



Melanoma Plasticity: Promoter of Metastasis and Resistance to Therapy

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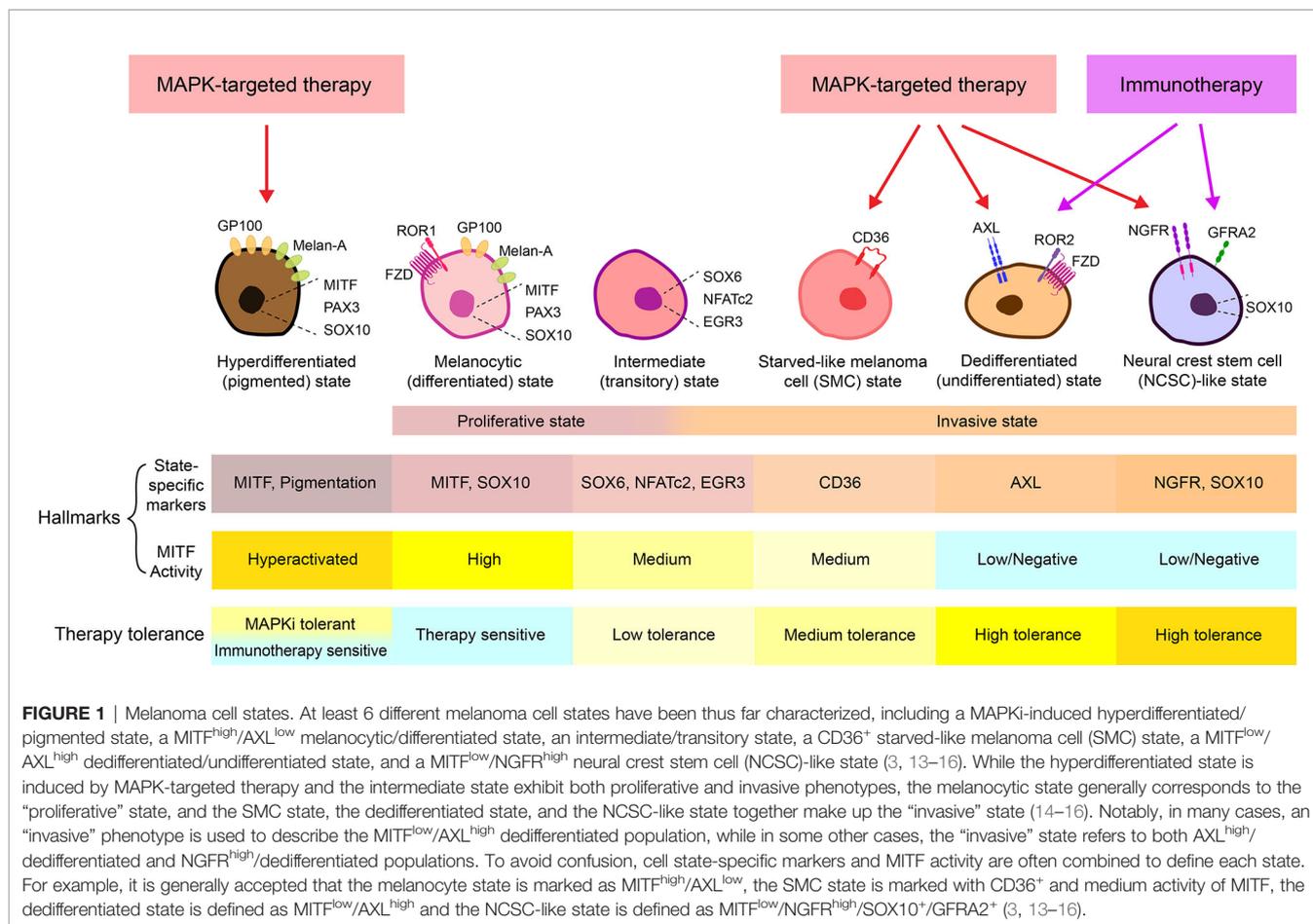
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Melanoma is the deadliest form of skin cancer. Although targeted therapies and immunotherapies have revolutionized the treatment of metastatic melanoma, most patients are not cured. Therapy resistance remains a significant clinical challenge. Melanoma comprises phenotypically distinct subpopulations of cells, exhibiting distinct gene signatures leading to tumor heterogeneity and favoring therapeutic resistance. Cellular plasticity in melanoma is referred to as phenotype switching. Regardless of their genomic classification, melanomas switch from a proliferative and differentiated phenotype to an invasive, dedifferentiated and often therapy-resistant state. In this review we discuss potential mechanisms underpinning melanoma phenotype switching, how this cellular plasticity contributes to resistance to both targeted therapies and immunotherapies. Finally, we highlight novel strategies to target plasticity and their potential clinical impact in melanoma.

Keywords: melanoma, phenotype switching, targeted therapy, immunotherapy, therapy resistance

INTRODUCTION

Melanoma is the deadliest form of skin cancer due to its high metastatic potential. Although MAPK-Kinase pathway (MAPK)-targeted therapies and immunotherapies have revolutionized the management of patients with metastatic melanoma, their clinical benefit is limited by the almost inevitable development of resistance and tumor recurrence. Metastasis and therapy resistance of numerous tumor types is associated with intratumoral heterogeneity and cancer cell plasticity (1–5). Melanoma has been well described to comprise phenotypically distinct subpopulations of cells. Gene expression analyses of cultured melanoma cells identified two predominant cell populations, exhibiting either ‘proliferative’ or ‘invasive’ phenotypes (6–9), reminiscent of the intratumoral heterogeneity present in patient-derived melanomas (10, 11). Throughout this review we will refer to a two-state system, the proliferative state/phenotype which is described as “differentiated”, “epithelial-like”, with high expression of microphthalmia-associated transcription factor (MITF) as a hallmark (MITF^{high}/AXL^{low}), while the invasive phenotype is described as “undifferentiated/dedifferentiated”, “mesenchymal-like”, with a marked expression of the receptor tyrosine kinase AXL (MITF^{low}/AXL^{high}) (10, 12, 13). Over the years, additional cell states have been defined with unique gene expression signatures, and differential therapeutic sensitivity and metastatic potential associated with each of these phenotypes (**Figure 1**) (14–16).



Similar mutations and gene expression patterns present in primary and metastatic melanomas suggest a mechanism independent of clonal evolution as a major driver of melanoma progression (17–21). An alternative concept of cancer stem cells provides one explanation for the phenotypic and functional heterogeneity among cancer cells in some tumors (2, 22). This model describes a hierarchy of intratumoral subpopulations comprising mainly tumor cells that do not form tumors when implanted into immunodeficient mice and a rare population of cancer cells with stem cell-like properties, which are thought to drive tumor progression, tumor dissemination, and therapy resistance (2, 22). Studies have shown however that all melanoma cells, and not just a small subset of stem-like cells, possess tumor initiating potential and can restore phenotypic heterogeneity when injected into immunodeficient mice, suggesting non-hierarchical plasticity of melanoma cells (23, 24). Notably, in the above-mentioned experimental settings, single melanoma cells were artificially implanted into immunocompromised mice, and subsequently formed tumors with restored heterogeneity (23, 24). However, for a tumor-initiating cell to drive metastasis, it needs to be invasive. In most cases, metastases are driven by several tumor cells in a cooperative manner and heterogeneity is therefore maintained and reprogrammed at the metastatic site (13, 22). Thus, a new paradigm termed “phenotype switching” emerged to better

describe the plasticity of melanoma cells and their stem-like behavior. This model predicts that melanoma metastasis and phenotypic heterogeneity is driven by specific gene expression programs rather than by the accumulation of irreversible genetic events. Microenvironmental conditions, coupled with melanoma cell-intrinsic pathways, regulate melanoma cell switching between a proliferative state or a mesenchymal-like invasive state (22). In line with this paradigm, the ‘proliferative, differentiated, and often therapy-sensitive’ and ‘invasive, dedifferentiated/undifferentiated, and often therapy-resistant’ melanoma cells can co-exist in bulk tumor tissues (10, 11) and are not defined by irreversible genetic lesions (6, 25). Rather, melanoma cells are phenotypically plastic, or ‘phenotype switch’, *in vitro* and *in vivo* (6, 15, 25, 26), *via* a process akin to the reversible epithelial-to-mesenchymal transition (EMT), which is characteristic of epithelial tumors.

Hyperactivation of tumor cell-intrinsic MAPK and PI3K signaling, microenvironmental stress conditions (i.e., hypoxia, nutrient limitation, and chronic inflammation) are commonly observed in melanomas of all genomic classifications. These varied growth conditions induce several stress adaptive pathways in melanoma cells, such as the HIF1 α pathway, p38 MAPK pathway, and integrative stress response (ISR), which are generally believed to be essential drivers of phenotype switching. Frontline therapies, such as MAPK-targeted therapy and

immunotherapy, can further induce these common stress signals. Together, these factors cooperate to promote melanoma phenotype switching, which will ultimately determine the invasive potential and therapeutic response regardless of their genomic mutations (14, 16). Therefore, blocking phenotypic switching is a promising and universal strategy in melanoma to prevent metastasis and overcome drug-resistance.

Here, we dissect mechanisms underpinning melanoma phenotype switching in response to a variety of stress conditions and their link to therapy resistance. We highlight recent studies demonstrating novel strategies to target plasticity and their potential clinical impact in melanoma. While there are a number of subtypes of melanoma, including acral and mucosal, in this review we focus on cutaneous melanoma, with a short discussion of recent reports of plasticity in uveal melanoma.

MECHANISMS UNDERPINNING MELANOMA PHENOTYPE SWITCHING

For the past 20 years, several studies on melanoma plasticity have focused on characterizing gene expression signatures and transcriptional programs of the proliferative versus the invasive states of melanoma cells (3, 13, 27). These studies have provided key insights into the molecular mechanisms driving phenotype switching and their relationship to metastasis and therapy resistance.

Overview of Phenotype Switching Associated Gene Signatures

The melanocyte lineage is derived from the neural crest through delamination and the epithelial-to-mesenchymal transition (EMT), both critical processes for embryonic morphogenesis and lineage differentiation. The invasive and proliferative phenotype gene signatures describe at least two distinct melanoma cell states resembling different phases of melanocyte lineage development, ranging from neural-crest stem cells (NCSCs) to highly differentiated melanocytes (**Figure 1**) (14, 16).

In the classic two-state system, ‘proliferative’ melanoma cells are thought to reflect those that proliferate rapidly in optimal low-stress conditions, such as within a suitable metastatic niche (28, 29). They exhibit a clear differentiation phenotype, marked with high expression of MITF, a master regulator of melanocyte lineage differentiation and hallmark of the proliferative melanoma cell phenotype (12). In line with the role of MITF in regulating the proliferative state, its upstream regulators (*SOX10*, *PAX3*, *EDNRB* and *CREB*) are often regarded as drivers of the invasive-to-proliferative switch, and its downstream targets (*MLANA*, *PMEL*, *DCT*, *TYRP*) are known markers of the proliferative signature (27, 30).

While MITF^{low}-marked undifferentiation/dedifferentiation is generally accepted as a key feature of the invasive phenotype, the ‘invasive’ gene signature appears to be more complex. Diverse signaling pathways that allow melanoma cells to adapt to a variety of high-stress conditions, have been associated with the invasive state. In line with the theory that phenotype switching is largely driven through microenvironmental-induced transcriptional

changes (3, 7, 8, 13, 28, 31), the proposed ‘invasive’ signature includes multiple extracellular factors, membrane receptors, transcriptional regulators, epigenetic factors, and their downstream targets and/or effectors, which are summarized in **Table 1**. Interestingly, proteins of the same family are sometimes found inversely expressed in melanoma cells with distinct phenotypes. For example, the switch in expression between pairs of closely related transcriptional regulators, such as ZEB2/ZEB1 (35–38), SOX10/SOX9 (32, 33), and LEF1/TCF4 (34), drive melanoma cells towards the proliferative and invasive phenotype, respectively.

It is worth mentioning that, apart from the conventional two-state system, several additional states, including the NGFR^{high}-marked neural crest stem cell (NCSC)-like state and an intermediate (transitory) state, have been proposed (14–16). While the NCSC-like state is also associated with low MITF expression, similar to the invasive/mesenchymal-like state, it is marked with high levels of NGFR, SOX10, AQP1, GFRA2, and RXR γ . This state of cells is highly enriched upon therapy and is thought to be a more drug-resistant version of the invasive state (14, 16). The intermediate state likely occurs when melanoma cells are switching between the proliferative and the invasive phenotypes. This transitory state is marked with concurrent enrichment of neural crest and pigmentation-associated gene sets and exhibit intermediate MITF activity (14–16). Notably, CD36 marks a unique population of this intermediate phenotype, termed the starved-like melanoma cells (SMCs) (16). These cells exhibit an altered metabolic gene signature and have increased tolerance to nutrient starvation and targeted therapy agents (3, 16, 77).

Nevertheless, the invasiveness-associated genes/proteins highlight the activation of multiple pathways that play central roles in cancer cell stress adaptation (**Table 1**). Melanoma switching to an ‘invasive’ phenotype can be experimentally driven by environmental stress signals summarized in **Table 2**. For example, melanoma cells cultured in conditions such as hypoxia (8, 55, 101), glucose and glutamine starvation (9, 57), presence of inflammatory cytokines such as TNF- α (7, 48, 86) and TGF β (102), ultimately switch to an invasive state. In addition, chronic exposure of cultured melanoma cells to BRAF and/or MEK inhibitors (54, 78) and melanocytic antigen-specific T cells (103) promotes the invasive switch, a phenotype that can be recapitulated *in vivo* (7, 104, 105). Here, we highlight essential signaling pathways that have been identified to drive melanoma phenotype switching and their roles in different stress conditions.

Pan-Signal Inducible Signaling Networks The BRN2 Signaling

BRN2 was one of the earliest transcription factors identified as a master regulator of melanoma invasion and metastasis (41–43), with its role in melanoma plasticity comprehensively demonstrated [reviewed by Fane et al. (43)]. Mutually exclusive expression of MITF and BRN2 was identified in patient melanomas and xenografts (41, 42). Mechanistically, BRN2 directly binds to the *MITF* promoter to repress its

TABLE 1 | Melanoma cell state-specific gene signature.

Signature	Type	Protein/Gene name & description
Proliferative Signature	Transcription factors (TFs)	MITF/ <i>MITF</i> : Hallmark of the proliferative melanoma signature (12, 14, 16) SOX10/ <i>SOX10</i> : Upstream TF of <i>MITF</i> (14, 16, 27, 30, 32, 33) PAX3/ <i>PAX3</i> : Upstream TF of <i>MITF</i> (27, 30) CREB/ <i>CREB1</i> : Upstream TF of <i>MITF</i> (27, 30) LEF1/ <i>LEF1</i> : β -catenin co-factor, suppresses TCF4 expression (34) ZEB2/ <i>ZEB2</i> : The ZEB1-to-ZEB2 switch promotes the proliferative switch (35–38)
	Receptors	EDNRB/ <i>EDNRB</i> : Upstream regulator of MITF, Endothelin-3 receptor (27, 30) ROR1: The ROR2-to-ROR1 switch promotes the proliferative switch (39)
	Extracellular ligands	Endothelin-3/ <i>EDN3</i> : Upstream regulator of MITF; EDNRB ligand (28, 40)
	MITF targets	Melan-A/ <i>MLANA</i> : Melanocytic antigen GP100/ <i>PMEL</i> : Melanocytic antigen Others: Tyrosinase/ <i>TYR</i> , TYRP2/ <i>DCT</i> , TYRP1/ <i>TYRP1</i>
Invasive Signature	Transcription factors	BRN2/ <i>POU3F2</i> : Pan-signal-induced regulator of phenotype switching (41–47) c-Jun/ <i>JUN</i> : Activator Protein-1 (AP-1) transcription factor subunit (48–50) JunB/ <i>JUNB</i> : AP-1 transcription factor subunit (51, 52) Fra1/ <i>FOSL1</i> : AP-1 transcription factor subunit (53) TEADs/ <i>TEAD1</i> , <i>TEAD2</i> , <i>TEAD3</i> , <i>TEAD4</i> (25, 54) HIF1 α / <i>HIF1A</i> : Key regulator of hypoxia-induced phenotype switching (8, 39, 55, 56) ATF4/ <i>ATF4</i> : Key regulator of starvation-induced phenotype switching (9, 57) NF- κ B/ <i>NFKB1</i> : Key regulator of inflammation-induced phenotype switching (48, 58–60) HOXA/ <i>HOXA</i> (61) ZEB1/ <i>ZEB1</i> : The ZEB2-to-ZEB1 switch promotes the invasive switch (35–38) SOX9/ <i>SOX9</i> (33) TCF4/ <i>TCF4</i> : β -catenin co-factor, inversely correlated with LEF1 (34) SOX10/ <i>SOX10</i> *: Neural crest stem cell TF, also marks the NCSC-like state (14) JunB/ <i>JUNB</i> : AP-1 transcription factor subunit; possibly negative regulation of the invasive phenotype (62) Fra2/ <i>FOSL2</i> : AP-1 transcription factor subunit; negative regulation of the invasive phenotype (63)
	Secretory factors/Extracellular ligands	WNT5A/ <i>WNT5A</i> (27, 64–66) TNF α / <i>TNF</i> (7, 48, 67) TGF β / <i>TGFB1</i> (6, 42, 68, 69) Others: IL-1/ <i>IL1</i> , IL-6/ <i>IL6</i> , CCL2/ <i>CCL2</i> , MMP-2/ <i>MMP2</i> , MMP-9/ <i>MMP9</i> , ANGPT2/ <i>ANGPT2</i> , IGFBP2/ <i>IGFBP2</i> , IGFBP6/ <i>IGFBP6</i> (70)
	Receptors	AXL/ <i>AXL</i> : Hallmark of the invasive melanoma signature (12, 14, 16, 27, 47, 64, 71) ROR2/ <i>ROR2</i> : WNT5A receptor (39, 66) NOTCHs/ <i>NOTCH1</i> , <i>NOTCH2</i> , <i>NOTCH3</i> , <i>NOTCH4</i> (45, 72–74) EGFR/ <i>EGFR</i> (75) PDGFR/ <i>PDGFRA</i> (76) CD36/ <i>CD36</i> : Hallmark of the starved-like melanoma cell (SMC) state (16, 77) NGFR/ <i>NGFR</i> : Hallmark of the NCSC-like phenotype (14, 16, 54, 78–80) GFRA2/ <i>GFRA2</i> : Marker of the NCSC-like state (16)
	Epigenetic regulators	BMI1/ <i>BMI1</i> (81) EZH2/ <i>EZH2</i> (72)
	Translational factors	eIF2 α (phospho Ser51)/ <i>EIF2A</i> : Upstream regulator of ATF4/ <i>ATF4</i> eIF4E (phospho Ser209)/ <i>EIF4E</i> : Upstream regulator of NGFR/ <i>NGFR</i>
	Others	RXR γ / <i>RXRG</i> : Nuclear receptor, driver of the NCSC-like state (16) AQP1/ <i>AQP1</i> : Marker of the NCSC-like state (16)

transcription (41), while increased MITF activity represses BRN2 through miR-211, which is derived from the MITF-target gene *TRPM1* (Figure 2) (44). It is tempting to speculate that a reciprocal regulation of BRN2 and MITF expression enables a swift switch between proliferative and invasive phenotypes, which is ultimately required for seeding and outgrowth of melanoma cells at secondary metastatic sites. BRN2 is generally accepted as a pan-signal inducible driver of

phenotype switching, activated by coordinated intrinsic oncogene-driven and extracellular factor-induced signaling networks, including the RAS/RAF/MAPK (106, 107), PI3K/PAX3 (108, 109), Wnt/ β -catenin (110), and TNF α /MYC (111, 112) pathways (Figure 2). For example, in oncogenic BRAF-driven melanomas, BRN2 expression is elevated through hyperactivated MAPK signaling, which transcriptionally represses the cGMP-specific phosphodiesterase PDE5A (106,

TABLE 2 | Stress-specific pathways driving melanoma phenotype switching.

Conditions	Factors	Master regulators	Signaling pathways/mechanisms
Metabolic stress	Hypoxia	HIF1 α	HIF1 α \rightarrow BHLHE40/BHLHB2 (-MITF) \rightarrow dedifferentiation (8, 55) HIF1 α \rightarrow WNT5A-ROR2 \rightarrow invasion (39, 66, 82) Akt \rightarrow NF- κ B (+HIF1 α) \rightarrow Notch1 \rightarrow phenotype switching (83, 84)
	Nutrient starvation	p-eIF2 α ATF4	p-eIF2 α (-eIF2B) \rightarrow ATF4 \rightarrow AXL \rightarrow invasion (9, 57) p-eIF2 α (-eIF2B) \rightarrow ATF4 (-MITF) \rightarrow dedifferentiation (9, 57)
	Oxidative stress	NRF2	NRF2 \rightarrow ATF4 (-MITF) \rightarrow dedifferentiation, inflammation (85)
Inflammation & cytokines	TNF α	BRN2 AP-1 (c-Jun)	TNF α \rightarrow BRN2 \rightarrow phenotype switching (Summarized in Figure 2) (86, 87) TNF α \rightarrow c-Jun \rightarrow phenotype switching (Summarized in Figure 2) (48, 88, 89)
	TGF β	ATF4 AP-1 (c-Jun) HIF1 α	TGF β \rightarrow ATF4 (-MITF) \rightarrow proliferation, differentiation (90, 91) TGF β \rightarrow c-Jun (or JunB) \rightarrow phenotype switching (Summarized in Figure 2) (52) TGF β (-IPHD2) \rightarrow HIF1 α \rightarrow phenotype switching (92)
	IL-1	NF- κ B AP-1	IL-1 \rightarrow phospho-I κ B \rightarrow NF- κ B \rightarrow phenotype switching (93) IL-1 \rightarrow JNK \rightarrow AP-1 (c-Jun) \rightarrow phenotype switching (94) IL-1 \rightarrow MMP-9 \rightarrow invasion (95, 96) IL-1 (-MITF) \rightarrow dedifferentiation (97)
	IL-6	WNT5A CA-IX	IL-6 \rightarrow MAPK \rightarrow WNT5A \rightarrow phenotype switching (98) IL-6 \rightarrow CA-IX \rightarrow phenotype switching (99, 100)

Extracellular Ligands.

Receptors.

Transcription factors.

Transcriptional repressors.

Epigenetic regulators.

Translational factors.

Others.

107). Consequently, accumulated cGMP leads to an increase in cytosolic calcium ions, which stimulates myosin light chain 2 (MLC2) phosphorylation, thereby inducing contractility and promoting invasion (106, 107). In addition, BRN2 and MITF activate and repress the NOTCH pathway, respectively (45, 129, 130). Activated NOTCH signaling subsequently drives melanoma dedifferentiation and invasion through the epigenetic regulator EZH2 (72, 113) (**Figure 2**).

The AP-1 and TEAD Transcription Factor Family

To better understand transcriptional regulators that drive melanoma phenotype switching, Verfaillie et al. found that the invasive gene signature (12, 26, 54, 64) was enriched for AP-1 and TEAD motifs, indicating an AP-1/TEAD-governed transcriptional landscape underpinning the invasive state (25).

AP-1 is a dimeric transcription factor composed of proteins belonging to the Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra1, and Fra2), and closely related transcription factor (ATF2, LRF1/ATF3 and B-ATF) subfamilies (117). AP-1 is activated by both intrinsic oncogene-driven and extrinsic stress-driven MAPK pathway hyperactivation (49, 115, 116), and other stimuli such as inflammatory cytokines and stress inducers (**Figure 2**) (48, 117, 118). For example, the WNT5A/ β -catenin-mediated non-canonical Wnt signaling, a well-known driver of melanoma phenotype switching, acts directly upstream of AP-1 (82, 118–120). In response to a variety of environmental stimuli, the extracellular ligand WNT5A interacts with ROR2 and Frizzled (Fzd)-family receptors, resulting in AP-1 activation (82, 118–120) and subsequently promotes melanoma cell invasion (65, 120). In addition, the AP-1 family member c-Jun

is highly active in melanoma cells, and its expression is negatively regulated by MITF, which binds to the *JUN* promoter and blocks its transcription (48, 50). In response to inflammation, TNF α induces the expression of c-Jun, when MITF is suppressed, leading to a switch to the invasive state (48). Hyperactivation of MAPK signaling in BRAF^{V600E}-mutant melanoma cells leads to increased c-Jun expression, resulting in mesenchymal-like phenotype and resistance to BRAF/MEK inhibitors (MAPKi) (49). MAPKi-sensitive cell lines develop adaptive resistance through *SPROUTY4* downregulation, which leads to increased abundance of c-Jun and a subsequent switch to the mesenchymal-like and drug-resistant state (49). Another AP-1 family member, Fra1 (*FOSL1*), downregulates MITF through its transcriptional target, the chromatin modifier HMGA1, and induces the expression of AXL, driving melanoma cells to the MITF^{low}/AXL^{high} state (53). Notably, while c-Jun and Fra1 promote the melanoma switch to the invasive phenotype, other AP-1 transcription factors, such as Fra2 (63) and perhaps JunB (62) inhibit this switch. These studies indicate that AP-1 mediates plasticity by the differing composition of its subunits, similar to the aforementioned ZEB2/ZEB1 and LEF1/TCF4 switch.

Although regulated downstream of various oncogenic pathways, including Wnt, TGF β , and EGFR signaling, the TEAD family of transcription factors (TEAD1-4) are best-known as final effectors of the Hippo pathway (122). Upon activation, YAP/TAZ translocate into the nucleus and bind to TEADs to promote transcriptional programs (123). Numerous studies demonstrate a cooperative transcriptional mechanism between AP-1 and TEADs (123–125). For example, YAP/TAZ/

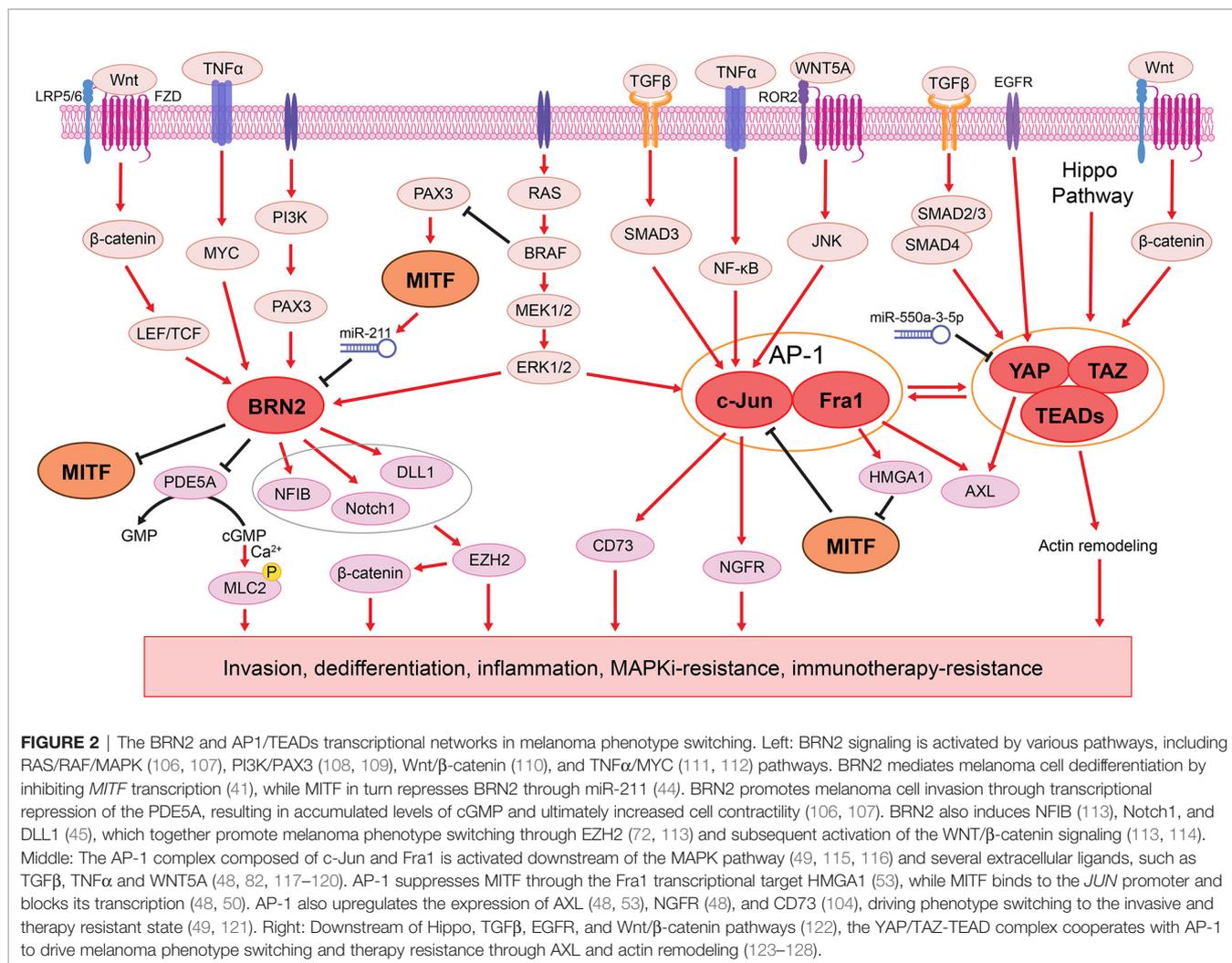


FIGURE 2 | The BRN2 and AP1/TEADs transcriptional networks in melanoma phenotype switching. Left: BRN2 signaling is activated by various pathways, including RAS/RAF/MAPK (106, 107), PI3K/PAX3 (108, 109), Wnt/ β -catenin (110), and TNF α /MYC (111, 112) pathways. BRN2 mediates melanoma cell dedifferentiation by inhibiting *MITF* transcription (41), while *MITF* in turn represses BRN2 through miR-211 (44). BRN2 promotes melanoma cell invasion through transcriptional repression of the PDE5A, resulting in accumulated levels of cGMP and ultimately increased cell contractility (106, 107). BRN2 also induces NFIB (113), Notch1, and DLL1 (45), which together promote melanoma phenotype switching through EZH2 (72, 113) and subsequent activation of the WNT/ β -catenin signaling (113, 114). Middle: The AP-1 complex composed of c-Jun and Fra1 is activated downstream of the MAPK pathway (49, 115, 116) and several extracellular ligands, such as TGF β , TNF α and WNT5A (48, 82, 117–120). AP-1 suppresses *MITF* through the Fra1 transcriptional target HMG1 (53), while *MITF* binds to the *JUN* promoter and blocks its transcription (48, 50). AP-1 also upregulates the expression of AXL (48, 53), NGFR (48), and CD73 (104), driving phenotype switching to the invasive and therapy resistant state (49, 121). Right: Downstream of Hippo, TGF β , EGFR, and Wnt/ β -catenin pathways (122), the YAP/TAZ-TEAD complex cooperates with AP-1 to drive melanoma phenotype switching and therapy resistance through AXL and actin remodeling (123–128).

TEAD-mediated *FOS* transcription increases AP-1 activity, which in turn contributes to the expression of YAP/TAZ downstream target genes (123). In addition, a significant overlap in the AP-1- and TEAD-mediated gene signatures is observed in invasive melanomas (25). Mechanistically, AXL is identified as a direct transcriptional target of YAP (126). Overexpression of YAP drives melanoma cell invasion and metastasis through induction of an invasive gene signature (127). Increased nuclear accumulation of YAP/TAZ leads to actin remodeling, which in turn confers BRAF inhibitor resistance in BRAF^{V600E}-mutant melanoma cells (Figure 2) (128). Simultaneous knockdown of all four TEADs in invasive melanoma cultures results in decreased invasiveness and increased sensitivity to MAPKi (25).

Metabolic Stress-Induced Plasticity

Limited availability of oxygen (hypoxia) and nutrients remain a challenge for most tumors. Numerous studies have demonstrated that hypoxia (8, 39, 55, 66, 83) and nutrient deprivation (9, 57) promote melanoma phenotype switching to

the invasive and dedifferentiated state (Table 2). In this state, slowly dividing melanoma cells show lower oxygen and nutrient consumption and are hence more “fit”, that is, able to survive under insufficient oxygenation and nutritional support. At the same time, these invasive cells have a better chance to migrate, and colonize new sites in the body where conditions are more optimal for their survival and growth.

HIF1 α : Hypoxia-Specific Driver of Phenotype Switching

Mechanistically, hypoxia drives melanoma phenotype switching in a HIF1 α -dependent manner (Table 2). Hypoxia induces the expression of HIF1 α , which regulates the expression of BHLHE40 (8, 55), WNT5A and ROR2 (39, 82), and Notch1 (83, 84). BHLHE40 is a transcriptional suppressor capable of binding to the *MITF* promoter to repress its transcription (8, 55). In response to hypoxia, ROR1-expressing melanomas adopt a ROR2-positive invasive phenotype. The tyrosine kinase receptor ROR2 drives invasion through its interaction with WNT5A and downstream non-canonical Wnt signaling (39, 66). In addition,

hypoxia induces Akt hyperactivation, which cooperates with HIF1 α to promote Notch1 expression, an effector that drives melanoma phenotype switching (45, 72, 83, 84). In non-melanoma cancers, hypoxia activates TGF- β signaling, which then cooperates with HIF1 α to promote invasion (131, 132). Finally, CA-IX is a metalloenzyme which is produced following hypoxic stress. In melanoma, CA-IX has been shown to be acidify the tumor microenvironment and participate in tumor growth, survival, invasion, and metastasis (**Table 2**) (99). CA-IX is druggable, and the inhibitor SLC-0111 has shown efficacy in inhibiting melanoma phenotype switching (**Table 3**) (100).

ATF4 in Nutrient Deprivation-Induced Phenotype Switching

Glucose and glutamine starvation drives melanoma phenotype switching through the ISR-associated transcription factor ATF4 (**Figure 3** and **Table 2**) (9, 57). Nutrient limitation leads to the phosphorylation of eIF2 α (p-eIF2 α), a hallmark of ISR. P-eIF2 α inhibits eIF2B activity, which diminishes global translation whilst increasing the translation of select mRNAs, including ATF4, to mitigate the effects of nutrient deprivation (9, 57, 147, 148). ATF4 activates *AXL* and represses *MITF* transcription, which subsequently slows down cell proliferation and drives a switch to the invasive phenotype (9). Notably, increased ATF4 activity alone is not sufficient to drive phenotype switching. Rather, it cooperates with the p-eIF2 α -mediated translational reprogramming (9).

Inflammation/Cytokine-Induced Plasticity

Inflammation was one of the first microenvironmental triggers identified to drive melanoma dedifferentiation (6, 7, 26). Studies focusing on the invasive switch of melanoma cells within primary tumors have found that stromal cells secrete pro- and anti-inflammatory factors such as TGF- β , TNF- α , MMPs, cytokines and WNT5A into the tumor microenvironment, all of which act to increase invasion and tumor cell dissemination. Curiously, melanoma cells in the invasive state produce pro-inflammatory factors, and in a paracrine manner stimulate proliferative melanomas to switch to the invasive state, a process termed “phenotype cooperativity” [reviewed by *Arozarena & Wellbrock* (13)].

TNF α

Tumor necrosis factor alpha (TNF α) is a soluble or membrane pro-inflammatory cytokine produced by macrophages, T-cells, and NK cells. In the case of melanoma, TNF α is involved in many of the mechanisms known to alter plasticity, leading to tumor escape by increasing adhesion molecules such as fibronectin (153). TNF α induces BRN2 signaling (87) and, in line with often mutually exclusive expression of BRN2 and MITF, it has been identified as an inhibitor of MITF and Melan A in 40 different melanoma cell lines (86). Interestingly, MITF expression can modify the inflammatory status of the tumor microenvironment by inhibiting c-Jun and subsequently decreasing TNF α (48). This results in a decrease in myeloid cell recruitment to the tumor, less inflammation and potentially less inflammation associated phenotype switching. These results

suggest MITF^{low}/c-Jun^{high} melanoma cells enhance phenotype switching by increasing TNF α production. Furthermore, the invasiveness pathway activated by AP-1 can be triggered by inflammatory cytokines from TNF family (88) and AP-1 can positively regulate TNF α production thereby amplifying phenotype switching (**Table 2**) (89). TNF α can also promote tumor cell dissemination by increasing angiogenesis (67, 154), the process whereby melanoma cells around blood vessels leads to metastasis without entry into the blood circulation. Tüting's team highlighted the role of TNF α in the enhancement of endothelial cell sprouting and promoted the pericyte-like expansion of co-cultured melanoma cells along such endothelial outgrowths. Co-culture of melanoma cells with endothelial cells composing the abluminal surface of blood vessels, was demonstrated to induce the expression of genes linked to cancer cell migration (CCL2, ICAM1), cancer progression (TRAF1, SERPINB2) or stem cell properties (PDGFB; CFDP1) (155). These data suggest the interaction between the abluminal surface of endothelial cells and melanoma cells could lead to a melanoma phenotype switch favoring metastasis.

TNF α can have additional unwanted effects on anti-tumor immune responses. First, TNF α can decrease the recognition of tumor cells by melanocytic antigen-specific CD8⁺ T-cells (7) and trigger the death of CD8⁺ T cells (156). Moreover, regulatory T-cells, which are generally considered a bad prognostic factor in melanoma (157), can strongly express TNFR2 on their surface, are the most potent Tregs and can survive longer than the others in the presence of TNF α (158).

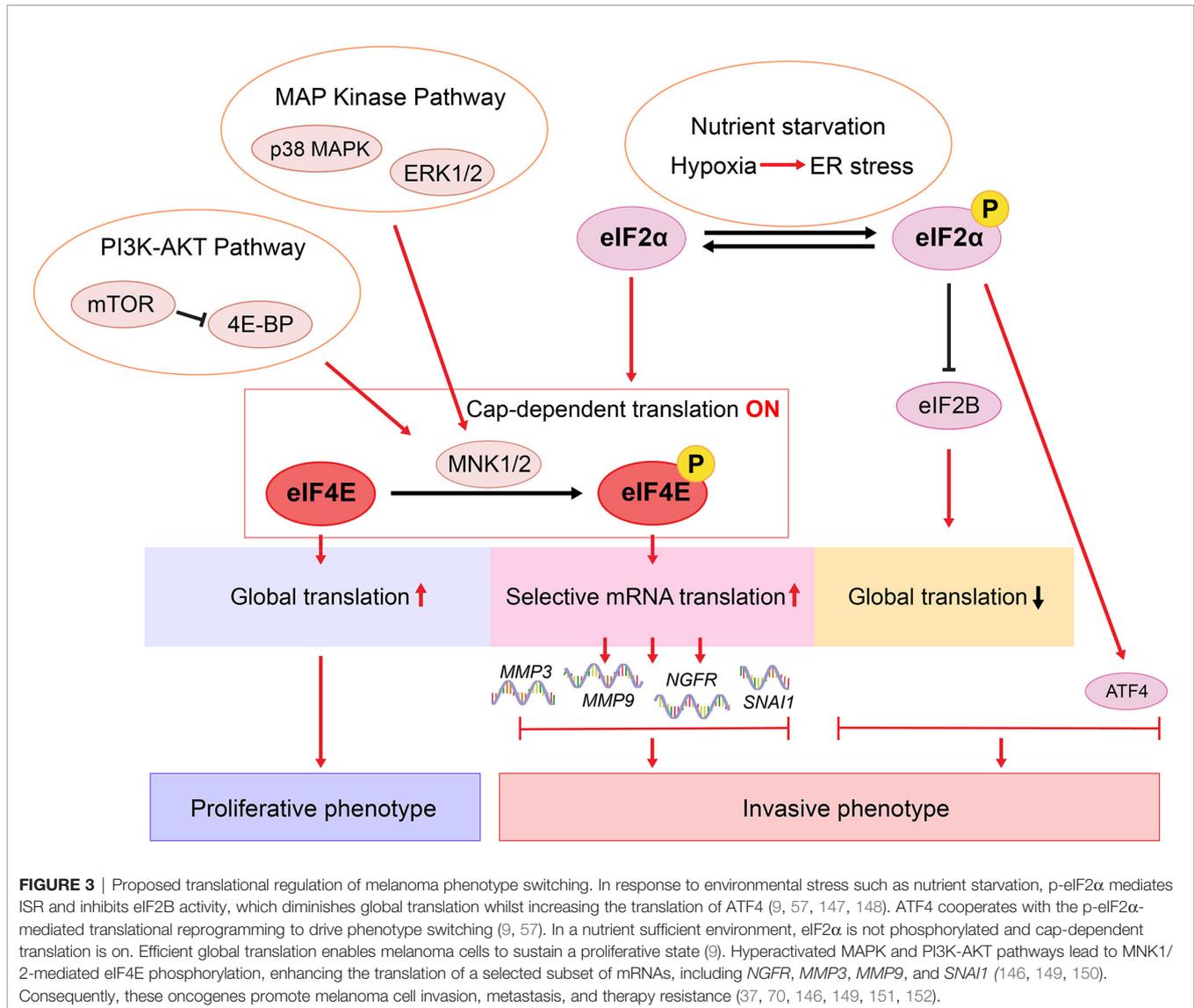
TGF β

Transforming growth factor β (TGF β) is a family of 40 proteins including TGF β , Activins and Nodal. TGF β is an anti-inflammatory cytokine important in the control of inflammation, its lack leads to lethal inflammation (159). TGF β is expressed by melanocytes, negatively regulating their proliferation. However, when melanoma initiates, tumor cells resist the anti-proliferative effects of TGF β while continually producing it (160). As described in the previous sections, phenotype switching can be induced in a number of ways and it is worth mentioning that TGF β is involved in most of these mechanisms, such as in the regulation of MITF expression or as a hypoxia-specific driver of phenotype switching. TGF β is a negative regulator of MITF leading to the shift from a proliferative to an invasive state (90). Furthermore, in triple negative breast cancer, arguably the most therapy resistant breast cancer, TGF β promotes ATF4 expression which is correlated with a poor prognosis (91, 161). TGF β has also been associated with the BRN2 invasive phenotype (42, 162), a pathway known to downregulate MITF. Moreover, TGF β activates the AP-1 pathway leading the production of c-Jun and JunB, respectively in epidermal keratinocytes and dermal fibroblasts (163), known to be involved in the process of phenotype switching and BRAF inhibitor resistance in melanoma (**Table 2**) (49, 51).

As we described above, hypoxia plays a central role in phenotype switching through the expression of HIF1 α . TGF β can trigger the expression of HIF1 α by selectively inhibiting PHD2 expression (**Table 2**) (92). However, TGF β is also directly

TABLE 3 | Therapeutic agents that target melanoma phenotype switching.

Target signaling	Agent	Description	Efficacy
WNT5A signaling	Anti-Fz5 antibody (120)	Frizzled-5 polyclonal antibody	Decrease melanoma cell invasion <i>in vitro</i>
	Gö 6983 (133)	PKC inhibitor	Block WNT5A-mediated inhibition of Melan-A and PAX3 <i>in vitro</i>
	C59 (134)	Soluble PORCN inhibitor	Decrease melanoma cell-derived WNT5A secretion Synergize with anti-CTLA-4 immunotherapy <i>in vivo</i>
AP-1 signaling	JNK-IN-8 (49)	c-Jun N-Terminal Kinase (JNK) inhibitor	Decrease melanoma cell migration <i>in vitro</i> Enhance the efficacy of vemurafenib <i>in vitro</i>
BRN2 signaling	DZNep (72)	EZH2 inhibitor	Increase melanosomes and pigmentation in melanoma cells Decrease melanoma cell invasion <i>in vitro</i>
	GSK343 (113)	EZH2 inhibitor	Increase MITF expression in melanoma cells Decrease melanoma cell migration <i>in vitro</i>
	GSK503 (135) AM404 (111, 136)	EZH2 inhibitor Inhibitor blocking NFATc2-DNA binding	Abolishes metastases formation <i>in vivo</i> Increase melanocytic differentiation markers, decrease BRN2, AXL, EZH2 and EMT markers, and decrease melanoma cell invasion <i>in vitro</i> AM404+GSK126 (EZH2i) reverse phenotype switching AM404+GSK126 induce apoptosis and sensitize melanoma cells to MAPKi
AXL ^{high} dedifferentiated state	Enapotamab vedotin (AXL-107-MMAE) (137, 138)	Human AXL antibody linked to monomethyl auristatin E (MMAE)	Display potent anti-tumor activity <i>in vivo</i> as single agent Synergize with MAPK inhibitors to inhibit tumor growth <i>in vivo</i> Synergize with anti-PD-1 immunotherapy <i>in vivo</i>
NGFR ^{high} NCSC-like state	Ganetespib (103)	HSP90 inhibitor	Decrease NGFR expression in T-cell therapy-resistant cells Restore melanoma cell sensitivity to T cell attack <i>in vitro</i> Restore tumor sensitivity to T-cell therapy <i>in vitro</i>
	17-AAG (103)	HSP90 inhibitor	Decrease NGFR expression in T-cell therapy-resistant cells Restore melanoma cell sensitivity to T cell attack <i>in vitro</i>
	AG-879 (103)	NGFR kinase inhibitor	Restore melanoma cell sensitivity to T cell attack <i>in vitro</i>
	HX531 (16)	RXR antagonist	Decrease NCSC-like populations upon BRAF/MEK inhibition Enhance the efficacy of dabrafenib+trametinib <i>in vivo</i> Delay resistance to dabrafenib+trametinib <i>in vivo</i>
	PF562271 (139)	Focal adhesion kinase (FAK) inhibitor	Decrease NCSC-like (GFRA2*) populations upon BRAF/MEK inhibition PF562271+HX531 combination delays the onset of therapy resistance further than HX531 alone <i>in vivo</i>
CD36 ⁺ SMC state (proposed)	Sulfosuccinimidyl oleate (SSO) (140–142)	CD36 inhibitor	Re-sensitize drug-resistant breast cancer cells to lapatinib <i>in vitro</i> Block high glucose-induced EMT in renal tubular epithelial cells Reduce <i>in vitro</i> proliferation and <i>in vivo</i> growth of colorectal cancer cells.
	Anti-CD36 mAb Clone JC63.1 (140, 143)	CD36-neutralizing antibody	Re-sensitize drug-resistant breast cancer to lapatinib <i>in vivo</i> Inhibit metastasis of oral squamous cell carcinomas <i>in vivo</i> Reduce the size of metastases of squamous cell carcinomas <i>in vivo</i>
	Anti-CD36 mAb Clone FA6.152 (143)	CD36-neutralizing antibody	Inhibit metastasis of oral squamous cell carcinomas <i>in vivo</i>
eIF4F complex	Silvestrol (144, 145)	eIF4A RNA helicase inhibitor	Selectively kill melanoma persister cells <i>in vitro</i> Inhibit the emergence of persister cells, combined with BRAFi and MEKi Synergistically inhibit melanoma cell growth <i>in vitro</i> , combined with BRAFi
	FL3 flavagline (145) 4EG inhibitor-1 (4EGI-1) (145)	eIF4A inhibitor Inhibitor blocking eIF4E-eIF4G interaction	Strong reduction of melanoma growth, combined with BRAFi <i>in vivo</i> Selectively kill vemurafenib-resistant melanoma cells <i>in vitro</i> Synergize with vemurafenib in inhibit melanoma cell growth <i>in vitro</i>
	SEL201 (146)	MNK1/2 kinase inhibitor	Decrease NGFR expression and increase MITF expression <i>in vitro</i> Decrease melanoma cell invasion <i>in vitro</i> Restore melanoma cell sensitivity to vemurafenib <i>in vitro</i> Sensitize melanoma to anti-PD-1 immunotherapy <i>in vivo</i>
	eFT508 (146)	MNK1/2 inhibitor	Sensitize melanoma to anti-PD-1 immunotherapy <i>in vivo</i>
Others	SLC-0111 (100)	CA-IX inhibitor	Revert mesenchymal stem cell (MSC)-mediated melanoma phenotype switching and vemurafenib resistance <i>in vitro</i>



involved in the process of vascularization by inducing the expression of macrophage inhibitory protein 1 and VEGF (164, 165).

Similar to the negative impact of TNF α on anti-tumor immunity, TGF β supports tumor immune-evasion responses by decreasing the activity of cytotoxic immune cells such as natural killer cells (166) or CD8⁺ T-cells (167), while favoring the generation of regulatory T-cells (153).

Interleukin-1 and Interleukin-6

Interleukin (IL)-1 is a major pro-inflammatory cytokine, mainly produced by macrophages (168), monocytes or neutrophils and can be triggered by the activation of the inflammasome (169). Two isoforms of IL-1 exist, IL-1 α and IL-1 β , which bind the same receptor IL-1R but are encoded by two different genes. Several reports highlight the important role of IL-1 in melanoma. A high concentration of IL-1 β has been detected in the plasma of

patients with melanoma compared to healthy donors (170). The consequences of this IL-1 over-production include (1) an increase in the phosphorylation of NF- κ B inhibitor (I κ B), thus freeing NF- κ B known to be involved in melanoma development (93) and (2) activation of stress-activated protein kinase/c-Jun N-terminal kinase (JNK) identified as a key factor in melanoma progression (94). IL-1 is also involved in the production of metalloproteinase 9 (MMP-9) (95), which plays an important role in the remodeling of extracellular matrix and enhancing tumor cell invasiveness (96). Furthermore, the level of circulating MMP-9 has been cited as a good candidate to evaluate the response to BRAF inhibitors in melanoma patients (70). In terms of plasticity, IL-1 α and β can downregulate the expression of MITF and melanocytic antigens, favoring melanoma dedifferentiation and phenotype switching (171). Lastly, IL-1 β can upregulate HIF1 α (97), known to inhibit MITF and increase melanoma invasiveness (55) (Table 2).

IL-6 is a pro-inflammatory cytokine family of ten proteins involved in the anti-viral immune response. IL-6 is a key factor in the acute phase of inflammation and participates in the establishment of chronic inflammation. IL-6 is produced by macrophages (172), T-cells, B-cells, and endothelial cells (173). IL-6 binds to the IL-6R to produce acute phase proteins such as inflammatory markers (e.g., serum amyloid A or complement factors) present in serum during inflammation. On the other hand, IL-6 is involved in the differentiation of Th17 (174), follicular helper T-cells (175), or in the proliferation and survival of CD8 T-cells (176). Similar to IL-1, IL-6 is abundant in the serum of patients with melanoma (177). IL-6 increases the invasiveness and motility of melanoma cells through the MAPK pathway which upregulates WNT5A (98), which as discussed above is a major regulator of phenotype switching.

Insight Into the Role of Translational Regulation of Cellular Plasticity

In the past decade, studies on melanoma phenotype switching have focused largely on mechanisms involving transcriptional reprogramming, through which intracellular cues and extracellular signals are integrated to enable rapid shifts in cell states. In recent years, emerging data suggest that translational reprogramming collaborates with epigenetic and metabolic programs to promote phenotypic plasticity of cancers (178). In eukaryotic cells, selective translation is regulated mostly, but not exclusively, at the translation initiation step, through the eIF4F complex and the ternary complex (179). In 2017, Falletta et al. and Ferguson et al. independently showed that phenotype switching is driven by an ISR-dependent translational reprogramming through the p-eIF2 α -eIF2B-ATF4 axis (9, 57). This ternary complex-mediated pathway is highly associated with nutrient starvation, resulting in increased ATF4 expression coupled with diminished global mRNA translation (9, 57, 147, 148) (**Figure 3** and **Table 2**). Importantly, while blocking p-eIF2 α impairs invasion, without translation reprogramming, increased ATF4 activity alone is not sufficient to drive phenotype switching (9). More recently, Huang et al. demonstrated a mechanism involving activation of the MNK1/2-eIF4E axis and cellular plasticity (146). As a key component of the eIF4F complex, eIF4E regulates cap-dependent mRNA translation initiation. Phosphorylation of eIF4E (p-eIF4E) by kinases MNK1/2 selectively enhances the translation of a subset of mRNAs encoding pro-invasive and pro-survival factors (180, 181). Blocking the MNK1/2-eIF4E axis reversed phenotype switching with impaired invasion, increased expression of MITF, and restored sensitivity to MAPK-targeted and immunotherapies (146). While we showed that the MNK1/2-eIF4E axis promotes the translation of *NGFR*, melanoma phenotype switching and therapy resistance (146), little evidence links *NGFR* inhibition with a fully reversed switch in melanoma cells (13, 79). These data suggest that more complex translational mechanisms are likely involved in driving melanoma plasticity. For example, in numerous models, p-eIF4E are shown to promote the translation of *MMP3*, *MMP9* and *SNAI1* (149, 150, 180), which are known drivers of

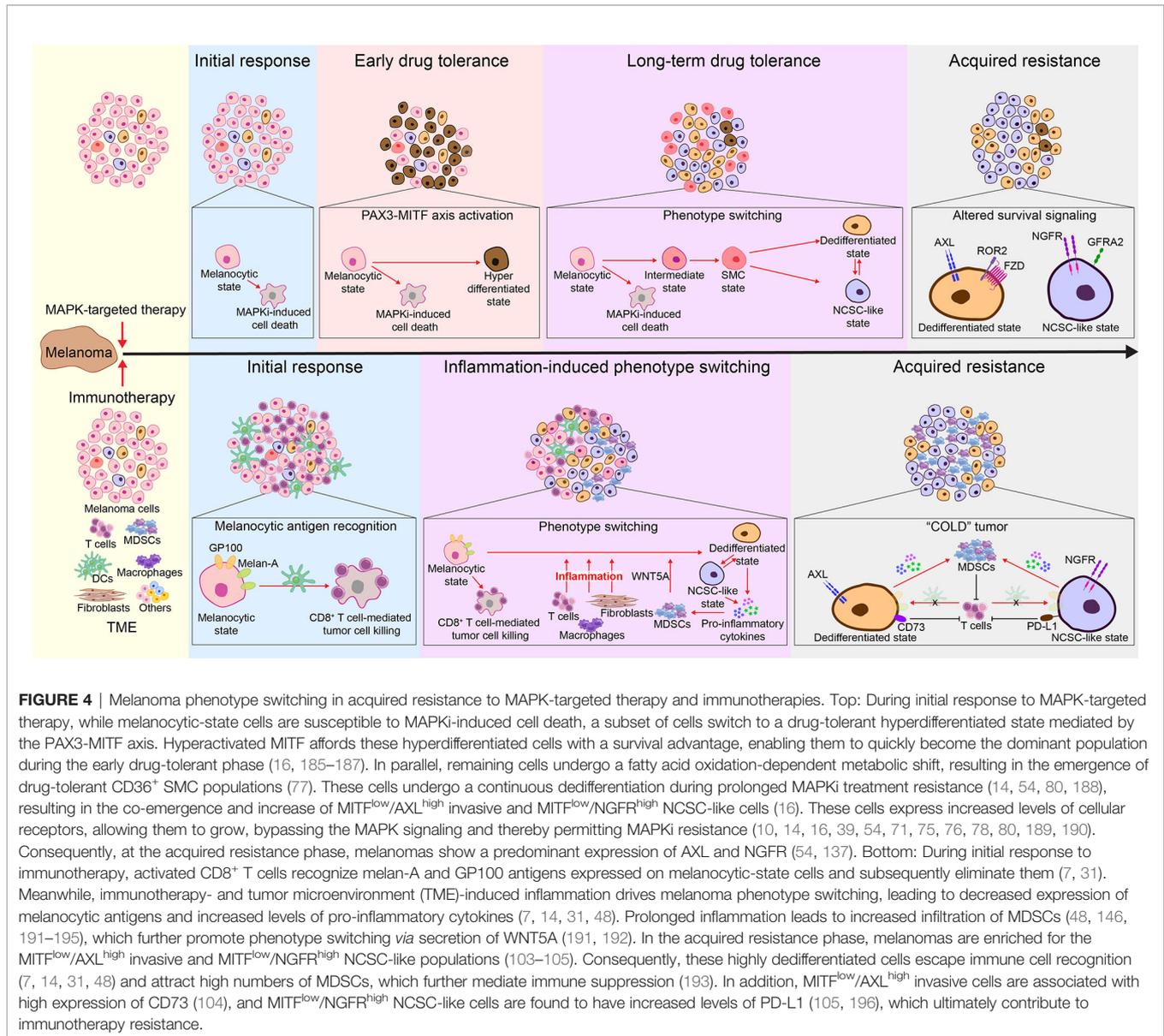
phenotype switching (37, 151, 152, 182, 183). Moreover, NODAL is shown to promote the invasive phenotype in melanoma (152) and it is regulated downstream of MNK1 signaling in breast cancer cells (184). Given that previous studies have largely focused on characterizing transcriptome changes between melanoma phenotypes (7–9, 25, 28, 57), it will be important to carry out high-throughput translational studies using pre-defined models of melanoma cell phenotype switching.

MELANOMA PLASTICITY CONTRIBUTES TO THERAPY RESISTANCE

Phenotype Switching and MAPK-Targeted Therapy Resistance

Chronic exposure of cultured melanoma cells to BRAF and/or MEK inhibitors leads to an initial response phase characterized by the induction of MITF and enrichment of MITF^{high} populations, followed by emergence of CD36⁺ SMC populations, which subsequently undergo continuous dedifferentiation, and a final state of acquired resistance marked by a predominance of slow-cycling NCSC-like NGFR^{high} cells (**Figure 4**) (54, 77, 78, 80, 185). In melanoma patients receiving MAPK-targeted therapies, similar patterns of consecutive transcriptional states have also been observed. Notably, during each phase of MAPKi-response and -resistance, different phenotypes (MITF^{high}/proliferative, ALX^{high}/invasive, and NGFR^{high}/NCSC-like) commonly coexist, while predominant population shifting occurs over the treatment course as a result of combined phenotype switching and MAPKi-mediated cell selection (16).

In the initial therapeutic response phase, an immediate activation of the PAX3-MITF-PGC1 α axis protects a subset of melanoma cells from MAPKi-induced cell death *via* MITF-mediated survival signaling, resulting in an early drug-tolerant state characterized by an enrichment of MITF^{high} hyperdifferentiated populations (16, 185–187). In parallel, the remaining melanoma cells undergo a fatty acid oxidation-dependent metabolic shift, resulting in a short-term emergence of drug-tolerant CD36⁺ SMC populations (77). The SMC-state cells express medium to low levels of MITF and subsequently undergo dedifferentiation, which allows these slow-cycling cells to persist until the (a) acquisition of new mutations that confer resistance (197), or (b) prolonged MAPKi exposure stabilizes this dedifferentiated state and leads to acquired resistance (14, 54, 80, 188). Notably, activation of ATF4 signaling can be detected in a small group of early MAPKi-tolerant cells, which exhibit a MITF^{low}/AXL^{high} invasive-like (dedifferentiated) phenotype (198). Thus, ATF4-mediated phenotype switching might occur early as melanoma cells are adapting to MAPKi therapy. Similarly, the MITF^{low}/NGFR^{high} NCSC-like cells also emerge during early MAPKi treatment (16, 139). The MITF^{high} hyperdifferentiated, CD36⁺ SMC, MITF^{low}/AXL^{high} dedifferentiated, and MITF^{low}/NGFR^{high} NCSC-like populations together make up a reservoir of early MAPKi-persister cells known as minimal residual disease, from which relapse inevitably arises (**Figures 1, 4**) (16, 139).



Following the continued dedifferentiation, MITF^{low}/AXL^{high} dedifferentiated and MITF^{low}/NGFR^{high} NCSC-like cells increase in number and often co-emerge within the same tumor (16). These cells express increased levels of several cellular receptors, such as AXL, ROR2, PDGFR, EGFR, NGFR, and GFRA2 (10, 14, 16, 39, 54, 71, 75, 76, 78, 80, 189). Mechanistically, these receptors, most being receptor tyrosine kinases, drive alternative survival signaling bypassing the MAPK pathway, thereby permitting MAPKi resistance (71, 189, 190). Consequently, at the acquired resistance and relapse stage, melanomas show a predominant expression of AXL and NGFR, along with an overall trend towards an AXL/AP-1/TEAD-driven gene signature (Figure 4) (54, 137). Importantly, as mentioned above, invasive melanoma cells communicate with proliferative melanoma cells through secretory factors to drive their switch to the invasive state. Therefore, an abundance of pre-existing invasive and NCSC-

like cells, which are prone to survive initial MAPKi treatment, could cooperate with BRAF/MEK inhibitors to accelerate the development of drug resistance mediated *via* phenotype switching.

Phenotype Switching and Immunotherapy Resistance

In this last decade, immunotherapies have revolutionized cancer treatment. Immune checkpoint blockade (ICB) targeting the PD-1/PD-L1 and the CTLA-4 axes have become the frontline therapy for patients with metastatic melanoma. In addition, adoptive T-cell transfer therapy is actively being tested in pre-clinical melanoma models. These therapies center on enhancing the function of cytotoxic T cells to improve anti-tumor immune responses (199). Consequently, primary and acquired resistance of melanoma to immunotherapies are linked with mechanisms

that ultimately lead to compromised cytotoxic T cell function (200, 201). Hugo et al. identified an innate anti-PD-1 resistance gene signature (IPRES) that is enriched in melanoma samples from anti-PD-1 immunotherapy non-responders (202). IPRES shares similarities with the invasive melanoma gene signature, including *AXL*, *ROR2*, *WNT5A*, *EGFR* and *PDGFRA*, as well as some other phenotype switching-associated genes, such as *TWIST2*, *MMPs*, *ANGPT2*, *IGFBP-6*, *SNAI1*, *CCL2* (146, 152, 202). In addition to intrinsic resistance, melanoma could also develop acquired resistance to both ICB and adoptive T cell transfer therapy *via* phenotype switching (**Figure 4**) (7, 14, 31, 103, 105). Mechanistically, immunotherapy-induced inflammation drives melanoma dedifferentiation, leading to decreased expression of melanocytic antigens, which impairs T-cell recognition and elimination of tumor cells (7, 14, 31). Moreover, melanoma of the invasive phenotype is associated with increased infiltration of myeloid-derived suppressor cells (MDSCs) (48, 146, 191–195). MDSCs drive melanoma cells to the invasive state, at least in part, *via* their secretion of WNT5A (191, 192), which in turn increases pro-inflammatory cytokine secretion by tumor cells and facilitates MDSC recruitment and function (48, 146). MDSCs mediate immune suppression and ultimately promote immunotherapy resistance (193). In addition, the cell surface enzyme CD73 (5' ectonucleotidase) is upregulated by AP-1 during therapy-induced phenotype switching (104), which in turn limits the anti-tumoral functions of T cells *via* adenosine receptor signaling (121).

Emerging evidence suggests a link between NGFR^{high} NCSC-like melanoma cells and immune escape of tumors (103, 105). Although the mechanism by which NGFR promotes immunotherapy resistance remains unclear, the BDNF-NGFR axis is suggested to contribute to T-cell therapy resistance (103), and a subset of NGFR-regulated cytokines are linked to immune suppression (146). Notably, NGFR^{high} expressing melanoma cells also showed increased PD-L1 expression (**Figure 4**) (105, 196), with NGFR and PD-L1 protein synthesis both being under translational control *via* the eIF4F complex (146, 203, 204). Again, these studies highlight the importance of examining the translational regulation of melanoma phenotype switching. Finally, it is worth mentioning that, although melanoma phenotype switching is an emerging mechanism underpinning immunotherapy resistance, its prognostic value in predicting responders is debatable. For example, in the case of desmoplastic melanoma where the dedifferentiation phenomenon is high (205), the mutation rate is also important as it leads to the apparition of neoantigens and a better response to anti-PD-1 (206).

UVEAL MELANOMA PLASTICITY AND IMMUNE SUPPRESSION

Whereas the role of cellular plasticity in cutaneous melanoma has been widely described, phenotype switching in non-skin melanomas, acral and uveal melanomas remains less clear (207). Uveal melanoma (UM) is a malignancy originated from the melanocytes in the uveal tract. The pathogenesis of UM differs from cutaneous

melanoma, where its molecular landscape and metastatic outcome presents different challenges (208). UM frequently express mutually exclusive mutations in *GNAQ* and *GNA11*. *GNAQ* and *GNA11* encode G-protein alpha-subunits that mediate signaling downstream from G-protein-coupled receptors, leading to the constitutive activation of diverse signaling pathways such as MAPK and PI3K/AKT/mTOR (209). UM rarely harbour mutations in *BRAF* and is thus not treatable with the BRAF-targeted therapies used in the management of cutaneous melanoma (210, 211). No treatment, including immune-targeted therapies, has shown efficacy in this high fatality ocular cancer. A unique characteristic of UM is the tendency to metastasize to the liver at early stages of the disease, where cells remain dormant until specific signals provided by the primary tumor, or the liver microenvironment, triggers cell proliferation (212).

Studies have been performed to determine potential prognostic factors and biomarkers in UM. In 2006, primary UM were clustered into two main classes related to their gene expression profile (also referred to as mRNA class) (213). Class 1 UM has low or medium metastatic potential, while the Class 2 UM present high metastatic potential with the deletion of chromosome 3. This classification also seems to relate to some characteristics of tumor cell plasticity. Whereas Class 1 UM present a melanocytic/neural crest phenotype, the Class 2 UM are characterized by the downregulated expression of melanocyte-specific and neural crest specification genes (e.g., *TYR*, *DCT*, *EDNRB*) and upregulated epithelial and cell adhesion markers (e.g., *EMP1/3*, *CDH1*). Whereas epithelial-derived tumors lose expression of *CDH1* (i.e., E-cadherin) as they gain invasive and metastatic potential, E-cadherin upregulation plays an important role in the dissemination of Class 2 UM. In that context, E-cadherin allows circulating tumour cells to survive once intravasation has been achieved, providing cell-cell interactions so the tumor cells avoid apoptosis (213).

Studies have been performed in recent years to better define the role of cellular plasticity (i.e. EMT) in UM. Repression of a number of transcriptional regulators of EMT, namely ZEB1, TWIST1, and SNAIL, results in decreased UM invasiveness (214). Other critical regulators of cutaneous melanoma phenotype switching are also important in UM. Class 2 UM tumours present with an upregulation of certain proteins and ligands related to the Notch pathway, triggering proliferation and invasion *in vitro* and *in vivo*, thus making it a possible druggable target (215). The expression of IGF-R, c-Fos and c-Jun in patients with Class 2 liver metastasis, indicates a role for plasticity in metastatic UM (216). Additional factors that might have a role in UM plasticity have been explored, such as HIFs and their role in regulating the expression of c-met and C-X-C chemokine receptor type 4 (CXCR4) (217). Moreover, expression of TGF- β and proinflammatory molecules like IL-6 and IL-8 in the liver microenvironment might enhance the survival of UM in the liver (218).

A major signaling pathway that has been proposed to have a pivotal role in UM proliferation and invasion, is the YAP pathway, which lies downstream target of *GNAQ* and *GNA11* aberrant signaling (219). Whereas the reliance of UM on YAP

signaling is still debated, in other cancers this signaling pathway has a central role in plasticity (220, 221). The role of YAP/TEADs and AP-1 cooperation was explored in UM tumors lacking expression of LATS1/2 and Kras activation. This study showed that YAP/TEADs and MAPK might cooperate to reinforce each other's signaling, where AP-1 upregulation or its factors (c-Jun) might have a role in increasing the transcriptional activity of YAP/TEADs (221).

Tumor progression attributed to cancer stem cells (CSCs) has also been explored in UM. CSCs have been described as small clusters of cells that can trigger tumor growth, proliferation and increased metastatic potential. It was found that UM cell lines show upregulation of putative markers of stem-like cells (e.g., CD44, CD133) but are not specific to a subset population of UM cells. The authors suggested UM plasticity might be explained by the neural crest origin of uveal melanocytes rather than presenting a specific hierarchy (222). Nevertheless, other studies showed that expression of nestin, CD166 and NGFR by specific clusters of cells provided a survival advantage, increased metastatic potential and migratory capacity of UM cells (223). Finally, it appears that NGFR also plays an important role in the plasticity and progression of UM. Primary choroidal UM express NGFR, whereas *in vitro* UM cell lines express NGFR when grown in a 3D matrix. In addition, vascular mimicry forming UM cell lines specifically express NGFR, indicating that there might be a possible role of NGFR in UM aggressiveness and resistance to therapy (105, 224). In summary, it is becoming evident that similar to cutaneous melanoma, the phenotypic states of UM will likely be an important mechanism underpinning metastasis. Future work employing single-cell level characterization of UM should enable a deeper understanding of the transitory cell states and phenotypic heterogeneity underlying this high fatality cancer.

EMERGING STRATEGIES TO TARGET MELANOMA PLASTICITY AND THEIR POTENTIAL CLINICAL IMPACT

Given the growing understanding of the regulatory networks that drive phenotype switching and its role in melanoma metastasis and therapy resistance, an important next step in research is whether this process is targetable and if so, how can we target it pharmacologically? One strategy is to direct melanoma cells towards the more therapy-sensitive proliferative/melanocytic state by blocking or reversing the invasive switch (225). In the past years, several therapeutic agents have been developed and tested (Table 3), among which the WNT5A-Protein Kinase C (PKC) pathway was among the first to be targeted (120, 133, 134). In 2002, a polyclonal antibody against the WNT5A receptor Frizzled-5 showed efficacy in decreasing melanoma cell invasion (120). Another early study showed that a pan-PKC inhibitor, Gö 6983, blocked WNT5A-mediated melanoma dedifferentiation *in vitro*, with increased expression of PAX3 and melan-A (133). Moreover, a soluble PORCN inhibitor C59, which functions by blocking WNT5A secretion, showed a synergistic effect with an anti-CTLA-4

antibody *in vivo* (134). Together, these studies provided early evidence that blocking phenotype switching is a promising strategy to sensitize melanoma to standard of care therapies.

More recently, several therapeutic agents that target the transcriptional/epigenetic reprogramming of phenotype switching have been tested. The JNK inhibitor JNK-IN-8, which reduces c-Jun phosphorylation, decreased melanoma cell migration and sensitized melanoma cells to BRAF inhibition (49). Small molecule inhibitors against EZH2, a key effector of the BRN2 signaling, restored differentiation and impaired invasion of melanoma cells *in vitro* (72, 113), and inhibited melanoma growth and metastasis in mouse models (135). Similarly, pharmacological inhibition of NFATc2, a transcription factor that acts upstream of BRN2, drove a melanoma cell switch to a more differentiated state with decreased invasiveness (111, 136). Importantly, combined inhibition of NFATc2 and EZH2 (i.e., AM404+GSK126, respectively) reversed melanoma phenotype switching overnight (136). That drug combination further induced apoptosis in treatment-naïve melanoma cells and restored drug sensitivity in MAPKi-resistant melanoma cells (136). Notably, AM404 alone reversed phenotype switching following a 6-day treatment (111, 136). These observations are in agreement with a paradigm that switching between cell phenotypes is driven by various signals over a prolonged period of time (13), and indicate that co-targeting multiple drivers of phenotype switching might be necessary to achieve optimal therapeutic efficacy.

An alternative strategy is to co-target different cell states of therapy resistance, namely, the SMC state, the dedifferentiated state, and the NCSC-like state. This is feasible due to specific markers for each cellular state (Figure 1). For example, enapotamab vedotin (AXL-107-MMAE), was designed to specifically target the AXL^{high} dedifferentiated melanoma cells (137). By eliminating the MITF^{low}/AXL^{high} therapy-tolerant and the MITF^{high}/AXL^{low} therapy-sensitive populations, respectively, enapotamab vedotin combined with MAPK inhibitors or with an anti-PD-1 antibody, cooperatively inhibited melanoma growth *in vivo* (137, 138). Several agents have shown efficacy targeting the MITF^{low}/NGFR^{high} NCSC-like populations, including HSP90 inhibitors (103), an NGFR kinase inhibitor AG-879 (103), an RXR antagonist HX531 (16), and a focal adhesion kinase (FAK) inhibitor PF562271 (139). As anticipated, blocking the NCSC-like state of cells led to enhanced/restored therapy sensitivity (16, 103, 139). The drug-tolerant SMC state is marked by the expression of CD36 (16, 77). It is therefore logical to target this population using CD36 inhibitors or neutralizing antibodies (3). Notably, several CD36-blocking agents have been identified and shown anti-tumor activities in different cancer types, including melanoma (140, 141, 143). Building on the concept that redirecting cell state switching might be necessary to achieve optimal therapeutic efficacy, the Marine group went on to show that melanoma lesions escaping a combination of MAPK-targeted therapy (dabrafenib+trametinib) and NCSC-directed therapy (HX531+PF562271) could be further targeted by ERK inhibitors (139).

Finally, accumulated evidence suggested that the mRNA translation machinery represents a therapeutic opportunity to modulate cancer cell plasticity and overcome therapy resistance (9, 144–146, 178). While agents targeting the eIF2 α -eIF2B axis/ternary

complex-mediated phenotype switching are yet to be explored, inhibitors targeting different eIF4F components have shown efficacy in preclinical melanoma models. For example, the eIF4A inhibitor silvestrol selectively killed MAPKi-tolerant melanoma cells (144). Combining MAPK inhibitors with an eIF4A inhibitor or with an inhibitor blocking the eIF4E-eIF4G interaction synergistically inhibited melanoma growth (144, 145). MNK1/2 inhibitors, which block eIF4E phosphorylation, decreased melanoma cell invasion, restored MITF expression and repressed NGFR expression in BRAFi-resistant cells, and cooperatively inhibited their growth in combination with vemurafenib (146). Importantly, MNK1/2 inhibitors are capable of sensitizing melanoma to anti-PD-1 immunotherapy in multiple melanoma mouse models (146).

CONCLUSIONS, PERSPECTIVES, AND OUTSTANDING QUESTIONS

Intratumoral heterogeneity and phenotype plasticity is essential for melanoma metastasis and therapy resistance. The phenotype switching model describes a cellular plasticity that enables melanoma cells to adapt to a variety of environmental stress signals including standard of care therapies. Emerging data suggest multiple phenotypic states of melanoma cells, representing a melanocyte differentiation gradient with different sensitivity to therapies, frequently coexisting throughout melanoma progression and throughout anti-cancer treatment (14, 16). At least six distinguished states have been identified, including a MITF^{high} hyperdifferentiated/pigmented state, a MITF^{high} differentiated/melanocytic state, an intermediate state, a CD36⁺ starved-like SMC state, an NGFR^{high} NCSC-like state and an AXL^{high} dedifferentiated state (**Figure 1**) (10, 12–16, 71, 77). Therefore, although most commonly used to describe the proliferative-to-invasive switch, the term “phenotype switching” actually describes cellular plasticity that enables melanoma cells to switch among all the distinct states. With accumulating studies carried out to understand the mechanism underpinning melanoma cell plasticity, it has become clearer to us that the switch between phenotypes is driven by a collaborative reprogramming of the transcriptional, the epigenetic, the translational, and the metabolic regulatory networks (13). In line with the complexity of these networks, blocking multiple drivers of the invasive switch is anticipated, and has been shown, to have better efficacy in reversing phenotype switching than blocking any single driver (111, 136, 139). Similarly, targeting an upstream regulator of the invasive switch has shown superior efficiency than targeting a downstream effector (9). Alternatively, a more direct strategy is to co-target multiple cell states, which has become feasible due to markers defining each cell state (**Figure 1** and **Table 1**). However, a real challenge is, while many state-specific makers have been identified, not all of them are real drivers of phenotype switching. For example, the fatty acid translocase CD36 is a hallmark of the SMC state, which is shown to have an altered metabolic profile (16). It is therefore tempting to speculate that CD36 is a functional driver of the

SMC state, perhaps by enabling more efficient uptake of fatty acids and hence confer a selective advantage during therapy (3, 143). However, a recent study suggested that while MAPKi-induced CD36⁺ melanoma cells have increased fatty acid oxidation (FAO), CD36 is not functionally involved in the FAO changes (77). Therefore, inhibitors or neutralizing antibodies against CD36 might not be able to eliminate the SMC population. Rather, a CD36 antibody-drug conjugate, similar to enapotamab vedotin, might be more effective. It is hence important to conduct more studies to distinguish drivers of phenotype switching from melanoma cell state-specific markers.

Another important aspect is the translational control of melanoma plasticity. Whereas the transcriptional, the epigenetic, and the metabolic reprogramming of melanoma phenotype switching have been comprehensively investigated (13), few studies highlight the translational regulation of this process (9, 146). As summarized in **Figure 3**, ATF4 needs to cooperate with reduced energy demand, exhibiting by an inhibition of global mRNA translation, to drive the invasive switch (9). However, blocking the upstream p-eIF2 α alone was sufficient to decrease invasion (9), indicating additional regulators downstream of p-eIF2 α . Moreover, under nutrient sufficient conditions, cap-dependent global translation is activated, driving melanoma cells to the proliferative state (9, 148). Our recent study suggested, even under such seemingly suitable conditions, melanoma cells could still switch to an invasive state through the MNK1/2-eIF4E axis (146). Notably, phosphorylation of eIF4E doesn't affect global mRNA translation (149), indicating this invasive switch occurs while maintaining high energy demand (178). Do these two distinct translational regulators lead to different invasive cell states? It is tempting to hypothesize that the p-eIF2 α -driven switch leads to a starved-like phenotype, resembling the CD36⁺ SMC state *in vivo* (16, 77), whereas the p-eIF4E-driven switch leads instead to the NGFR^{high} NCSC-like state (14, 16, 79). Further investigation is needed to demonstrate the role of these two translational factors in mediating melanoma phenotype switching.

Finally, uveal melanoma is a complex disease where the role of cell plasticity has not been sufficiently explored. Although exhibiting different genomic mutations from cutaneous melanoma, *GNAQ* and *GNA11* mutations in UM ultimately lead to the hyperactivation of MAPK and PI3K signaling (209). Similar microenvironmental stress conditions such as hypoxia, nutrient limitation, and chronic inflammation are also commonly observed in UM. Thus, UM cells are likely to undergo similar stress-induced phenotypic plasticity. Indeed, many genes that are known to determine invasiveness of cutaneous melanoma cells have also been reported to drive UV progression, such as transcription factors AP-1 and TEADs (219–221), EMT-like markers ZEB1, TWIST1, and SNAIL (214), Notch signaling, and extracellular factors TGF- β , IL-6 and IL-8 (218). These data suggest a potentially significant overlap in plasticity regulators between cutaneous melanoma and UM. Notably, no targeted therapy or immunotherapy has shown efficacy in UM management. Several clinical trials using

new molecules, inhibitors alone or in combination are being developed based on preclinical work. MEK inhibitors when combined with dacarbazine or other chemotherapy agents failed to improve progression free survival in UM (226). However, new combination treatments co-targeting MEK with PKC, FAK, GNAQ/11 and other main pathways such as YAP have revealed promising new therapeutic vulnerabilities that can be exploited in a clinical manner (NCT03875820) (227–229). Remarkably, a state-of-the-art immunotherapy agent, Tebentafusp, is showing clinical benefit in patients with advanced UM. Tebentafusp is a molecule that consists in a TCR targeting domain (specific for an antigen of interest) and single-chain variable fragment (scFv) anti-CD3 effector domain. The soluble TCR is designed to bind to GP100, presented by HLA-A*02:01 UM, where the CD3⁺ effector domain will later bind and activate CD3⁺ T cells (230). One might speculate that, similar to immunotherapy resistance in cutaneous melanoma, that the loss of GP100 in UM cells through phenotype switching could lead to Tebentafusp resistance. Therefore, blocking UM

phenotype switching is expected to overcome drug resistance, and potentially reduce liver metastasis and sensitize UM cells to future therapies.

AUTHOR CONTRIBUTIONS

FH and FS wrote the manuscript. RF wrote sections of the manuscript. FH made figures and tables. SR supervised this study and revised the manuscript. All authors contributed to the article and approved the submitted version.

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