ERBB4 is a Driver of *BRAF* WT Melanomas

by

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Abstract

Approximately 50% of metastatic melanomas harbor a *BRAF* V600 mutation which causes elevated RAF/MEK/ERK pathway signaling. These tumors are treated using a combination of a BRAF and MEK inhibitor which targets the canonical RAF/MEK/ERK pathway. However, the other ~50% of metastatic melanomas which possess wild-type (WT) *BRAF* alleles are just as aggressive as *BRAF* V600 mutant tumors but have no targeted therapeutic available beyond immune checkpoint inhibitors.

ERBB4 (HER4) is a receptor tyrosine kinase that is closely related to the epidermal growth factor receptor (EGFR/ERBB1/HER1), ERBB2 (Neu/HER2), and ERBB3 (HER3). EGFR and ERBB2 are well-established oncogenes and therapeutic targets in multiple tumor types. Our *in silico* analyses of *BRAF*-WT tumor genomes suggest that increased transcription of *ERBB4* drives *BRAF* WT melanomas via cooperation with elevated RAS/RAF/MAPK pathway signaling via mutation in a *RAS* or *NF1* gene. Therefore, we obtained a panel of *BRAF* WT melanoma cell lines that harbor *RAS* or *NF1* mutations and showed that ERBB4 signaling is both sufficient and necessary for clonogenic proliferation and ERBB4 is necessary for anchorage-independent colony growth.

Our *in silico* analysis found that *ERBB4* mutant alleles appear to be associated with increased stimulation of the PI3K/Akt canonical pathway and also cooperate with *NF1* or *RAS* gene mutations. Our *in silico* analyses have determined which *ERBB4* mutants found in *BRAF* WT melanomas are priority candidate tumor drivers. We selected nine high-priority mutations and one known gain-of-function positive control mutation and introduced them to the MEL-JUSO *BRAF* WT, ERBB4-dependent melanoma cell

line. We found that introduction of some of these mutations causes increased oncogenic activity greater than WT *ERBB4*.

ERBB4 is known to be a context-dependent oncogene and tumor suppressor where EGFR-ERBB4 and ERBB2-ERBB4 heterodimers are oncogenic and ERBB4-ERBB4 homodimers are tumor suppressive. We introduced the constitutively homodimerized *ERBB4* Q646C mutant to the MEL-JUSO cell line and found that indeed, MEL-JUSO cells do respond to the Q646C mutant with tumor suppressor activity.

Together these experiments suggest that there exists a novel class of *BRAF* WT, ERBB4-dependent melanomas where ERBB4 function is tightly regulated to mitigate ERBB4 homodimer-mediated tumor suppressor activity and that these melanomas may be effectively treated with a combination MEK inhibitor and ERBB receptor inhibitor.

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List of Abbreviations

ATCC	American Type Culture Collection
BaF3	Mouse pro-B-lymphocyte cell line
BRAF	BRAF member of RAF proteins
BRAF	Human gene that encodes BRAF protein
BSA	Bovine serum albumin
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor tyrosine kinase
EGFR	Gene that encodes for EGFR receptor tyrosine kinase
ErbB	ErbB family of receptor tyrosine kinases
ERBB	Genes that encode for the ErbB family of receptor tyrosine kinases
ErbB1	A.k.a. EGFR
ErbB2	Receptor tyrosine kinase ErbB2
ERBB2	Gene that encodes for the ErbB2 receptor tyrosine kinase
ErbB3	Receptor tyrosine kinase ErbB3
ERBB3	Gene that encodes for the ErbB3 receptor tyrosine kinase
ErbB4	Receptor tyrosine kinase ErbB4
ERBB4	Gene that encodes for the ErbB4 receptor tyrosine kinase
ERK	Extracellular signal-related kinase
FDA	US Food and Drug Administration
GAP	GTPase activated protein
HEK 293T	Human embryonic kidney 293 cell line with SV40 T-antigen

HER2	A.k.a. ErbB2
HER3	A.k.a. ErbB3
HER4	A.k.a. ErbB4
HRAS	Gene that encodes for the HRAS protein
HRP	Horseradish peroxidase
IC_{50}	Inhibitor concentration that reduces response by half
IL3	Interleukin 3
KRAS	Gene that encodes for the KRAS protein
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated kinase
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
NCI	National Cancer Institute
Neu	A.k.a. ErbB2
NF1	Gene that encodes for the NF1 protein
NIH 3T3	Mouse embryonic fibroblast cell line
NRAS	NRAS member of RAS proteins
NRAS	Gene that encodes for the NRAS protein
NRG1β	Neuregulin 1beta
NRG2β	Neuregulin 2beta
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
PI3K	Phosphoinositide 3-kinase

RAF	RAF kinase family
RAS	RAS protein family
RPMI	Roswell Park Memorial Institute
RTK	Receptor tyrosine kinase
shRNA	Short hairpin RNA
SKCM	Skin cutaneous melanoma
TCGA	The Cancer Genome Atlas
TMB	Tumor Mutational Burden

Chapter 1: Introduction

1.1. Metastatic Melanoma

Melanoma of the skin makes up ~5% of all new cancer cases in the United States. Melanoma has close to a 100% survival rate when detected in its early stages. However, late-stage metastatic melanomas have a dramatically smaller survival rate of around 30% [1, 2]. Over the past few decades, our understanding of the driving mechanisms of melanoma has vastly improved. However, select groups of melanomas exhibit driving forces that are either poorly understood, difficult to target, or both. As expected, melanoma patients who fall into these categories have poorer prognoses. Here we present the current first-line therapeutic strategy space for various categories of late-stage melanomas and leverage our existing knowledge of ERBB family receptor function to develop a hypothesis regarding ERBB4 function in melanoma.

1.2. Melanoma Signaling Mechanisms and Associated Treatment Strategies

The recommended first-line therapy for late-stage melanomas largely centers around the RAS/RAF/MAPK signaling pathway function, as this pathway is commonly dysregulated in melanoma [3]. Therefore, per the 2022 NCCN guidelines, stage III and IV melanomas are recommended to undergo mutational testing for actionable targets. This includes the testing of *BRAF* and *NRAS* [4, 5].

The RAS/RAF/MAPK pathway regulates cell proliferation and migration, apoptosis, and survival signaling [6]. In brief, receptor tyrosine kinases (RTKs) dock guanine nucleotide exchange factors (GEFs) at binding sites on their cytoplasmic tail, thereby holding them at the plasma membrane. Upon binding to the RTK, these GEFs

conformationally change so that they can bind the GTPase, RAS, and facilitate GTP binding to RAS. The binding of GTP to RAS causes a conformational switch that puts RAS in its active state [7]. The bound GTP is then hydrolyzed such that GTP becomes GDP. This hydrolysis event resulting in GDP-bound RAS switches RAS to its inactive state. Hydrolysis of bound GTP to GDP can be either facilitated by the intrinsic GTPase activity of RAS or catalyzed by GTPase-activated proteins (GAPs), such as NF1 [8]. When RAS is in its GTP-bound, active state, it is available to activate RAF proteins. RAF proteins are serine-threonine kinases which, when activated, dimerize, and canonically activate another serine-threonine kinase, mitogen-activated protein kinase 1/2 (MEK 1/2), which activates a third serine-threonine kinase, extracellular signal-regulated kinase 1/2 (ERK 1/2). ERK activation initiates many downstream cellular signaling mechanisms, including a feedback loop that regulates RAS activity. Together, MEK and ERK proteins are also defined as MAPK proteins [8, 9]. Many melanomas are addicted to the signaling of this pathway via mutations in *RAS*, *NF1*, or *BRAF*. Gain-of-function oncogenic RAS mutations cause a fundamental change in the intrinsic ability of RAS to hydrolyze bound GTP such that RAS is more often in its active state. Loss-of-function mutations in *NF1* decrease the amount of GAP activity, thereby also increasing the amount of time RAS is in its active state. Finally, gain-of-function RAF mutations alter the conformation of RAF proteins such that it is constitutively active. In melanoma, *RAS* mutations and *RAF* mutations (most commonly in *NRAS* and *BRAF* isoforms of RAS and RAF) are known to occur in a mutually exclusive manner [10].

1.2.1. RAS-dependent Melanoma Treatment Strategies

Mutations in *RAS* and *NF1* cause elevated RAS signaling, which is associated with a more aggressive phenotype. Melanomas which harbor these mutations are particularly difficult to treat. The RAS family of protein isoforms includes NRAS, HRAS, and KRAS. *NRAS* is the most mutated RAS gene found in melanomas, although mutated *HRAS* and *KRAS* play driving roles in some melanomas and various other cancers [6].

NRAS-mutated melanomas make up ~20-40% of melanomas. *NRAS* hotspot mutation sites include Q61 as the most common, with G12 and G13 mutations occurring less often [6, 10-12]. *NRAS* mutant melanomas have been treated with a MEK inhibitor plus immune checkpoint inhibitors and these treatments have mostly failed to improve overall patient survival, leaving *NRAS* mutant melanoma patients without any accepted targeted therapeutic strategy beyond immune checkpoint inhibition [10]. However, there is a considerable amount of pre-clinical and clinical work regarding the treatment of *NRAS* mutant melanoma patients with MEK inhibitors in combination with various other targeted therapeutics [6, 10].

RAS stimulates the catalytic subunit of phosphoinositide 3-kinase (PI3K), p110α. In fact, an active p110α binding site is required for mutant RAS-driven tumorigenesis *in vivo* [13]. Activation of the PI3K signaling pathway also requires the activation of the PI3K regulatory subunit, p85. The regulatory and catalytic subunits must both be active to stimulate signaling. To determine whether RAS stimulation of p110α is important to tumor genesis and progression, Downward and colleagues developed cell models and mouse models which harbored mutant p110α that is unable to bind RAS [13]. They found that these mutants disrupted growth factor mediated signaling of the PI3K pathway

in vitro. Cells expressing mutant p110α also exhibited significantly less Akt phosphorylation, a molecule phosphorylated downstream of PI3K activity. Furthermore, these cells exhibited a decrease in PIP_3, an indicator of PI3K activity. In mice expressing RAS-activating mutants, the co-expression of a p110α loss-of-function mutant caused a significant increase in apoptosis occurrence compared to those that co-express WT p110α [13]. This suggests that RAS activation of the PI3K p110α subunit is important for tumor maintenance and growth. Furthermore, inducing the loss of p110α interaction with KRAS in KRAS-driven lung cancers in mice caused tumor regression and tumor stasis. Treating these mice with a MEK inhibitor caused greater tumor regression [14]. Thus, combination therapy using a PI3K inhibitor and MEK inhibitor may be useful for treating RAS mutant tumors.

Although it appears that RAS plays a role in activating both the PI3K and RAF/ERK/MAPK signaling pathways in some cancers, there is evidence that there are other mechanisms of PI3K pathway activation where it is not an effector of RAS. In KRAS-driven colorectal cancer cell lines, silencing mutant KRAS does not cause a decrease in Akt phosphorylation, suggesting that other signaling mechanisms are responsible for PI3K/Akt signaling, such as RTK signaling [15]. Thus, although mutant RAS exhibits some PI3K pathway stimulation activity, some tumors also rely on other mechanisms to cause increased PI3K activity. Despite the conflicting evidence regarding the origin of PI3K pathway stimulation in RAS mutant tumors, various clinical trials are underway to evaluate the treatment of RAS-mutant tumor patients with PI3K pathway inhibitors in combination with MEK inhibitors [6, 16].

The *RAS* mutant cancer research field has most recently focused on developing *RAS* mutant-specific inhibitors of RAS. Thus far, the only FDA-approved RAS inhibitor targets the KRAS protein resulting from the *KRAS* G12C mutation. However, this mutation is not associated with melanoma but is associated with lung and colorectal cancers [6]. This KRAS inhibitor functions by covalently binding to a site formed by the mutation from a glycine to a cysteine residue on KRAS. These inhibitors have had some therapeutic success, although long-term results have not yet been observed.

1.2.2. RAF-dependent Melanoma Treatment Strategies

Mutations in *BRAF* cause elevated RAF/MAPK signaling and the treatment of these *BRAF* mutant melanomas with BRAF inhibitors in combination with MEK inhibitors has mostly been therapeutically successful.

The most common *BRAF* mutation is the *BRAF* V600 mutation which occurs in ~50% of melanoma patients. Roughly 80% of *BRAF* mutant patients carry the *BRAF* V600E mutation, while ~10-20% carry *BRAF* V600K or some other less common nonsynonymous mutation at that site [1, 10]. Patients who present with advanced melanomas which harbor one of these *BRAF* V600 mutations are treated with a combination therapy of a BRAF inhibitor such as dabrafenib or vemurafenib and a MEK inhibitor such as cobimetinib or trametinib. This treatment strategy has resulted in a significant improvement in the overall survival of *BRAF* mutant metastatic melanoma patients [1, 10, 12]. These *BRAF* mutations often coincide with an inactivating mutation in *PTEN*, a tumor suppressor gene that inhibits signaling by PI3K in the PI3K/AKT/mTOR signaling pathway, thereby increasing signaling by PI3K [1].

1.2.3. Triple-WT Melanoma Treatment Strategies

Triple wild-type melanomas are those that do not harbor *BRAF*, *NRAS*, or *NF1* mutations. They make up less than 10% of melanomas and are more common in acral and mucosal melanoma subtypes. Their study is out of the scope of this work [3, 10, 17].

1.2.4. Immune Checkpoint Inhibitor Use in Melanoma

Immune checkpoint inhibitors have been particularly successful at improving the overall survival of patients diagnosed with advanced melanoma. NCCN guidelines suggest the use of immune checkpoint inhibitors as a first-line therapy for melanoma patients with unresectable or metastatic disease [5]. In a phase 3 study of melanoma patients diagnosed with advanced melanoma (CheckMate 067 trial), the combination therapy of nivolumab and ipilimumab (CTLA-4 inhibitor) resulted in an increased 3-year overall survival rate. Patients given the combination therapy had a 3-year OS of 58% as opposed to 52% when given nivolumab alone and 34% when given ipilimumab alone [18]. This study exemplifies the importance of identifying the correct immune checkpoint inhibitor. Because of this study, the NCCN guidelines were adjusted to suggest that ipilimumab should not be given alone and only in combination with nivolumab.

Although the clinical utility of immune checkpoint inhibitors is broad in melanoma, there is still a need to define biomarkers for their most efficacious use and targets for combination therapy to increase the efficacy of immune checkpoint inhibitors. Increased tumor mutational burden (TMB) is a predictive biomarker for immunotherapeutic response in non-melanoma solid tumors [19]. However, likely due to melanoma's inherent elevated TMB, this has not proven to be an infallible biomarker for effective immune checkpoint inhibitor use in melanoma [1, 16]. Quantifying programmed death ligand 1 (PD-L1) expression has been employed broadly as an indicator of the

clinical efficacy of immune checkpoint therapy where elevated expression is associated with success. Although this trend has been observed, it has not been proven to predict therapeutic success consistently. The measure of tumor-infiltrating lymphocytes (TILs) has been evaluated as a measure of therapeutic response with the expectation that more TILs would be associated with increased therapeutic response. However, recent work has found that these TILs are largely a heterogeneous mixture of both cytotoxic T cells which increase the efficacy of immune checkpoint inhibitors, regulatory T cells (Tregs) which decrease the efficacy of immune checkpoint inhibitors, and exhausted T-cells [1]. In general, melanoma is known to create an increased number of exhausted T-cells, which are not associated with a better therapeutic response to immune checkpoint inhibition [16]. Furthermore, patients who harbor a *BRAF* V600 mutation and an inactivating *PTEN* mutation have been shown to have decreased antitumoral immune cells leading to decreased immune targeted therapeutic efficacy [1]. This suggests that the increased activation of the RAF/MAPK pathway in combination with the PI3K pathway is associated with immune cell evasion. Unfortunately, for those patients who do not harbor a *BRAF* V600 mutation, immune therapy is the only therapeutic option [10].

1.3. The Gap in Melanoma Treatment

Although there have been great improvements in the development of targeted therapeutics and identification of actionable biomarkers in melanomas, there remains a significant gap in targeted treatment opportunities, specifically for *BRAF* WT melanoma patients [10].

1.3.1. Biomarkers for Treatment are Likely Found in the RAS/RAF/MAPK or PI3K/Akt Signaling Pathways

One of the main difficulties in identifying driving alterations in melanomas is the high mutation rate associated with melanomas [16]. Therefore, melanomas have thus far only been classified by well-understood oncogenic driver mutations that occur in large percentages of melanoma patients, such as the aforementioned *BRAF*, *NRAS*, and *NF1* mutations.

Because increased activation of the MAPK pathway is common in advanced *BRAF* WT melanomas, there has been an increase in studies of combination therapies with chemotherapy and targeted MAPK inhibitors [6]. In one such study, the combination of paclitaxel and trametinib was associated with improved progression-free survival and overall response rate but was not associated with improved overall survival [20]. The PI3K/Akt pathway has also recently become a candidate for targeted therapeutics in melanoma. Because melanomas tend to rely on the RAS/RAF/MAPK pathway and there is evidence that melanomas also rely on the PI3K/Akt signaling pathway, molecular targets and biomarkers of therapeutic success likely lie upstream or downstream of either of these pathways [6, 21].

1.4. Receptor Tyrosine Kinases in Cancer

Receptor Tyrosine Kinases (RTKs) make up a large family of more than 50 identified proteins in humans that share similar structures and signaling mechanisms. RTKs are common therapeutic targets for many cancer types and stimulate many signaling pathways [22, 23]. These transmembrane receptors are generally stimulated by

receptor-specific ligand binding at an extracellular ligand binding domain, which then prompts receptor dimerization and induces a conformational change to an "active" receptor state. In the active state, the kinases then phosphorylate tyrosine residues within the cytoplasmic domain. Tyrosine phosphorylation may occur as autophosphorylation or as a trans-phosphorylation event between multiple dimerized or oligomerized receptor monomers. Phosphorylated tyrosine residues then recruit effector molecules that have a SRC homology domain 2 (SH2) or phosphotyrosine binding domain (PTB) to bind and initiate downstream signaling pathways [24, 25]. An analysis of RTK tyrosine phosphorylation in melanoma cell lines found in the Yale SPORE (Specialized Program of Research Excellence) in Skin Cancer determined that several families of RTKs exhibit greater phosphorylation in melanoma compared to normal melanocytes suggesting that they are more active. These families include the Insulin Receptor family, ERBB family, MET family, and TAM family [26]. Many RTKs exhibit overlapping signaling mechanisms, and there is increasing evidence of multiple feedback and feedforward networks made up of multiple RTKs, protein tyrosine kinases (PTKs), and other proteins [22].

RTKs are associated with many signaling pathways, such as the PI3K/Akt pathway, RAS/RAF/MAPK pathway, and JAK/STAT pathway, among many others. RTK activation of the PI3K signaling pathway is linked to a broad set of cell regulatory systems, many of which are implicated in the oncogenic activity of a multitude of cancer types, including melanoma [23, 25, 27]. RTKs activate the PI3K regulatory subunit, p85, via binding of p85 to its phosphotyrosines. The p85 subunit harbors two SH2 binding domains, thereby allowing its binding to phosphorylated RTKs [28]. The p85 subunit

binds an active catalytic subunit, p110 (as described in section 1.2.1.), to form a heterodimer and create the PI3K complex. This complex is inactive due to p85-mediated inhibition of p110. Binding the p85/p110 complex to an RTK phosphotyrosine relieves p85-mediated inhibition of p110, thus allowing PI3K signaling [29]. Active PI3K canonically phosphorylates a second messenger, PIP_2, to become PIP_3 which then recruits Akt to the plasma membrane, thereby allowing various effector molecules to phosphorylate Akt causing specific downstream signaling events [30]. The PI3K/Akt signaling axis is implicated broadly in oncogenic signaling. The lipid phosphatase, PTEN, is a tumor suppressor that dephosphorylates PIP_3, thereby inhibiting PI3K-mediated PIP_2 activation. Loss-of-function mutations in *PTEN* result in increased PI3K signaling activity [31].

1.4.1. The ERBB Family of RTKs

The ERBB receptor tyrosine kinase family has been particularly well studied in the oncology field. This family consists of the epidermal growth factor receptor (EGFR/ERBB1/HER1), ERBB2 (HER2, neu), ERBB3 (HER3), and ERBB4 (HER4) [32]. These receptors are mainly expressed at the cell membrane. When unbound to ligand, they exist in a conformational equilibrium that shifts between the open and closed extracellular domain conformations. As the closed conformation is more stable, ~95% of non-ligand bound ERBB receptors are in the closed conformation at any point. Ligand binding stabilizes the receptors in the open conformation. Receptors in the open conformation have exposed dimerization motifs at extracellular domains II and IV (Figure 1). When exposed, the dimerization motifs of two receptor monomers interact to form an ERBB receptor heterodimer or homodimer. The extracellular domains of the

receptor monomers dimerize in a symmetric manner whereas the intracellular domains dimerize in an asymmetric manner. The asymmetric intracellular dimerization event designates one monomer as the regulatory/substrate monomer and the other monomer as the catalytic monomer [32]. The catalytic monomer then trans-phosphorylates the carboxy-terminal tyrosine residues of the regulatory monomer. The phosphorylation of these residues induces conformational changes, which create docking sites for various effector molecules that harbor SH2 or PTB domains and subsequently trigger downstream signaling cascades (Figure 2) [32].

ERBB receptor-ligand binding is a source of signaling specificity. ERBB receptor ligands are members of the EGF family of peptide growth factors, including amphiregulin (AREG), betacellulin (BTC), EGF, epigen, epiregulin (EREG), heparin-binding EGF-like growth factor (HBEGF), neuregulin1 (NRG1), neuregulin2 (NRG2), neuregulin3 (NRG3), neuregulin4 (NRG4), and transforming growth factor α (TGFα). Additionally, NRG1 and NRG2 have functionally distinct splicing isoforms, NRG1α, NRG1β, NRG2α, and NRG2β. Although ERBB2 does not bind any of these growth factors and has no known ligand, EGFR, ERBB3, and ERBB4 exhibit variable ligand binding affinities that allow for signaling specificity (Figure 2) [32].

Variable receptor dimerization circumstances result in signaling specificity as well. Active (open conformation) ERBB receptors can dimerize with any member of the ERBB family, creating homodimers or heterodimers. Variation in ERBB receptor dimerization partners and subtle variation in the position of receptor monomers within a dimer, causes functionally distinct variations in the phosphorylation of the regulatory monomer. ERBB3 exhibits very low levels of kinase activity and is therefore described as

"kinase-dead" (Figure 2). This lack of kinase activity means ERBB3 mainly functions as a regulatory monomer within a heterodimer rather than as a catalytic monomer and/or in a homodimer. ERBB2 is a preferred dimerization partner for all three other ERBB receptors but is well-established as a preferred oncogenic dimerization partner for ERBB3 [32-34].

1.4.2. ERBB Family Alterations in Cancer

The ERBB family of receptors has been studied for their importance to tumorigenesis, tumor maintenance, and metastasis; particularly in solid tumors [35]. EGFR and ERBB2 are well-established therapeutic targets in multiple tumor types. Both receptors exhibit oncogenic activity due to increased signaling. This increase in signaling activity can result from receptor mutation, increased receptor expression, or increased ligand activity. However, ERBB3 and ERBB4 function in tumors has been comparatively less thoroughly evaluated.

1.4.3. EGFR and ERBB2 Alterations in Cancer

EGFR and ERBB2 are among the most frequently amplified genes in all cancer types [36]. Amplification of the *EGFR* gene resulting in increased EGFR expression frequently occurs in glioblastoma (GBM) and is associated with angiogenesis-independent invasiveness and increased secretion of VEGFA, an angiogenic factor [37]. Amplification of the *ERBB2* gene resulting in increased ERBB2 expression frequently occurs in breast, ovarian, bladder, and gastric tumors [27, 36, 38, 39]. *EGFR* and *ERBB2* are also frequently mutated in some cancers. These mutations are often single-point missense mutations, frameshifts, or deletions.

In melanoma, EGFR has been shown to have some oncogenic activity, whereas ERBB2 appears to have very little involvement in tumor progression despite its well-documented involvement in many other epithelial tumor types. In a phospho-proteomic screen of melanoma cell lines, EGFR was identified as being significantly more active in melanoma as opposed to normal melanocytes [26]. Additionally, it has been observed that acquired overexpression of EGFR is a potential mechanism of BRAF and MEK inhibitor resistance found in *BRAF* V600 mutant melanomas. Thus, using EGFR inhibitors to overcome BRAF and MEK inhibitor resistance is being explored [40, 41]. There is a limited understanding of ERBB2 function in melanoma as it's generally not overexpressed or mutated. However, inhibition of ERBB2-ERBB3 heterodimeric signaling has been suggested as a method to treat melanomas which are both *BRAF* and *NRAS* WT [42].

1.4.3.1.EGFR Mutations in Cancer

The *EGFRvIII* mutation is a deletion of amino acids 6-273 (exons 2-7) with the addition of a new glycine residue between amino acids 5 and 274. This results in the loss of the ligand binding domain and a constitutively active receptor. *EGFRvIII* and *EGFR* WT are often jointly amplified in GBM. This amplification causes increased stochastic EGFR homodimerization, resulting in elevated EGFR homodimer signaling [39].

Single point mutations R108K, A289V/D/T, and G598D are found in a large proportion of glioblastomas, although not as often as the *EGFRvIII* mutation. Similar to the *EGFRvIII* mutation, these point mutations result in a shift in the conformational equilibrium of EGFR in the cell to favor the open, active extracellular domain conformation, thereby allowing for more EGFR dimerization [39]. Interestingly,

mutations are thought to occur after the amplification of *EGFR* WT, and therefore mutations are often found in cases where EGFR is already over-expressed. Furthermore, EGFRvIII kinase activity is not required when both *EGFRvIII* and *EGFR* WT are expressed, and therefore EGFRvIII mainly functions as a substrate for WT EGFR [36, 39].

EGFR mutations such as L858R and the in-frame exon 19 deletion are commonly found in non-small cell lung cancers (NSCLCs) but not in GBM [39]. *EGFR* mutations occur in up to 30% of NSCLCs. Exon 19 deletions and the L858R mutation make up 40-45% of *EGFR* mutations found in NSCLCs. Like the mutations found in glioblastomas, these mutations shift the conformational equilibrium of the receptor. This shift causes a preference for an active conformation of the kinase domain. For example, the L858R mutation causes conformational rearrangement of the activation loop in the kinase domain which indirectly destabilizes the inactive state of the receptor kinase domain and prefers asymmetric intracellular domain dimerization, thereby promoting receptor activation [43, 44].

1.4.3.2.ERBB2 Mutations in Cancer

ERBB2 mutations are the second most commonly mutated ERBB receptor behind *EGFR* in all human tumors [27]. *ERBB2* mutation frequency ranges from 5-13% in a multitude of cancers, including bladder, cervical, colorectal, esophageal, skin, and uterine cancers [45]. *ERBB2* mutations mainly occur in the extracellular and kinase domains, and several hotspot mutation sites cause increased activation of the receptor or enhanced kinase activity [27]. Unlike *EGFR* amplification and mutation in glioblastomas, *ERBB2* mutation occurs in a mutually exclusive manner with increased ERBB2 expression. This

suggests that either increased ERBB2 expression or *ERBB2* mutation cause sufficient levels of increased ERBB2 signaling and that there is no benefit to selecting for both elevated expression and *ERBB2* mutation. Indeed, both overexpression of ERBB2 and *ERBB2* mutations have been shown to induce similar phenotypes and cause increased oncogenicity both *in vivo* and *in vitro*. Both overexpression of ERBB2 and *ERBB2* mutations have been shown to initiate mechanisms of drug resistance as well. However, some of the less common *ERBB2* point mutations do not demonstrate an oncogenic phenotype. Therefore, not all *ERBB2* mutations are activating and some passenger mutations exist. Thus, ERBB2 overexpression and *ERBB2* mutation are independent driver events [45]. Because there is a larger set of oncogenic *ERBB2* mutations compared to *EGFR*, *ERBB2* mutations are defined in terms of classes. Class I mutations are point mutations in the extracellular domain, transmembrane domain, and juxtamembrane domain. Class II mutations are defined as insertion mutations in the kinase domain. Class III mutations are defined as point mutations in the kinase domain. The frequency of each class of mutation varies across different tumor types suggesting selective pressure for specific alterations. *ERBB2* mutations tend to co-occur with mutations in *ERBB3*, *RAF1*, *PIK3CA*, and *PIK3R2* and tend to be mutually exclusive with *KRAS* alterations [45].

1.4.3.3.EGFR Targeted Therapeutics Indicated by *EGFR* Alterations

EGFR-targeted tyrosine kinase inhibitors (TKIs) are approved for use in some cancers. Gefitinib and afatinib are first- and second-generation small molecule ATP-competitive inhibitors that bind to the kinase domain of EGFR. These inhibitors have had a lot of success in treating NSCLCs with the L858R mutation. As previously described in section 1.4.3.1, the gain-of-function EGFR L858R mutation causes a conformational

change in the kinase domain activation loop, resulting in increased receptor dimerization. This creates a tumor with "oncogene addiction" where it relies mainly on EGFR signaling. However, this conformational change also causes decreased ATP binding affinity. This decrease in binding affinity allows gefitinib and afatinib to compete for binding at the ATP binding site, thereby inhibiting the kinase activity of the receptor [43, 44, 46]. Parenthetically, WT EGFR is poorly inhibited by gefitinib due to its inability to compete with ATP binding [47]. Therefore, the presence of the *EGFR* L858R mutation indicates increased sensitivity to first and second-generation TKIs, gefitinib, erlotinib, and afatinib.

Following treatment with a TKI such as gefitinib, afatinib, or erlotinib, many tumors develop the *EGFR* T790M mutation, which results in drug resistance by reportedly increasing ATP binding affinity and simultaneously decreasing TKI binding affinity. This effectively attenuates the drug's ability to compete with ATP for binding [43, 46]. Roughly 55% of resistant tumors acquire the T790M mutation, although it's important to note that there are numerous other methods by which a tumor acquires TKI resistance [43, 46]. To treat this resistance, the 3rd generation TKI, AZD9291/ Osimertinib, was developed as an irreversible inhibitor of EGFR. Osimertinib has had great initial success in overcoming TKI resistance and is now used as first-line treatment for NSCLCs with activating *EGFR* L858R or exon 19 deletion mutations [48, 49]. Unfortunately, it's recently been observed that Osimertinib-resistance develops via the development of the *EGFR* C797S mutation in some patient populations [50, 51]. Osimertinib and other non-covalently binding EGFR TKIs, interact with EGFR at the cysteine residue at EGFR amino acid 797. However, upon mutation of that residue to a

serine, the binding affinity decreases and causes drug resistance [46, 50, 51]. Various combinatorial treatment strategies are currently being evaluated, including concurrent inhibition of EGFR downstream signaling such as MEK and ERK signaling [48]. A fourth-generation TKI, EAI045, has very recently been developed to overcome resistance to osimertinib by binding to the allosteric site outside of the ATP-binding domain. It is effective mainly in combination with cetuximab [41, 50].

EGFR-targeted therapies have not been particularly successful in treating EGFR-amplified or mutant glioblastomas. One of the main obstacles has been poor blood-brain barrier permeability of drugs for treating GBM. As such, clinical trials are underway to improve the bioavailability of EGFR-targeted therapies by delivering these drugs to the tumor using a nanoparticle or liposome-based delivery system [52]. Various clinical trials are underway to evaluate small molecule TKIs for the treatment of GBM. Osimertinib has moderately improved responsiveness as a single-agent therapy in phase I and II clinical trials. However, most EGFR-targeted inhibitors have not shown promising results thus far. It's hypothesized that alternative signaling pathways circumvent EGFR signaling and result in poor response to EGFR-targeted monotherapy [38, 39, 52].

1.4.3.4.ERBB2 Targeted Therapeutics Indicated by *ERBB2* Alterations

Monoclonal antibodies trastuzumab and pertuzumab are approved therapies for *ERBB2* amplified (HER2+) breast cancers [53]. ERBB3 is essential as a heterodimerization partner of ERBB2 in these breast cancer as well [54]. Trastuzumab binds to domain IV on ERBB2 and is known to mainly disrupt ERBB2 homodimer formation and signaling. Pertuzumab binds to domain II of ERBB2 and may disrupt some ERBB2 homodimer formation but is also effective at blocking ERBB2-ERBB3

heterodimer formation [53-55]. Thus, trastuzumab and pertuzumab have complementary mechanisms of action. When HER2+ breast cancer patients are treated with a combination of trastuzumab, pertuzumab, and docetaxel, they have improved progression-free survival, overall survival, and duration of response over treatment with trastuzumab plus docetaxel alone [53, 56, 57]. Small molecule TKIs such as lapatinib, neratinib, pyrotinib, and tucatinib bind ERBB2's intracellular domain and show some success in combination with trastuzumab in HER2+ breast cancers and in patients who previously progressed while being treated with trastuzumab. Many of these TKIs bind to sites that are conserved among EGFR, ERBB2, and ERBB4. Hence, there are some off-target effects. Neratinib and Pyrotinib are both irreversible inhibitors known to target EGFR, ERBB2, and ERBB4 [58]. Tucatinib is a reversible inhibitor that is only known to target EGFR and ERBB2 [58]. Lapatinib is a reversible inhibitor that also targets EGFR, ERBB2, and ERBB4 but inhibits ERBB4 kinase activity to a lesser extent than EGFR and ERBB2. Lapatinib binds the inactive form of ERBB4 near the activation loop of the kinase domain [58, 59]. Although ERBB2 inhibition is mainly approved for use in tumors that exhibit amplified *ERBB2*, there is some evidence that targeting ERBB2 may be helpful in treating *ERBB2*-mutant tumors. However, no small molecule inhibitor has been approved for tumors that harbor a point mutation in *ERBB2* [45, 60].

1.4.4. ERBB3 Alterations in Cancer

Our understanding of ERBB3 function in cancers is much more limited compared to EGFR and ERBB2. However, *ERBB3* has recently emerged as an important oncogene and putative therapeutic target in many cancers. Because ERBB3 has a deficient kinase domain, it requires a heterodimerization partner to stimulate signaling pathways. As

previously discussed, ERBB3 is the known preferred oncogenic dimerization partner for ERBB2, making it an intriguing putative target for ERBB2-dependent breast cancers. ERBB3 has also been suggested to mediate EGFR- and ERBB2-targeted therapy resistance. Although *ERBB3* mutations don't occur as often as *EGFR* or *ERBB2* mutations, they appear to be most common in breast, gastric, ovarian, colon, glioblastoma, squamous carcinomas, and head and neck cancers. Recently, more *ERBB3* mutations have been characterized in various cancer types, and several seem to confer oncogenic properties. However, thus far, ERBB3 has not been approved as a biomarker for therapeutic assignment [27, 61].

The study of ERBB3 function in melanoma has only recently become of some interest, and ERBB3 has been hypothesized to be a driver of melanoma progression. Elevated expression of ERBB3 and its ligand, NRG1, is correlated with poor survival in metastatic melanoma patients [62, 63]. Furthermore, silencing of ERBB3 in *BRAF* mutant melanoma cell lines resulted in fewer metastases formation in mice [62]. Unlike EGFR and ERBB2, ERBB3 can directly bind the p85 PI3K regulatory subunit without requiring adaptor proteins. Silencing ERBB3 in *BRAF* mutant melanoma cell lines causes decreased Akt phosphorylation in the presence of NRG1 stimulation. Stimulation of both *BRAF* WT and *BRAF* mutant cell lines with NRG1 causes increased Akt phosphorylation. Therefore ERBB3 is thought to mainly signal via a NRG1/ERBB3/PI3K/Akt canonical signaling pathway [62]. It's important to note that both ERBB3 and ERBB4 are stimulated by NRG1 and directly bind the p85 PI3K regulatory subunit [32].

1.4.5. ERBB4 Alterations in Cancer

The role *ERBB4* plays in human cancers is ambiguous, as it does appear to function as a context-dependent oncoprotein and tumor suppressor. However, some work has been done to pool the field's knowledge and elucidate ERBB4's putative roles in various cancer types [32]. ERBB4 expression is generally downregulated in aggressive tumors, but elevated expression associated with poorer prognosis has been documented in some tumor types such as brain, colorectal, gastric, head and neck, lung, ovarian, and thyroid cancers, and therefore it's likely that ERBB4 functions as an oncoprotein in those cancers [32]. ERBB4 appears to be required for ERBB2 oncogenic function in some breast cancers [64]. ERBB4 has also been shown to have oncogenic function in pancreatic cancers [65]. However, ERBB4 exhibits tumor suppressive activity when constitutively homodimerized via the *ERBB4* Q646C mutant in breast, pancreatic, and prostate tumor cell lines [65-68].

Unlike *EGFR* and *ERBB2*, *ERBB4* mutations are distributed across the entirety of the *ERBB4* gene in multiple different tumor types, and therefore there are no identifiable hotspot mutation sites indicating oncogenic selection [69]. Thus, there has yet to be a consensus in the field regarding the identification of putative driver *ERBB4* mutations. Several gain-of-function ERBB4 mutations have been identified in lung cancers (Y285C, D595V, D931Y, and K935I). These mutations cause an increase in both basal and NRG1 stimulated ERBB4 phosphorylation when expressed in NIH 3T3 cells or COS-7 cells [70]. In a screen of *ERBB4* mutations found in melanoma patients, several *ERBB4* mutations were found to be gain-of-function and oncogenic when transformed into NIH3T3 cells or SK-MEL-2 human melanoma cells (E317K, E452K, E542K, R544W, E563K, E836K, and E872K). Additionally, melanoma patient-derived cell lines which

endogenously express *ERBB4* mutants (R393W and E563K) responded with greater efficacy to the EGFR/ERBB2 inhibitor lapatinib than those with an endogenous WT *ERBB4* gene [71]. Subsequently, a clinical trial for the treatment of *ERBB4* mutant melanoma patients with lapatinib started recruitment but failed due to a lack of patient accrual [27, 72]. Conversely, ERBB4 mutations found in lung (G802dup) and colorectal (D861Y) cancers are loss-of-function and result in decreased ERBB4 phosphorylation upon ligand stimulation compared to WT ERBB4 when transfected into the MCF-7 and 32D cell lines [73]. Given the conflicting evidence regarding ERBB4 function in cancer, *ERBB4* status is not considered in the therapeutic intervention of any cancer type.

1.5. The Lack of ERBB Exploration in *BRAF* WT Melanoma

Thus far, very little work has been done to study ERBB receptor function in *BRAF* WT melanoma specifically [21, 72]. Despite being established oncogenes in numerous other tumor types, EGFR and ERBB2 have not been validated as oncogenes in *BRAF* WT melanoma. Upregulation in EGFR expression has, however, been suggested as a mechanism of resistance to BRAF and MAPK inhibition in *BRAF* mutant melanomas. Thus, initial clinical studies with EGFR inhibitors have been directed toward the treatment of *BRAF*-mutant melanomas [16, 41]. EGFR has been suggested to be overexpressed in *BRAF* WT melanomas, although this claim has not been sufficiently explored [21].

ERBB3 is reportedly overexpressed and more commonly phosphorylated in melanoma cell lines compared to normal melanocytes. ERBB3 overexpression has also been associated with a poorer prognosis in melanoma. ERBB3 function has been studied

in the context of *BRAF* mutant and dual *BRAF* and *NRAS* WT melanomas. However, ERBB3 has not been thoroughly studied in *BRAF* WT, *NRAS,* or *NF1* mutant melanomas [23, 26, 63].

ERBB4 has gained some attention as a putative driver of melanoma but has never been studied specifically in the context of *BRAF* WT melanomas. It has been reported that *ERBB4* has a mutation rate of ~15-20% in all melanomas [3, 71]. These mutations are reported to coexist with *BRAF* and *NRAS* mutations [3, 74].

1.6. Conclusions: Applying Lessons From EGFR and ERBB2 to ERBB4 in *BRAF* WT Melanoma

BRAF WT melanomas are a particularly understudied subpopulation of melanoma patients. There is a paucity of effective therapeutic options and an equal lack of distinct biomarkers for the few available therapeutic options. Furthermore, the therapeutic options available for these patients does not afford them the same positive outcomes as the targeted treatments available to *BRAF* V600 mutant patients. Many of these *BRAF* WT patients harbor gain-of-function *NRAS* mutations or loss-of-function *NF1* mutations and their tumors are particularly aggressive.

Based on the evidence that many melanomas rely not only on RAS/RAF/MAPK signaling but also PI3K/Akt signaling, the PI3K/Akt signaling pathway presents a viable targetable pathway for all melanoma patients, including *BRAF* WT melanoma patients. However, there is a need to identify the mechanisms by which the PI3K/Akt signaling pathway is initiated to define patient populations that would most greatly benefit from

PI3K pathway inhibition. Furthermore, the most effective therapeutic strategy for PI3K pathway inhibition needs to be elucidated.

ERBB4 is known to stimulate the PI3K pathway and there is some evidence that ERBB4 may in fact drive some subset of melanomas, including *BRAF* WT melanomas. Therefore, it is in the best interest of the *BRAF* WT melanoma patient population that we determine whether ERBB4 functions as a driver of *BRAF* WT melanomas and could be a putative therapeutic biomarker or target.

Given that the ERBB family of receptors share many structural and functional similarities, it is likely that we can leverage our knowledge regarding EGFR, ERBB2, and ERBB3 function to elucidate possible roles of ERBB4 in *BRAF* WT melanoma. Elevated expression patterns are common driving events of ERBB receptors in cancer. Overexpressed WT EGFR, ERBB2, and ERBB3 are implicated in multiple cancers and this overexpression has proven to be a helpful biomarker for therapeutic indication in several instances. Therefore, it is likely that if ERBB4 is indeed oncogenic in *BRAF* WT melanoma, it may be due to elevated expression.

Mutations in *EGFR* and *ERBB2* are also common driving events in various tumor types, as are mutations that develop as drug-resistance mechanisms. Therefore, mutations in *ERBB4* may also cause increased oncogenic activity. Furthermore, *ERBB4* mutations may be acquired as a mechanism of drug resistance. However, *ERBB4* does not exhibit classic “hotspot” type mutations like *EGFR* in NSCLC. Instead, *ERBB4* mutations are scattered across the gene with little recurrence among specific patient populations. This suggests that *ERBB4* driving mutations may need to be classified like ERBB2 mutations, which are defined by mutation location and type. It is important to note that although

EGFR and *ERBB2* mutations have different mutation patterns, most oncogenic mutations result in a similar characteristic where the receptor's conformational equilibrium is shifted towards the open conformation. As such, oncogenic mutations in *ERBB4* may exhibit the same conformational equilibrium change.

1.7. Discussion

The main function of ERBB4 which sets it apart from the other members of the ERBB receptor family is its context-dependent oncogenic and tumor suppressor activity. Given this phenomenon, ERBB4 function in tumors is likely to be tightly regulated to control its tumor suppressor activity. ERBB4 is poorly expressed in melanoma making it easy to conclude that ERBB4 must function as a tumor suppressor and therefore expression is downregulated for the purposes of depressing its tumor suppressor activity. However, it is equally possible that ERBB4 expression is downregulated to exploit ERBB4's oncogenic activity by optimizing the chances that when ERBB4 dimerizes, it forms an ERBB4-EGFR or ERBB4-ERBB2 heterodimer rather than a homodimer. Regulation of ERBB4 ligand expression could also be used to optimize ERBB4 activation. A tumor cell's ability to manage these stochastic ERBB4 dimerization events may be a key driving trait adapted to promote tumorigenesis and tumor progression.

As was previously noted, *ERBB4* mutations occur in up to 20% of melanomas. Interestingly, very few of these are frameshifts, deletions, or truncating mutations. This lack of classic loss-of-function variants in melanoma suggests that ERBB4 is not functioning as a tumor suppressor. If indeed ERBB4 were functioning as a tumor suppressor, we would expect to see both a decrease in ERBB4 expression and a greater

proportion of loss-of-function mutation events. Instead, many of these mutations are point mutations, and many of them do not recur in large patient population analyses. This observation would suggest that ERBB4 is phenotypically irrelevant and harbors mainly passenger mutations. This hypothesis does have some merit, as decreased expression would suggest a tumor's lack of prioritization of energy expenditure on *ERBB4* transcription, and the non-hotspot mutation pattern would suggest a lack of pressure for any specific ERBB4-mediated function. However, this is unlikely based on the existing research on ERBB4 function in melanoma and in other tumor types. What is more likely is that there are indeed some passenger mutations but that there are also an appreciable number of oncogenic mutations which cause increased heterotypic ERBB4 signaling. Like oncogenic mutations found in EGFR and ERBB2, oncogenic ERBB4 mutations may cause a conformational change to prefer the open conformation, thereby creating the same phenotypic effect that increased ERBB4 expression would confer where ERBB4 is more likely to heterodimerize with EGFR or ERBB2. Alternatively, *ERBB4* mutations in the extracellular domain could alter ligand binding affinities to prefer ligand-specific downstream signaling events that are preferred for tumor development and growth. *ERBB4* mutations within the intracellular domain could also optimize oncogenic ERBB4 function by altering the intracellular active conformation such that the asymmetrical dimerization structure causes specific tyrosine residue phosphorylation events that trigger tumor-promoting downstream signaling events.

Several factors aid the effective treatment of EGFR and ERBB2-dependent melanomas. First, there are developed monoclonal antibodies, such as cetuximab, trastuzumab, and pertuzumab, which bind their target receptor and consequently limit

specific activating events such as receptor dimerization and ligand binding. Small molecule inhibitors such as gefitinib and lapatinib were designed to bind EGFR and ERBB2 (respectively) and have some therapeutic effects. However, gefitinib treatment often results in the acquisition of drug resistance mechanisms. Subsequent generations of EGFR-targeted small molecule inhibitors also result in the development of drug resistance mechanisms. Lapatinib and other small molecule TKIs are designed to bind to regions of the ERBB2 receptor intracellular domain which are conserved in EGFR and ERBB4, leading to some off-target effects. However, due to the relatively high concentration of target receptors present in the tumor cells for which these drugs are indicated, the drug binds to its target more often than it binds to off-target proteins.

Treatment of the putative class of ERBB4-dependent tumors, however, is more complicated. There is no approved monoclonal antibody or small molecule TKI specific for ERBB4. ERBB4 also exhibits low concentrations of receptor in the cell, therefore treating ERBB4-dependent melanomas with a reversible small molecule TKI with off-target effects for ERBB4, such as lapatinib, would likely require an unmanageable or toxic dose range.

A more effective therapeutic strategy for treating ERBB4-dependent *BRAF* WT melanoma may come from inhibiting ERBB4 dimer partners. Given that ERBB4 oncogenic activity requires ERBB4-EGFR or ERBB4-ERBB2 heterodimerization, EGFR and ERBB2 monoclonal antibodies, such as cetuximab or pertuzumab which block receptor dimerization, could be effective at also limiting ERBB4 heterodimer-mediated oncogenic activity. Similarly, using small-molecule TKIs may add some therapeutic benefit despite off-target effects. Given that the irreversible TKI, osimertinib has been the

most effective TKI in overcoming TKI-resistant lung cancers, ERBB4-dependent melanomas may also exhibit some therapeutic benefit to being treated with an irreversible TKI that binds somewhat promiscuously in the ERBB family. Whether the drug binds ERBB4 or its heterodimerization partner, ERBB4-heterodimer-mediated signaling would be inhibited. Neratinib is an irreversible inhibitor of EGFR, ERBB2, and ERBB4 and has been FDA-approved for use in HER2+ breast cancers in the adjuvant and metastatic setting. Unfortunately, side effects of neratinib have limited the dosage range [58].

Given that melanomas appear to rely on both RAS/RAF/MAPK pathway signaling and PI3K/Akt signaling, and given that ERBB4 is known to stimulate the PI3K pathway, ERBB4-dependent melanomas may be most effectively treated by combination therapy with MAPK inhibitors and PI3K pathway inhibitors. PI3K pathway inhibition may be achieved via multiple methods including direct PI3K inhibition (Alpelisib), dimer partner monoclonal antibody inhibition (Cetuximab or Pertuzumab), or irreversible ERBB small molecule inhibitor (Neratinib).

1.8. Figures

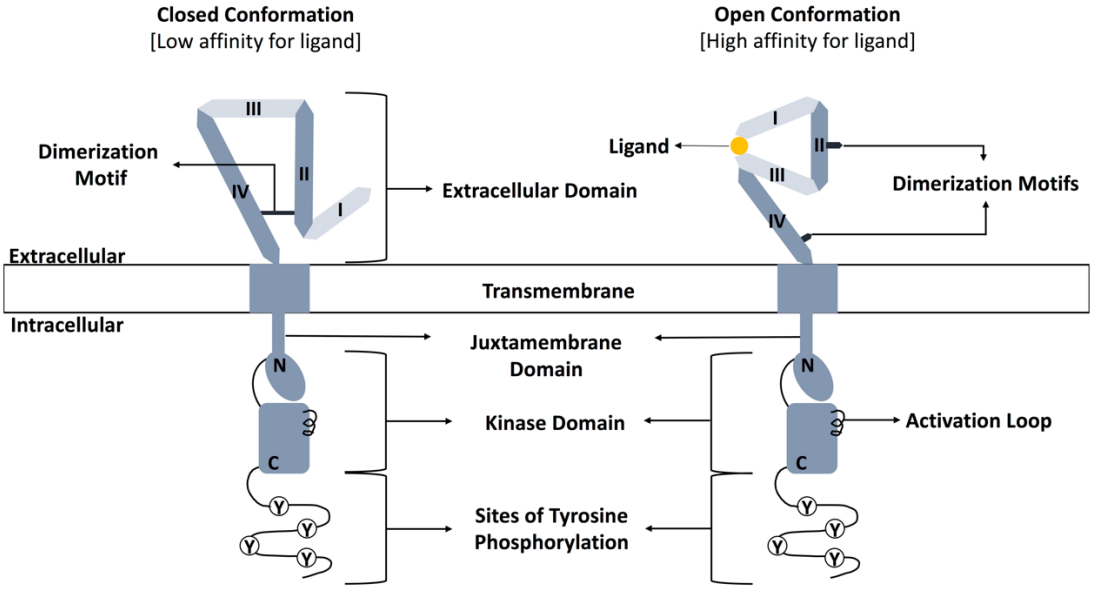

Figure 1. The extracellular domains of ERBB receptors generally exist in an equilibrium between the closed conformation that has low affinity for ligand and buried dimerization motifs and the open conformation that has high affinity for ligand and has exposed dimerization motifs. Adapted from [32].

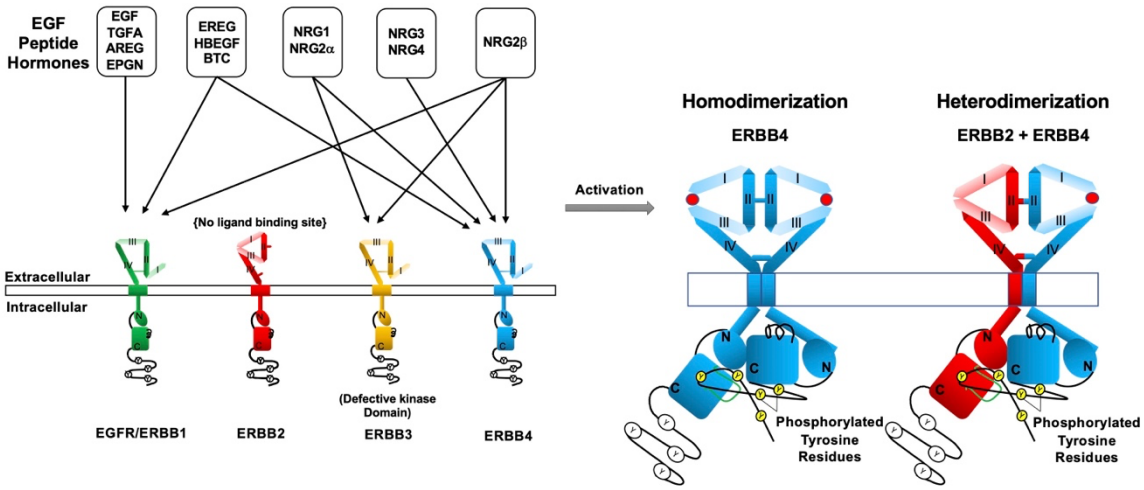

Figure 2. ERBB ligands stimulate ERBB receptor signaling via ERBB receptor homodimerization and heterodimerization. Adapted from [32].

Chapter 2: Wild-type ERBB4 is a driver of *BRAF* WT melanoma cell lines

2.1. Introduction

BRAF WT patients make up ~50% of melanoma patients and are currently left without targeted therapeutic options beyond immune checkpoint inhibitor therapy. The 5-year survival rate of *BRAF* WT patients treated with a combination of immune checkpoint inhibitors is 48%. It's anticipated that this outcome would improve if patients were treated with appropriate targeted therapies in combination with immune checkpoint inhibitors [1, 10, 12, 16]. Therefore, there is a need to identify effective drug targets to inhibit tumor-promoting pathways in *BRAF* WT melanomas so that these metastatic *BRAF* WT melanoma patients can be treated in combination with a targeted therapy and immune checkpoint inhibitor.

Many *BRAF* WT melanomas appear to rely on both the RAS/RAF/MEK/ERK signaling pathway and the PI3K/Akt signaling pathway [16, 75-77]. Mutations in *RAS* and *RAF* isoforms (most often *NRAS* and *BRAF*) occur in a mutually exclusive manner [10]. Mutations in *NRAS* occur in 20-40% of all melanomas with very little overlap with *BRAF* mutations. Thus, a majority of *BRAF* WT melanomas harbor a *RAS* mutation. Unfortunately, these tumors do not respond to MEK inhibitors alone [6, 10]. It's hypothesized that this lack of response to MEK inhibitors is partly due to an increase in activation of the PI3K/Akt signaling pathway which would circumvent the need for functional MEK signaling. Indeed, there is evidence of increased Akt phosphorylation in the presence of an Erk inhibitor and increased Erk phosphorylation in the presence of an mTOR inhibitor in melanoma cell lines suggesting the presence of a synergistic feedback mechanism where the PI3K/Akt/mTOR and RAS/RAF/MEK/ERK pathways work

together to propel tumor development [76]. Combination-targeted therapy of MEK and PI3K/mTOR has proven to be more effective than single-agent therapy in models of melanoma and it's suggested to be a synergistic response [6, 75-77]. The MEK inhibitor, cobimetinib, has been approved for use in *BRAF* V600 mutant melanomas. The FDA-approved PI3K inhibitor, alpelisib, has proven difficult to treat patients with due to adverse events and toxicities [78]. Therefore, the identification of biomarkers that segregate the *BRAF* WT patient population by indicated therapeutic targets is needed to eliminate inappropriate treatment of patients and to improve *BRAF* WT patient outcomes.

The ERBB family of receptor tyrosine kinases is made up of the epidermal growth factor receptor (EGFR/ERBB1/HER1), ERBB2 (HER2, neu), ERBB3 (HER3), and ERBB4 (HER4) [32]. ERBB receptors are single-pass transmembrane proteins with an extracellular ligand binding domain, a transmembrane domain, an intracellular kinase domain, and tyrosine residues near the c-terminus which can be phosphorylated to initiate downstream signaling. These receptors exist in a conformational equilibrium between the open (active) conformation and the closed (inactive) conformation in which the closed conformation is most stable. Upon ligand binding, ERBB receptors (except ERBB2, which does not bind ligand) are stabilized in the open conformation. Receptors in the open conformation dimerize such that the extracellular domains form a symmetrical dimer, and the intracellular domains form an asymmetrical dimer that fosters trans-phosphorylation of one receptor's tyrosine residues by the other receptor's kinase activity. Thus, one receptor is designated the substrate receptor monomer and the other is the catalytic receptor monomer.

The ERBB4 receptor has multiple transcriptional splicing isoforms which confer signaling specificity [32]. There are two different splicing sites in the *ERBB4* transcript which each result in two different isoforms. One splice site is in the extracellular juxtamembrane region resulting in the JMa and JMb isoforms [79]. The second site is in the cytoplasmic region carboxyl-terminal to the kinase domain, resulting in the Cyt1 and Cyt2 isoforms [80]. Thus, there are four *ERBB4* isoforms, JMa-Cyt1, JMa-Cyt2, JMb-Cyt1, and JMb-Cyt2. The JMa and JMb isoforms vary in that the JMb isoform lacks a TNF-converting enzyme cleavage site which allows for ERBB4 intracellular domain (4ICD) cleavage and its subsequent intracellular trafficking and signaling activity. The JMa isoform is fully functional in its ability to cleave the 4ICD [32]. The Cyt1 and Cyt2 isoforms vary in that the Cyt2 isoform lacks a short section of amino acids that contain the phosphorylation site at Tyrosine 1056 which binds the p85 regulatory subunit of PI3K. This is the only p85 binding site on ERBB4 and therefore the Cyt2 isoform is defective in its ability to couple to the PI3K signaling pathway [32]. The Cyt2 isoform also lacks a WW domain binding motif (PPAY) resulting in a decrease in WW protein signaling such as the WWOX tumor suppressor protein [32]. Furthermore, the Cyt1 amino acid sequence that is missing in the Cyt2 isoform is required for the oncogenic activities of ERBB4 [32, 81].

Gene amplification of ERBB family receptors drives oncogenic activity in various tumor types, including *EGFR* amplification in glioblastomas and *ERBB2* amplification in breast cancers [27, 36, 38, 39]. Although ERBB4 gene amplification is not a validated clinical biomarker for tumor aggressiveness, ERBB4 amplification has been reported as an indicator of aggressiveness in colorectal, stomach, head and neck, ovarian, and thyroid

cancers as well as in anaplastic large-cell lymphoma and childhood medulloblastoma [32].

ERBB4 function has been evaluated in a wide range of tumor types with the conclusion that it functions as a context-dependent oncogene and tumor suppressor whereby ERBB4-EGFR and ERBB4-ERBB2 heterodimers exhibit oncogenic activity and ERBB4 homodimers exhibit tumor suppressor activity [32]. In melanoma, ERBB4 has been suggested to have some oncogenic activity, but ERBB4 has not been studied explicitly in the context of *BRAF* WT melanoma [71, 74]. While ERBB4 may play a minor role in melanoma overall, it may play a much more central role in tumorigenesis and/or progression in the absence of an activating *BRAF* mutation [74]. Therefore, we have evaluated ERBB4's oncogenic role in *BRAF* WT melanoma and have determined that some *BRAF* WT melanomas are indeed ERBB4-dependent. These melanomas may benefit from treatment with an inhibitor of ERBB4 signaling in combination with a MEK inhibitor.

2.2. Results

2.2.1. *BRAF* WT Melanomas Do Not Appear to be Less Aggressive Than *BRAF* V600 Mutant Melanomas.

The Cancer Genome Atlas – Skin Cutaneous Melanoma (TCGA-SKCM) dataset contains patient clinical and biospecimen data for 470 cases of skin cutaneous melanomas. We analyzed the TCGA-SKCM dataset to determine whether the group of skin cutaneous melanoma patients whose tumors possess *BRAF* WT alleles presented with greater or equally aggressive tumors compared to the group of melanoma patients

whose tumors possess a gain-of-function *BRAF* V600 mutant allele, therefore suggesting that the *BRAF* WT melanomas found in the TCGA-SKCM dataset are a relevant cohort of patients in which to identify putative clinically relevant tumor drivers (Table 1a).

BRAF WT melanomas account for a slightly greater percentage of cases in the TCGA-SKCM dataset than *BRAF* V600 mutant melanomas. Chi-square analysis indicates that a slightly ($p = 0.1252$) greater percentage of *BRAF* WT melanoma patients had died by the completion of the dataset than *BRAF* V600 mutant melanoma patients (Table 1b). Moreover, chi-square analysis indicates that the AJCC pathologic stage at diagnosis of the *BRAF* WT melanomas was not significantly different ($p = 0.6842$) from the AJCC pathologic stage at diagnosis of the *BRAF* V600 mutant melanomas (Table 1c). Therefore, in TCGA-SKCM, *BRAF* WT melanomas do not appear to be less aggressive than *BRAF* V600 mutant melanomas. This is consistent with findings in other literature [1, 10, 12, 16]. Hence, these *BRAF* WT melanomas pose a significant clinical problem. Given the lack of therapeutic strategy available for this subset of tumors, there is a need to identify biomarkers and therapeutic targets for these tumors. Furthermore, the TCGA-SKCM dataset appears to represent an appropriate group of patients in which we can identify these putative biomarkers and targets.

2.2.2. Elevated ERBB4 Expression is Positively Correlated With *RAS* or *NF1* Mutations.

Gain-of-function *RAS* gene family mutations are common in melanoma, and they do not co-occur with *BRAF* mutations [11]. The *RAS* family isoform, *NRAS,* is the second most mutated driver occurring in ~30-40% of skin cutaneous melanomas [10, 11]. Loss-

of-function mutations in *NF1* occur in about 14% of skin cutaneous melanomas, and these mutations often result in an increase in RAS protein signaling [11].

RTKs typically initiate RAS pathway signaling. Hence, we predicted that elevated ERBB4 expression (which is likely to cause elevated ERBB4 signaling) would be inversely correlated with gain-of-function *RAS* gene mutations or loss-of-function *NF1* mutations in *BRAF* WT melanomas of the TCGA-SKCM dataset. *ERBB4* transcription, *RAS* mutation, and *NF1* mutation data were available for 178 *BRAF* WT melanomas. Based on our analysis of ERBB4 mRNA expression data in the TCGA-SKCM, *BRAF* WT dataset, we defined elevated expression as being >0.12 as defined by the genomic data commons (GDC) HT-Seq workflow (Figure 3) [82]. Contrary to our hypothesis, chi-square analysis indicates that elevated ERBB4 expression (22 melanomas – 12% of the total) is positively correlated ($p = 0.0057$) with a gain-of-function *RAS* gene mutation or a loss-of-function *NF1* mutation in these *BRAF* WT melanomas (Table 2). This correlation suggests that ERBB4 is critically involved in *RAS* and *NF1* mutant melanomas and that ERBB4 signaling is not a mechanism by which the RAS pathway is initiated. Instead, it appears that elevated ERBB4 signaling and elevated RAS pathway signaling cooperate to drive *BRAF* WT melanomas.

2.2.3. Commercially Available *BRAF* WT Melanoma Cell Lines Appear to be Appropriate for Analyses of ERBB4 Function

We obtained a panel of *BRAF* WT melanoma cell lines to identify a model system in which ERBB4 oncogenic signaling can be assessed. In an effort to ensure rigorous and reproducible work, we have used the Broad Institute Cancer Cell Line Encyclopedia (CCLE) [83] to identify six commercially available *BRAF* WT melanoma cell lines.

RNAseq data from the Broad Institute CCLE indicate that these cell lines do not contain gain-of-function mutations in *BRAF* or *PIK3CA* (coding for PI3K catalytic subunit p110α), nor loss-of-function mutations in *PTEN* (coding for a PI3K pathway inhibiting phosphatase); however, they do contain mutations in *NRAS*, *HRAS*, or *NF1* (Tables 3a and 3b). Therefore, these cell lines endogenously exhibit mutations that are known to cause increased RAS signaling activity but not mutations known to cause increased PI3K signaling activity. Endogenous *ERBB4* mutations are present in the MeWo (M766I, R488R, S449F), SK-MEL-2 (R50C), and COLO 792 (G730K, G730R, M313I) cell lines (Table 3b).

RNAseq data from the Broad Institute CCLE (Table 3c) also indicate that these cell lines exhibit different patterns of ERBB gene transcription and ERBB4 ligand gene transcription. Furthermore, there does not appear to be any correlation between these patterns of gene expression and the absence or presence of an *ERBB4* mutation (Tables 3b and 3c).

2.2.4. The MeWo *BRAF* WT Melanoma Cell Line Expresses the JMa-Cyt1 ERBB4 splicing isoform

To determine which ERBB4 splicing isoform is appropriate to study in the context of our *BRAF* WT melanoma cell line panel, we verified the endogenous ERBB4 isoform present using PCR. We hypothesized that the full-length, ERBB4 JMa-Cyt1 isoform would be present in *BRAF* WT melanoma cell lines. To determine which ERBB4 splicing isoform is endogenously present in the human melanoma cell lines, we performed PCR on each cell line's cDNA using primers that are diagnostic for isoform presence. Due to poor endogenous expression of ERBB4 in some of the cell lines, we

were unable to reproducibly detect the endogenous ERBB4 isoform in all the cell lines. However, we were able to reproducibly confirm that the MeWo cell line expresses the JMa-Cyt1 isoform (Figure 4). Therefore, we chose to study ERBB4 function in *BRAF* WT melanoma cell lines in the context of the *ERBB4* JMa-Cyt1 isoform.

2.2.5. ERBB4 is Sufficient and Necessary for the Clonogenic Proliferation of MEL-JUSO, MeWo, IPC-298, and SK-MEL-2 Human Melanoma Cell Lines.

We have previously used clonogenic proliferation assays to measure the phenotypic effects of ERBB4 signaling on human prostate, breast, and pancreatic tumor cell lines [64, 65, 84]. Briefly, we infected MEL-JUSO, MeWo, IPC-298, and SK-MEL-2 *BRAF* WT melanoma cells (Table 3a) with a recombinant amphotropic retrovirus that expresses wild-type *ERBB4* (LXSN-ERBB4-WT), a recombinant amphotropic retrovirus that expresses the *ERBB4* K751M dominant-negative mutant (LXSN-ERBB4-DN), the vector control amphotropic retrovirus (LXSN), or a mock virus preparation. Because the LXSN recombinant retroviral vector contains a neomycin resistance gene, we selected infected cells using G418.

We observed that infection of all four cell lines with LXSN-ERBB4-WT results in greater clonogenic proliferation than infection with the LXSN control retrovirus. Likewise, infection of MEL-JUSO, MeWo, and IPC-298 cells with LXSN-ERBB4-DN results in less clonogenic proliferation than infection with the LXSN control retrovirus (Figure 5).

Using the method described in section 2.4.4. for the MEL-JUSO cell line, we determined that the LXSN-ERBB4-WT retrovirus exhibited a 390% clonogenic proliferation efficiency relative to the LXSN control retrovirus which was normalized to

a 100% clonogenic proliferation efficiency. This increase in clonogenic proliferation efficiency is statistically significant (t-test, p=0.002, n = 9) (Table 4). The LXSN-ERBB4-DN retrovirus exhibited 35.3% clonogenic proliferation efficiency relative to the LXSN control retrovirus. This decrease in clonogenic proliferation efficiency is statistically significant (t-test, $p = 5.14x10^{-7}$, n = 10) (Table 4).

Using the method described in section 2.4.4. for the MeWo cell line, we determined that the LXSN-ERBB4-WT retrovirus exhibited a 564% clonogenic proliferation efficiency relative to the LXSN control retrovirus which was normalized to a 100% clonogenic proliferation efficiency. This increase in clonogenic proliferation efficiency is statistically significant (t-test, $p=1.73x10^{-2}$, n = 5) (Table 4). The LXSN-ERBB4-DN retrovirus exhibited 28.5% clonogenic proliferation efficiency relative to the LXSN control retrovirus. This decrease in clonogenic proliferation efficiency is statistically significant (t-test, $p = 3.19x10^{-4}$, n = 5) (Table 4).

Using the method described in section 2.4.4. for the IPC-298 cell line, we determined that the LXSN-ERBB4-WT retrovirus exhibited a 1340% clonogenic proliferation efficiency relative to the LXSN control retrovirus which was normalized to a 100% clonogenic proliferation efficiency. This increase in clonogenic proliferation efficiency is statistically significant (t-test, p = 0.0257, n = 5) (Table 4). The LXSN-ERBB4-DN retrovirus exhibited 64.3% clonogenic proliferation efficiency relative to the LXSN control retrovirus. This decrease in clonogenic proliferation efficiency is statistically significant (t-test, p = 0.0084, n = 5) (Table 4).

Using the method described in section 2.4.4. for the SK-MEL-2 cell line, we determined that the LXSN-ERBB4-WT retrovirus exhibited a 208% clonogenic

proliferation efficiency relative to the LXSN control retrovirus which was normalized to a 100% clonogenic proliferation efficiency. This increase in clonogenic proliferation efficiency is statistically significant (t-test, $p = 0.0491$, $n = 4$) (Table 4). The LXSN-ERBB4-DN retrovirus exhibited 50.4% clonogenic proliferation efficiency relative to the LXSN control retrovirus. This decrease in clonogenic proliferation efficiency is not statistically significant (t-test, $p = 0.0213$, $n = 5$) (Table 4).

We also analyzed the phenotypic effects of ectopic ERBB4 expression on HMCB and COLO-792 *BRAF* WT melanoma cell lines, thereby completing our panel of six *BRAF* WT melanoma cell lines. We found that ectopic expression of WT ERBB4 or the DN ERBB4 mutant had no effect on clonogenic proliferation efficiency in the HMCB cell line (data not shown). We also found that our retroviral delivery system was ineffective in the COLO 792 cell line, so we could not evaluate the effects of ERBB4 function in that cell line.

These results indicate that *ERBB4* is both sufficient and necessary for the clonogenic proliferation of the MEL-JUSO, MeWo, IPC-298, and SK-MEL-2 *BRAF* WT melanoma cell lines. Hence, targeting ERBB4 or its signaling effectors may be an effective strategy for treating BRAF WT melanomas exhibiting elevated ERBB4 signaling due to elevated ERBB4 expression.

2.2.6. ERBB4 is Necessary for Anchorage Independent Growth of the MEL-JUSO Human Melanoma Cell Line.

We have previously used anchorage independence assays to measure the phenotypic effects of ERBB4 signaling in breast tumor cell lines [64]. Briefly, we seeded stably infected MEL-JUSO cell lines that express the vector control (LXSN), *ERBB4* WT

(LXSN-ERBB4-WT), or the *ERBB4* K751M dominant-negative mutant (LXSN-ERBB4-DN) in a semi-solid medium. We measured the diameter of the resulting colonies and compared the diameters using a t-test (Figure 6).

The colonies resulting from the MEL-JUSO LXSN cell line had an average diameter of 83 μm and a median of 78 μm. The colonies resulting from the MEL-JUSO LXSN ERBB4 WT cell line had an average diameter of 83 μm and a median of 77 μm. The colonies resulting from the MEL-JUSO LXSN ERBB4 DN cell line had an average diameter of 69 μm and a median of 63 μm. The MEL-JUSO LXSN ERBB4 WT colonies were not significantly different from the MEL-JUSO LXSN colonies (t-test, $p = 0.483$). The MEL-JUSO LXSN ERBB4 DN colonies were significantly different from the MEL-JUSO LXSN colonies (t-test, $p = 1.93 \times 10^{-4}$). This is representative of 3 trials where greater than 100 randomly chosen colonies were measured for each cell line per trial (Table 5).

2.3. Discussion

2.3.1. *ERBB4* Appears to Drive *BRAF* WT Melanomas

Our analysis of the TCGA-SKCM dataset suggests that ERBB4 is indeed a driver of *BRAF* WT melanomas. Our analysis also showed that elevated endogenous ERBB4 expression has a significant positive correlation with *NF1*/*RAS* gene mutations. Assuming that elevated ERBB4 expression causes elevated ERBB4 signaling, we postulate that elevated ERBB4 signaling cooperates with elevated RAS signaling to drive *BRAF* WT melanomas.

Given that our analysis of the TCGA-SKCM, *BRAF* WT melanoma dataset showed that some melanomas may be dependent on ERBB4-mediated signaling, we hypothesized that ectopic expression of *ERBB4* WT would enhance the oncogenic phenotype of *BRAF* WT melanoma cell lines and that ectopic expression of an *ERBB4* DN mutant would diminish the oncogenic phenotype of *BRAF* WT melanoma cell lines. Indeed, we found that the ectopic expression of *ERBB4* WT stimulates clonogenic proliferation of the MEL-JUSO, MeWo, IPC-298, and SK-MEL-2 *BRAF* WT melanoma cells greater than the control. In contrast, the *ERBB4* DN (K751M) mutant inhibits clonogenic,c proliferation of MEL-JUSO, MeWo, and IPC-298 *BRAF* WT melanoma cells. Stable expression of the *ERBB4* DN mutant also inhibited anchorage-independent growth of the MEL-JUSO cell line. These results indicate that ERBB4 is both sufficient and necessary for the malignant activity of some *BRAF* WT melanoma cell lines. Interestingly, the HMCB cell line did not respond to ectopic expression of *ERBB4* WT or *ERBB4* DN and therefore may serve as a control for ERBB4-independent oncogenic signaling in *BRAF* WT melanoma.

2.3.2. ERBB4 Signaling Cooperates with Elevated RAS Signaling in *BRAF* WT Melanomas and May be Treated Using a Combination of Targeted Therapeutics

Our data suggest that elevated ERBB4 signaling cooperates with elevated RAS signaling to drive the proliferation of *BRAF* WT melanomas (Figure 7). We predict that ERBB4-dependent, *BRAF* WT melanomas will respond to a combination of a MEK inhibitor with an inhibitor of ERBB4 signaling activity, such as Lapatinib or Neratinib, which are small molecule ERBB receptor inhibitors that have some affinity for ERBB4.

Future work will need to determine whether WT ERBB4 signaling occurs via receptor heterodimerization. If so, inhibition of ERBB4 signaling activity may be best achieved by targeting ERBB4's heterodimerization partner via an anti-EGFR or anti-ERBB2 monoclonal antibody. Work will also need to be done to elucidate the signaling mechanism by which ERBB4-dependent, *BRAF* WT melanomas function. Therapeutics which target this pathway may also be effective.

2.4. Methods

2.4.1. Accession and Analysis of TCGA-SKCM Data

Clinical and biospecimen data was downloaded from The Cancer Genome Atlas – Skin Cutaneous Melanoma (TCGA-SKCM) dataset accessed at https://portal.gdc.cancer.gov/projects/TCGA-SKCM. We obtained the following data for all 470 cases in the SKCM dataset: gender, race, ethnicity, vital status, age at diagnosis, AJCC pathologic stage at diagnosis, primary site, days to death, tumor type sequenced, copy number variation for *ERBB4*, mutation status of *BRAF*, *HRAS, NRAS, KRAS, NF1, EGFR, ERBB2, ERBB3, ERBB4, PIK3CA, PTEN*, and expression of AKT1, AKT2, AKT3, PTEN, PIK3CA, EGFR, ERBB2, ERBB3, and ERBB4. The R statistical computing and graphics environment software (https://www.r-project.org) was used to reorganize the dataset. Statistical analyses were performed using GraphPad Prism (https://www.graphpad.com) and Microsoft Excel (https://office.microsoft.com/excel).

2.4.2. Cell Lines and Cell Culture

Mouse C127 fibroblasts and the ψ2 and PA317 recombinant retrovirus packaging cell lines are generous gifts of Daniel DiMaio (Yale University). These cells were

cultured essentially as described previously [85]. The MEL-JUSO [86] and IPC-298 [87] human melanoma cell lines were obtained from DSMZ (Braunschweig, Germany) and were cultured as recommended. The HMCB [88], MeWo [89], and SK-MEL-2 [90] melanoma cell lines were obtained from the American Type Culture Collection (ATCC – Manassas, VA) and were cultured as recommended. The COLO-792 melanoma cell line [91] was obtained from Sigma Aldrich (St. Louis, MO) and was cultured as recommended. Cell culture media, serum, and supplements were obtained from Cytiva (Marlborough, VA). G418 was obtained from Corning (Corning, NY). Genetic and mRNA expression data for the cell lines were obtained from the Broad Institute Cancer Cell Line Encyclopedia (CCLE, https://sites.broadinstitute.org/ccle/) [92].

2.4.3. ERBB4 Transcriptional Splicing Isoform Validation

Total RNA was extracted from MeWo cells using TRIzol Reagent obtained from Invitrogen (Waltham, MA). The cDNA synthesis was performed using the OneTaq RT-PCR Kit (NEB). The cDNA was amplified for the juxtamembrane (JMa or JMb) and the cytoplasmic region (Cyt1 or Cyt2) isoforms region using the following primers obtained from IDT (Coralville, IA):

JMa/b forward – GTGGAAAAATGTCCAGATGGCT

JMa/b reverse – CTGTTTCCAAGAATCTTCTCAAGGC

Cyt1/2 forward – GAAGAGGATTTGGAAGATATGATGGATGC

Cyt1/2 reverse – CAAAAATCTCAGCAGTAGCACCCT

The PCR reaction for diagnosing the JMa or JMb isoform results in an amplicon approximately the size of 310 base pairs indicating the presence of the JMa isoform or an amplicon approximately the size of 280 base pairs indicating the presence of the JMb

isoform. The PCR reaction for diagnosing the Cyt1 or Cyt2 isoform results in an amplicon approximately the size of 280 base pairs indicating the presence of the Cyt1 isoform or an amplicon approximately the size of 232 base pairs indicating the presence of the Cyt2 isoform. As a positive control, we also performed PCR for the amplification of GAPDH using pre-designed primers also obtained from IDT which results in an amplicon of 452 base pairs. PCR products were run on an 8% acrylamide gel and imaged on a ChemiDoc MP (BioRad).

2.4.4. Recombinant Retroviruses

Briefly, the recombinant amphotropic retroviruses LXSN [93], LXSN-ERBB4 (ERBB4 WT) [94], and LXSN-ERBB4-K751M (ERBB4 DN) [67] were packaged using the ψ2 ecotropic retrovirus packaging cell line, and the PA317 amphotropic retrovirus packaging cell line [95] as previously described [84, 96].

2.4.5. Clonogenic Proliferation Assays

C127, MEL-JUSO, MeWo, IPC-298, and SK-MEL-2 cells were infected essentially as described [84, 97] with 500, 3000, 3000, 20000, and 20000 infection units of each amphotropic retrovirus (respectively). Infected cells were selected using G418 at a concentration of 800 ug/mL in all cell lines. Selection medium was changed every 4 days. The resulting colonies of G418-resistant cells were stained using Giemsa, and colonies were counted. Staining occurred at the following time points: 8 (C127), 13 (MEL-JUSO), 14 (MeWo), 11 (IPC-298), and 17 (SK-MEL-2) days in selection medium. C127 infections served as a control for viral titer as they do not endogenously express ERBB4 or respond to ERBB4 signaling [97]. Tissue culture plates were digitized, and recombinant retroviral titer for each infection was calculated by dividing the number of

colonies by the infection volume. The recombinant retroviral titer of the melanoma cell lines was then divided by the corresponding titer in the C127 cell line to determine the clonogenic proliferation efficiency of the melanoma cell line [84]. Clonogenic proliferation efficiencies for each cell line were normalized such that the LXSN clonogenic proliferation efficiency was 100%. A t-test was used to determine whether the efficiency of clonogenic proliferation was significantly different from the control LXSN retrovirus. Significance was defined by a p-value <0.05 (1-tailed).

2.4.6. Generation of MEL-JUSO Cell Lines That Stably Express LXSN, LXSN ERBB4 WT, and LXSN ERBB4 DN

MEL-JUSO cells were infected with amphotropic retrovirus harboring the pLXSN, pLXSN-ERBB4-WT or pLXSN-ERBB4-DN construct at a multiplicity of infection less than 1 infectious unit per 10 cells. After incubation with the viruses, infected cells were selected using G418 at a concentration of 800 ug/mL. The resulting colonies of G418-resistant cells were pooled and maintained in medium with a G418 concentration of 500 ug/mL.

2.4.7. Anchorage Independence Assay

Anchorage independence assays were conducted using the MEL-JUSO LXSN, MEL-JUSO LXSN ERBB4 WT, and MEL-JUSO LXSN ERBB4 DN stable cell lines described in section 2.4.5. Each cell line was seeded at a density of $2x10^4$ cells per 60mm dish in a semisolid medium that consisted of RPMI-1640 supplemented with 10% FBS, 500 ug/mL G418, and 0.3% low melting point (LMP) agarose. Additional 0.3% LMP agarose medium was added to the plates every 4 days to keep the agarose medium from drying out. Fourteen days later, 9 microscopic fields were selected at random and

photographed. Using Image J (https://imagej.nih.gov), we measured the diameter of each colony based on a 1mm distance standard. For each cell line, ~100 colonies were measured per trial. We performed a total of 3 independent trials; therefore, a total of ~300 colonies were measured for each cell line. We pooled all the diameter measurements from the three trials for each cell line and performed a t-test to determine the statistical difference between the colony sizes of the two experimental conditions.

2.5. Tables

	Cases	% of Total Cases	*BRAF* V600 Mutant Cases	% of *BRAF* V600 Mutant Cases	*BRAF* WT Cases	% of *BRAF* WT Cases
Gender	470		210		227	
Male	290	61.70%	126	60.00%	141	62.11%
Female	180	38.30%	84	40.00%	86	37.89%
Race	470		210		227	
White	447	95.11%	203	96.67%	211	92.95%
Asian	12	2.55%	2	0.95%	10	4.41%
Black or African American	1	0.21%	0	0.00%	1	0.44%
Not Reported	10	2.13%	5	2.38%	5	2.20%
Ethnicity	470		210		227	
Hispanic or Latino	11	2.34%	6	2.86%	4	1.76%
Not Hispanic or Latino	446	94.89%	197	93.81%	217	95.59%
Not Reported	13	2.77%	7	3.33%	6	2.64%
Vital Status	470		210		227	
Alive	249	52.98%	119	56.67%	112	49.34%
Dead	221	47.02%	91	43.33%	115	50.66%
Not Reported	0	0.00%	0	0.00%	0	0.00%
Age at Diagnoses	470		210		227	
<30 (10950 days)	21	4.47%	19	9.05%	2	0.88%
30-49 (10951-17885 days)	106	22.55%	59	28.10%	46	20.26%
50-64 (17886-23360 days)	157	33.40%	80	38.10%	70	30.84%
65+ (>23360 days)	178	37.87%	48	22.86%	106	46.70%
Not Reported	8	1.70%	4	1.90%	3	1.32%
AJCC Pathologic Stage	470		210		227	
0	7	1.49%	2	0.95%	5	2.20%
I	30	6.38%	18	8.57%	12	5.29%
IA	18	3.83%	6	2.86%	10	4.41%
IB	29	6.17%	13	6.19%	14	6.17%
II	30	6.38%	14	6.67%	12	5.29%
IIA	18	3.83%	8	3.81%	8	3.52%
IIB	28	5.96%	5	2.38%	19	8.37%
IIC	64	13.62%	29	13.81%	32	14.10%
III	41	8.72%	22	10.48%	15	6.61%
IIIA	16	3.40%	8	3.81%	6	2.64%
IIIB	46	9.79%	20	9.52%	25	11.01%
IIIC	68	14.47%	25	11.90%	40	17.62%
IV	23	4.89%	12	5.71%	10	4.41%
Not Reported	52	11.06%	28	13.33%	19	8.37%
AJCC Pathologic Stage	390		182		208	
0	7	1.79%	2	1.10%	5	2.40%
I, IA, IB	77	19.74%	37	20.33%	36	17.31%
II, IIA, IIB, IIC	140	35.90%	56	30.77%	71	34.13%
III, IIIA, IIIB, IIIC	171	43.85%	75	41.21%	86	41.35%
IV	23	5.90%	12	6.59%	10	4.81%

Table 1a. Demographic and clinicopathologic characteristics of *BRAF* V600 mutant and *BRAF* WT melanoma cases in the TCGA-SKCM dataset

	BRAF V600X	*BRAF* WT	Total
Alive	119	112	231
Dead	91	115	206
Total	210	227	437

p = 0.1252

Table 1b. Survival among *BRAF* V600 mutant and *BRAF* WT melanoma cases in the TCGA-SKCM dataset. Statistical analysis is a chi-squared test.

		BRAF V600X	*BRAF* WT	Total
AJCC Pathologic Stage at Diagnosis	0	2	5	7
	I, IA, IB	37	36	73
	II, IIA, IIB, IIC	56	71	127
	III, IIIA, IIIB, IIIC	75	86	161
	IV	12	10	22
Total		182	208	390

p = 0.6842

Table 1c. AJCC pathological stage among BRAF V600 mutant and BRAF WT melanoma cases in the TCGA-SKCM dataset. Statistical analysis is a chi-squared test.

	Elevated *ERBB4* mRNA Expression	*ERBB4* mRNA Expression NOT Elevated	Total
***RAS* or *NF1* Mutation**	21	104	125
***RAS* and *NF1* WT**	1	52	53
Total	22	156	178

p = 0.0057

Table 2. Elevated ERBB4 expression is correlated with a gain-of-function RAS gene mutation or a loss-of-function *NF1* mutation in *BRAF* WT melanomas. Statistical analysis is a chi-squared test.

Cell Line	Species	Lineage	Origin	Disease
IPC-298	Human	Skin	Primary	Melanoma
MEL-JUSO	Human	Skin	Primary	Melanoma
MeWo	Human	Skin	Metastasis: Lymph Node	Malignant Melanoma
HMCB	Human	Skin	Primary	Melanoma
SK-MEL-2	Human	Skin	Metastasis: Thigh	Malignant Melanoma
COLO 792	Human	Skin	Metastasis: Brain	Malignant Melanoma

Table 3a. *BRAF* WT human melanoma cell lines.

Cell Line	*ERBB4* Mutation 1	*ERBB4* Mutation 2	*ERBB4* Mutation 3	*PIK3CA* Mutation	*PTEN* Mutation	*BRAF* Mutation	*NRAS* Mutation	*HRAS* Mutation	*NF1* Mutation 1	*NF1* Mutation 2
IPC-298							Q61L			
MEL-JUSO							Q61L	G13D	L1779P	
MeWo	M766I	R488R	S449F			L255L			Q1336*	R2053R
HMCB									Q535H	
SK-MEL-2	R50C						Q61R			
COLO 792	G730K	G730R	M313I						W1236R	Splice Site

Table 3b. *BRAF* WT melanoma cell lines' mutation data according to Broad Institute's DepMap Public 22Q4 records [92].

Cell Line	EGFR mRNA Expression	ErbB2 mRNA Expression	ErbB3 mRNA Expression	ErbB4 mRNA Expression	NRG1 mRNA Expression	NRG2 mRNA Expression	NRG3 mRNA Expression	NRG4 mRNA Expression	HBEGF mRNA Expression	BTC mRNA Expression	EREG mRNA Expression	ACTB mRNA Expression	GAPDH mRNA Expression
IPC-298	0.07	3.58	6.18	0.00	0.03	1.36	1.04	0.33	1.76	0.00	0.01	11.79	12.74
MEL-JUSO	0.97	3.87	5.95	0.12	3.67	2.02	0.10	1.02	6.42	0.26	0.04	12.13	12.90
MeWo	1.43	4.74	6.59	0.69	1.01	0.28	0.07	0.58	2.05	0.46	0.03	11.45	11.95
HMCB	0.00	6.12	8.12	0.93	6.00	0.11	0.23	0.58	3.49	0.89	0.45	11.09	11.74
SK-MEL-2	0.06	3.18	6.74	0.01	0.77	0.73	2.13	0.81	4.49	0.00	0.00	10.85	11.47
A388	7.76	4.57	3.63	0.00	4.67	0.01	0.00	2.20	5.84	1.34	2.81	10.75	12.44
COLO 792	0.15	5.28	6.70	0.01	0.45	1.16	0.68	0.08	2.49	0.07	0.08	11.74	12.02

Table 3c. *BRAF* WT melanoma cell lines' mRNA expression data for ERBB receptors, ERBB4 receptor ligands, and housekeeping genes (ACTB and GAPDH) according to Broad Institute's DepMap Public 22Q4 records. Reported values are inferred from RNA-seq data after a log2 transformation using a pseudo-count of 1 [92].

	Percent Efficiency of Clonogenic Proliferation Relative to Vector		
Cell Line	**Vector Control**	**ERBB4 WT**	**ERBB4 DN**
MEL-JUSO	100%	390% n = 9 *p = 0.002*	35.3% n = 10 *p = 5.14E-7*
MeWo	100%	564% n = 5 *p = 0.0173*	28.5% n = 5 *p = 0.0003*
IPC-298	100%	1340% n = 5 *p = 0.0257*	64.3% n = 5 *p = 0.0084*
SK-MEL-2	100%	208% n = 4 *p = 0.0491*	50.4% n = 5 *p = 0.0213*

Table 4. Percent efficiency of clonogenic proliferation of LXSN, LXSN-ERBB4-WT, and LXSN-ERBB4-DN retroviral vector infection in the MEL-JUSO, MeWo, IPC-298, and SK-MEL-2 cell lines. P values represent a T-test compared to the vector control.

	Vector	WT	DN
Average (um)	83	83	69
SEM (um)	3	3	2
Median (um)	78	77	63
P-value: T-test (Relative to Vector)		0.483	1.932E-04
N (colonies)	324	323	326

Table 5. Average, standard error of the mean (SEM), and median of diameters of MEL-JUSO LXSN (Vector), MEL-JUSO LXSN ERBB4 WT (WT), and MEL-JUSO LXSN ERBB4 DN (DN) anchorage-independent colonies. Diameters of measured colonies from the MEL-JUSO LXSN ERBB4 WT, and MEL-JUSO LXSN ERBB4 DN cell lines were compared using a t-test relative to the diameters of measured colonies from the MEL-JUSO LXSN cell line. This is representative of 3 trials where the total number of colonies counted across all three trials is represented (N).

2.6. Figures

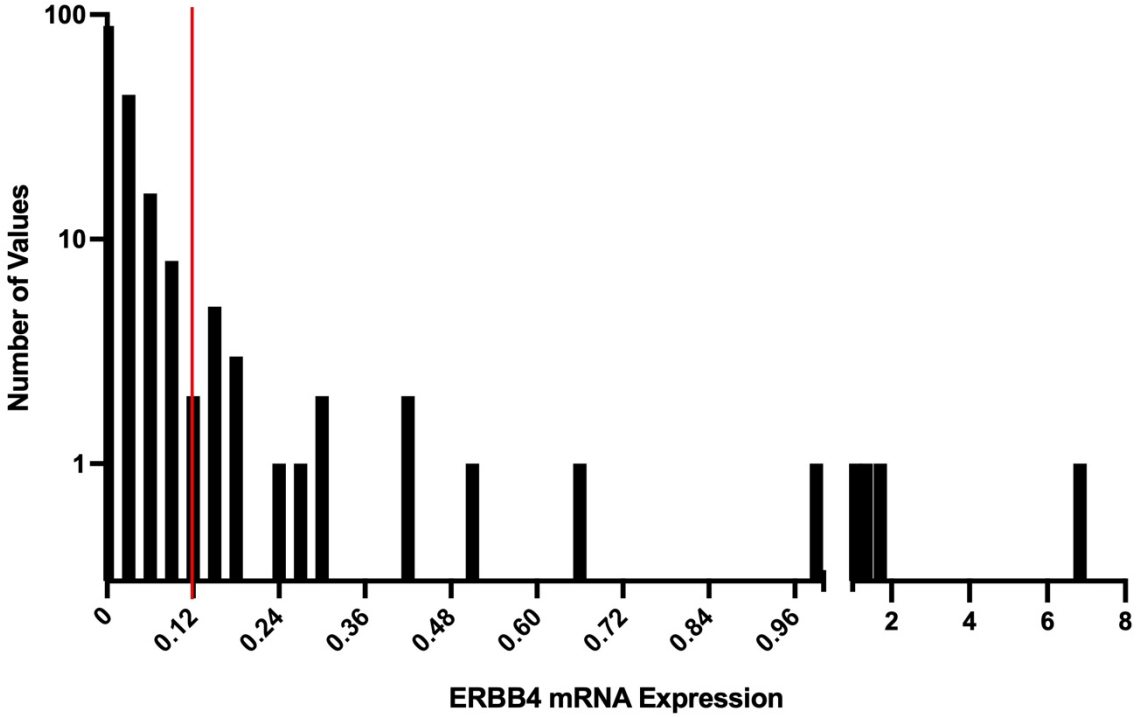

Figure 3. Histogram of ERBB4 mRNA expression data downloaded from the TCGA-SKCM dataset. This represents cases found in *BRAF* WT melanomas. Elevated expression is defined as being >0.12 as indicated by the red line. ERBB4 mRNA expression values are representative of the GDC HT-Seq workflow [82].

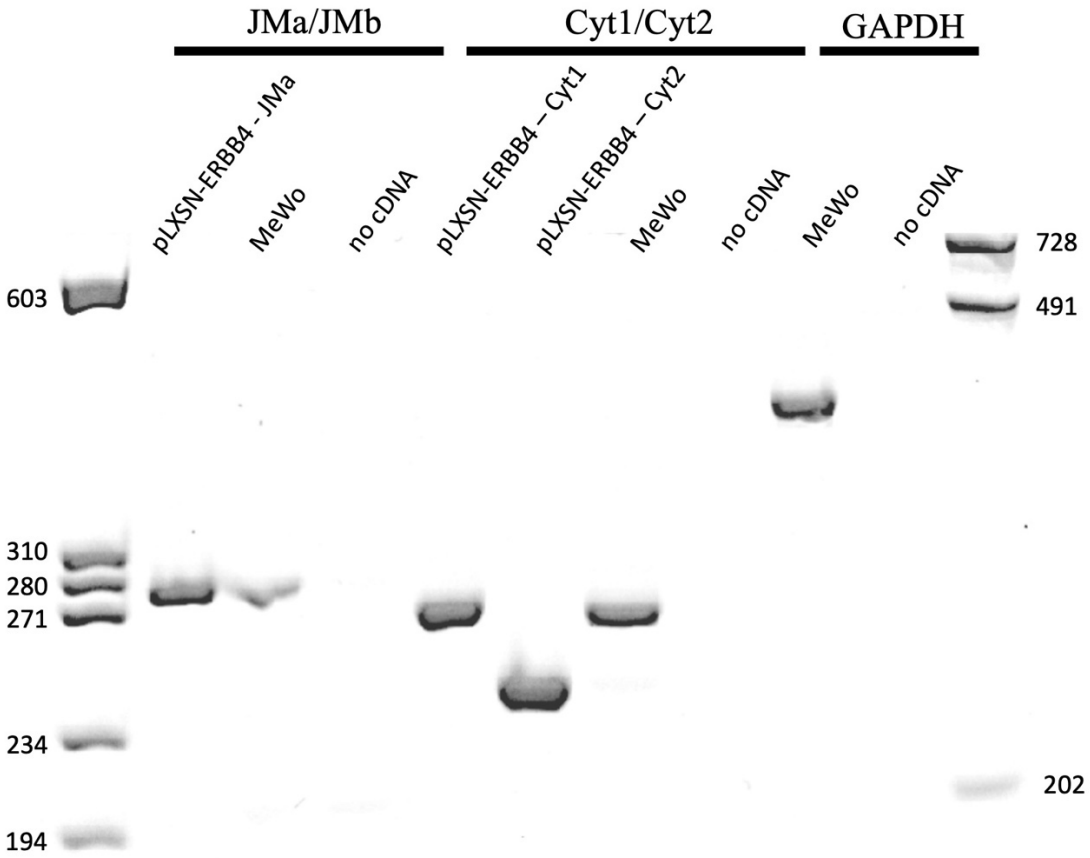

Figure 4. PCR amplified MeWo cDNA for the validation of endogenous *ERBB4* transcriptional splicing isoforms JMa/JMb and Cyt1/Cyt2.

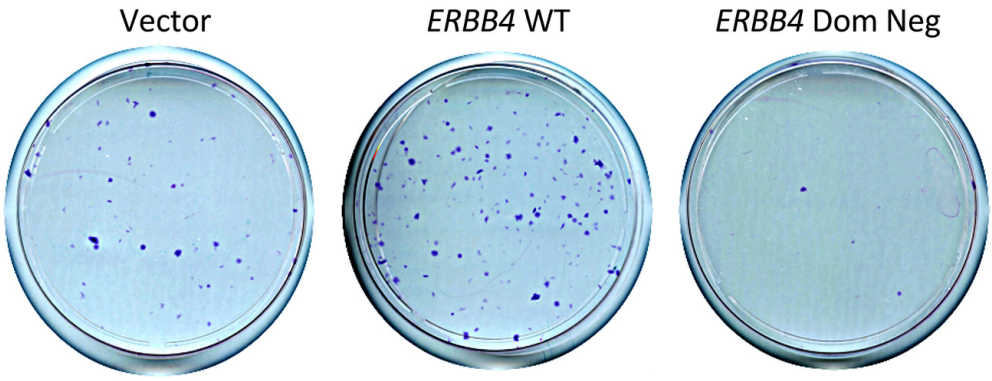

Figure 5. Clonogenic proliferation of MEL-JUSO cells following stable infection with LXSN, LXSN-ERBB4-WT, and LXSN-ERBB4-DN retroviral vectors.

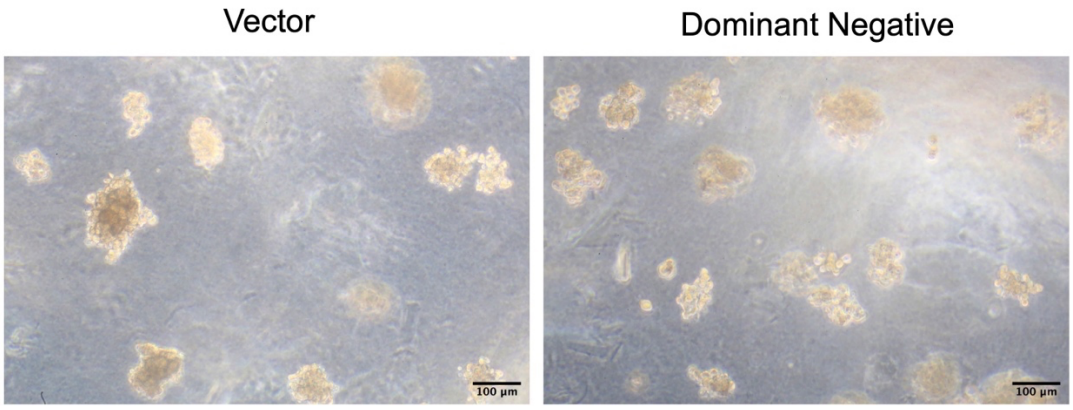

Figure 6. Anchorage-independent colony growth of MEL-JUSO LXSN (Vector) and MEL-JUSO LXSN ERBB4 DN (Dominant Negative) cell lines.

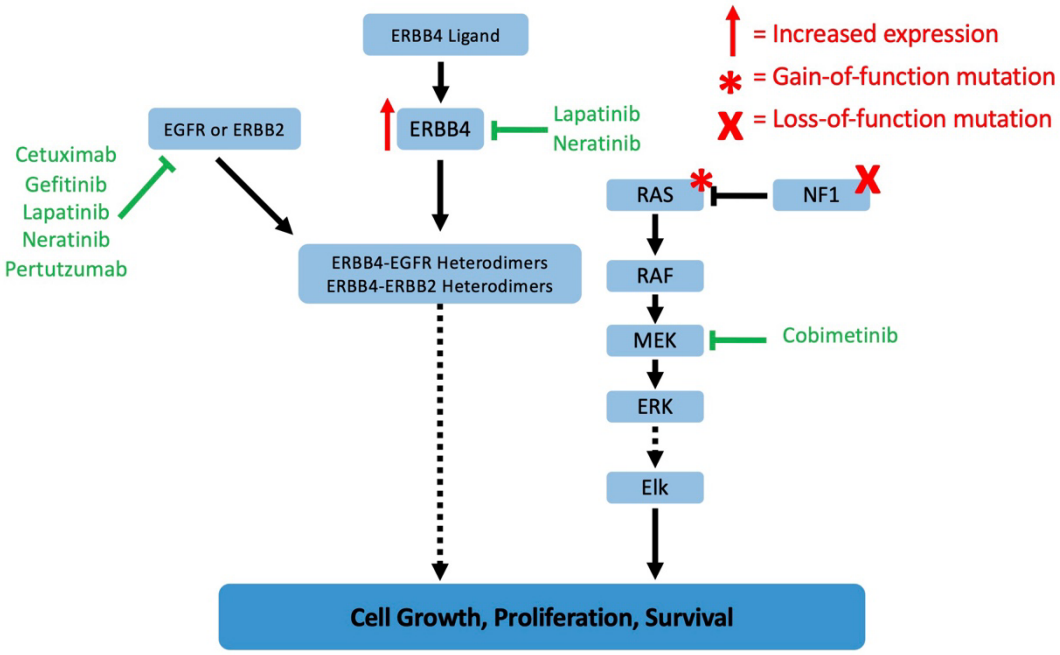

Figure 7: In some *BRAF* WT melanomas, elevated ERBB4 expression causes signaling that cooperates with elevated RAS/MAPK pathway signaling (caused by a gain-of-function *RAS* or loss-of-function *NF1* mutation) to drive the proliferation of *BRAF* WT melanomas. These melanomas may be treated with a combination MEK inhibitor and ERBB receptor inhibitor.

Chapter 3: *ERBB4* Mutant Alleles May Drive *BRAF* WT Melanomas

3.1. Introduction

Mutations in *ERBB4* occur in 15-20% of melanomas and have been described as oncogenic in several melanoma model systems [3, 71, 74]. We have shown previously that elevated expression of WT *ERBB4* in *BRAF* WT human melanoma cell lines is associated with an oncogenic phenotype (chapter 2). We also identified three human *BRAF* WT melanoma cell lines (MEL-JUSO, IPC-298, and MeWo) in which *ERBB4* is both sufficient and necessary for clonogenic proliferation. Additionally, we found that *ERBB4* is necessary for anchorage independent growth of the MEL-JUSO cell line. This suggests that ERBB4 signaling is oncogenic in *BRAF* WT melanoma, and therefore, we hypothesize that *ERBB4* mutations also cause increased oncogenic ERBB4 signaling.

ERBB4 mutations in melanoma are not known to exhibit any hotspot mutations [71]. In a study of a cohort of melanoma patients, *ERBB4* mutations E317K, E452K, E542K, R544W, E563K, E836K, and E872K were identified as gain-of-function [71]. The introduction of these *ERBB4* mutations to HEK 293T cells resulted in an increase in ERBB4 phosphorylation and ERBB4 kinase activity. Furthermore, introducing these *ERBB4* mutations to NIH 3T3 cells and SK-MEL-2 (*BRAF* WT melanoma cell line) cells caused an increase in cell transformation and anchorage-independent growth [71]. This same study found that patient-derived melanoma cell lines that harbored endogenous *ERBB4* mutations (E542K/E872K and R544W) exhibited increased basal ERBB4 phosphorylation. Finally, these patient-derived melanoma cell lines that harbor endogenous *ERBB4* mutations responded to treatment with lapatinib with greater potency than did *ERBB4* WT melanomas [71]. This led to a clinical trial studying lapatinib's use

to treat *ERBB4* mutant melanomas. However, this trial failed due to poor patient accrual [27, 72]. Furthermore, to our knowledge, this work has yet to be replicated and the tumor-derived cell lines are unavailable.

Several factors have limited the study of ERBB4 in melanoma. Despite documented cases of oncogenic *ERBB4* mutations, the diverse spread of *ERBB4* mutations across the gene makes it difficult to identify oncogenic ERBB4 mutations without experimental validation on a case-by-case basis. Furthermore, the diversity of oncogenic *ERBB4* mutations suggests that there is also a variety of mechanisms by which oncogenic *ERBB4* mutations function. To progress our knowledge regarding oncogenic mutant ERBB4 in melanoma, we need to identify trends surrounding *ERBB4* mutant occurrence in *BRAF* WT melanomas to develop a prioritization scheme that uses molecular tumor information to identify putative *ERBB4* driver mutations.

ERBB4 mutations have not yet been evaluated for oncogenic activity, specifically in the *BRAF* WT melanoma context. Signaling mechanisms in *BRAF* WT melanomas are poorly understood; thus, these melanomas have no targeted therapeutic available beyond immune checkpoint inhibitors. Identifying *ERBB4* mutations that drive *BRAF* WT melanomas could lead to the identification of effective drug targets for patients with *ERBB4* mutant, *BRAF* WT melanoma. Here we conduct an analysis of The Cancer Genome Atlas – Skin Cutaneous Melanoma (TCGA-SKCM) dataset and propose a scheme for identifying putative *ERBB4* driver mutations found in *BRAF* WT melanoma patients. We then conducted a phenotypic screen of putative ERBB4 driver mutations in an ERBB4-dependent melanoma cell line to identify those that confer greater oncogenic activity than *ERBB4* WT.

3.2. Results

3.2.1. ERBB4 Non-Synonymous Mutations Occur at a Non-Stochastic Frequency.

The Cancer Genome Atlas – Skin Cutaneous Melanoma (TCGA-SKCM) dataset contains patient clinical and biospecimen data for 470 cases of skin cutaneous melanoma [98]. We analyzed the TCGA-SKCM dataset and found that *ERBB4* missense mutant alleles are slightly more common in *BRAF* WT melanomas than *BRAF* V600 mutant melanomas (15.4% and 11.4%, respectively). Therefore, we hypothesize that *ERBB4* mutant alleles are selected in *BRAF* WT melanomas.

The ratio of non-synonymous (missense, stop gained, frameshift) to synonymous (synonymous, stop retained) mutations is an indicator of selection for mutant alleles by tumor cells. Generally speaking, if the ratio of non-synonymous to synonymous mutant alleles (N/S ratio) in a particular gene is greater than 1, it is indicative of selection for non-synonymous mutant alleles in that gene [99].

Because of the exposure of skin cells to ultraviolet (UV) light, melanomas carry numerous cytosine to thymine mutations, many of which function as passengers. This precludes using an N/S cutoff of 1 to identify genes for which mutant alleles function as drivers [100]. Thus, we have compared the N/S ratio for *ERBB4* in the 227 *BRAF* WT melanomas of the TCGA-SKCM dataset against the N/S ratio for genes (*ARMC4, CFTR, ERC2, SLIT2,* and *SLIT3)* that are roughly the same size as *ERBB4* and that from the literature do not appear to function as melanoma drivers.

The N/S ratio for *ERBB4* is 5.33, which is significantly greater (p=0.003) than the average N/S ratio for the five control genes (2.44) (Table 6). Moreover, the N/S ratio for the *KRAS* and *NRAS* oncogenes and the *CDKN2A*, *NF1*, *RB*, and *TP53* tumor suppressor

genes is also greater than the average N/S ratio for the five control genes. In contrast, the N/S ratio for EGFR, ERBB2, and ERBB3 genes are not significantly greater than the average N/S ratio for the five control genes. These results suggest that mutations in *ERBB4*, but not mutations in *EGFR*, *ERBB2*, or *ERBB3*, function as drivers in *BRAF* WT melanomas.

3.2.2. *ERBB4* Mutations That Affect Functionally Conserved Amino Acid Residues are More Likely to be Drivers.

Using the TCGA-SKCM, *BRAF* WT melanoma dataset, we identified 40 *ERBB4* missense mutations that are not coincident with an *ERBB4* stop-gained mutation or splice site mutation. One of the *ERBB4* mutant alleles was found in three tumor samples (R711C), and the other 39 *ERBB4* mutant alleles were each only found in a single tumor sample (Figure 8). Therefore, there is only one single, weak mutational hotspot near the beginning of the ERBB4 intracellular kinase domain, and we must employ other approaches to identify and prioritize candidate driver mutations.

The conservation of a particular amino acid residue across functionally related proteins suggests that residue is critical for the shared function of the related proteins. Thus, we hypothesized that *ERBB4* melanoma driver mutations are more likely to affect conserved residues.

Many members of the epidermal growth factor family of peptide hormones bind to both ERBB3 and ERBB4 (Figure 2) [101]. Moreover, ERBB3 dimerization and ERBB4 dimerization are regulated by an identical mechanism (Figure 2) [32]. We have identified fifteen ERBB4 extracellular domain (ECD) I-IV amino acid residues that are affected by *ERBB4* missense mutations found in the TCGA-SKCM *BRAF* WT melanoma

data set. Thus, we hypothesized that these fifteen residues are conserved in the ERBB3 ECDs I-IV. However, only eleven residues affected by *ERBB4 BRAF* WT melanoma missense mutations are conserved in the ERBB3 ECDs I-IV, which falls well short of a statistically significant correlation (p=0.3763) (Table 7a). Nonetheless, these eleven *ERBB4* missense mutant alleles (G85S, R106C, R196C, P331S, G340E, A383T, S418F, T422I, E452K, R491K, and P517A) are assigned a priority point to help identify the best candidates for *ERBB4* driver mutant alleles in *BRAF* WT melanomas.

Only ERBB4 and EGFR directly bind ligands and form homodimers that undergo ligand-dependent receptor cross-phosphorylation (ERBB2 does not bind ligand, and ERBB3 lacks sufficient kinase activity) [101]. We have identified thirteen ERBB4 tyrosine kinase domain amino acid residues affected by *ERBB4* missense mutations found in the TCGA-SKCM *BRAF* WT melanoma data set. We hypothesized that these thirteen residues are conserved in the EGFR tyrosine kinase domain. Indeed, all thirteen residues affected by *ERBB4 BRAF* WT melanoma missense mutations are conserved in the EGFR tyrosine kinase domain. However, this distribution falls just short (p=0.0565) of a statistically significant correlation (Table 7b). Nonetheless, these thirteen *ERBB4* missense mutant alleles (R711C, G741E, P759L, P800L, D813N, N814T, D861N, L864P, P925S, G936E, P943S, E969K, and R992C) are assigned a priority point to help identify the best candidates for *ERBB4* driver mutant alleles in *BRAF* WT melanomas.

Finally, three *ERBB4* missense mutant alleles (R106C, G741E, L864P) found in the TCGA-SKCM *BRAF* WT melanoma data set correspond to gain-of-function alleles in *EGFR* (R108K, G735S, and L858R, respectively) or *ERBB2* (L866M corresponds to *ERBB4* L864P). As a result, these three *ERBB4* missense alleles (Table 8) are assigned a

priority point to help identify the best candidates for *ERBB4* driver mutant alleles in *BRAF* WT melanomas.

3.2.3. *ERBB4* Missense Mutants are Significantly More Common in TCGA-SKCM *BRAF* WT Melanoma Cases in Which There are *RAS* or *NF1* Nonsynonymous Mutations.

The *RAS* family of protein isoforms includes *NRAS*, *HRAS*, and *KRAS*. In melanoma, gain-of-function *BRAF* mutations (such as the *BRAF* V600 mutation) and *NRAS* mutations (most commonly the *NRAS* Q61 mutation) occur in a mutually exclusive manner [10]. Furthermore, gain-of-function *NRAS* mutations and loss-of-function *NF1* mutations frequently occur in *BRAF* WT melanomas and cause increased RAS pathway signaling and a more aggressive phenotype. Overall, *NRAS* is the most mutated *RAS* isoform gene found in melanomas [6]. Gain-of-function *RAS* gene mutations occur in about 30% of skin cutaneous melanomas, and loss-of-function mutations in *NF1* occur in about 20% of skin cutaneous melanomas [6, 10-12, 102].

Receptor tyrosine kinases typically stimulate RAS pathway signaling. Hence, in the *BRAF* WT melanomas of the TCGA-SKCM dataset, we predicted cases that contain an ERBB4 missense mutation would be less likely to contain a nonsynonymous mutation in a *RAS* gene or *NF1*. Surprisingly, 29 cases in the TCGA-SKCM *BRAF* WT melanoma dataset contain an *ERBB4* missense mutation, as well as a nonsynonymous mutation in a *RAS* gene or *NF1* (Table 9). This statistically significant correlation ($p = 0.0189$) suggests that ERBB4 signaling cooperates with RAS signaling to drive *BRAF* WT melanomas. Hence, we have assigned a priority point to the 33 *ERBB4* mutant alleles (E33K, G85S, R106C, D150N, P331S, A383T, S418F, T422I, E452K, R491K, P517A,

G549S, P572L, F662L, R711C, P759L, P800L, D813N, D861N, P925S, G936E, E969K, R992C, P1080L, P1117L, R1127K, R1139Q, R1142Q, P1165L, E1187D, P1276S, P1282S, and P1300S) found in the 29 cases that contain a nonsynonymous mutation in a *RAS* gene or *NF1*.

3.2.4. *ERBB4* Missense Mutations are Significantly More Likely in Cases Where There is a *RAS* or *NF1* Nonsynonymous Mutation as Well as No Other Apparent Cause of Increased PI3K Signaling.

The evolution of tumors to the development of a more aggressive tumor requires an accumulation of driver mutations [103]. Therefore, *ERBB4* driver mutations are more likely to be found in cases with other driver mutations. Melanomas which rely on the RAS/RAF/MEK/ERK pathway, such as RAS mutant melanomas, are suggested to also rely on activation of the PI3K pathway. Therefore, we hypothesized that ERBB4 mutations would preferentially occur in cases with both a *RAS*/*NF1* mutation and a PI3K signaling pathway activating alteration. Thus, we tested whether *ERBB4* missense mutations are more likely to occur in cases where there is a *RAS* gene or *NF1* mutation and a PI3K pathway-associated driver event (increases in *PIK3CA* transcription, gain-of-function mutations in *PIK3CA*, decreases in *PTEN* transcription, or loss-of-function mutations in *PTEN*) (Table 10). Twenty-five cases in the TCGA-SKCM *BRAF* WT melanoma dataset contain an *ERBB4* missense mutation, a nonsynonymous mutation in a *RAS* gene or *NF1*, but do not exhibit an event that would cause increased PI3K pathway signaling. Four cases in the TCGA-SKCM *BRAF* WT melanoma dataset contain an *ERBB4* missense mutation, a nonsynonymous mutation in a *RAS* gene or *NF1*, and exhibit an event that would cause increased PI3K pathway signaling. Thirty-five cases in

the TCGA-SKCM *BRAF* WT melanoma dataset are *ERBB4* WT, harbor a nonsynonymous mutation in a *RAS* gene or *NF1*, and exhibit an event that would cause increased PI3K pathway signaling. Ninety-three cases in the TCGA-SKCM *BRAF* WT melanoma dataset are *ERBB4* WT, harbor a nonsynonymous mutation in a *RAS* gene or *NF1*, but do not exhibit an event that would cause increased PI3K pathway signaling. Four cases in the TCGA-SKCM *BRAF* WT melanoma dataset harbor an *ERBB4* missense mutation and are *RAS* and NF1 *WT*. Sixty-one cases in the TCGA-SKCM *BRAF* WT melanoma dataset are *ERBB4, RAS,* and *NF1* WT. The status of PI3K pathway signaling activity was not considered in the 65 cases that are *RAS,* and *NF1* WT.

Contrary to our hypothesis, a chi-squared analysis of this data found an inverse correlation between the occurrence of *ERBB4* mutations and activating PI3K pathway events in *RAS*/*NF1* mutant melanomas (chi-squared, $p = 0.0166$) (Table 10). This suggests that mutant *ERBB4* is responsible for activating the PI3K pathway as a substitution for other causes of PI3K pathway activation. Hence, we have assigned a priority point to the 29 *ERBB4* mutant alleles (E33K, G85S, R106C, D150N, P331S, A383T, S418F, T422I, E452K, R491K, P517A, G549S, R711C, P759L, P800L, D813N, D861N, P925S, G936E, E969K, R992C, P1080L, R1127K, R1139Q, P1165L, E1187D, P1276S, P1282S, and P1300S) found in the 25 cases that contain a nonsynonymous mutation in a *RAS* gene or *NF1* as well as no apparent cause of ERBB4-independent increased PI3K pathway signaling.

3.2.5. ERBB4 Mutations and Elevated ERBB4 Transcription Appear to Independently Drive BRAF WT Melanomas.

In silico analyses of the TCGA-SKCM *BRAF* WT melanoma dataset suggest that elevated *ERBB4* transcription cooperates with elevated RAS signaling (caused by a gain-of-function *RAS* gene mutation or a loss-of-function *NF1* mutation) to drive *BRAF* WT melanomas. Furthermore, *in vitro* evaluation of the phenotypic effects of elevated ERBB4 expression suggest that ERBB4 signaling is both sufficient and necessary for malignant activity in *BRAF* WT melanoma cell lines (chapter 2). Thus, *ERBB4* mutant alleles may require elevated *ERBB4* transcription to drive *BRAF* WT melanomas. In the 178 cases in the TCGA-SKCM *BRAF* WT dataset for which *ERBB4* transcription and mutation data are available, 17 cases contain an *ERBB4* missense mutation but do not exhibit elevated *ERBB4* transcription, 19 cases do not contain an *ERBB4* missense mutation but do exhibit elevated *ERBB4* transcription, and three cases contain an *ERBB4* missense mutation and exhibit elevated *ERBB4* transcription (Figure 9). These data suggest that *ERBB4* mutations and elevated *ERBB4* transcription drive *BRAF* WT melanomas in an independent manner. Therefore, we have assigned a priority point to each of the 17 *ERBB4* mutations that are not coincident with elevated *ERBB4* transcription (R106C, D150N, A383T, S418F, R491K, P517A, G549S, F662L, R711C, P759L, P800L, D861N, G936E, E969K, R1142Q, P1276S, P1300S). None of these mutations occur more than once in those 17 cases. There are three mutations in the three cases that exhibit both an ERBB4 mutation and elevated ERBB4 transcription. None of these mutations occur more than once in those three cases (P572L, R1127K, E1187D).

3.2.6. We Have Prioritized *ERBB4* Mutant Alleles as Candidate Drivers of BRAF WT Melanomas.

Based on the *in silico* data presented in Table 7a, Table 7b, Table 8, Table 9, Table 10, Figure 8, and Figure 9, we have established and applied criteria for prioritizing *ERBB4* mutant alleles as candidate drivers of *BRAF* WT melanomas (Table 11). Interestingly, the 3 mutations exhibited in cases with a co-occurring ERBB4 mutation and elevated ERBB4 expression (Figure 9) are not found among the top-ranked candidates.

3.2.7. Prioritized *ERBB4* Mutant Alleles Stimulate Clonogenic Proliferation of a *BRAF* WT Melanoma Cell Line.

We have shown that ectopic expression of wild-type *ERBB4* (*ERBB4* WT) significantly stimulates the clonogenic proliferation of MEL-JUSO, MeWo, IPC-298, and SK-MEL-2 human *BRAF* WT melanoma cell lines. Moreover, ectopic expression of the dominant-negative *ERBB4* K751M (*ERBB4* DN) mutant significantly inhibits the clonogenic proliferation of MEL-JUSO, MeWo, and IPC-298 cell lines and modestly inhibits the clonogenic proliferation of SK-MEL-2 cells. These results indicate that *ERBB4* is both sufficient and necessary to drive some *BRAF* WT melanoma cell lines; they also suggest that we can use the MEL-JUSO, MeWo, and IPC-298 cell lines to identify *ERBB4* mutant alleles that function as *bona fide* drivers of *BRAF* WT melanomas (chapter 2).

Given that the MeWo and SK-MEL-2 cell lines harbor endogenous *ERBB4* mutations that may confound our analysis of the effects of ectopic expression of *ERBB4* mutants, and given that the IPC-298 cell line exhibits a low clonogenic proliferation efficiency, we chose to use the MEL-JUSO *BRAF* WT melanoma cell line as a model system for evaluating the high priority *ERBB4* mutations. The MEL-JUSO cell line

exhibits moderate relative endogenous ERBB4 expression and does not harbor any endogenous *ERBB4* mutations [83].

We hypothesized that high-priority mutations identified using our mutation prioritization scheme (Table 11) would be most likely to function as drivers. We also postulated that *ERBB4* driver mutations would confer greater oncogenic activity than *ERBB4* WT. We selected the following *ERBB4* mutations for phenotypic analysis: G85S, R106C, S418F, E452K, R711C, G741E, P759L, D861N, and R992C. As a positive control, we selected the Y285C *ERBB4* mutant, which was found in non-small cell lung carcinoma (NSCLC) samples and stimulates ligand-dependent and -independent ERBB4 signaling and increased ERBB4 heterodimerization with ERBB2 [32, 70]. We evaluated the phenotype of the *ERBB4* mutants using a clonogenic proliferation assay in the MEL-JUSO cell line. Confirming our previous findings from Chapter 2, the introduction of *ERBB4* WT caused significantly greater clonogenic proliferation than the vector control in the MEL-JUSO cell line (Tables 12 and 13). The *ERBB4* G85S, Y285C, G741E, and D861N mutants caused significantly greater clonogenic proliferation than *ERBB4* WT. The G85S mutant exhibited a clonogenic proliferation efficiency of 162% relative to WT (t-test, $p = 0.003$, $n = 10$). The Y285C mutant exhibited a clonogenic proliferation efficiency of 161% relative to WT (t-test, $p = 0.007$, $n = 9$). The G741E mutant exhibited a clonogenic proliferation efficiency of 140% relative to WT (t-test, $p = 0.018$, $n = 10$). The D861N mutant exhibited a clonogenic proliferation efficiency of 119% relative to WT (t-test, $p = 0.042$, $n = 9$). Interestingly, the R711C mutant exhibited an average clonogenic proliferation efficiency of 174% compared to WT across 9 trials. However, due to the large degree of variability among individual trials, the t-test resulted in a p-

value of 0.052, making the difference between R711C and WT not statistically significant. Regardless, the R711C mutant should be considered for future study. The *ERBB4* R106C, S418F, E452K, P759L, and R992C mutants did not exhibit significantly greater clonogenic proliferation relative to WT in the MEL-JUSO cell line (Table 12). These data suggest that the G85S, R711C, G741E, and D861N mutations may be *bona fide* drivers of *BRAF* WT melanomas. Furthermore, the fact that the Y285C mutation was identified in an NSCLC but conferred significant oncogenic activity in a *BRAF* WT melanoma model system suggests that oncogenic mutations identified in other cancers may also be oncogenic in *BRAF* WT melanomas.

3.2.8. The *ERBB4* Q646C Mutant Causes Decreased Clonogenic Proliferation of a *BRAF* WT Melanoma Cell Line

We have previously shown that the *ERBB4* Q646C mutation causes constitutive homodimerization and tumor suppressive activity in multiple tumor types [65-67]. Therefore, we hypothesized that the MEL-JUSO cell line would also respond to ERBB4 constitutive homodimerization with a tumor-suppressive phenotype. Indeed, we found that the introduction of the *ERBB4* Q646C mutant causes a significant decrease in the clonogenic proliferation efficiency of the MEL-JUSO cell line to 37% efficiency relative to Vector Control (t-test, $p = 0.036$, $n = 2$) (Table 12). This suggests that ERBB4 functions as a context-dependent tumor suppressor in *BRAF* WT melanoma.

3.3. Discussion

3.3.1. Specific Molecular Characteristics May Identify ERBB4 Mutant Alleles That Function as Drivers of BRAF WT Melanoma.

One of the main obstacles to studying *ERBB4* mutations in melanoma is the inability to easily identify the best candidates for study. To solve this issue, we analyzed the TCGA-SKCM, *BRAF* WT melanoma dataset and identified several characteristics which oncogenic *ERBB4* mutations may exhibit and developed a prioritization scheme to narrow down putative *ERBB4* driving mutations. It appears that ERBB4 driving mutations may be more likely to occur at sites that: are functionally conserved in either the EGFR kinase domain or the ERBB3 ECD, correspond to known gain-of-function mutant alleles in *EGFR* and *ERBB2* in other tumor types, co-occur with a gain-of-function *RAS* or loss-of-function *NF1* mutation, do not co-occur with any other apparent cause of increased ERBB4-independent PI3K signaling, and occur more than once within a population of *BRAF* WT melanoma patients.

Due to the high volume of individual *ERBB4* mutations found in *BRAF* WT melanomas, continuing to develop and validate this prioritization scheme is clinically useful. For example, *ERBB2* mutations cause oncogenic signaling in various tumor types and occur most frequently in bladder, cervical, colorectal, esophageal, skin, and uterine cancers. Like *ERBB4* mutations, *ERBB2* mutations are spread across the gene despite conferring similar malignant phenotypes. Therefore, *ERBB2* mutations are classified in the clinical setting by location and mutation type. These classifications are used for therapeutic selection [45]. If the prioritization scheme presented here for *ERBB4* mutations is further specified and rigorously validated, it is possible that despite the wide

distribution of mutation sites, *ERBB4* mutations can also be categorized such that certain classes of mutations may serve as biomarkers to indicate therapeutic strategy.

3.3.2. *ERBB4* Mutant Alleles, which are Prioritized Candidate Drivers of *BRAF* WT Melanomas, Appear to Drive the Proliferation of *BRAF* WT Melanoma Cell Lines.

Thus far, we have validated *ERBB4* G85S, Y285C, G741E, and D861N as drivers of MEL-JUSO cell malignancy. The *ERBB4* E452K, R711C, P759L, and R992C mutations may also confer malignant activity. However, more work needs to be done to thoroughly validate their biological effect *in vivo*. The *ERBB4* E452K mutation was identified in a separate melanoma patient population and has previously been shown to exhibit greater basal levels of ERBB4 kinase activity and tyrosine phosphorylation in HEK 293T cells. The *ERBB4* E452K mutant also conferred greater transformation ability and anchorage independent growth in NIH3T3 cells and SK-MEL-2 cells [71]. The *ERBB4* R106C mutant was initially identified as one of the higher ranked mutants in our prioritization scheme (rank 5) (Table 11). However, it did not confer any oncogenic advantage over WT *ERBB4* in the MEL-JUSO cell line. This mutation's function may be contextually dependent and may be oncogenic in a different *BRAF* WT melanoma model system. The *ERBB4* S418F mutant did not confer any oncogenic advantage over WT *ERBB4* in the MEL-JUSO cell line.

Confounding factors for which cannot be accounted when studying malignant effects of an *ERBB4* mutant in a *BRAF* WT melanoma cell-based model system include but are not limited to: effects of variable dimer partner/ERBB family receptor expression, variable endogenous ligand expression, and tumor environmental factors. However, *in*

vitro and *in vivo* study limitations make our TCGA-SKCM analysis more important as we can use statistical trends identified there to choose the best model systems for mutant evaluation.

3.3.3. ERBB4 Functions as a Context-dependent Tumor Suppressor

We expressed the constitutively homodimerized *ERBB4* Q646C mutant in the ERBB4-dependent MEL-JUSO cell line and found that it causes a significant decrease in clonogenic proliferation. This suggests that despite requiring ERBB4 function, the MEL-JUSO cell line does respond to ERBB4 homodimer-mediated tumor suppressor activity. Thus far, we have postulated that the relative lack of ERBB4 expression in melanoma is not due to a lack of ERBB4 driving function but due to tight regulation of ERBB4 expression to limit the stochastic formation of ERBB4 homodimers. Our finding that ERBB4 homodimers do indeed cause a tumor suppressive phenotype corroborates our working hypothesis regarding ERBB4 expression regulation in melanoma. In the future, it will be important to study the limit to which increased WT or mutant ERBB4 expression causes a change in phenotype due to a shift in the regulation of stochastic dimer formation. Furthermore, future work identifying the mechanisms by which ERBB4 expression is regulated in *BRAF* WT melanoma should be done.

3.3.4. Strategies for Treating ERBB4-dependent, *BRAF* WT Melanomas.

Here we demonstrate that in the TCGA-SKCM *BRAF* WT melanoma dataset, *ERBB4* mutants are correlated with nonsynonymous mutations in a *RAS* gene or *NF1*. This surprising observation suggests that ERBB4 does not stimulate RAS signaling in *BRAF* WT melanomas. Instead, it appears that ERBB4 signaling cooperates with RAS signaling to drive *BRAF* WT melanomas.

Here we demonstrate that in the TCGA-SKCM *BRAF* WT melanoma dataset, *ERBB4* mutations that co-occur with a *RAS* or *NF1* mutation are inversely correlated with events predicted to cause ERBB4-independent activation of the PI3K pathway (mutation of *PIK3CA*, increased *PIK3CA* transcription, mutation of *PTEN*, or decrease in *PTEN* transcription). Thus, we have postulated that, in *BRAF* WT melanomas, *ERBB4* gain-of-function mutants substitute for other methods of PI3K pathway activation by causing increased PI3K pathway signaling which cooperates with elevated RAS signaling to drive the proliferation of these tumor cells (Figure 10). This suggests that *BRAF* WT melanomas which harbor both an oncogenic *ERBB4* mutation and a *RAS* or *NF1* mutation may be treated with an inhibitor of ERBB4-mediated stimulation of the PI3K pathway in combination with a MAPK inhibitor. We believe that the most suitable RAS pathway inhibitor would be a MEK inhibitor (Trametinib, Binimetinib, Selumetinib, or Cobimetinib). The toxicity of the PI3K and mTOR inhibitors (Alpelisib, Sirolimus, Everolimus, Temsirolimus) may preclude the use of these drugs in this application. Although the FDA has approved no specific ERBB4 inhibitors, *ERBB4* mutants are likely to drive *BRAF* WT melanomas through signaling by ERBB4-EGFR or ERBB4-ERBB2 heterodimers. Therefore, drugs which target EGFR (Gefitinib or Cetuximab), ERBB2 (Lapatinib or Pertuzumab), or a combination of EGFR, ERBB2, or ERBB4 (Neratinib) may be suitable (Figure 9). Future work is needed to evaluate these agents.

3.4. Methods

3.4.1. Accession and Analysis of TCGA-SKCM Data

Clinical and biospecimen data was downloaded from The Cancer Genome Atlas – Skin Cutaneous Melanoma (TCGA-SKCM) dataset accessed at https://portal.gdc.cancer.gov/projects/TCGA-SKCM. We obtained the following data for all 470 cases in the SKCM dataset: gender, race, ethnicity, vital status, age at diagnosis, AJCC pathologic stage at diagnosis, primary site, days to death, tumor type sequenced, copy number variation for *ERBB4*, mutation status of *BRAF*, *HRAS, NRAS, KRAS, NF1, EGFR, ERBB2, ERBB3, ERBB4, PIK3CA, PTEN*, and mRNA expression of AKT1, AKT2, AKT3, PTEN, PIK3CA, EGFR, ERBB2, ERBB3, and ERBB4. The R statistical computing and graphics environment software (https://www.r-project.org) was used to reorganize the dataset. Statistical analyses were performed using GraphPad Prism (https://www.graphpad.com) and Microsoft Excel (https://office.microsoft.com/excel).

3.4.2. Creating the *BRAF* WT Dataset

All 227 *BRAF* WT cases were segregated from the TCGA-SKCM dataset for analysis. Cases in which there were *ERBB4* stop-gained or splice-site mutations were removed from the *BRAF* WT dataset. This resulted in the removal of 5 cases, thereby creating a *BRAF* WT dataset that consists of a total of 222 cases. Two of the 5 removed cases also harbored *ERBB4* missense mutation(s). Thus, the three *ERBB4* missense mutations identified in these two cases (M958I, R47Q, G573D) were not considered in the analysis of putative driving *ERBB4* missense mutations in *BRAF* WT melanoma (Sections 3.2.2.-3.2.6.). Each of the three mutations only occurred once. All *ERBB4*

mutations, regardless of coincident stop-gained or splice-site mutation, are included in the N:S ratio analysis for section 3.2.1.

3.4.3. Cell Lines and Cell Culture

Mouse C127 fibroblasts and the ψ2 and PA317 recombinant retrovirus packaging cell lines are generous gifts of Daniel DiMaio (Yale University). These cells were cultured essentially as described previously [85]. The MEL-JUSO [86] human melanoma cell lines were obtained from DSMZ (Braunschweig, Germany) and were cultured as recommended. Cell culture media, serum, and supplements were obtained from Cytiva (Marlborough, VA). G418 was obtained from Corning (Corning, NY). Genetic and mRNA expression data for the cell lines were obtained from the Broad Institute Cancer Cell Line Encyclopedia (CCLE, https://sites.broadinstitute.org/ccle/) [92].

3.4.4. Mutagenesis of DNA Constructs

The recombinant retroviral expression construct pLXSN-ERBB4 has been described previously [94]. We used pLXSN-ERBB4 as the parent plasmid for site-directed mutagenesis using the Q5 Site Directed Mutagenesis Kit (NEB). Mutagenesis was performed essentially as recommended by the manufacturer.

3.4.5. Recombinant Retroviruses

Briefly, the recombinant amphotropic retroviruses LXSN, LXSN-ERBB4 (ERBB4 WT), LXSN-ERBB4 G85S, LXSN-ERBB4 R106C, LXSN-ERBB4 Y285C, LXSN-ERBB4 S418F, LXSN-ERBB4 E452K, LXSN-ERBB4 R711C, LXSN-ERBB4 G741E, LXSN-ERBB4 P759L, LXSN-ERBB4 D861N, and LXSN-ERBB4 R992C were packaged using the ψ2 ecotropic retrovirus packaging cell line, and the PA317 amphotropic retrovirus packaging cell line as previously described [84].

3.4.6. Clonogenic Proliferation Assays

C127, MEL-JUSO cells were each infected with 500 amphotropic retroviral infectious units, essentially as described [84, 97]. After incubation with the viruses, infected cells were selected using G418 at a concentration of 800 ug/mL. The resulting colonies of G418-resistant cells were stained using Giemsa 8 and 14 days later (respectively), and colonies were counted. C127 infections served as a control for viral titer as they do not endogenously express ERBB4 or respond to ERBB4 signaling [97]. Tissue culture plates were digitized, and clonogenic proliferation efficiency was calculated by first calculating the recombinant retroviral titer for each infection and dividing the recombinant retroviral titer in each of the melanoma cell lines by the corresponding recombinant retroviral titer in the C127 cell line [84]. A paired t-test was used to determine whether the efficiency of clonogenic proliferation was significantly different from the efficiency of clonogenic proliferation of the LXSN-ERBB4-WT retrovirus. Significance was defined by a p-value <0.05 (1-tailed).

3.5. Tables

	Non-synonymous	Synonymous	N/S Ratio	
			Mean	Std Dev
Control Genes			2.44	1.23
ARMC4	60	13	4.62	
CFTR	37	18	2.06	
ERC2	53	28	1.89	
SLIT2	50	26	1.92	
SLIT3	49	29	1.69	
ERBB4 Gene			N/S Ratio	One Sample T-test Against Control Genes
ERBB4	48	9	5.33	p = 0.003
Oncogenes			N/S Ratio	
KRAS	12	1	12.00	
NRAS	126	1	126.00	
Tumor Suppressor Genes			N/S Ratio	
CDKN2A	25	6	4.17	
NF1	59	15	3.93	
RB1	13	2	6.50	
TP53	35	3	11.67	
ERBB1-3 Genes			N/S Ratio	
EGFR	25	10	2.50	
ERBB2	5	2	2.50	
ERBB3	6	5	1.20	

Table 6. The ratio of *ERBB4* nonsynonymous to synonymous (N/S) mutations in the TCGA-SKCM *BRAF* WT melanoma dataset

	Site of *ERBB4* Mutation	Not Site of *ERBB4* Mutation	Total
Conserved in ERBB3 ECDs	11	351	362
Not Conserved in ERBB3 ECDs	4	214	218
Total	15	565	580

p = 0.3763

Table 7a. *ERBB4* missense mutations that affect residues of the extracellular domains (ECDs) are somewhat (but not significantly) conserved in the ERBB3 extracellular domains. Statistical analysis is a chi-squared test.

	Site of *ERBB4* Mutation	Not Site of *ERBB4* Mutation	Total
Conserved in EGFR kinase domain	13	226	239
Not Conserved in EGFR kinase domain	0	64	64
Total	13	290	303

p = 0.0565

Table 7b. *ERBB4* missense mutations that affect residues of the kinase domain are somewhat (but not significantly) conserved in the EGFR kinase domain. Statistical analysis is a chi-squared test.

ERBB4 Mutation	Corresponding Mutation in *EGFR*	Corresponding Mutation in *ERBB2*	Function
R106C	R108K		Gain-of-function Oncogenic
G741E	G735S		Gain-of-function Oncogenic
L864P	L858R	L866M	Gain-of-function Oncogenic

Table 8. *ERBB4* missense mutations that affect residues that correspond to sites of oncogenic mutations in *EGFR* or *ERBB2*

	ERBB4 Missense Mutation	*ERBB4* WT	Total
RAS or *NF1* Mutation	29	128	157
RAS and *NF1* WT	4	61	65
Total	33	189	222

$p = 0.0189$

Table 9. In cases of the TCGA-SKCM, *BRAF* WT dataset, *ERBB4* missense mutations are significantly correlated with a *RAS* or *NF1* nonsynonymous mutation. Statistical analysis is a chi-squared test.

		ERBB4 Missense Mutation	*ERBB4* WT	Totals
RAS or *NF1* Mutation	*PIK3CA* Mutation or Increase in PIK3CA Expression or *PTEN* Mutation or Decrease in PTEN Expression	4	35	39
	No *PIK3CA* Mutation or Increase in PIK3CA Expression and no *PTEN* Mutation or Decrease in PTEN Expression	25	93	118
RAS and *NF1* WT	No Consideration of *PIK3CA* /*PTEN* Mutation/Expression Status	4	61	65
Totals		33	189	222

p = 0.016

Table 10. In cases of the TCGA-SKCM, *BRAF* WT dataset, ERBB4 missense mutations are significantly correlated with a *RAS* or *NF1* mutation AND no other apparent cause of increased PI3K activity. Statistical analysis is a chi-squared test.

ERBB4 Mutation	Table 7a	Table 7b	Table 8	Table 9	Table 10	Figure 8	Figure 9	Rank
R106C	1		1	1	1		1	5
R711C		1		1	1	1	1	5
A383T	1			1	1		1	4
S418F	1			1	1		1	4
R491K	1			1	1		1	4
P517A	1			1	1		1	4
P759L		1		1	1		1	4
P800L		1		1	1		1	4
D861N		1		1	1		1	4
G936E		1		1	1		1	4
E969K		1		1	1		1	4
G85S	1			1	1			3
D150N				1	1		1	3
P331S	1			1	1			3
T422I	1			1	1			3
E452K	1			1	1			3
G549S				1	1		1	3
D813N		1		1	1			3
P925S		1		1	1			3
R992C		1		1	1			3
P1276S				1	1		1	3
P1300S				1	1		1	3
E33K				1	1			2
F662L				1			1	2
G741E		1	1					2
L864P		1	1					2
P1080L				1	1			2
R1127K				1	1			2
R1139Q				1	1			2
R1142Q				1			1	2
P1165L				1	1			2
E1187D				1	1			2
P1282S				1	1			2
R196C	1							1
G340E	1							1
P572L				1				1
N814T		1						1
P943S		1						1
P1117L				1				1
R114Q								0
Total: 40	11	13	3	33	29	1	17	

Table 11. Prioritization of candidate *ERBB4* driver mutant alleles in the TCGA-SKCM *BRAF* WT melanoma dataset. Columns refer to tables and figures found in the manuscript and the mutations listed in the table are from the values circled in red in those tables and figures.

Retrovirus	Average Efficiency of Clonogenic Proliferation Relative to LXSN ERBB4 WT	1 Tailed, 1 sample T-test (P) Compared to WT	N
Vector Control	33%	0.002	3
ERBB4 WT	100%	-	11
ERBB4 G85S	162%	0.003	10
ERBB4 R106C	98%	0.400	11
ERBB4 Y285C	161%	0.007	9
ERBB4 S418F	93%	0.197	10
ERBB4 E452K	105%	0.291	10
ERBB4 Q646C*	37%	0.036	2
ERBB4 R711C	174%	0.052	9
ERBB4 G741E	140%	0.018	10
ERBB4 P759L	119%	0.196	10
ERBB4 D861N	119%	0.042	9
ERBB4 R992C	122%	0.068	10

Table 12. Clonogenic proliferation efficiency of LXSN (Vector Control), LXSN-ERBB4-WT, LXSN-ERBB4-candidate priority mutants, and the LXSN-ERBB4-Q646C mutant in the MEL-JUSO cell line. Highlighted p-values are statistically significant ($p < 0.05$).
* The ERBB4 Q646C mutant average efficiency of clonogenic proliferation and t-test is relative to vector control.

3.6. Figures

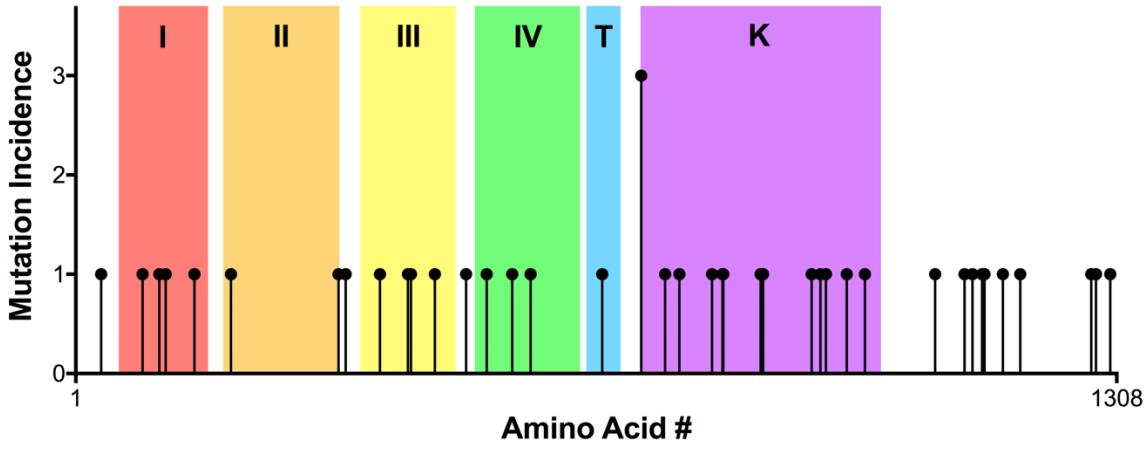

Figure 8. In *BRAF* WT melanomas of the TCGA-SKCM dataset, *ERBB4* nonsynonymous missense mutations are evenly distributed across the entire *ERBB4* coding region. Roman numerals indicate subdomains of ERBB4's extracellular region. The "T" domain represents the transmembrane domain. The "K" domain represents the intracellular kinase domain.

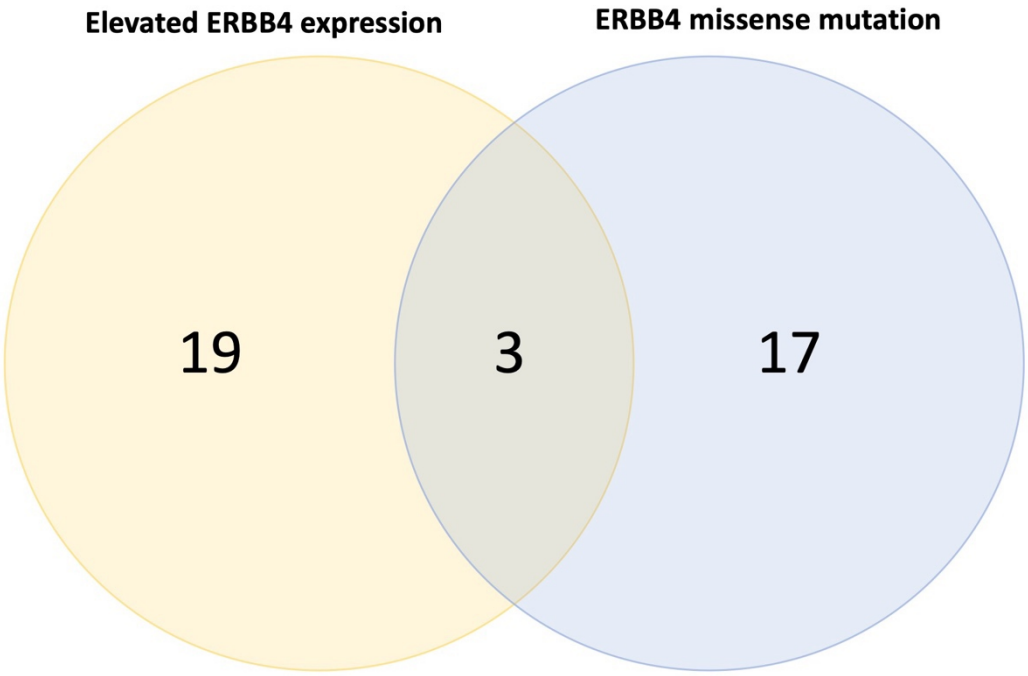

Figure 9. Within the 178 *BRAF* WT cases in the TCGA-SKCM dataset, an *ERBB4* missense mutation and elevated *ERBB4* transcription appear to be largely mutually exclusive.

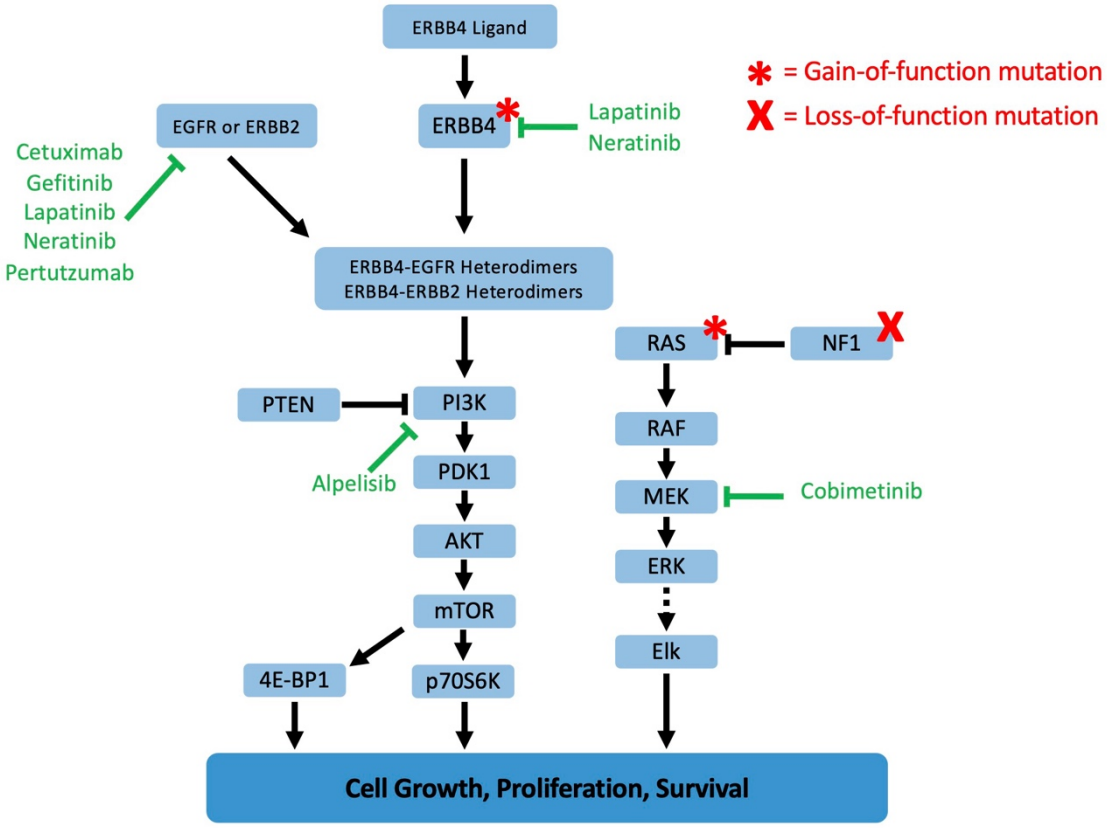

Figure 10. In some *BRAF* WT melanomas, mutant ERBB4 stimulates the PI3K pathway by causing increased ERBB4-EGFR or ERBB4-ERBB2 heterodimer formation and cooperates with elevated RAS/MAPK pathway signaling (caused by a gain-of-function *RAS* or loss-of-function *NF1* mutation) to drive the proliferation of *BRAF* WT melanomas. These melanomas may be treated with a combination MEK inhibitor and PI3K or ERBB receptor inhibitor.

Chapter 4: Conclusions and Future Work

BRAF WT melanomas appear to be a significant clinical problem as they have no available targeted therapeutic available beyond immune checkpoint inhibitors and make up ~50% of the metastatic melanoma population. Many of these melanomas harbor a *RAS* or *NF1* mutation resulting in elevated RAS/RAF/MEK/ERK signaling. Unfortunately, MEK inhibitors are not successful in treating these melanomas. This is in part due to increases in PI3K/Akt pathway signaling, and as such, PI3K and MEK inhibitor combinations appear to be more successful in treating some *RAS* mutant melanomas.

ERBB4 is a receptor tyrosine kinase and a member of the ERBB receptor family which also includes EGFR, ERBB2, and ERBB3. Effects of ERBB4 signaling have not been evaluated specifically in *BRAF* WT melanomas. Our analysis of the TCGA-SKCM dataset showed that elevated ERBB4 expression appears to be positively correlated with the presence of a *RAS* or *NF1* mutation in *BRAF* WT melanomas. Therefore, we hypothesized that ERBB4 signaling cooperates with RAS pathway signaling in *BRAF* WT melanomas that harbor a RAS pathway activating mutation.

We obtained a panel of *BRAF* WT melanoma cell lines and determined that the introduction of the *ERBB4* WT gene caused a significant increase in clonogenic proliferation of the MEL-JUSO, MeWo, IPC-298, and SK-MEL-2 cell lines. We also determined that the introduction of the *ERBB4* K751M, kinase-inactive, mutant functioned as a dominant negative (DN) and resulted in a significant decrease in clonogenic proliferation of the MEL-JUSO, MeWo, and IPC-298 cell lines. Therefore, we determined that ERBB4 is both sufficient and necessary for the clonogenic proliferation of some *BRAF* WT melanoma cell lines.

We created MEL-JUSO cell lines which stably expressed the LXSN vector control gene, LXSN-ERBB4-WT gene, and the LXSN-ERBB4-DN gene and evaluated their ability to grow in semi-solid medium. We found that the MEL-JUSO LXSN ERBB4 DN cell line exhibited a significant decrease in anchorage-independent colony growth. However, WT *ERBB4* had no effect on the MEL-JUSO cell line's ability to grow in an anchorage independent manner. Therefore, we concluded that ERBB4 is necessary for anchorage-independent growth of the MEL-JUSO *BRAF* WT melanoma cell line.

ERBB4 mutations are known to function in an oncogenic manner in multiple cancer types, including melanoma. However, until now, *ERBB4* mutations had not been evaluated for oncogenic activity specifically in the context of *BRAF* WT melanoma. We calculated the nonsynonymous to synonymous mutation ratio of *ERBB4* in *BRAF* WT cases found in the TCGA-SKCM dataset and determined that the N/S ratio indicated non-random selection for *ERBB4* mutation. Therefore, we developed a prioritization strategy for identifying putative driver mutations by analyzing mutations occurring in the TCGA-SKCM, *BRAF* WT dataset. We prioritized mutations which occurred more than once in the TCGA-SKCM, *BRAF* WT dataset. We prioritized mutations which occurred at sites conserved in the *EGFR* kinase domain or the *ERBB3* ligand binding domain. We also prioritized mutations which occurred at sites conserved in *EGFR* or *ERBB2* that are validated oncogenic mutation sites. Paralleling our findings regarding elevated ERBB4 expression, we found that the occurrence of *ERBB4* missense mutation positively correlates with the occurrence of a *RAS* or *NF1* mutation. This suggested once again that ERBB4 signaling cooperates with elevated RAS signaling. Therefore, we prioritized mutations which occurred in cases that also contained a *RAS* or *NF1* mutation. We

hypothesized that oncogenic ERBB4 signaling would be more likely to occur in aggressive melanomas. Because melanomas are known to rely on both the RAS/RAF/MEK/ERK signaling pathway and the PI3K/Akt signaling pathway, we hypothesized that ERBB4 driver mutations would be more likely to occur in those tumors which exhibit both elevated RAS and PI3K pathway signaling. However, we found that ERBB4 mutation was more common in *RAS* or *NF1* mutant cases which did not harbor another apparent cause of increased PI3K activity. This suggests that ERBB4 stimulates the PI3K pathway in place of some other alteration which would cause elevated PI3K signaling. Therefore, we prioritized *ERBB4* mutations that occurred in cases where there was a *RAS* or *NF1* mutation and no other apparent cause of increased PI3K signaling. Because elevated ERBB4 expression and *ERBB4* mutation appear to function similarly, we evaluated the TCGA-SKCM, *BRAF* WT dataset for overlap between the occurrence of increased ERBB4 expression and *ERBB4* mutation. We found that there were only 3 cases (of those where both expression and mutation data were available) in the dataset that exhibited both elevated ERBB4 expression and an *ERBB4* mutation. This suggests that indeed, elevated ERBB4 expression and *ERBB4* mutation are independent tumor-driving events that may function in similar ways such that there lacks selection for both alterations to be coincident in a tumor. We prioritized *ERBB4* mutations that are not coincident with elevated ERBB4 expression. Taken all together, we created a strategy where we identified high-priority putative driver *ERBB4* mutations.

We made retroviral vectors to deliver 9 high-priority candidate driver *ERBB4* mutant genes to the ERBB4-dependent MEL-JUSO cell line and evaluated their ability to confer greater clonogenic proliferation efficiency than WT *ERBB4*. We found that the

G85S, G741E, and D861N mutations identified in the *BRAF* WT dataset cause a significant increase in clonogenic proliferation efficiency and thus are likely drivers of melanoma. We also identified the R711C mutant as a likely driver of melanoma due to the large increase (though not statistically significant) in average clonogenic proliferation efficiency. We developed a retroviral vector for the gain-of-function *ERBB4* Y285C mutation which was identified in a non-small cell lung cancer. We hypothesized that its gain-of-function activity would cause increased clonogenic proliferation in a *BRAF* WT melanoma cell line. Indeed, we found that it caused significantly greater clonogenic proliferation compared to WT *ERBB4.* To our knowledge, this is the first time that the *ERBB4* Y285C mutation was evaluated for phenotype in a cancer cell line.

ERBB4 is known to be poorly expressed in melanoma and as such the position that ERBB4 "overexpression" could cause oncogenic activity has historically been overlooked. We postulated that low ERBB4 receptor density within a cell is not due to a lack of need for ERBB4 signaling but rather due to a regulatory mechanism that controls the stochastic dimerization events of ERBB4 such that it is more likely that ERBB4 dimerizes with EGFR or ERBB2 to initiate oncogenic signaling events. For our hypothesis to be true, it would imply that melanomas respond to ERBB4 homotypic tumor suppressive signaling. To test this, we introduced the constitutively homodimerized *ERBB4* Q646C mutant and evaluated its effect on clonogenic proliferation efficiency. Indeed, we found that the *ERBB4* Q646C mutation caused a significant decrease in clonogenic proliferation of the MEL-JUSO cell line. Thus, we have confirmed that ERBB4 is indeed a context-dependent oncogene and tumor suppressor in some *BRAF* WT melanomas. Therefore, melanomas have likely developed

mechanisms to limit ERBB4-mediated tumor suppressor activity. Furthermore, this suggests that oncogenic ERBB4 signaling is likely due to ERBB4 heterodimerization.

Now that we have established that some *BRAF* WT melanomas exhibit an ERBB4-dependent phenotype, we can leverage our understanding of ERBB4 signaling to elucidate the mechanism by which ERBB4 functions as a driver of *BRAF* WT melanoma and subsequently identify effective drug targets for the treatment of *BRAF* WT melanoma.

It appears that oncogenic ERBB4 functions via heterotypic signaling. Identifying which ERBB4 heterodimerization partners are responsible for this signaling could help identify a therapeutic target for these ERBB4-dependent, *BRAF* WT melanomas. If ERBB4-EGFR heterodimers are responsible for oncogenic signaling, treatment with an anti-EGFR monoclonal antibody such as cetuximab may be therapeutically beneficial. If ERBB4-ERBB2 heterodimers are responsible for oncogenic signaling, treatment with an anti-ERBB2 monoclonal antibody such as pertuzumab may be more appropriate. Furthermore, ERBB4 heterodimerization partner preferences may vary depending on whether a tumor is *ERBB4* WT or *ERBB4* mutant. Different *ERBB4* mutants may also exhibit individual preferences for dimer partners. Finally, the EGFR and ERBB2 receptor expression is likely to be relevant as well. *BRAF* WT melanomas likely respond differently to ERBB4 overexpression or mutation dependent on the endogenous expression of EGFR and ERBB2. In the future, we will evaluate ERBB4 heterodimerization preferences in various ERBB4-dependent, *BRAF* WT melanoma cell lines by evaluating phenotypic changes in oncogenic activity following the introduction of silencing RNAs specific for EGFR or ERBB2 or dominant negative mutations for

EGFR and ERBB2. We will also treat ERBB4-dependent, *BRAF* WT melanoma cell lines with EGFR and ERBB2-targeted monoclonal antibodies to evaluate their efficacy.

Given our findings from the TCGA-SKCM, *BRAF* WT dataset that suggest that ERBB4 mutant melanomas cause increased PI3K signaling activity and that ERBB4 mutants cooperate with elevated RAS signaling, we hypothesize that melanoma cell lines that express *ERBB4* driver mutations will exhibit decreased potency for PI3K pathway inhibitors. Furthermore, we hypothesize that the potency of PI3K inhibitors may improve when given in combination with a MEK inhibitor. Treatment of these cell lines with an EGFR or ERBB2 targeted monoclonal antibody in combination with a MEK inhibitor may also prove effective at treating *ERBB4* dependent, *BRAF* WT melanomas, and we postulate that this combination may improve MEK inhibitor potency.

ERBB4 ligands have not yet been studied for functional relevance in *BRAF* WT melanomas. However, ERBB4 ligands couple to distinct signaling and biological activities [101, 104]. Therefore, we hypothesize that ERBB4 ligand expression is adapted to promote ERBB4-dependent tumor progression in *BRAF* WT melanomas. We will be evaluating the importance of ERBB4 ligands by silencing them individually in ERBB4-dependent, *BRAF* WT melanoma cell lines. Additionally, we will evaluate the oncogenic phenotype associated with introducing the ERBB4 ligand, NRG2β, and the ERBB4 partial agonist, NRG2b Q43L mutant [105].

This work has been fundamental in establishing a panel of ERBB4-dependent, *BRAF* WT melanoma cell lines and developing a pilot driver mutation screening method. Future work will be directed towards understanding the mechanistic underpinnings of ERBB4-dependent activity in *BRAF* WT melanomas and expanding our screen of *ERBB4*

mutations to be more thorough in the identification and validation of oncogenic *ERBB4* mutations. This future work will result in the identification of biomarkers for the use of various targeted therapeutics such as EGFR and ERBB2 targeted monoclonal antibodies, PI3K inhibitors, and MEK inhibitors in *BRAF* WT melanoma patients.

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