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# Zebrafish MITF-low melanoma subtype models reveal transcriptional subclusters and MITF-independent residual disease

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**Running title:** MITF-low melanoma subtypes and residual disease in zebrafish

**Conflict of interest:** All authors declare no conflict of interest

## Abstract

The MITF-low melanoma transcriptional signature is predictive of poor outcomes for patients but little is known about its biological significance and animal models are lacking. Here, we used zebrafish genetic models with low activity of Mitfa (MITF-low) and established that the MITF-low state is causal of melanoma progression and a predictor of melanoma biological subtype. MITF-low zebrafish melanomas resembled human MITF-low melanomas and were enriched for stem and invasive (mesenchymal) gene signatures. MITF-low activity coupled with a p53 mutation was sufficient to promote superficial growth melanomas, while BRAF<sup>V600E</sup> accelerated MITF-low melanoma onset and further promoted the development of MITF-high nodular growth melanomas. Genetic inhibition of MITF activity led to rapid regression; recurrence occurred following reactivation of MITF. At the regression site, there was minimal residual disease that was resistant to loss of MITF activity (termed MITF-independent cells) with very low-to-no MITF activity or protein. Transcriptomic analysis of MITF-independent residual disease showed enrichment of mesenchymal and neural crest stem cell signatures similar to human therapy-resistant melanomas. Single-cell RNA-seq revealed MITF-independent residual disease was heterogeneous depending on melanoma subtype. Further, there was a shared subpopulation of residual disease cells that was enriched for a neural crest G0-like state that pre-existed in the primary tumor and remained present in recurring melanomas. These findings suggest that invasive and stem-like programs coupled with cellular heterogeneity contribute to poor outcomes for MITF-low melanoma patients, and that MITF-independent subpopulations are an important therapeutic target to achieve long-term survival outcomes.

## **Significance**

This study provides a useful model for MITF-low melanomas and MITF-independent cell populations that can be used to study the mechanisms that drive these tumors as well as identify potential therapeutic options.



## Introduction

Melanoma is the most lethal form of skin cancer, and its incidence continues to rise rapidly. Despite the significant progress in targeted therapy against BRAF mutations and MEK activity, and in immune therapy, most patients with metastatic melanoma still succumb to the disease because they do not respond to therapy or they develop drug resistance (1). Genetic mutations define subgroups of melanoma (BRAF, RAS or NF1 mutated, or Triple-Wild type (WT)) that can help predict a positive response to targeted therapy, however overall this genomic classification cannot predict patient survival (2).

In contrast, gene expression profiling of melanomas has revealed transcription-based melanoma subtypes that have significance for disease outcome and may be important for patient care (3). The TCGA melanoma dataset revealed three transcriptionally distinct subtypes that are enriched for immune genes, for keratinocyte genes, or have low expression of the melanocyte-inducing transcription factor (MITF) and its targets as well as high expression of neuronal genes (called the MITF-low signature) (2).

The TCGA MITF-low signature in primary and metastatic melanomas shares over 75% overlap with a MITF-low proliferation transcriptional subtype identified in the Lund dataset of stage IV metastatic melanomas (4). The TCGA MITF-low and Lund MITF-low proliferation groups are further found in the primary (Leeds), stage III (Lund) and metastatic (Bergen) transcriptome melanoma datasets (5-7). Together, these data support the concept that MITF-low transcriptional activity defines a melanoma biological subtype (8). Importantly, outcomes for patients with melanomas that express the TCGA MITF-low signature or the Lund MITF-low proliferative signature genes are amongst the poorest for survival and metastasis (8). However, whether the MITF-low signature is simply a marker for poor outcomes or whether it is causative remains an open question.

Here, we use zebrafish genetics coupled with analysis of human melanoma transcriptomes to determine the biological significance of the MITF-low classification, and reveal a pre-existing

melanoma cell subpopulation that is resistant to loss of MITF activity and has very low-to-no MITF activity toward its target genes (termed MITF-independent cells). These cells remain at the site of residual disease and could be an important drug target.

## **Materials and methods**

### **Husbandry**

Zebrafish were maintained in accordance with UK Home Office regulations, UK Animals (Scientific Procedures) Act 1986, under project license 70/8000 and P8F7F7E52. All experiments were approved by the Home office and AWERB (University of Edinburgh Ethics Committee).

### **Zebrafish melanoma models**

Zebrafish were genotyped using DNA extracted from fin clipped tissue by PCR to establish the mutant allele status *tp53*<sup>M214K</sup> or *mitfa-BRAF*<sup>V600E</sup> and grown in temperature-controlled systems as described (9). Briefly, for melanoma induction, or recurrence, fish were transferred to glass tanks at room temperature (24-26°C), while for melanoma regression fish were transferred to tanks with heaters that kept the temperature at 32°C.

### **Histology**

Fish samples were collected, fixed and processed as described (9). The detailed method is described in Supplementary Material and Methods. Antibodies and their dilutions are listed in **Supplementary Table S1**.

### **Low number and single cell RNA-seq library preparation**

For low cell number and single cell RNA-Seq, the Smart-seq2 protocol was used with minor modifications as described previously (10) and detailed in Supplementary Materials and Methods.

### **Read mapping and differential gene expression analysis of bulk and low number RNA-seq**

Reads were aligned to the Ensembl *Danio rerio* reference genome version GRCz11 (Ensembl V92, 20178) that included coding sequences for *EGFP* and *BRAF*<sup>V600E</sup> using STAR (V 2.5.1b, (11)). For quality control and pre-processing, quantification of mapped reads per gene was calculated using HTseq (12). Genes that were not expressed in any cells were excluded. Data were not normalized for any variable based on histological evaluation. The raw read counts were loaded in the R Deseq2 package (V 1.20.0, (13)). For bulk samples, genes with fewer than 10 reads across the samples were excluded. Quality control exclusion criteria for low cell number RNA-seq samples were the following: samples with fewer than 100,000 unique-mapped reads and fewer than 1,000 genes detected (minimum one read per gene) were excluded. For differential gene expression analysis, genes displaying fewer than 10 counts across all samples for bulk RNA-seq or that did not express with at least 1 count in a minimum of 3 samples for low number RNA-seq were excluded.

### **Single cell RNA-seq analysis**

Single cell RNA-seq analysis was performed using adapted pipeline from Lun et al. (14). Detailed description of the analysis is found in Supplementary Materials and Methods.

### **Pathway analysis**

Genes upregulated from DESeq2 (bulk samples), or scDE (single cell) were selected based on FDR<0.05 and ranked by Log2FC (Log2FC>0) for input to the R ClusterProfiler package (15). To examine pathway over-representations based on literature available datasets (abbreviated

by L: **Supplementary Table S2**, all genes have been manually converted to zebrafish orthologues using zfin.org database), or using KEGG (K:) and GO-BP (G:) databases. Pathways with  $p_{\text{adj}} < 0.05$  (hypergeometric test, BH adjusted) were considered to be significant, listed in Supplementary tables with subsets visualized using dot-plots.

Gene set functional enrichment analysis (GSEA) (16) identified enriched pathways at  $\text{FDR} < 0.05$  (Kolmogorov–Smirnov test). The matrix of all read counts was used to compare against literature-based datasets (**Supplementary Table S2**) using gene set permutation settings. FDR values and leading-edge genes of enrichment plots shown in Figures are listed in **Supplementary Table S3**.

### qRT-PCR

Detailed method is described in Supplementary Materials and Methods with primers listed in **Supplementary Table S4**.

Data availability: RNA-seq data are deposited in the GEO database (GSE130037 and GSE136900).

For additional Materials and Methods, please see Supplementary Information.

## Results

### **MITF-low activity co-operates with p53 mutations and BRAF<sup>V600E</sup> in melanoma**

The *MITF* gene is highly conserved and in zebrafish there are two orthologous *mitf* genes (*a* and *b*) whereby *mitfa* is required for the development of all body melanocytes (17). A genetic temperature sensitive *mitfa* mutation (*mitfa<sup>vc7</sup>*) in zebrafish has enabled us to test the impact of altering MITF activity in development and show that low MITF activity co-operates with BRAF<sup>V600E</sup> in melanomagenesis (9,18,19). The conditional *mitfa<sup>vc7</sup>* mutant allele results from a

single nucleotide T-to-A mutation in intron 6 that disrupts splicing in a temperature sensitive manner (18,20). Analysis of the splicing variants of *mitfa*<sup>vc7</sup> indicates that at permissive temperatures (25°C) lower levels of wild type *mitfa* RNA are generated (defined as a MITF-low state), and that these transcripts are further reduced with increasing water temperatures (**Fig. 1A**) (9,20). Thus, this conditional control of *mitfa* RNA enables us to regulate wild type *mitfa* expression in zebrafish development and cancer.

In the TCGA data, an MITF-low transcriptional signature is found in 18% of cutaneous melanomas and across all four TCGA cutaneous melanoma genomic groups (**Fig. 1B**). *TP53* (p53) is frequently mutated in human melanoma (18% of cutaneous melanoma; TCGA), and these mutations co-operate with BRAF<sup>V600E</sup> mutations to promote melanoma in zebrafish and mouse models (21,22). We aimed to model the MITF-low transcriptional state using genetic mutation combinations present in MITF-low melanomas in patients. We explored the human melanoma genetic and transcriptomic TCGA dataset and found *TP53* mutations or low levels of *TP53* expression in BRAF mutant and non-BRAF-mutant melanomas, including a minor subset that have the MITF-low transcriptional signature (**Fig. 1B**). To test the potential for p53 mutations to co-operate with MITF-low activity in nevi and melanoma we generated zebrafish homozygous for *mitfa*<sup>vc7</sup> and *tp53* (p53<sup>M214K</sup>) mutations. The impact of the conditional *mitfa*<sup>vc7</sup> activity on zebrafish stripe formation can be seen in **Fig. 1C** (left panels) whereby zebrafish grown at <25°C (MITF-low activity state) develop fewer melanocytes in a stripe pattern while animals grown at 32°C lack sufficient functional MITF activity to develop body melanocytes.

Strikingly, we found that the *mitfa*<sup>vc7</sup>;p53<sup>M214K</sup> animals developed large darkly pigmented melanocyte lesions by 9 months that progressed into melanoma with 73% incidence (n= 94/129; **Fig. 1C, D**). These data reveal an unexpected co-operation between MITF-low activity and p53 mutations in melanoma initiation that is independent of MAPK activation. To address the impact of the BRAF<sup>V600E</sup> mutation on *mitfa*<sup>vc7</sup>;p53<sup>M214K</sup> zebrafish, we crossed the *mitfa*<sup>vc7</sup>;p53<sup>M214K</sup> zebrafish to zebrafish expressing human BRAF<sup>V600E</sup> from the *mitfa* promoter

(23). This promoter is not regulated by Mitfa protein levels, and thus BRAF<sup>V600E</sup> expression is not altered by levels of Mitfa in the *mitfa*<sup>vc7</sup> mutant (9). BRAF<sup>V600E</sup> dramatically accelerated the onset of melanoma, and gave rise to melanomas with both superficial growth and exophytic nodular growth patterns, and could be either pigmented or unpigmented (**Fig. 1C-E**). Taken together these results reveal that BRAF<sup>V600E</sup>, MITF-low activity and p53 mutations have an independent and co-operative impact on melanoma initiation and growth.

### **Superficial and nodular growth melanomas differ in mode of invasion**

Next, we examined the pathology of the melanoma subtypes. Histological examination showed all tumors to be highly cellular and express Melan A (**Supplementary Fig. S1A, B**; n=15/15). Melanomas from the *mitfa*<sup>vc7</sup>;p53<sup>M214K</sup> fish originated from the skin, grew in a superficial growth pattern, and were highly invasive into the muscle, penetrating the tissue to deeper levels, around the internal organs and towards the spinal cord (**Fig. 2A-C**). We term these melanomas double superficial (DS) because they have two engineered genetic mutations and grow in a superficial growth pattern. The melanomas in the *Tg(mitfa:BRAF<sup>V600E</sup>); mitfa*<sup>vc7</sup>;p53<sup>M214K</sup> fish grew in either a superficial or a highly proliferative nodular growth pattern (referred to as triple superficial (TS) or triple nodular (TN)) (**Supplementary Fig. S1C, D**). Both the superficial and nodular growth melanomas were highly invasive but they differed in the nature of invasion. We measured from the level of the background contour of the fish to the deepest invasive cell and found no difference between the superficial and nodular growth melanomas in invasion depth. In contrast, there was a marked difference in the mode of invasion: superficial growth melanomas showed a single-to-few cell wide stream of highly infiltrative cancer cells between muscle fibers, while nodular growth melanomas showed a more confluent invasive growth pattern often leading to deterioration of surrounding tissues (**Fig. 2D-F**).

Notably, despite heavy pigmentation in the DS and TS tumors at the surface site, melanoma cells of the invasive region were unpigmented (**Fig. 2A-C**). DS melanomas showed little to no activation of the MAPK-signaling pathway in the superficial part of the tumor, but the

unpigmented invasive compartments expressed high levels of phospho-ERK in a heterogeneous pattern (**Fig. 2G, H**). To relate this finding to human melanoma, we asked whether MAPK signaling pathways are associated with an increase in invasive properties in TCGA triple-WT melanomas. We found a significant positive correlation between the invasive signature genes and MAPK-signaling signature genes (**Supplementary Fig. S1E**) indicating that MAPK-signaling is associated with melanoma invasion in both zebrafish and human triple-WT melanomas. This is consistent with the findings of Shain *et al.* (24) in which genetic and transcriptomic activation of the MAPK signaling pathway increases with disease progression.

### **Melanoma subtypes cluster by genotype and directionality of growth**

To analyse the three melanoma models, we dissected the DS, TS and TN tumors from melanoma-bearing fish and performed bulk RNA-seq (**Fig. 3A, Supplementary Fig. S2A-C**). Principal component analysis divided the melanoma transcriptomes into three groups (**Fig. 3B**). Along the PC1 axis, melanomas separated according to the direction of growth (superficial or nodular), and along the PC2 axis melanomas separated according to the presence or absence of BRAF<sup>V600E</sup>. Although pigmented nodular growth melanomas are found between the BRAF<sup>V600E</sup> superficial growth and the BRAF<sup>V600E</sup> unpigmented nodular growth melanomas in the PCA (**Fig. 3B**), they clustered together with BRAF<sup>V600E</sup> unpigmented nodular growth melanomas by hierarchical clustering (**Supplementary Fig. S3A**).

### **MITF transcriptional states distinguish superficial and nodular growth melanomas**

We quantified mRNA expression of *mitfa* between DS and TS superficial growth melanomas and found no significant difference in its expression (**Fig. 3C**). Indeed, we found that most MITF target gene expression levels were similar in both DS and TS melanomas suggesting that the BRAF<sup>V600E</sup> mutation alone does not significantly alter the overall MITF-dependent transcriptome of MITF-low tumors (**Fig. 3C, Supplementary Fig. S3B**). In contrast, there was a marked increase in *mitfa* expression in TN growth melanomas (**Fig. 3C**). This change was

also accompanied by an increase in *BRAF*<sup>V600E</sup> mRNA expression from the *mitfa* promoter in addition to an increase in expression of MITF target genes involved in proliferation and differentiation (*cdk2*, *dct*, *tyr*, *mc1r*, *tyrp1a/b*).

Given that the *mitfa*<sup>vc7</sup> mutant is a splice-site mutation, the increase in *mitfa* expression by RNA-seq might simply reflect an increase in aberrantly spliced non-functional transcripts. To test this, we designed primers that specifically amplified correctly spliced *mitfa* exons and found a significant increase in functional wild type *mitfa* transcripts in the TN melanomas compared with DS and TS melanomas (**Fig. 3D, E**). This was associated with a modest increase in Mitfa protein in TS melanomas, and a robust increase in Mitfa protein in the TN melanomas (**Fig. 3F, G**). Based on these data, we classified DS melanomas as MITF-low melanomas and TN melanomas as MITF-high melanomas. Some TS melanomas had higher RNA and protein levels of Mitfa, however we also classified these as MITF-low melanomas because they shared a strong similarity of MITF target gene expression with DS melanomas and clustered together by unsupervised clustering (**Fig. 3C; Supplementary Fig. S3A, B**). Further, MITF has been proposed to function as a repressor at some gene promoters (25) and we find de-repression of some of these genes in MITF-low melanomas, including genes involved in stem cell states (*klf4*, *klf5a*, *klf9*, *erbb3*) and metabolism (*acsf2*, *apodb*, *mboat1*) (**Supplementary Fig. S3B**). These results suggest that DS and TS MITF-low melanomas are transcriptionally distinct from TN MITF-high melanomas in MITF activity.

In human melanoma, *MITF* gene expression is controlled by neural crest transcription factors including PAX3 and BRN2 (also known as POU3F2) that act in a reciprocal manner to control high and low *MITF* gene expression, respectively (26). Increased *mitfa* gene expression in *BRAF*<sup>V600E</sup> nodular growth melanomas was accompanied by an increase in *pax3a* expression, while *mitfa* low expression level in *BRAF*<sup>V600E</sup> melanomas with superficial growth was associated with upregulation in *brn2b* (*pou3f2b*) expression. Both *pax3a* and *brn2b* (*pou3f2b*) were down-regulated in (non-*BRAF*<sup>V600E</sup>) MITF-low p53<sup>mut</sup> melanomas (DS, **Fig. 3C**). This



suggests that a Brn2b-Pax3a reciprocal mechanism might be active only in BRAF<sup>V600E</sup> melanomas and be responsible for a switch from low to high *mitfa* RNA expression.

### **Zebrafish and human MITF-low melanomas share invasive and mesenchymal programs**

We sought to relate our findings in zebrafish melanoma to MITF-low melanomas from patients. We established the genes that were differentially expressed between superficial growth (low *mitfa* expression) and nodular growth (higher *mitfa* expression) zebrafish melanomas, and then compared these with the genes that were differentially expressed between 40 human TCGA melanomas with the lowest and 40 melanomas with the highest *MITF* mRNA expression (**Fig. 3H**). MITF-low superficially growing zebrafish melanomas (both with or without BRAF<sup>V600E</sup>) shared marked and significant similarity to human melanomas with low *MITF* mRNA expression (**Fig. 3I**) such as mesenchymal and invasive programs, retinoic acid metabolic processes, as well as neural crest cell development, migration and differentiation signatures that include genes involved in stem cell and mesenchymal fates (**Fig. 3I, Supplementary Tables S2, S5**). These results show that zebrafish MITF-low melanomas model human MITF-low transcriptional signature melanomas at the molecular level.

Nodular growth melanomas showed less resemblance to the 40 human melanomas with the highest *MITF* mRNA expression levels, however some pathways were significantly shared between nodular zebrafish melanomas and human melanomas with the highest *MITF* RNA expression level, including the Lund MITF-high pigmentation cluster (**Supplementary Fig. S4A-B, Supplementary Table S6**). These data indicate that zebrafish MITF-low superficial growth melanomas model the TCGA MITF-low melanomas while zebrafish MITF-high nodular melanomas more closely align with the Lund MITF-high pigmentation melanomas at the transcriptional level.

### **Subtype-specific transcriptional signatures stratify zebrafish melanomas**

Next, we performed a pairwise comparison of differential mRNA expression from each zebrafish melanoma subtype to identify the molecular signatures that define them (**Fig. 4A, Supplementary Table S7**). DS melanomas expressed high levels of WNT and mesenchymal genes (**Fig. 4A-D**), as well as the vascular genes *pdgfra* and *kdr* that are orthologous to human *PDGFRA* and *KDR* and are characteristic of human Triple-WT melanomas (**Supplementary Fig. S4C, D**). Surprisingly, the TS melanomas showed upregulation of neuronal genes, including the neuronal gene set specifically upregulated in MITF-low TCGA transcriptional clusters (**Fig. 4A, D**). TN melanomas that were enriched for MITF program activation expressed proliferation genes and a neural crest formation program (**Fig. 4A-D**). This MITF-high state includes expression of *sox10*, *foxd3*, *pax3*, and *tfap2* genes, as well as *crestin* (a zebrafish neural crest marker gene) that is expressed in melanoma initiating cells in zebrafish BRAF<sup>V600E</sup> p53<sup>mut</sup> melanomas (27) (**Fig. 4B-D**).

Neural crest genes were expressed in all melanoma subtypes. Nevertheless, the levels and gene signatures differed between them. The superficial growth melanomas expressed neural crest signatures that are broadly stem-like (e.g. *erb2*, *erb3a/b*) and mesenchymal (e.g. *sox9*, *twist1a/1b/3*) while the nodular growth melanomas expressed genes involved in developmental programs (e.g. *sox10*, *foxd3*, *mitfa*) (**Supplementary Fig. S4E**). We validated this finding using a zebrafish specific Sox10 antibody, and found highest Sox10 protein expression in the TN melanomas (**Fig. 4E, F**). These results indicate that more than one neural crest cell lineage specification program can be active in melanoma, perhaps reflecting activation of some of the recently described gene networks that characterize neural crest stage and fate decisions (28).

### **Discovery of melanoma subpopulations resistant to MITF activity loss**

*MITF* is a lineage dependent oncogene (29) and we have previously demonstrated that BRAF<sup>V600E</sup> MITF-low melanomas depend on MITF activity for survival (9). We asked whether our three new melanoma models presented here also depended on MITF activity for survival. Indeed, tumors in all three melanoma models rapidly regressed by apoptosis when MITF was

eliminated by switching to the restrictive temperature (32°C), and recurred at the permissive temperature (<26°C) (**Fig. 5A, B** and **Supplementary Fig. S5A, B**). In all fish, the melanomas recurred at the same sites as the initial tumor site. These experiments indicate that *MITF* is a lineage survival oncogene for the bulk of melanoma with and without *BRAF*<sup>V600E</sup> mutations, and in melanomas with *MITF*-low or *MITF*-high activity.

The dramatic loss of tumor followed by regrowth in the same site led us to hypothesize that a subpopulation of melanoma cells remains at the regression site and can resist loss of *MITF* activity. To address this, we crossed our melanoma prone zebrafish (TS and TN) to zebrafish that carry a transgene that expresses *GFP* from the *mitfa* promoter in order to follow *GFP* positive (+) cells during melanoma regression. As explained previously, we chose this *mitfa* promoter because it is not regulated by *Mitfa* protein, and therefore the expression of *BRAF*<sup>V600E</sup> or *GFP* does not change as a result of altering *Mitfa* protein activity in our *mitfa*<sup>vc7</sup> conditional allele (9). We found *GFP* expression was clearly detected in the bulk of the melanoma prior to regression (**Fig. 5C**).

Strikingly, despite no overt evidence of melanoma on the animal, confocal imaging revealed small clusters of *GFP*+ melanoma cells at the original tumor site following regression (**Fig. 5D**). Clusters of *GFP*+ cells were specific to the regression site and were not found in matched healthy tissue from the same animal (**Fig. 5D**), and non-melanoma melanocyte progenitor *GFP*+ cells were only sparsely located elsewhere on the fish skin (**Supplementary Fig. S5C, D**). Sectioning the fish at the melanoma regression site confirmed the presence of small clusters of *GFP*+ cells (**Fig. 5E**).

These results show that *MITF* is a lineage oncogene in *BRAF* dependent and independent melanomas, and reveal subpopulations of melanoma cells that remain at the regression site and are resistant to *MITF* loss. This residual disease cell population (termed *MITF*-independent cells) likely contribute to melanoma recurrence, and may represent an important new target for therapeutic interventions in melanoma.

## **MITF-independent cells share gene expression signatures with human melanoma residual disease states**

We next sought to understand the nature of residual disease cells remaining at the tumor site after melanoma regression. To this end we sorted small cell clusters (<50 cells) of GFP+ cells from primary tumor and residual disease from superficial (TS) and nodular (TN) growth melanomas (**Fig. 6A, Supplementary Fig. S6A, B**) and generated RNA-sequencing libraries using the Smart-Seq2 protocol (10). Analysis of primary and regressed cell clusters showed a number of differentially expressed genes with significant upregulation in the primary tumor (**Fig. 6B**). Expression of MITF target genes was strongly suppressed in melanoma cells residing at the regression site (**Fig. 6C**). Mitfa protein expression was almost undetectable at the regression site, while residual disease cells maintained the expression of Sox10 protein (**Fig. 6D, E**). GSEA analysis revealed that MITF-independent residual disease cells are enriched in mesenchymal and invasive gene expression signatures (e.g. as defined by Cheng, Hoek and Kandel, **Supplementary Table S2-S3**), and in the neural crest stem cell (NCSC) signature identified in residual disease following BRAF plus MEK inhibitor treatment (30) (**Fig. 6F, G; Supplementary Table S3**).

To establish whether these cell states are relevant to human melanoma, we analyzed an RNA-Seq dataset of patients before BRAF plus MEK inhibitor treatment and following resistance (31,32), and found that the MITF-independent residual disease cells share similar molecular expression signatures with human therapy-resistant melanomas (**Fig. 6G**). This enrichment is specific for melanoma samples because matched GFP+ melanocyte progenitor cells from unaffected tissue of the same fish are not enriched for these gene sets (**Fig. 6G**). Thus, the MITF-independent residual disease cell state is specific to melanoma, and does not exist in non-melanoma melanocytes, and is not a result of the temperature-shift to turn off MITF activity.

## **The MITF-independent cell state pre-exists in primary melanoma**

Minimal residual disease cells are rare, and so we performed deep plate-based (Smart-Seq2) sequencing to investigate the residual disease state at the single cell resolution. To investigate the residual disease state at the single cell resolution, we profiled the transcriptomes of 332 GFP+ single cells from superficial (TS) and nodular (TN) growth melanomas and residual disease from corresponding subtypes, as well as from TN melanoma recurring disease (n= 43, 80, 63, 61 and 85 single cells respectively, **Supplementary Fig. S6C**). We clustered cells based on similarities of their transcriptional profiles using the unsupervised Louvain algorithm on principal component reduction and visualised using Unifold Manifold Approximation and Projection (UMAP). We identified 6 clusters (numbered 0-5) (**Fig. 7A**). Clusters 0 and 2-5 were primarily composed of cells from a predominant but not exclusive single experimental condition (**Fig. 7B**). In contrast, cluster 1 contained cells from all experimental conditions revealing a cell state common to all melanoma subtypes and stages (**Fig. 7B**).

All residual disease states had very low-to-no MITF target gene expression confirming the MITF-independent state of these cells. We investigated whether the MITF-independent cell state was specific to the residual disease cells or whether an MITF-independent state pre-existed in the primary tumor. Strikingly, while MITF target genes were generally upregulated in the primary tumor cells (even at low levels) (**Fig. 7C**) a subpopulation of cells in the primary tumors showed markedly reduced MITF target gene expression (**Fig. 7C**), indicating that the MITF-independent state is already present in the primary tumors. A pre-existing MITF-independent subpopulation was also present in melanomas and residual disease from a smaller additional single cell dataset we generated (**Supplementary Fig. S6D, S7A, B**). All cells continued to express *sox10* perhaps as a means to bypass the dependence on MITF activity for survival (**Fig. 7D; Supplementary Fig. S7C**). Thus, while the bulk of the tumor is dependent on MITF activity for survival, a MITF-independent state is present as a subpopulation and is resistant to loss of the MITF lineage factor.

### **Exclusive and shared cell states in residual and recurrent disease**

Interestingly, we identified distinct cell populations in residual disease that were specific to melanoma subtype. Superficial melanoma residual disease cells (cluster 4) were enriched for invasive and mesenchymal genes, and expressed some of the genes of the Rambow-neural crest stem cell signature, compared with the superficial primary tumor (cluster 3) (**Fig. 7E, Supplementary Fig. S7D, Supplementary Table S8**). In contrast, nodular melanoma residual disease (cluster 5) was enriched for genes involved in purine metabolism and the neural crest developmental programme (Kaufman\_zfin\_NC; (27)) compared to the primary nodular tumor (cluster 0) (**Fig. 7F, Supplementary Table S8**). Enhanced expression of and dependency on nucleotide metabolism is a feature of the neural crest and melanoma (33,34). Expression of purine metabolism genes may represent a Sox10+ MITF-independent neural crest metabolic state. Alternatively, purine metabolism genes are highly expressed in xanthophores, a closely related zebrafish neural crest derived pigment cell type (35), and expression of purine metabolism pathways may represent differentiation toward this neural crest cell fate to maintain survival.

Finally, we explored the cell state in nodular melanomas in the early stages of recurrence (10 days following reactivation of *Mitfa*). We found recurrent melanoma cells enriched for MITF target genes and protein translation genes (**Fig. 7G, Supplementary Table S8**). Despite the apparent shift away from the neural crest and purine metabolism pathways expressed in residual disease and towards MITF pathway activity, recurrent disease did not show gene expression associated with the cell cycle that is present in the primary nodular melanomas (**Fig. 7G**). Thus, early recurrent disease may need to first activate MITF transcriptional and protein translation pathways prior to activation of a proliferative phase to contribute to tumor regrowth.

Strikingly, all experimental conditions (primary, residual and recurrent disease) shared a common cell state (cluster 1) that expresses neural crest genes including enrichment for *erbb2* and *zeb2a*. Further, we found particular enrichment of genes recently described in a G0-like

neural stem cell state (36) such as the epigenetic factors *arid1b*, *kmt2c*, the cell cycle inhibitor p27 (*cdkn1bb*) and ribosomal components (**Fig. 7H, I**). Some cells in cluster 1 also express high levels of the melanoma slow cycling cell marker JARID1B (encoded by the *kdm5ba* gene in zebrafish) (37). These experiments show that residual disease generated through MITF loss leads to multiple MITF-independent cell states both exclusive and shared with primary tumors. Further, MITF depletion leads to a common neural crest G0-like state (NC\_G0-like\_ribosomal) that exists in primary, regressed and recurrent disease stages that may be vital for melanoma survival.

## Discussion

Gene expression signatures defined by the TCGA and Lund subtypes are predictive for survival across multiple independent datasets indicating that gene expression profiling is a clinically relevant biomarker for patient stratification (2,4-8). Moreover, immune based transcriptional signatures can be predictive for response to immune therapies (38,39). Of particular importance, the Lund MITF-low proliferation melanoma subtype and the TCGA MITF-low signature predicts among the worst outcomes for patients (8). However, animal models of melanomas defined by these transcriptional signatures are lacking in part because little is known about how these subtypes are established.

Here, we have used a temperature sensitive conditional *mitfa*<sup>vc7</sup> mutant allele to model the MITF-low melanoma transcriptional state. We discover that the MITF-low state is highly invasive (mesenchymal) and exhibits plasticity toward a proliferative MITF-high state. Further, we discover a shared MITF-independent cell state in residual disease that pre-exists in primary melanomas. Together, these cell states may partially explain the poor outcomes for patients with MITF-low melanomas.

We define this MITF-low state in zebrafish by reduced levels of wild type *mitfa* RNA transcript and Mitfa protein (**Fig. 3**) (9,20). The zebrafish MITF-low activity results in reduced expression

of pigmentation genes, and shares stem and mesenchymal gene expression patterns with the human TCGA MITF-low and Lund MITF-low proliferation gene expression clusters. Although the TCGA MITF-low transcriptional signature includes expression of neuronal genes, we demonstrate that the MITF-low state can also be characterized by a vasculogenic signature uncoupled from neuronal genes (i.e. DS melanomas). Taken together, zebrafish MITF-low melanomas are relevant models for the human MITF-low melanoma transcriptional gene expression cluster.

BRAF<sup>V600E</sup> is not sufficient on its own to increase *mitfa* expression and many BRAF<sup>V600E</sup> positive melanomas maintain a MITF-low gene expression signature. How *mitfa* expression is significantly increased in only some BRAF<sup>V600E</sup> melanomas in our zebrafish models is not known, but our results demonstrate that nodular growth melanomas have the potential to increase *sox10* and *mitfa* RNA and protein expression, and express genes similar to the Lund MITF-high pigmentation subtype. We term these MITF-high melanomas, and find an increase in wild type *mitfa* mRNA and Mitfa protein expression is accompanied by an increase in BRAF<sup>V600E</sup> expression because the BRAF<sup>V600E</sup> transgene is under the control of a *mitfa* promoter fragment. While we cannot differentiate between the impact of Mitfa and BRAF<sup>V600E</sup> activity on nodular growth pathology, our models reveal the important finding that the MITF-low state is unstable with the potential to increase *mitfa* and Mitfa target gene expression.

While melanomas can express high or low levels of MITF overall, cells can be heterogeneous for MITF within the tumor itself (40) and cells with low MITF are resistant to MAPK pathway inhibition (41). Single-cell RNA analysis indicates that cells that express high or low *MITF* RNA levels can coexist within an individual melanoma tumor supporting the concept that cells can switch their MITF transcriptional states *in vivo* (30,31,42-44). Our results suggest a Pax3a-Brn2b rheostat may regulate this switch in MITF gene expression from a low to high state specifically in BRAF<sup>V600E</sup> melanomas (26,40,45). Understanding how MITF-low melanomas shift to MITF-high melanomas, and *vice versa*, is important because the Lund MITF-low proliferation transcriptional signature is enriched in melanomas post therapy and this state



confers intrinsic and acquired resistance to MAPK pathway inhibition in cell lines and in patient tumors (5,41). Notably, patients can relapse on targeted therapy with individual melanoma clones that are both MITF-high and MITF-low, underscoring the importance of the transcriptional state switch in patients (41).

Our work shows that neural crest programs are active in both the superficial and nodular growth subtypes, but that the neural crest signatures that characterize the superficial growth melanomas (MITF-low melanomas) are enriched in mesenchymal and invasive genes (e.g. *sox9b*, *twist 1a/b/3*) while nodular growth melanomas (MITF-high melanomas) are enriched in developmental program genes (e.g. *mitfa*, *sox10*, *foxd3*, *pax3a*). Recent single cell RNA-sequencing studies of the mouse neural crest reveal early migratory transcriptional states followed by branched fate transcriptional trajectories that co-express bipotent cell fates prior to fate commitment (46). While these states are not as markedly defined in our melanomas as found in development, our results extend the understanding of neural crest pathways in melanoma by showing that more than one neural crest gene expression program can be active depending on melanoma subtype.

MITF activity is considered a drug target because MITF is a lineage survival oncogene and turning off MITF activity has little impact upon other cell types (29). Regression followed by recurrence in our zebrafish MITF on-off-on models has similarities to the regression and recurrence of patient tumors following BRAF inhibitor therapy in which both diseases progress through undetectable minimal residual disease. Through live imaging and immunohistochemistry of zebrafish at the melanoma regression site, we visualized small cell clusters of residual disease. Our model predicts these cell populations would be resistant to MITF inhibitors. Further, it seems likely that these cells contribute to disease recurrence, although we cannot exclude that other melanoma cells exist at the regression site that do not express GFP and may be transformed in the context of the tumor regression micro-environment for expansion and growth (for example as reported in (47)). Lineage-tracing studies in this model system, and more broadly in the study of residual disease, will be critical

to determine which cells at the residual disease site maintain malignancy and contribute to disease relapse.

Our results here show that residual disease has very low-to-no MITF transcriptional activity, very low-to-undetectable Mitfa protein and is resistant to MITF as a lineage survival oncogene. Recently, Rambow and colleagues (30) identified four subpopulations of cells in residual disease following BRAF and MEK inhibitor treatment including invasive cells and NCSCs with "very low to undetectable" expression of *MITF* or its target genes. MITF-independent cells express genes that are enriched for the invasive and NCSC states indicating that these signatures can define both the BRAF plus MEK inhibitor resistant cells and MITF-independent cells. One explanation for this result is that MAPK pathway inhibitors lead to the direct loss of MITF activity in residual disease, and therefore resemble the same cell state as experimental loss of MITF as seen in our models. An alternative explanation is that tumor regression itself (caused by BRAF/MEK inhibitors or MITF loss) changes the microenvironment such that the extant MITF-independent subpopulation undergoes a transcriptional shift toward invasive/NCSC programs (e.g. TS residual disease), or even toward differentiation programs (e.g. TN residual disease) depending on the primary tumor subtype.

The MITF-independent state that is shared between all disease conditions (cluster 1) maintains expression of neural crest markers *sox10* and *foxd3* with specific enrichment of *erbb2* and *zeb2a*. Further, cluster 1 shows up-regulation of ribosomal genes together with the cycle cell inhibitor p27 (*cdkn1bb*) and *arid1b* which together are enriched in a G0-like state (36). Notably, a few cells in cluster 1 strongly express the slow-cycling cell gene *jarid1b* (*kdm5ba*) (37). This G0-like state may have important clinical implications for patients because G0-like cells can be enriched following therapy (48) and have been reported to require JARID1B to exert a stem cell-like function (49). Lineage tracing experiments will be important to directly test if the extant cluster 1 cells in the primary tumor are the same cells in residual disease, and how they contribute to recurrent disease. Future cell transplantation studies will enable the

characterization of phenotypic differences among residual disease cell subpopulations, such as tumor initiation or invasion potential, or differentiation states.

In summary, our results prove the MITF-low transcriptional signature reflects an underlying biological state and reveals melanoma transcriptional heterogeneity within this subtype which may contribute to poor outcomes for patients. Further, given MITF is an important drug target we reveal an extant melanoma cell subpopulation that resists loss of MITF activity and may contribute to disease perdurance. Some of the MITF-independent residual disease may be sensitive to therapy. For example, RXR-gamma inhibitors can target melanoma cells in the NCSC state (30) and mitochondrial respiration inhibitors can target the slow-cycling JARID1B<sup>High</sup> cells (50). Our work shows further complexity for the targeting of residual disease because we find residual disease states that are shared by all disease stages and those that are exclusive to tumor subtype. These findings underscore the importance of targeting both the bulk of the tumor and residual disease cells for long-term survival outcomes.

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## Figure legends

**Fig. 1. MITF-low activity and p53 mutations co-operate in melanoma**

- A. Graphical overview of MITF activity based on temperature changes in *mitfa*<sup>vc7</sup> mutant fish compared to wild type.
- B. Pie chart of MITF-low melanoma subtypes and associated mutations in TCGA cutaneous melanoma samples.
- C. Images of wild type, *mitfa*<sup>vc7</sup> mutant, *mitfa*<sup>vc7</sup>; *p53*<sup>M214K</sup> double mutant and *Tg(mitfa:BRAF<sup>V600E</sup>); mitfa*<sup>vc7</sup>; *p53*<sup>M214K</sup> zebrafish at different temperatures. At 25°C (permissive conditions), wild-type fish show normal stripe pattern, while *mitfa*<sup>vc7</sup> fish show reduced pigmentation (green arrow). *mitfa*<sup>vc7</sup>; *p53*<sup>M214K</sup> fish develop pigmented (black) melanomas (black arrow) and *Tg(mitfa:BRAF<sup>V600E</sup>); mitfa*<sup>vc7</sup>; *p53*<sup>M214K</sup> develop unpigmented (blue arrow) and pigmented (black and red arrows) melanomas.
- D. Survival curves for *mitfa*<sup>vc7</sup> (green), *p53*<sup>M214K</sup> (dark blue), *mitfa*<sup>vc7</sup>; *p53*<sup>M214K</sup> (red) and *Tg(mitfa:BRAF<sup>V600E</sup>); mitfa*<sup>vc7</sup>; *p53*<sup>M214K</sup> (light blue) zebrafish. \*\*\*\**p*<0.0001 (Log-rank test).
- E. Bar chart showing percentage of pigmented, unpigmented and both melanoma types.

**Fig. 2. Superficial and nodular growth melanomas are highly invasive**

- A.** H&E stained sections of *mitfa*<sup>vc7</sup>; *p53*<sup>M214K</sup> (double superficial, DS) and *Tg(mitfa:BRAF<sup>V600E</sup>); mitfa*<sup>vc7</sup>; *p53*<sup>M214K</sup> (triple superficial, TS, triple nodular, TN pigmented and unpigmented). Scale bar = 1 mm.
- B.** Close-up H&E images of the surface area of melanoma, scale bar = 50  $\mu$ m.
- C.** Close-up H&E images of the invasive component of melanoma, scale bar = 20  $\mu$ m.
- D.** Drawing showing the experimental approach for quantification of melanoma invasion depth (left, red lines; quantified in **E**) and width of invasion stream at the edge of invasion (right, yellow box left and black lines with arrows; quantified in **F**), blue dashed lines follow invasive edge.
- E.** Dot-boxplot showing the invasion depth calculated as ratio between melanoma thickness (a) and distance to the midline (b). ns= not significant (Dunn test), n=4 (DS, TS) and 5 (TN) fish.
- F.** Dot-boxplot showing the cell stream width at the invasion edge measured as distance between two neighboring muscles (average of 5 measures per fish); ns = not significant, \*\*p<0.01 (Dunn test), n=4 (DS, TS) and 5 (TN) fish.
- G.** Immunohistochemistry (DAB staining) of *mitfa*<sup>vc7</sup>; *p53*<sup>M214K</sup> superficially growing (left) and invasion (right) parts of bleached melanoma sections, stained with anti-phospho-ERK antibody. Unbleached corresponding H&E section in **H** shows no pigmentation in the invasive component; scale bar = 50 $\mu$ m. See also **Supplementary Fig. S1**.

**Fig. 3. MITF-low melanomas model human melanomas with low *MITF* expression**

- A. Schematic of zebrafish melanoma phenotypes used for whole tumor bulk RNA-seq.
  - B. Principal component analysis plot showing three separate clusters of primary melanomas, *mitfa*<sup>vc7</sup>; *p53*<sup>M214K</sup> (double superficial, DS) and two clusters for *Tg(mitfa:BRAF<sup>V600E</sup>); mitfa*<sup>vc7</sup>; *p53*<sup>M214K</sup> (triple superficial, TS and triple nodular, TN); ellipse represents 95% confidence interval (multivariate t-distribution).
  - C. Heat map of gene expression for *mitfa*, *pax3a*, *pou3f2b* (*brn2b*), human *BRAF<sup>V600E</sup>* transgene and MITF target genes (as defined in Hoek *et al.*, 2008).
  - D. Schematic of *mitfa* pre-mRNA with the *vc7* mutation indicated. Location of aberrant splicing changes (red asterisk) and qPCR-product of correctly spliced variant (grey and yellow line) are indicated.
  - E. Dot-boxplot showing qPCR of the *mitfa* correctly spliced variant using two sets of primers (exon 6-7, grey; exon 5-7, yellow). \*\*p<0.01 (Dunn test).
  - F. Representative images showing IF-IHC staining of Mitf protein across different melanoma subtypes counterstained with DAPI. Scale bar = 20  $\mu$ m.
  - G. Violin dotplot showing Mitf nuclear fluorescence intensity normalized to the size of nucleus (Corrected Total Cell Fluorescence; CTCF). Each dot represents one nucleus, n=4 fish per condition. \*\*\*\* p< 0.0001 (Dunn test).
  - H. Differential expression analysis between superficial and nodular zebrafish melanomas compared to differential expression analysis between 40 patients with lowest *MITF* and highest *MITF* expression. Venn diagrams of shared upregulated genes (left) and shared pathways (right) (p<0.05, Benjamini-Hochberg = BH test). \*\*\*\*p<0.0001 (hypergeometric distribution with Holm correction).
  - I. Selection of 11 pathways from the Venn diagram intersect is shown in the dotplot (GO-BP, KEGG and literature based). Dot size represents the fold enrichment of the pathway compared to expected expression. Color shows adjusted p-value (BH test).
- See also **Supplementary Tables S2, S5-S6** and **Supplementary Figs. S2-S4**.

**Fig. 4. Transcriptional sub-clusters define MITF-low and MITF-high melanomas**

- A.** Dot plot of pathway analysis showing a selection of significant pathways (GO-BP, KEGG and literature based – **Supplementary Table S2**) in a pairwise comparison between melanoma model clusters. Dot size represents the fold enrichment of the pathway compared to expected expression. Color shows adjusted p-value (Benjamini-Hochberg test = BH). DS=double superficial, TS = triple superficial, TN = triple nodular
- B.** Violin plots showing examples of differences in neural crest markers expression (*sox10*, *crestin*) and Wnt pathway (*wnt7aa*, *tcf7l2*) followed by their validation by qPCR using the same samples used for RNA-seq. ns= not significant, \*  $p < 0.05$ , \*\*\*\*  $p < 0.0001$ , BH test.
- C.** Violin plots showing validation by qPCR of genes from **B** using the same samples used for RNA-seq, ns= not significant, \*  $p < 0.05$ , \*\*  $p < 0.01$ , Dunn test.
- D.** Schematic of zebrafish melanoma classification with a heatmap of five selected genes of a pathway(s) specific for the cluster (red=high, blue=low).
- E.** Representative images showing IF-IHC staining of Sox10 protein across different melanoma subtypes counterstained with nuclear marker (DAPI). Scale bar = 20  $\mu\text{m}$ .
- F.** Violin dotplot showing Sox10 nuclear fluorescence intensity normalized to the size of nucleus (Corrected Total Cell Fluorescence, CTCF). Each dot represents one nucleus, n=4 fish per condition. \*\*\*\*  $p < 0.0001$  (Dunn test).

See also **Supplementary Table S7** and **Supplementary Fig. S4**.

**Fig. 5. Imaging of residual disease cells at the site of melanoma regression**

- A.** Representative images of zebrafish with melanoma at  $<26^{\circ}\text{C}$ , after 2 month upshift to  $32^{\circ}\text{C}$  to regress the tumor followed by a 1 month downshift back to  $<26^{\circ}\text{C}$  for tumor regrowth.
- B.** Line graphs of four zebrafish showing tumor size over time during regression and recurrence.
- C.** Brightfield and fluorescence images of zebrafish with melanoma in the head region at  $<26^{\circ}\text{C}$  and 2 months after upshift to  $32^{\circ}\text{C}$ . Black arrows indicate melanoma before and after regression. White squared box indicate residual disease cells enlarged in **D**.
- D.** Representative images of standard deviation intensity projection of confocal z-stack acquisition of the melanoma regression site (white square box region (**C**); left) and healthy tissue from the same fish (right). White arrows point to the residual GFP+ cells, scale bar =  $100\ \mu\text{m}$ ,  $n = 6$  fish.
- E.** Histopathology image shows DAB immunohistochemistry on a bleached transverse section of the fish after 2 months at  $32^{\circ}\text{C}$  and stained with anti-GFP antibody. The black box is enlarged to show the staining in more detail. One of the cell clusters stained in brown is highlighted in square box and displayed at higher magnification to the right. Black arrows depict GFP positive clusters. B – brain, e – eye; scale bars =  $100\ \mu\text{m}$  and  $10\ \mu\text{m}$ . See also **Supplementary Fig. S5**.

**Fig. 6. MITF-independent cells mark residual disease and show decrease in Mitfa activity**

- A.** Schematic of low input Smart-seq2 RNAseq design for cells at the regression site, in matched healthy tissue and in the primary tumor.
- B.** Volcano plot shows differentially expressed genes in primary and regressed melanomas with color change denoting genes with  $FDR < 0.05$  (Benjamini-Hochberg test). Orange dots indicate significant genes expressed in the primary tumor, green dots denote significant genes in regressed tissue.
- C.** Enrichment plot showing decrease of MITF target genes expression in regressed tumor compared to primary melanoma with  $NES = 1.60$  and  $FDR = 0.05$  (Kolmogorov–Smirnov=KS test).
- D.** Representative images showing IF-IHC staining of Mitfa protein in primary tumor and regression site (=Reg) counterstained with nuclear marker (DAPI). White arrows point to very low-to-no Mitfa expressing cells at residual disease. Scale bar = 20  $\mu\text{m}$ ,  $n = 4$  fish.
- E.** Representative images showing IF-IHC staining (of consecutive section to **D**) of Sox10 protein between primary tumor and regression site counterstained with nuclear marker (DAPI). Scale bar = 20  $\mu\text{m}$ ,  $n = 4$  fish.
- F.** Enrichment plots of Mesenchymal ( $NES = -1.93$ ) and NCSC ( $NES = -1.82$ ) signatures specifying genes that are enriched at the regression site relative to the primary tumor.  $FDR$ : Mesenchymal = 0.004, NCSC = 0.01 (KS test).
- G.** Heatmap showing the average  $\log_2$  fold change of genes defining pathways (**Supplementary Table S3**) within primary and regressed tumors relative to overall average values across all samples – primary, regressed tissue and matched unaffected tissue from regressed fish. The same analysis was performed for pre and post BRAFi and MEKi treatment patients and merged into one heatmap.

See also **Supplementary Figs. S6-S7** and **Supplementary Tables S2-S3**.

**Fig. 7. Single cell analysis shows distinct and shared cell states in residual disease**

- A.** Uniform Manifold Approximation and Projection (UMAP) of primary, regressed and recurring *GFP+* positive melanoma single cells (n=332 cells) showing 6 different clusters.
- B.** UMAP representation of **A** highlighting the origin of the single cells: primary superficial (blue), primary nodular (red), regressed superficial (purple), regressed nodular (yellow) and recurring nodular (green). Grey dotted line shows cluster 1 containing a mix of origins.
- C.** UMAP representation of **A** based on the AUC scores of MITF target genes (Hoek *et al.*, 2008) indicated with color change from blue (negative) to orange (positive).
- D.** UMAP representation of **A** with color change from grey (negative) to red (positive) based on log<sub>10</sub> mRNA expression of *sox10*.
- E-G.** Dot plots of pathway analysis show a selection of upregulated significant pathways in clusters with predominant regressed or recurring populations. From left to right: cluster 4 (**E**), cluster 5 (**F**) and cluster 2 (**G**). Dot size represents the fold enrichment of the pathway compared to expected expression. Color shows adjusted p-value (BH test).
- H.** UMAP representation from **A** with color change from grey (negative) to red (positive) based on the AUC scores of selected genes (denoted as NC\_G0-like\_ribosomal gene set) highlighted in red dashed rectangle in **I**.
- I.** Dot plot of selected genes showing average expression among the clusters, with cluster 1 highlighted by grey dotted box. Red dashed rectangle denotes genes used for NC\_G0-like\_ribosomal gene set shown in **H**. Dot size corresponds to the percentage of expressing cells within the cluster.

See also **Supplementary Figs. S6-S7 and Supplementary Tables S2 and S8.**

















