



# BRN2 and MITF together impact AXL expression in melanoma

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## Abstract

The inverse relationship between transcription factor MITF and receptor tyrosine kinase AXL has received much attention recently. It is thought that melanoma tumors showing AXL<sup>high</sup>/MITF<sup>low</sup> levels are resistant to therapy. We show here that a population of cells within melanoma tumors with extremely high expression of AXL are negative/low for both MITF and the transcription factor BRN2. Depletion of both transcription factors from cultured melanoma cell lines produced an increase in AXL expression greater than depletion of MITF alone. Further, re-expression of BRN2 led to decreased AXL expression, indicating a role for BRN2 in regulation of AXL levels unrelated to effects on MITF level. As AXL has been recognized as a marker of therapy resistance, these cells may represent a population of cells responsible for disease relapse and as potential targets for therapeutic treatment.

## KEYWORDS

POU3F2, therapy resistance, transcription factor

## 1 | BACKGROUND

Microphthalmia-associated transcription factor (MITF) is a key melanocytic transcription factor with well-documented roles in normal melanocytes and during melanoma progression.<sup>1</sup> Its binding motif is found within 20 kb of over 9000 genes with MITF directly linked to regulation of at least 465 genes from a broad range of processes from cellular senescence to mitosis.<sup>2</sup> MITF is often linked to a phenomenon described as phenotype switching where cells reversibly switch between proliferative (MITF expressing) and invasive (reduced MITF level) states during metastasis.<sup>3</sup> The invasive state characterized by loss of MITF may gain expression of the POU domain transcription factor BRN2 (encoded by *POU3F2*).<sup>4,5</sup>

Despite the requirement for both MITF and BRN2 to be present in a tumor population for efficient melanoma metastasis to occur,<sup>6</sup> expression of MITF and BRN2 is generally accepted to be mutually exclusive at the cellular level and inversely correlated in melanoma tumors, xenografts and 3D cultured cells but not 2D culture.<sup>4,5,7</sup> The

reciprocal relationship between MITF and BRN2 is maintained by both direct binding of BRN2 to the *MITF* promoter and the indirect suppression of BRN2 by MITF through miR-211.<sup>5,8</sup>

Another noteworthy inverse relationship is that which exists between MITF and the receptor tyrosine kinase AXL.<sup>9</sup> This relationship has drawn much interest with AXL<sup>high</sup>/MITF<sup>low</sup> cells being described as resistant to targeted MAPK inhibitor therapy.<sup>10,11</sup> It was also reported that invasion in MITF<sup>low</sup> cells is dependent upon AXL and the transcription factor FRA-1.<sup>9,11</sup>

With MITF having an inverse relationship with both BRN2 and AXL, we sought to investigate the relationship between the three proteins further to characterize their contribution to melanoma heterogeneity.

## 2 | QUESTIONS ADDRESSED

What is the relationship between MITF, AXL and BRN2 in melanoma cells?

### 3 | EXPERIMENTAL DESIGN

#### 3.1 | Analysis of publically available data

The Gerber dataset, GSE81383,<sup>12</sup> was downloaded from NCBI's Gene Expression Omnibus. The normalized read count was converted to  $\log_{10}$ , values of 0 were assigned  $-2 \log_{10}$ , and GraphPad Prism version 8.2.1 was used for linear regression analysis. For pooling of data, the cut-off value between low and high expression was set to 2 for *AXL*, 0.1 for *POU3F2* and 1.6 for *MITF*.

#### 3.2 | Cell culture

Melanoma cell lines were maintained in RPMI-1640 with 10% FBS (Life Technologies). Routine mycoplasma tests were always negative, and short tandem repeat profiling confirmed cell line identity.

#### 3.3 | Inducible knockdown and expression

For both knockdown and over-expression cell lines, we used the BLOCK-iT Lentiviral expression system (Life Technologies). We have previously described the over-expression of *BRN2* and depletion of *MITF* and *BRN2*.<sup>6,13</sup> We have further modified the same system to simultaneously express shRNA's targeting *MITF* and *BRN2*.

#### 3.4 | Western blot analysis

Cell lysates (30  $\mu$ g protein) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Antibodies used in this study were as follows: anti-*MITF* (12590; Cell Signaling Technologies [CST]), anti-*BRN2* antibody (12137; CST), anti-*AXL* antibody (8661; CST) and anti-*GAPDH* (R&D Systems). Where required, bands were quantified by densitometry using ImageJ version 1.52a and analysed by linear regression analysis using GraphPad Prism version 8.2.1 (GraphPad Software, San Diego, CA).

#### 3.5 | Quantitative reverse transcription PCR

RNA was extracted from cells using the QIAGEN RNeasy plus kit, and 2  $\mu$ g of total RNA was reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) following the manufacturer's protocol. The CFX384 Real-Time System (Bio-Rad Laboratories) was used for qRT-PCR with SYBR Green PCR master mix (Applied Biosystems). The *AXL* primer sequences were (5'-3'); Forward: ACCTACTCTGGCTCCAGGATG; Reverse: CGCAGGAGAAAGAGGATGTC *GAPDH* primers have been described previously.<sup>6</sup>

### 4 | RESULTS

To investigate the relationship between *MITF*, *BRN2* and *AXL*, we used a publically available gene expression dataset to explore expression correlations between *MITF*, *BRN2* (*POU3F2*) and *AXL*. The Gerber dataset (GSE81383) consists of single-cell RNA-seq of 307 cells from three early passage melanoma patient biopsies.<sup>12</sup> An inverse correlation between *MITF* and *AXL* in this dataset has been reported previously.<sup>14</sup> Similarly, we found a strong inverse relationship between *MITF* and *AXL* (Figure 1A,  $R^2 = 0.5648$ ,  $p = <0.0001$ ) and between *MITF* and *POU3F2* (Figure 1B,  $R^2 = 0.2127$ ,  $p = <0.0001$ ) using linear regression analysis of the  $\log_{10}$  normalized read count (read count values of 0 were arbitrarily assigned a value of  $-2$ ). Interestingly, a positive correlation was also seen between *AXL* and *POU3F2* (Figure 1C,  $R^2 = 0.3562$ ,  $p = <0.0001$ ).

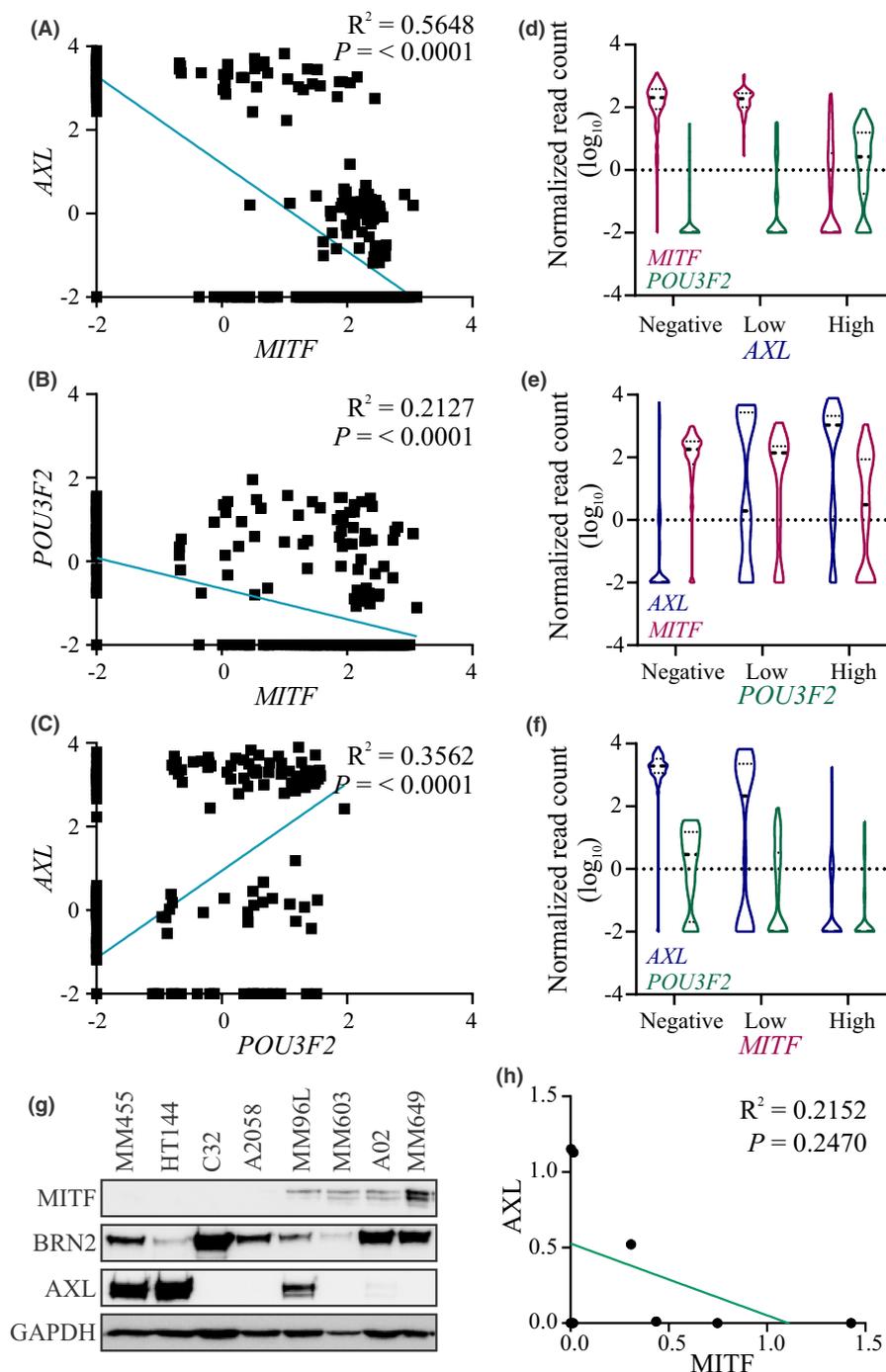
Cells appeared to cluster into three distinct groups on the basis of *AXL* expression (Figure 1A, C). We therefore grouped data on the basis of *AXL* expression (negative, low, high) and graphed the  $\log_{10}$  normalized read count of *MITF* and *POU3F2* (Figure 1D). Interestingly, while *AXL* negative or low cells maintained the inverse relationship between *MITF* and *POU3F2*, this was lost in *AXL* high cells. Similarly, separation of cells into three groups on the basis of *POU3F2* expression showed an inverse relationship between *MITF* and *AXL* in the *POU3F2* negative group only (Figure 1E). These results highlight the complexity of the relationship between these three proteins.

Importantly, *MITF* and *POU3F2* expression is not entirely mutually exclusive, even at single-cell level.<sup>15</sup> A proportion of *MITF* negative cells were also negative for *POU3F2* while cells expressing high levels of *POU3F2* had great variability in the level of *MITF* expression (Figure 1E, F). We also noted a population of cells that had high expression of *AXL* and low or negative expression of both *MITF* and *POU3F2* (Figure 1D). This was further evident when *AXL* high cells were graphed with connecting lines and coloured on the basis of *POU3F2* expression (Figure S1).

Western blot analysis of a panel of 8 metastatic melanoma-derived cell lines (Figure 1G) revealed an inverse relationship between *MITF* and *AXL* (Figure 1H) although due to low sample numbers this was not statistically significant. No correlation was seen between *MITF* and *BRN2* or *AXL* and *BRN2* (Figure S2A, B). However, the limitation of the Western blot analysis is that levels of *MITF* and *BRN2* are known to vary between cells grown in 2D culture and 3D cultured cells or patient samples.<sup>16</sup>

Given the results from single-cell RNA-seq where a proportion of *AXL* high cells were negative for *MITF* and *BRN2* expression, we wished to examine the effect of reduction of these transcription factors on *AXL* expression. Using doxycycline-inducible shRNA expression, we were able to deplete *MITF* and *BRN2* in two metastatic melanoma cell lines (A02 and MM649) by the addition of 50 ng/ml doxycycline to culture media for 96 h (Figure 2A, B). *AXL* protein level increased upon depletion of *MITF* either alone or combined with depletion of *BRN2*. Interestingly, the level of *AXL* was much

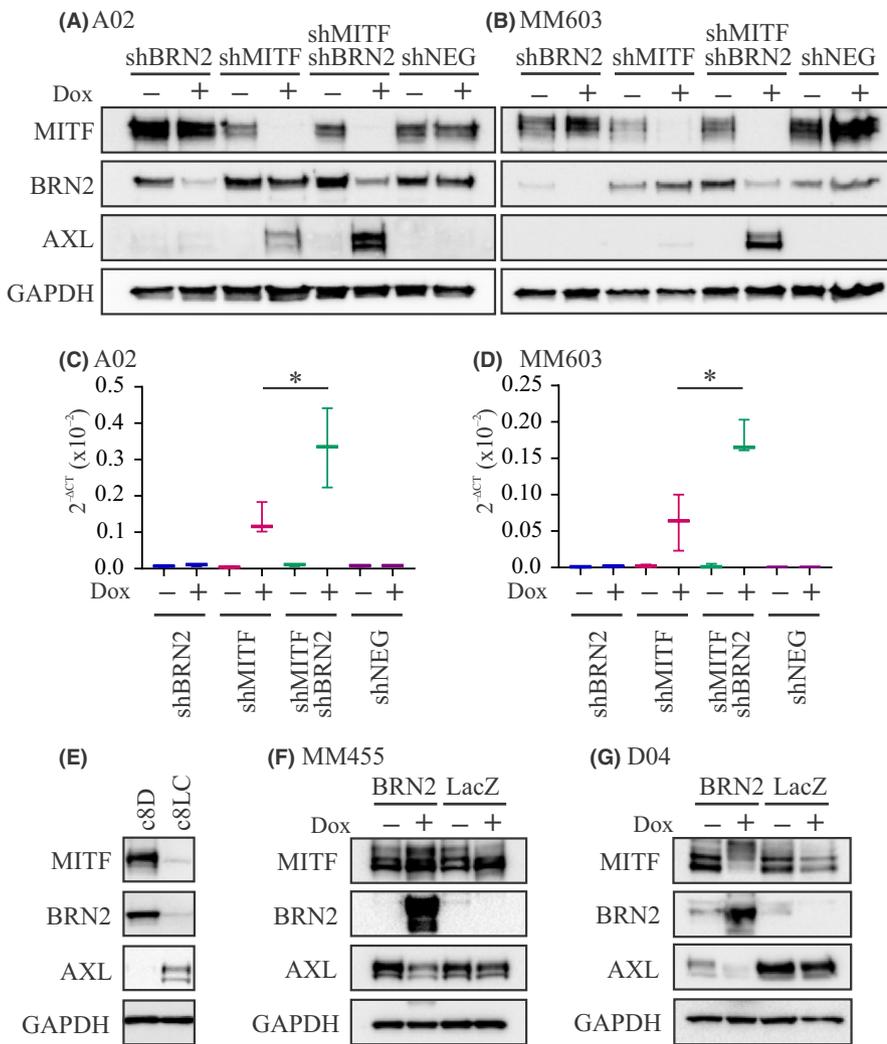
**FIGURE 1** Heterogeneous expression of *MITF*, *POU3F2* and *AXL* in single melanoma cells (A–C) Linear regression analysis of *MITF*, *POU3F2* and *AXL* expression in the Gerber dataset (GSE81383) from single-cell RNA sequencing of short-term patient biopsy cultures.<sup>12</sup> Normalized read count is expressed as  $\log_{10}$ , and values of 0 were assigned as 0.01 ( $-2 \log_{10}$ ). D–F, Separation of cells on the basis of the  $\log_{10}$  normalized read count into negative, low and high groups. Negative =  $-2$ ; low =  $<2$  (D, *AXL*, blue),  $<0.1$  (E, *POU3F2*, green) or  $<1.6$  (F, *MITF*, pink); high =  $>2$  (d, *AXL*),  $>0.1$  (e, *POU3F2*) or  $>1.6$  (F, *MITF*). Broken line = median, dotted lines = quartiles (G) Western blot showing expression of *MITF*, *BRN2* and *AXL* in cultured melanoma cell lines. (H) Quantification of Western blot shown in panel g, and analysis of correlation between *MITF* and *AXL* by linear regression analysis ( $R^2 = 0.2152, p = 0.2470$ )



higher in cells depleted of both *BRN2* and *MITF* when compared to cells depleted of *MITF* alone (Figure 2A, B). Analysis of the same cell lines by qRT-PCR showed a significant increase in *AXL* transcript when *MITF* and *BRN2* were depleted in tandem compared to only *MITF* depletion (Figure 2C,  $p = 0.0393$ , D,  $p = 0.0109$ ). In addition, MM96Lc8LC cells, a line negative for constitutive expression of both *BRN2* and *MITF*,<sup>16,17</sup> also showed an upregulation of *AXL* protein level compared to the control cells that express both transcription factors (MM96Lc8D) (Figure 2E). Further, re-expression of *BRN2* in metastatic melanoma cell lines resulted in decreased *AXL* expression but variable changes to *MITF* expression (Figure 2F, G). Both cell lines have extremely low expression of *MITF* (allowing expression of

*AXL*); however in MM455 cells, *MITF* expression increased slightly on expression of *BRN2* (Figure 2F) while the reverse was true for D04 (Figure 2G).

Taken together, these results indicate a role for both *MITF* and *BRN2* in the transcriptional regulation of *AXL*. The relationship between *MITF* and *AXL* in melanoma is well reported.<sup>9–11</sup> Interestingly, expression of *MITF* in immortalized melanocytes caused increased expression of *AXL*.<sup>18</sup> Mechanistically, it appears that neither *MITF* nor *BRN2* directly repress *AXL* expression. Analysis of the promoter region of *AXL* and published ChIP-seq data do not show potential binding of either transcription factor.<sup>2,19</sup> However, indirect control of *AXL* level may be mediated by induction of miRNA expression by



**FIGURE 2** BRN2, as well as MITF, impacts AXL expression. Western blot analysis of MITF, BRN2 and AXL expression in A02 (A) and MM603 (B) melanoma cell lines expressing shRNA against MITF, BRN2 or both. Analysis of AXL expression by qRT-PCR in (C) A02 and (D) MM603 cells expressing shRNA as indicated, data represent mean and SD of 3 biological replicates. \* $p < 0.05$ , paired-t-test (E) Western blot analysis of MITF, BRN2 and AXL in clones of the MM96L cell line; MM96Lc8LC (c8LC) and MM96Lc8D (c8D). Western blot analysis of inducible BRN2 expression in (F) MM455 and (G) D04 melanoma cell lines

MITF and BRN2. MITF has been reported to induce expression of a number of miRNAs, including miR-24-3p,<sup>2,20</sup> while BRN2 binds at the promoter regions of miR-101, miR-103, miR-7-5p and miR-214-5p<sup>19</sup>; highly conserved binding sites for each miRNA are present in the AXL 3' UTR. Further analysis of data from 74 Stage III melanoma patient tissues (GSE54467, GSE59334)<sup>21</sup> showed a significant positive correlation between MITF mRNA and miR-24-3p level, while a negative correlation was observed between miR-24-3p and AXL mRNA expression (Figure S3), supporting the notion of this control. The relationship between MITF and BRN2 is complex and generally thought to be inversely correlated.<sup>5</sup> We, however, show here that expression of MITF and BRN2 is not always mutually exclusive or inversely correlated. The transcription factor PAX3 has been reported to regulate expression of both MITF and BRN2 and may contribute to the complexity of the relationship between these two important transcription factors.<sup>22,23</sup> Further analysis of the Gerber single-cell dataset revealed a positive correlation between PAX3 and MITF ( $R^2 = 0.03067$ ,  $p = 0.0021$ ) with a negative correlation between PAX3 and AXL ( $R^2 = 0.02914$ ,  $p = 0.0027$ ); however, there was no correlation between POU3F2 and PAX3 ( $R^2 = 0.00021$ ,  $p = 0.8006$ ). Further experimental validation of these mechanistic relationships is required.

## 5 | CONCLUSIONS

These results highlight that in the extremely heterogeneous melanoma tumor environment protein expression patterns are tremendously complicated. Traditional "bulk" analysis of expression within a tumor could result in only the major contributing cell populations being identified. This was highlighted in a recent study where two different MITF<sup>low</sup> populations were identified in a patient-derived xenograft model of minimal residual disease.<sup>24</sup> One population was associated with an EMT signature and was decreased in residual disease (compared to untreated) while the de-differentiation-associated population was enriched at the same stage. Crucially, as upregulation of AXL is currently recognized as a marker of resistance to therapy the presence of MITF<sup>low</sup>/BRN2<sup>low</sup>/AXL<sup>high</sup> cells could be responsible for disease relapse, or indicate a requirement for closer monitoring of patients during treatment.

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## CONFLICT OF INTEREST

The authors state no conflict of interest.

## AUTHOR CONTRIBUTIONS

JLS and GMB designed the study. JLS and HMN performed the research. JLS and GMB analysed the data and wrote the manuscript. All authors revised the manuscript.

## DATA AVAILABILITY STATEMENT

All data used in the current study are available from the Gene Expression Omnibus (GEO), number GSE81383. Accessible from <https://www.ncbi.nlm.nih.gov/geo/>

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Figure S1. Melanoma tumors contain a population of AXL high cells with low/no expression of MITF and POU3F2. The log<sub>10</sub> normalized read count for AXL high cells from the Gerber dataset was graphed. Single cells are connected by lines. Colors represent POU3F2 expression, blue =negative, green = low, pink = high.

Figure S2. Western blots of MITF, BRN2 and AXL expression in melanoma cell lines were quantified by densitometry and analyzed by linear regression analysis. No correlation was found between (a) MITF and BRN2 (R = 0.01483, P = 0.7739) or (b) AXL and BRN2 (R = 0.2032, P = 0.2623).

Figure S3. Expression analysis of 74 Stage III melanoma patient samples revealed a positive correlation between (a) MITF and miR-24-3p (R = 0.05346, P = 0.0475) and an inverse 2 correlation between (b) AXL and miR-24-3p (R = 0.07753, P = 0.0163).

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