1).

We next used PANTHER Gene Ontology (GO) and Ingenuity® Pathway Analysis (IPA) to identify significantly over-represented processes and pathways in the MiGS. GO analysis revealed a prevalence of significant terms (FDR < 0.05) related to “morphogenesis,” “melanin biosynthesis,” and “neurogenesis”/“neurotransmitter regulation” (Figure 2a). Terms related to “melanin processes” had equally high fold-enrichment; for example, 4 out of the 15 genes associated with “melanin biosynthetic process” are present in the MiGS (*Tyrp1*, *Mc1r*, *Oca2*, and *Pmel*). IPA highlighted canonical pathways related to “neuroinflammation,” “synaptogenesis,” and “neuron receptor signaling” (Figure 2b). These terms are primarily driven by a host of receptors and ligands within the gamma-aminobutyric acid (GABA), N-methyl-D-aspartate (NMDA)/Glutamate, Ephrin, and Semaphorin families.

3.2 | Predicted transcriptional regulators of the MC1R-inhibited gene signature include TBX3

To better identify the GRN regulating pheomelanin synthesis, we examined differentially expressed transcription factors, as well as predicted molecular upstream regulators of gene regulatory networks. The MiGS contains 22 annotated transcription and

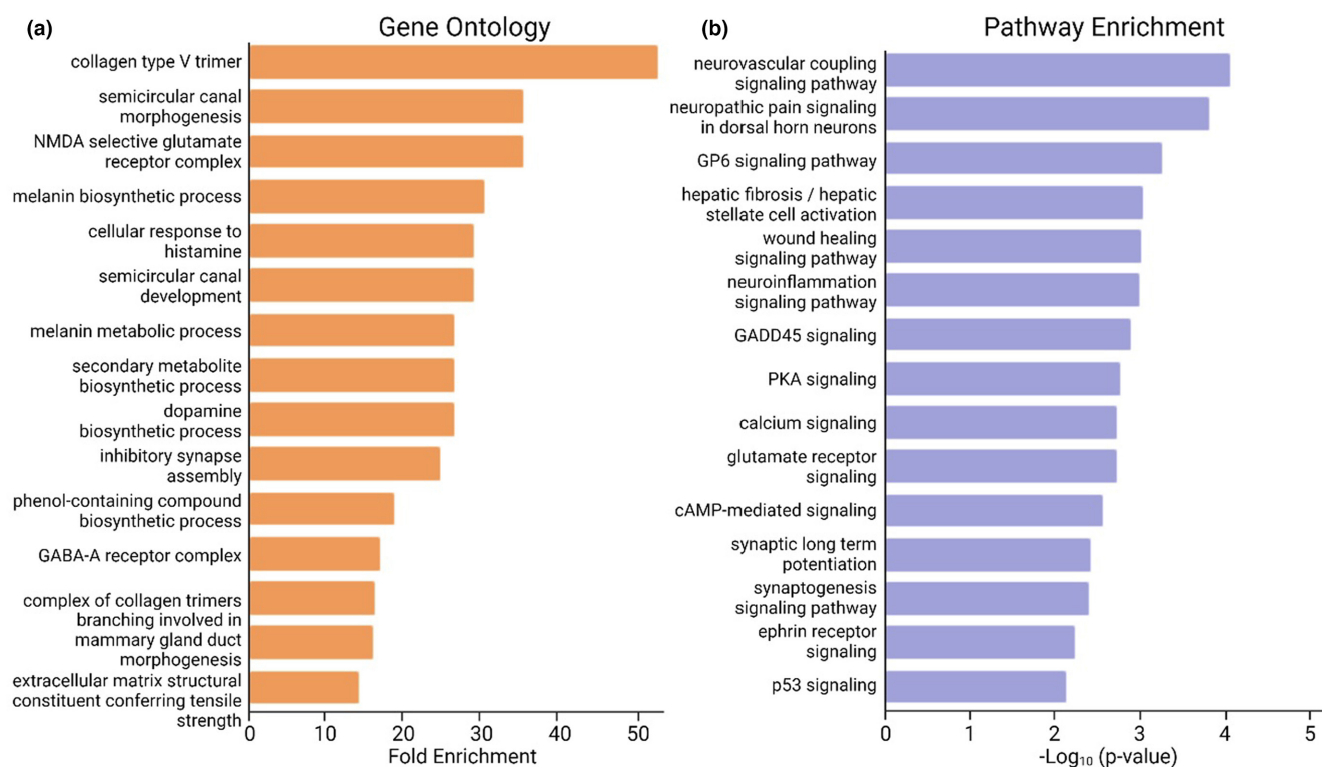


FIGURE 2 Enriched processes and pathways present in the MiGS. (a) Top 15 significant GO terms ranked by fold-enrichment (FDR < 0.05). (b) Top 15 significant pathway terms from IPA ranked by significance (p-value < 0.05).

co-transcription factors (Hu et al., 2019) (Figure 3a). Many of these have established relationships to melanocyte and pigment cell biology, including neural crest development (*Hmga2*, *Id4*, *Mef2c*, *Tbx3*, and *Tfap2b* [Hu et al., 2015; Kee & Bronner-Fraser, 2001; Lopez et al., 2018; Macri et al., 2016; Seberg et al., 2017]), melanocyte differentiation (*Mef2c* [Agarwal et al., 2011]), melanogenesis (*Id4*, *Irf4*, and *Tbx3* [Chhabra et al., 2018; DiVito et al., 2015; Imsland et al., 2016; Wang et al., 2020]), oxidative stress protection (*Nr4a* [Yin et al., 2014]), and melanoma (*Hmga2*, *Irf4*, *Nfactc2*, *Nr4a*, *Tbx3*, and *Zeb1* [Boyd et al., 2013; Chen et al., 2017; Heppt et al., 2018; Lu et al., 2010; Moon et al., 2017; Peres et al., 2021; Peres et al., 2010; Perotti et al., 2016; Perrot et al., 2013; Rodriguez et al., 2008; Smith et al., 2011; Zhang et al., 2018]). Of note, the *microphthalmia-associated transcription factor* (*Mitf*), a master melanocyte regulatory transcription factor (Goding & Arnheiter, 2019), was not identified as transcriptionally altered. Collectively, these data suggest that pheomelanin synthesis is not simply a result of eumelanin synthesis inactivation, rather this process involves multiple complex pathways downstream of MC1R signaling loss.

IPA was further used to predict upstream regulators which may be driving expression of these transcription factors and the MiGS as a whole (Figure 3b). The top 10 predicted regulators include three proteins—SMAD4, GDF2, and SMAD1—that are all related to TGF β /BMP signaling. These factors are expressed by multiple cell types of the hair follicle which border melanocytes (Rezza et al., 2016). Importantly, TGF β signaling can regulate melanin synthesis, with activation of the pathway resulting in hypopigmentation (Murakami et al., 2009). All differentially expressed transcription factors merit further exploration in MiGS regulation; however, we first chose to prioritize transcriptional targets downstream of TGF β /BMP signaling for further validation. Among these was *Tbx3*, a T-box family member activated by the TGF β signaling pathway (Khan et al., 2020; Li et al., 2013). Importantly, *Tbx3* showed significantly increased expression in both RHC (lethal yellow and recessive yellow) samples compared to nonagouti (Figure 3a). Additionally, TBX3 has previously been implicated in pigmentation of Dun horses (Andersson, 2020; Imsland et al., 2016); however, how TBX3 mediates this phenotype is unclear. Beyond this, TBX3 plays key roles in human melanomas as a regulator of epithelial-to-mesenchymal transition in melanoma invasion (Rodriguez et al., 2008), and acts as an anti-senescence transcription factor thus promoting cellular proliferation (Bennett & Medrano, 2002; Ha et al., 2008; Khan et al., 2020; Peres et al., 2010; Vredevelde et al., 2012; Wagstaff et al., 2022). Therefore, TBX3 was selected for further exploration as a candidate transcription factor for regulation of MiGS genes.

3.3 | TBX3 binds at T-box and E-box motifs

If TBX3 plays a role in establishing a cellular state favorable for pheomelanogenesis, we hypothesized that TBX3 would bind to, and therefore regulate expression of, a subset of the MiGS. Thus, we set out to establish direct TBX3 target candidates to prioritize

for validation. To our knowledge, there are no global binding studies (i.e., Chromatin Immunoprecipitation-sequencing [ChIP-seq]) of TBX3 in a melanocyte specific context. Therefore, we performed ChIP-seq in 501mel cells to identify global targets of TBX3 binding. In our hands, commercially available antibodies against TBX3 proved inadequate for IP-pulldown. To address this issue, we used the piggyBac transposase system to create a stable doxycycline inducible cell line with TBX3 tagged with a 3xHA epitope at the carboxy terminus (Figure S4a). We opted for this method as it has previously been used to produce high-quality ChIP-seq datasets (Laurette et al., 2015; Louphrasitthiphol et al., 2020; Lu et al., 2021). We choose the 501mel cell line as these cells are widely characterized, easy to transfect and, most importantly, they have intact MC1R and endogenously express TBX3 (Figure S4b). In our studies, doxycycline induced the level of TBX3 to physiologically relevant levels (Figure S4b) which were appropriately detected in the nucleus via immunofluorescence (Figure S4c). We performed ChIP-seq on two biological replicates and utilized MACS2 for peak calling (q -value cutoff ≤ 0.01). To identify common peaks between the two replicates, an intersection filter was applied requiring an 80% overlap of coordinates between the two replicates, resulting in the identification of 20,449 common peaks (Figure S4d).

To determine the biological significance of these peaks, we used Genomic Regions Enrichment Annotation Tool (GREAT) (McLean et al., 2010) to associate 10,000/20,449 peaks with the GREAT assigned or curated regulatory elements of 7109 genes (Methods; Table S5). We next assessed the binding distribution of these 10,000 peaks relative to gene transcriptional start sites (TSS). Promoter region binding within ± 5 kb of the TSS comprised only 12.68% of peaks, and the vast majority were situated ± 20 kb from the TSS, suggesting TBX3 binds distal enhancer elements (Figure 4a,b). GO enrichment for the 7109 candidate TBX3 gene targets included multiple terms related to pigmentation/melanogenic pathways, as well as multiple terms associated with EMT, of which TBX3 is a well-known regulator (Figure 4c). Phenotype association analyses showed that pigmentation-related phenotypes were the most abundant for both human and mouse with a particular emphasis on hypopigmentation/abnormal melanocyte activity (Figure 4d).

We next used MEME-ChIP to assess motif enrichment within the TBX3 peaks. De novo motif analysis revealed that the top 10 sequences (JASPAR CORE vertebrates non-redundant v2), ranked by e -value, were not only associated with the expected T-box related elements, but also with E-box related motifs (Figure 5a). Interestingly, the palindromic canonical E-box sequence CACGTG is bound by basic-Helix-Loop-Helix (bHLH) and bHLHZip transcription factors including MITF and its related family members. This sequence shares a 3bp GTG with the well-described core of the T-box element (AGGTGTGA) bound by T-box family members (Khan et al., 2020; Strub et al., 2011; Washkowitz et al., 2012). Furthermore, TBX3 represses *CDH1* at a CAGGTGT site which shares 5bp with the canonical E-box (Rodriguez et al., 2008). Interestingly, a TBX3 peak does not appear at this site in our ChIP dataset; however, TBX3 and MITF share two peaks within intron 2, and another peak 1.6 kb upstream

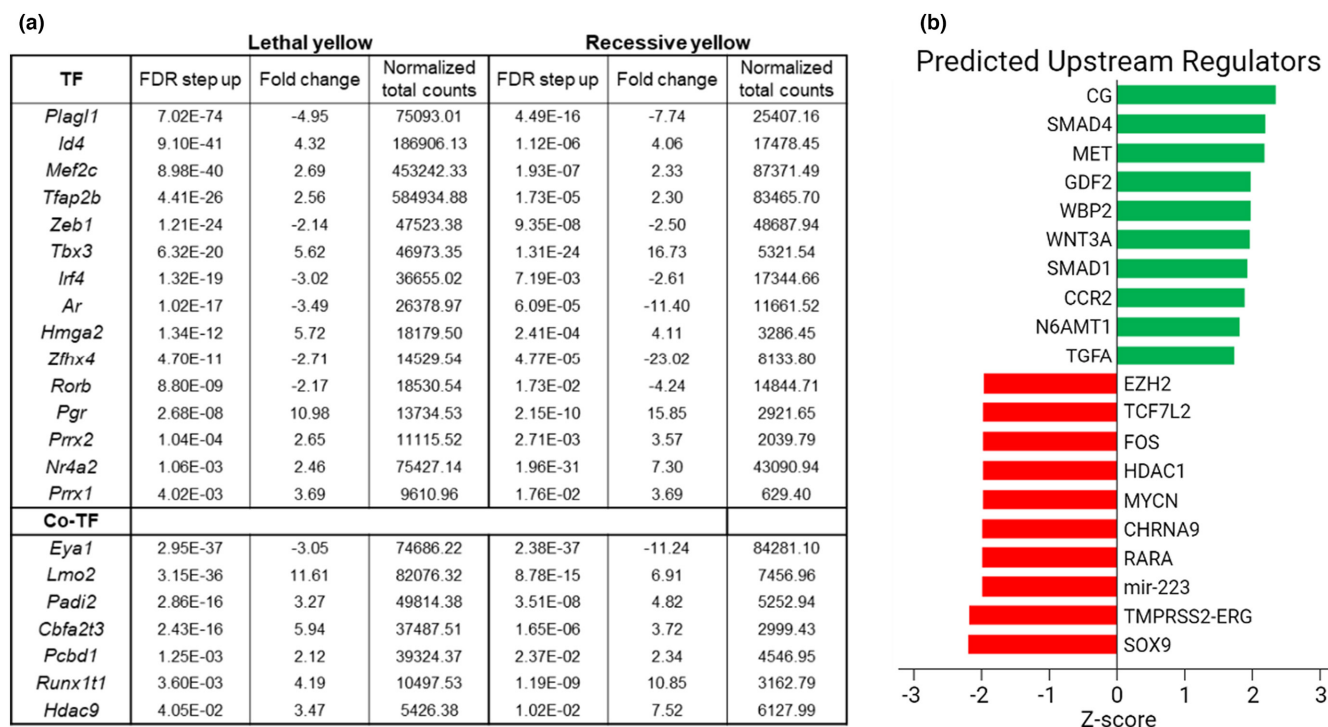


FIGURE 3 Identifying MiGS regulatory factors. (a) List of all differentially expressed transcription/co-transcription factors found within the MiGS and ranked by FDR from the nonagouti/lethal yellow pairwise differential analysis. (b) Top 10 predicted activated (green) and inhibited (red) regulators of the MiGS gene list ranked by Z-score.

of the promoter region (Figure S4d). Collectively, T-box and E-box motifs were found in the majority of TBX3-bound input sequences (8320/10,000; 83.20%) with the E-box motifs occurring more frequently than the T-box motifs (69.17% vs. 45.92%, respectively) (Figure 5b).

Importantly, as the E-box motif is the primary recognition sequence associated with MITF (Goding & Arnheiter, 2019), we wanted to interrogate the degree of shared genome-wide binding sites between TBX3 and MITF. To assess for common occupancy of TBX3 and MITF, we intersected our 20,449 TBX3 peaks with 12,137 called peaks from a previously published human MITF ChIP dataset in 501mel cells (Strub et al., 2011). This yielded 4758 common peaks which were assessed with MEME-ChIP for motif enrichment. The top 10 sequences (JASPAR CORE vertebrates non-redundant v2), ranked by e-value, were all E-box related motifs (Figure 5c) centrally located within the TBX3 bound peaks (Figure 5d). The frequency of TBX3 binding at centrally located E-box motifs also known to be occupied by MITF suggests that TBX3 and MITF may compete for binding at a set of shared regulatory elements.

To query the set of TBX3 and MITF dual candidate target genes, we utilized the same GREAT parameters as our TBX3 analysis to identify 7096 MITF candidate target genes associated with peaks from the MITF ChIP dataset (Strub et al., 2011) (Table S6). We then identified the mouse homologs for both the TBX3 and MITF target gene sets to allow comparison to the MiGS gene list derived from in vivo mouse experiments (Table S7 and S8). The intersection of TBX3 (6499/7109) and MITF (6661/7096) gene target lists identified

a shared set of 4072 genes, representing 62.6% of the TBX3 genome-wide target list (Figure 6a). Of these, 59 are found on the MiGS (59/216; 27.3%), suggesting that a portion of the MiGS is directly regulated by both TBX3 and MITF and that this portion is similar or very slightly enriched ($p = .04$) relative to the genome-wide portion of shared TBX3 and MITF targets (4072 shared targets/18,778 annotated genes = 21.6% of genes genome wide) (Figure 6b/Table S9). Importantly, TBX3 and MITF shared binding sites at notable pigmentation genes, with four of these having well-described MITF regulatory elements *IRF4*, *MC1R*, *OCA2*, and *TYRP1* (Aoki & Moro, 2002; Gautron et al., 2021; Praetorius et al., 2013; Visser et al., 2012) (Figure 6c). Collectively, these data suggest that TBX3 can directly bind at E-box motifs associated with MiGS genes, thus suggesting a potential regulatory role in melanogenesis via modulation of MITF-mediated gene regulation either indirectly, simply blocking MITF's ability to bind, or directly, by actively repressing transcription at bound targets.

3.4 | TBX3 functions as a transcriptional activator and repressor

Our bioinformatic analyses identified 7109 genes in which TBX3 binding is associated with cis-regulatory elements. This constituted multiple MiGS genes, including important genes related to melanogenesis. Given the large number of genome-wide TBX3 binding sites, it is unlikely that TBX3 directly regulates all of the identified gene

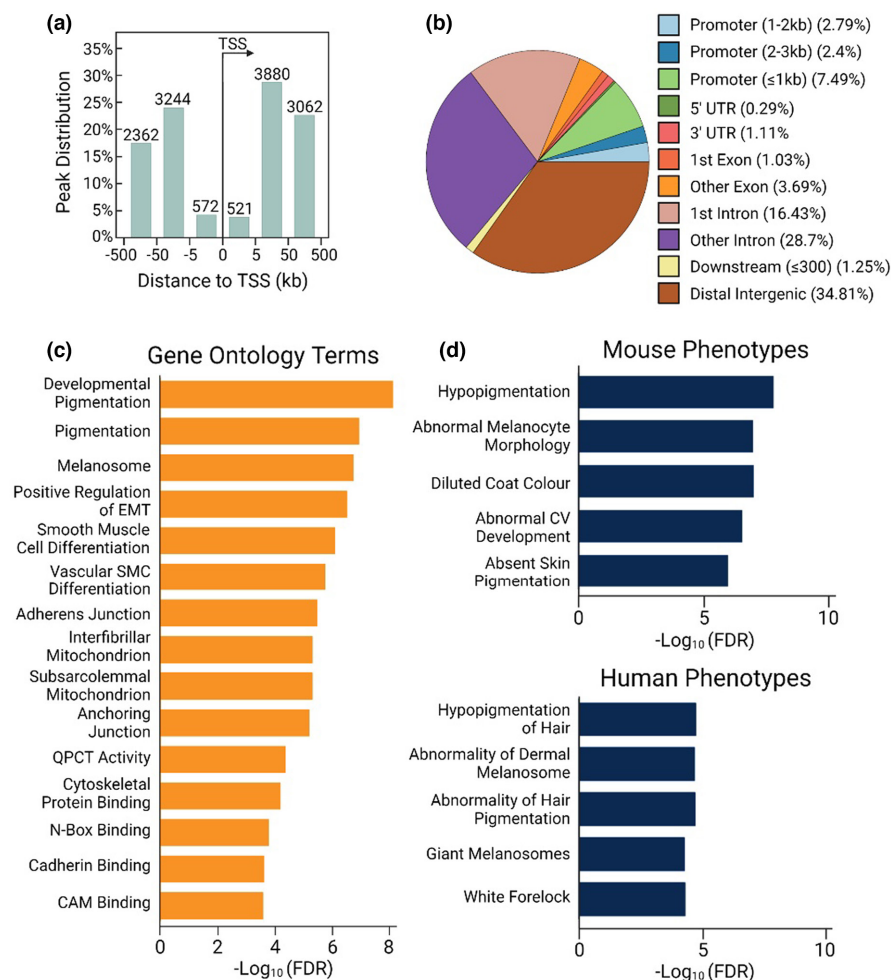


FIGURE 4 ChIP-seq analysis implicates TBX3 as a regulator of melanogenesis. (a) Position of called TBX3 peaks relative to the TSS. (b) ChIPseeker plot identifying genomic regions in which TBX3 is bound. (c) Top 15 GO terms of genes associated with TBX3 ChIP target genes ranked by $-\log_{10}$ of the FDR. (d) Top 5 predicted phenotype associations (human and mouse) from TBX3 ChIP gene by $-\log_{10}$ of the FDR.

candidates. Therefore, we sought additional evidence that TBX3 directly influences expression of these candidates, especially those within the MiGS. Traditionally, TBX3 has been shown to function as a transcriptional repressor; however, it is known to include an activator domain (Khan et al., 2020; Washkowitz et al., 2012). It is therefore possible that TBX3 can either activate or repress these candidate target genes. To address these points, we performed bulk RNA sequencing in 501mel human melanoma cells following the transient reduction of TBX3, to determine (1) which putative target genes were most likely to be regulated by TBX3 and (2) if TBX3 repressed or activated these targets.

We performed transient knockdown of *TBX3* in 501mels for 24 h, in triplicate, with two distinct siRNAs (TBX3_13.6 and TBX3_13.8) and a non-coding control. At 24 h, we collected lysates, harvested RNA, and then submitted samples for bulk RNA-seq. We then performed differential analysis and identified significant DEGs ($p_{\text{adj}} < .05$) that were responsive to TBX3 depletion (Table S10). We next intersected DEGs from the two small interfering RNAs and removed genes in which FC occurred in contradictory directions, resulting in 1420 positive FC genes (+FC) and 1340 negative FC genes (−FC) (Figure 7a). To identify a subset of these genes that were most likely to be regulated as direct targets of TBX3 binding, we intersected the DEG list with our TBX3 ChIP peak associated target genes, which

identified a total of 1091 targets (623 +FC, 468 −FC) that show both responsiveness to TBX3 depletion as well as TBX3 ChIP-seq binding sites within cis-regulatory elements of target genes as defined by our GREAT analysis (Table S11).

3.5 | TBX3 regulates MiGS and cell cycle genes

We next performed Gene Set Enrichment Analysis (GSEA) on the candidate TBX3 regulated targets to identify significant ($\text{FDR} < 0.05$) KEGG terms associated with the +FC and −FC genes. Terms for the +FC list include those related to TBX3's known role in melanoma, such as "TGF β signaling pathway," "focal adhesion," and "ECM receptor interaction" (Figure 7b). Importantly, "melanogenesis" was one of the most significant terms. Enriched terms for −FC genes include those consistent with TBX3's proliferative and anti-senescent functions, such as "cell cycle," "MAPK signaling pathway," and "p53 signaling pathway" (Figure 7b). Specifically, this includes cell cycle progression genes *CDK1* and *CDK2* (Table S11). Lastly, we wanted to assess which MiGS targets were specifically bound and regulated in our in vitro system. We first converted the 1091 +/− FC genes bound and regulated by TBX3 to their known mouse homologs, yielding 1041 genes. These were intersected with the 98 genes

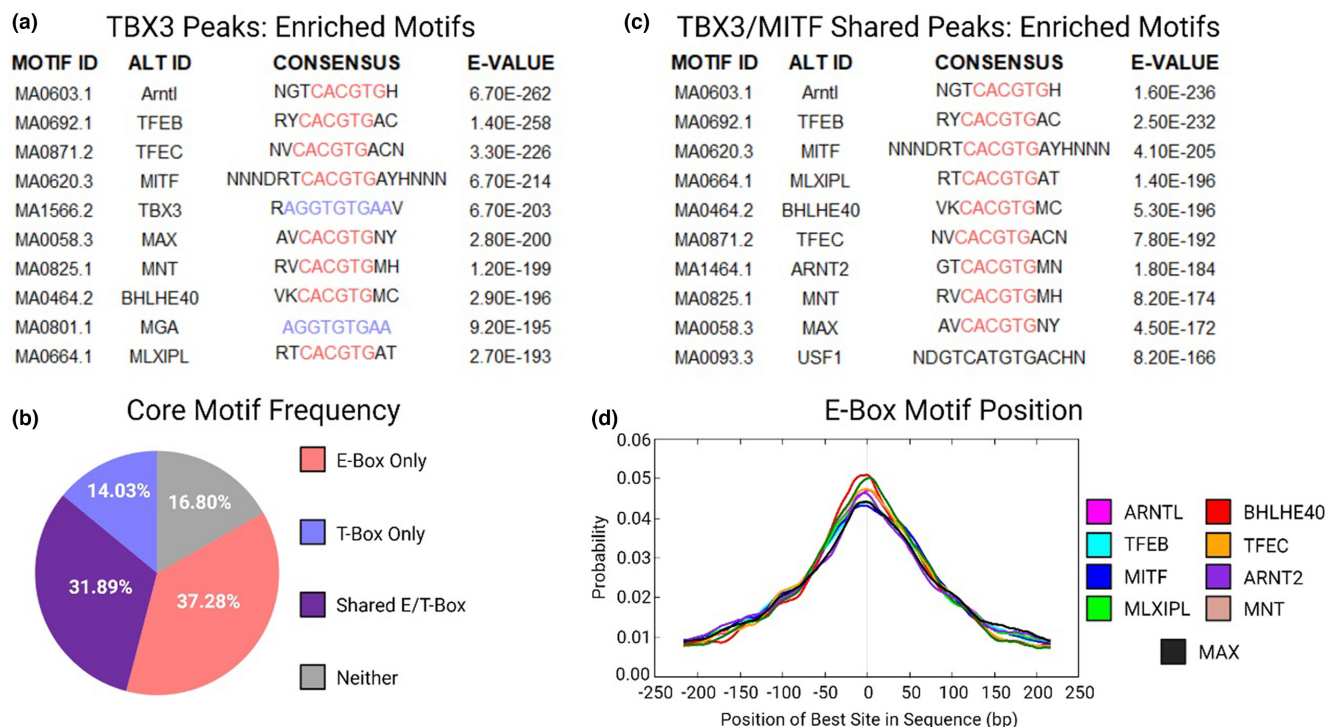


FIGURE 5 TBX3 binds MITF targets at E-boxes. (a) The top 10 motifs enriched within TBX3 ChIP peaks show over-representation of core E-box (red) and T-box (purple) sequences (b) The majority of TBX3 ChIP peaks contain either a T-box motif (14%), E-box motif (27%), or both (32%). (c) Top motifs of shared MITF and TBX3 peak sites (E-box = red; T-box = purple). (d) CentriMo plot showing top 9 centrally bound motifs.

comprising TBX3 targets of the MiGS, of which 23 were present (Figure 7c). Collectively, these data suggest that another role, apart from modulation of MITF-mediated gene regulation, is that TBX3 may regulate melanogenesis via transcriptional activation and/or repression of MiGS genes.

4 | DISCUSSION

Coding variants in *MC1R* are associated with the RHC trait. However, the GRNs downstream of *MC1R* remain poorly understood. Through a high-resolution, single-cell RNA-sequencing approach, we have identified a MiGS in melanocytes comprising both known- and new-candidate genes which may regulate important biological processes in melanocytes including, but not limited to, melanogenesis and melanomagenesis. One such candidate is *Tbx3* which we show can bind and regulate a subset of MiGS genes. Interestingly, we have shown that the transcriptional regulatory activities of TBX3 include both activation and repression of candidate genes. Furthermore, this function is not restricted to DNA binding via the T-box motif, rather it includes binding the canonical CACGTG E-box, which is the main recognition sequence of the melanocyte master regulatory transcription factor MITF.

The vast majority of genes comprising the MiGS have no previously established ties to pigmentation phenotypes (205/216; 94.9%). A common starting approach toward investigation of a novel gene

list involves identifying over-enriched gene families and pathways. One such MiGS-enriched family encompasses neurogenesis and neurotransmitter regulation and is primarily driven by receptors and ligands within the gamma-aminobutyric acid (GABA), N-methyl-D-aspartate (NMDA)/Glutamate, Ephrin, and Semaphorin families. Mature melanocytes show morphological similarities with neurons in that they are highly dendritic, which enables contact with multiple keratinocytes for melanin transfer. Furthermore, normal human melanocytes have GO enrichment for "neuron ensheathment and synapse" (Belote et al., 2021). Interestingly, melanocytes that predominantly produce pheomelanin in the presence of ASIP lack dendricity (Le Pape et al., 2008). Likewise, inhibition of glutamate signaling through NDMA receptors has been shown to inhibit melanocyte filopodia attachment to keratinocytes as well as reduce melanosome transfer efficacy (Ni et al., 2016). A recent study has shown that GABA signaling between keratinocytes and melanoma cells occurs through neuronal like synapses, and further shows that this communication promotes melanoma initiation (Tagore et al., 2021). Interestingly, in this context, keratinocytes showed increased GABA receptor expression while melanoma cells did not. Collectively, increased expression of these gene families may reflect both intrinsic and extrinsic signaling to promote melanocyte development, dendrite formation, and melanosome transfer abilities.

Previous work has shown that the benzodiazepine Diazepam, which works to increase the action of GABA, increases both melanogenesis and melanocyte dendricity (Enkhtaivan & Lee, 2021).

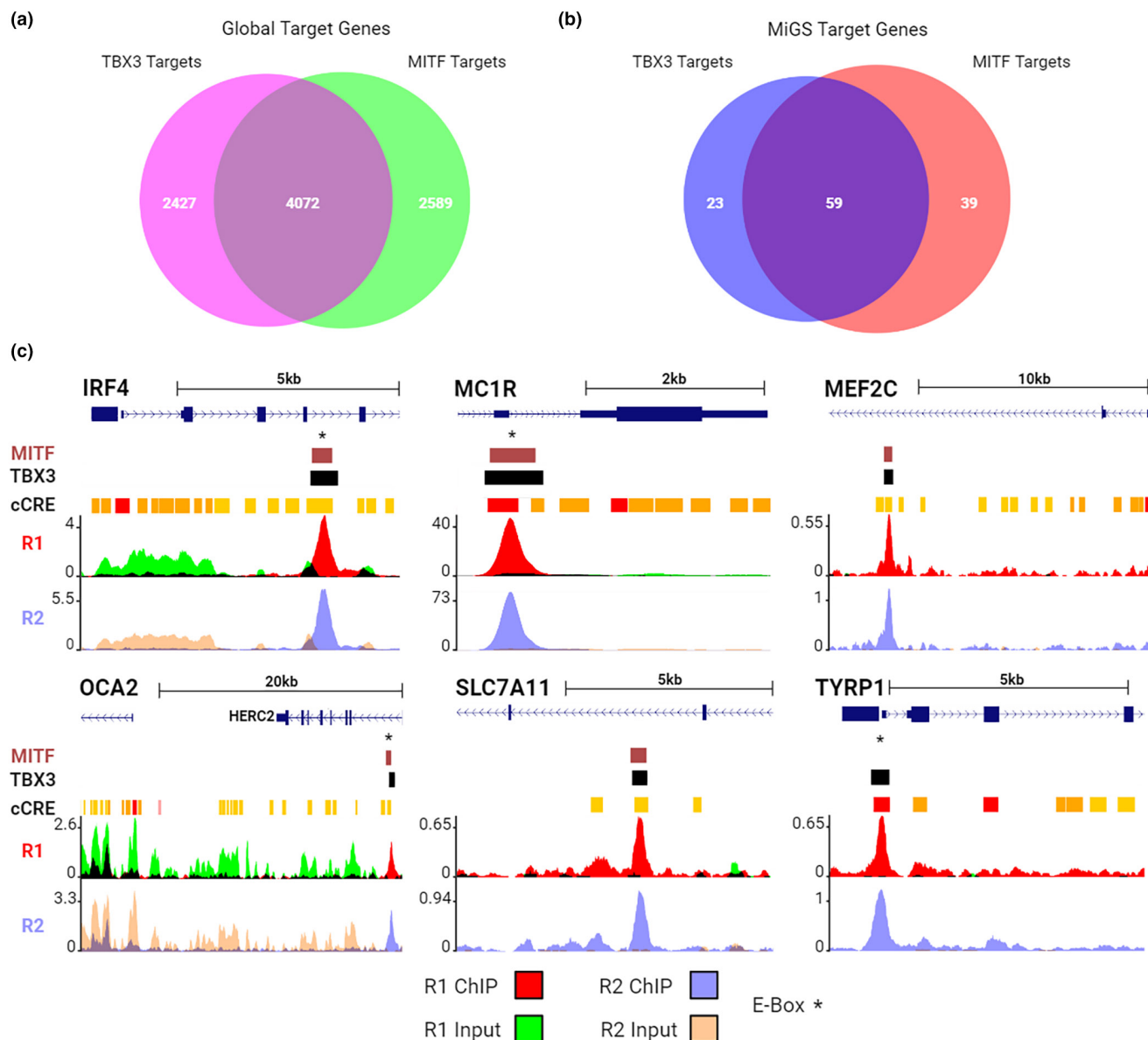


FIGURE 6 TBX3 binds the E-box of pigmentation gene targets. (a) Intersection showing the number of murine genes (4072) associated with ChIP peaks in both our TBX3 dataset and a published MITF dataset. (b) Intersection of MiGS genes identified as targets of both TBX3 and MITF. (c) UCSC browser view of TBX3 and MITF ChIP peaks associated with pigmentation genes. Browser tracks are as follows: TBX3=replicated binding sites in our dataset; MITF=ChIP peaks from Strub et al; cCRE=encode annotated candidate cis-regulatory elements. The location of verified MITF regulatory elements containing E-box motifs are indicated (*) (refs in text).

Likewise, NMDA receptors appear to promote melanin synthesis and tyrosinase activity in the cuttlefish, *Sepia officinalis* (Enkhtaivan & Lee, 2021; Palumbo, 2003). In addition, glutamate receptor genes, especially *Grm1*, are expressed in human melanomas, and ectopic expression of *mGluR1* in melanocytes leads to oncogenic transformation and metastatic spread with 100% penetrance (Eddy & Chen, 2021). Ephrins play an essential role in regulating biological processes such as cell-cell attachment, cell shape, and motility (Janes et al., 2014). Along with Semaphorins, they are expressed in neural crest cells (which give rise to melanocytes) and are of vital importance in regulating neural crest migration (Theveneau & Mayor, 2012). Genes within these families also show differential

expression in melanocytes treated with ASIP, suggesting that this treatment results in dedifferentiation toward a neural crest-like state (Le Pape et al., 2009). Our data further validate this idea and suggest that MC1R acts downstream of ASIP to effect these changes, as loss of MC1R in primary melanocytes drives similar gene expression changes.

Transcription factors play an essential role in the regulation of gene expression. The most well-characterized of these in melanocytes is *Mitf*. This gene encodes a master regulatory transcription factor implicit in melanocyte specificity, proliferation, survival, migration, differentiation, and melanogenesis, and whose expression has been reported to lie downstream from multiple signaling

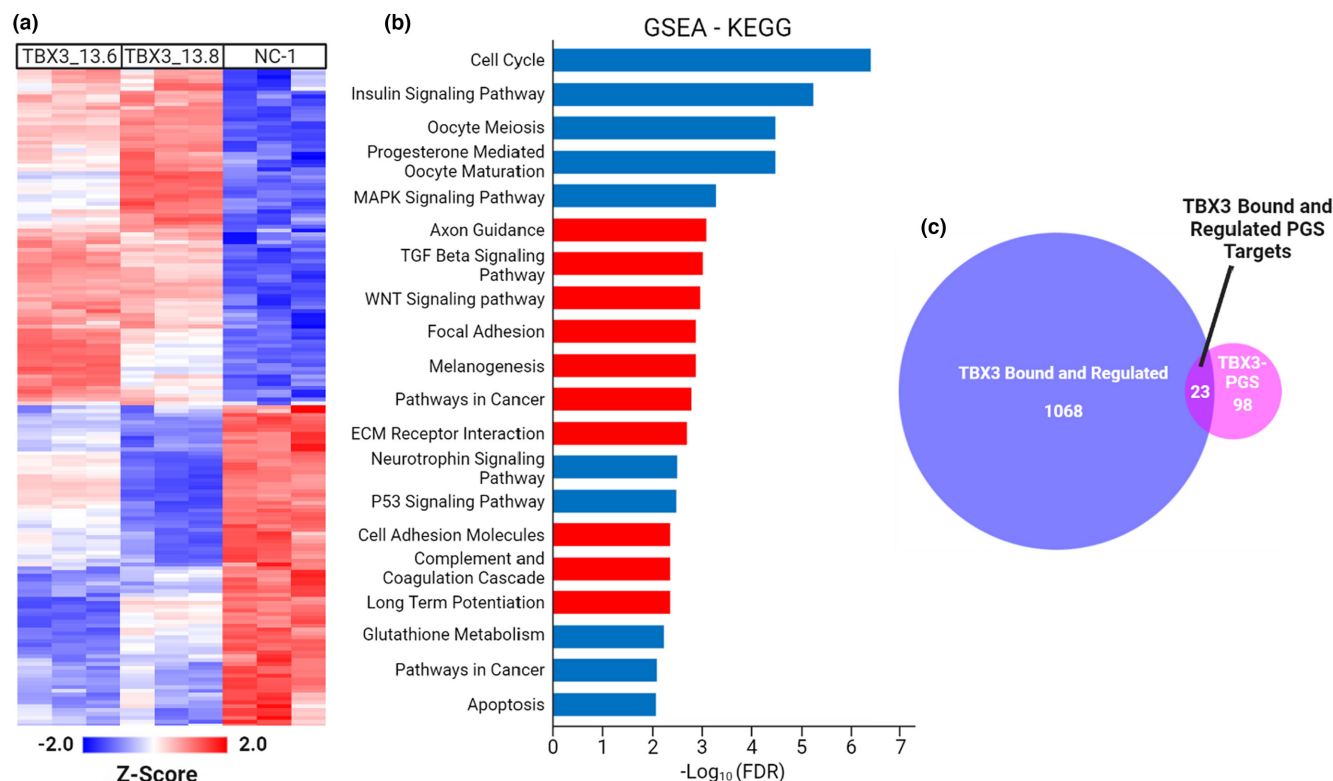


FIGURE 7 TBX3 binds to and regulates MiGS genes. (a) Heatmap depicting relative gene expression of 2780 DEG (Adjusted p -value $< .05$) from the TBX3 knockdown samples against a non-coding control. (b) Top KEGG terms ranked by $-\log_{10}$ of the p -value for upregulated (green) and downregulated (red) genes. (c) Intersection of DE TBX3 gene targets against MiGS TBX3 targets.

pathways, including MC1R (Arnheiter, 2010; Levy et al., 2006). *Mitf* does not exhibit differential expression in our study, nor do many of its known post-transcriptional/translational modifiers, such as MAPKs, *Gsk3b*, *p300*, and *Stat3*, although changes in the activity of these signaling molecules may not be apparent in the gene expression profile. Similarly, other well-characterized transcription factors in melanocyte biology, including *Sox10*, *Pax3*, *Lef1*, and *Creb*, which can all regulate *Mitf*, also exhibit no differential expression. Importantly, changes in the activity of these signaling molecules cannot be confirmed without further proteomic and enzymatic studies. However, the absence of many of these genes from the MiGS potentially supports the notion that primary transcriptional changes driving MiGS expression, and pheomelanin synthesis, in the MC1R-inhibited signaling state are not necessarily mediated through reduced *Mitf* transcription but instead through transcriptional regulation of other factors that could modulate the DNA binding or post-transcriptional regulation of MITF.

One such factor identified in the MiGS that we selected for further analysis on was the T-box transcription factor family member, *Tbx3*. This candidate stood out for the following reasons: (1) it is activated by TGF β /BMP signaling (Li et al., 2013; Li et al., 2014; Washkowitz et al., 2012); (2) it has high fold-enrichment in melanocytes from both pheomelanin-producing mouse genotypes (Figure 3a); (3) it is found across multiple terms from our GO analysis; (4) it has known functional roles in developmental states of melanocytes (Imsland et al., 2016; Lopez et al., 2018; Wang et al., 2020);

(5) it regulates developmental processes across multiple tissue/cell types, including heart (Mesbah et al., 2012; Mohan et al., 2020; Singh et al., 2012), lungs (Lüdtke et al., 2016), limbs (Frank et al., 2013; Sheeba & Logan, 2017), inner ear (Kaiser et al., 2021), and neural crest (Lopez et al., 2018; Mesbah et al., 2012), and (6) is highly expressed in a multitude of cancers, especially melanoma, where its primary roles appear to be in metastasis by activation of EMT, at least in part through down-regulation of E-cadherin, as well as suppression of senescence (Boyd et al., 2013; Dong et al., 2018; Peres et al., 2010; Peres et al., 2021; Peres & Prince, 2013; Rodriguez et al., 2008).

The differential expression of *Tbx3* in wildtype and MC1R-inhibited melanocytes, along with genome-wide binding of TBX3 to T- and E-box elements in melanoma cells, suggested that TBX3 may transcriptionally regulate both MiGS- dependent and independent MITF targets in melanocytes with suppressed MC1R signaling. Through a multi-omics approach, including ChIP and bulk RNA-seq, we have shown that TBX3 can (1) bind and (2) regulate genes within the MiGS. Although TBX3 is often viewed as a transcriptional repressor, it has been shown to activate gene expression of putative target genes (Khan et al., 2020). Likewise, our data strongly suggest that TBX3 can both activate or repress target genes. These targets include genes with known associations to melanogenesis and pigmentation phenotypes. An intriguing example of TBX3-mediated activation is *Slc7a11*. This gene encodes the cystine/glutamate exchanger (xCT) which imports

cystine into the cell where it is reduced into two molecules of cysteine, a necessary component for pheomelanin synthesis (Chintala et al., 2005; Hoekstra, 2006). The increased pool of cysteine provides an environment that is favorable for pheomelanin synthesis. Increased expression of *Slc7a11* by TBX3 suggests that pheomelanin synthesis could be a more active process than previously appreciated. The importance of cysteine goes beyond pheomelanin synthesis, as it is an essential factor of the glutathione system and oxidative stress regulation as well as ferroptosis inhibition (Chintala et al., 2005; Poltorack & Dixon, 2022). Following TBX3 knockdown, we observed a significant reduction of *SLC7A11* expression, with associated enrichment of pathways associated with "glutathione metabolism" and "apoptosis." Given the cytotoxicity of pheomelanin, regulation of cysteine levels within melanocytes is critical and presents a paradox where cysteine is essential for regulating oxidative stress; however, too much can exacerbate cell stress through pheomelanin accumulation.

TBX3 is implicated in senescence bypass partly through its ability to repress cell cycle checkpoint genes such as *CDKN2B* (Khan et al., 2020). Our TBX3 knockdown data support this, with *CDKN2B* showing significantly increased expression. Furthermore, our data also support a role for TBX3 in senescence bypass through activation of cell cycle progression genes, where the loss of TBX3 yields decreased expression of *CDK1* and *CDK2* (Table S11). Willmer et al. have shown that TBX3 is important in S-phase transition in part due to post-translational phosphorylation via *CDK2* (Willmer et al., 2015). More recent work from this group suggests a more complex relationship, whereby TBX3 itself promotes *CDK2* expression (Damerell et al., 2021). Our data further support this model and suggest a further way in which TBX3 can contribute to oncogenic transformation by facilitating senescence bypass. These observations may pave the way in explaining why redheads with diminished MC1R function may be predisposed to melanoma.

The T-box family members comprise five sub-groups, with TBX3 as a member of the TBX2 sub-group. TBX2 and TBX3 share 95% homology within the DNA binding domain (Washkowitz et al., 2012). TBX2, which canonically binds the T-box, can also bind the E-box motif, and functions as both an activator and repressor (S. Lu et al., 2021). Our data support the idea that TBX3 also binds to, and regulates gene expression, via the E-box motif. However, unlike TBX2, the type of motif bound does not seem to influence its action (Figure S5a). E-box site recognition of TBX3 can possibly be explained with protein structural studies. As mentioned previously, the canonical E-box and T-box elements share a 3bp (GTG) region. This corresponds to bases 3–5 of the T-box (AGGTGTGA). Consequently, this 3bp core significantly affects TBX3's ability to bind DNA, whereas the flanking base pairs are deemed less crucial (Coll et al., 2002). As a monomer, TBX3 can also bind to either the major or minor groove (Coll et al., 2002). Therefore, while bound in the minor groove, it is possible that TBX3 may interact with other transcription factors bound in the major groove, including MITF. The degree to which TBX3 modulates gene expression is most likely dependent on several factors, including cell type, differentiation state,

co-factor availability, chromatin accessibility, and biological state (e.g., methylated and acetylated). Variation across these factors could explain why more putative genes targets regulated by TBX3 in human melanoma cells in vitro are not represented in the in vivo healthy murine melanocyte MiGS.

In this study, we aimed to profile the transcriptome of inhibited-MC1R murine melanocytes which mimic the human RHC trait. To this end, we have identified a set of differentially expressed genes (MiGS) that merit further investigation toward better understanding the complex function of GRNs in melanocytes. This signature includes the melanoma-associated transcription factor, *Tbx3*. Up-regulation of TBX3/TBX3, and consequent suppression of senescence, in melanocytes lacking functional MC1R prior to acquisition of activating mutations in BRAF or NRAS would therefore provide a potential mechanism by which people with RHC exhibit predisposition to melanoma. More work will need to be done along these lines, particularly in RHC human samples. Finally, we believe the MiGS provides an important set of candidate genes which merit further follow-up regarding all aspects of melanocyte biology including, but not limited to, regulation of melanocyte development, cell signaling, melanogenesis, and melanomagenesis.

AUTHOR CONTRIBUTIONS

William J. Pavan, Dawn E. Watkins-Chow, H. Matthew Berns, Pedro Moura-Alves, and Colin R. Goding designed the research study; H. Matthew Berns, Dawn E. Watkins-Chow, Sizhu Lu, Pakavarin Louphrasitthiphol, Kevin M. Brown, and Tongwu Zhang acquired the data; H. Matthew Berns, Dawn E. Watkins-Chow, Kevin M. Brown, and Tongwu Zhang analyzed data; H. Matthew Berns, Dawn E. Watkins-Chow, Colin R. Goding, and William J. Pavan wrote the manuscript; H. Matthew Berns, Dawn E. Watkins-Chow, Sizhu Lu, Pakavarin Louphrasitthiphol, Colin R. Goding, Pedro Moura-Alves, and William J. Pavan edited the manuscript; and all authors approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no competing interests to declare.

DATA AVAILABILITY STATEMENT

Original data created for the study are deposited in Gene Expression Omnibus (GEO) data repository as follows: TBX3 ChIP-seq (GSE223256), bulk RNA-seq (GSE236917), and single-cell RNA-seq (GSE236918).

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