Developing an Isoform-Specific Knock Out Mouse to Investigate the Roles of Non-Canonical Signaling by ErbB4 In Vivo

by

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Dedication

This is for

Jack Finnegan Doherty

with all my heart

Doctor Daddy

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Abstract

ErbB4 is a receptor tyrosine kinase (RTK) of the epidermal growth factor receptor (EGFR) family that regulates numerous biological processes, from development to physiological function and degeneration. ErbB4 is important in many aspects of neurodevelopment including astrogenesis, neural migration, dendrite formation and synapse maturation. ErbB4 is also necessary for normal development of the heart, mammary glands, immune cells, and lungs. Furthermore, ErbB4 plays important roles in brain function and synaptic transmission in structures such as the hippocampus, amygdala, nucleus accumbens and substantia nigra. ErbB4, and its cognate ligand neuregulin 1 (NRG1) are both schizophrenia susceptibility genes. Mice lacking ErbB4 expression during development or in adulthood display 'schizophrenia-like behavior,' indicating that ErbB4 plays important roles in both development and maintenance of normal brain function.

ErbB4, like all RTKs, signals through canonical signaling via phosphorylation-mediated signaling cascades. ErbB4 can also signal through a non-canonical mechanism whereby ErbB4 is cleaved and releases an intracellular domain (E4ICD) that shuttles to the nucleus and regulates gene transcription. Non-canonical signaling by ErbB4 requires an isoform-specific inclusion of a cleavage site in the extracellular juxtamembrane domain (EJD). Alternative splicing of the *ERBB4* can generate two different ErbB4 isoforms: ErbB4-JMb, which is uncleavable, and ErbB4-JMa, which includes a cleavage site required to signal through the non-canonical mechanism. Non-

canonical signaling by ErbB4-JMa has been implicated in the regulation of cortical, cardiac, mammary gland, pulmonary and immune cell development.

While ErbB4-JMa was the first RTK identified to be capable of non-canonical signaling, it is now known that most RTKs can be cleaved and utilize direct ICD signaling. However, most studies of this non-canonical signaling by RTKs, including ErbB4, have been performed in vitro. Loss-of-function mouse models, in which non-canonical RTK signaling is abrogated, have not yet been developed to study non-canonical RTK signaling in vivo.

The central aim of this dissertation is to create a novel mutant mouse in which non-canonical signaling by ErbB4-JMa is abolished and to phenotypically assess the roles of ErbB4 signaling in the intact organism. To accomplish this, I used germline CRISPR/Cas9 gene editing of mouse *ERBB4* exon 16a to target ErbB4-JMa and create an isoform-specific knock out mouse, designated ErbB4-JMa$^{-/-}$. In this mutant mouse expression of ErbB4-JMa, and hence non-canonical E4ICD signaling, is eliminated, while ErbB4-JMb canonical signaling is intact. I first confirmed that formation of E4ICD was abolished due to the absence of ErbB4-JMa transcription. I then used the ErbB4-JMa$^{-/-}$ mouse to showed that non-canonical signaling by ErbB4-JMa regulates the expression of GFAP during cortical development, consistent with previous results. Next, I used an unbiased gene-discovery approach to identify genes that are regulated by ErbB4 during cortical development. I found that the regulation of two genes, CRYM and DBi, is dependent on ErbB4-JMa. Finally, I used the ErbB4-JMa$^{-/-}$ mouse to assess whether non-canonical ErbB4 signaling plays a role in ErbB4-related developmental phenotypes. I found that ErbB4-JMa signaling is not required for the development of the

heart, mammary glands, sensory ganglia or cortical interneurons, but may regulate the development of immune cells. Together these findings show that the ErbB4-JMa$^{-/-}$ mouse is a robust tool to study the role of non-canonical ErbB4-JMa signaling in vivo.

CHAPTER 1: Introduction

1.1: Overview

Receptor tyrosine kinases (RTKs) are among the most important biological signaling systems. RTKs play numerous important roles in development, physiology, and degeneration. RTKs are primarily known to signal through the canonical mechanism. Briefly, RTKs are activated by their cognate ligand, which leads to dimerization, autophosphorylation and phosphorylation of downstream signaling molecules. Some RTKs are also capable of signaling through a non-canonical mechanism, where following ligand binding the RTK is cleaved to create a soluble intracellular domain (ICD) that can shuttle to the nucleus to regulate transcription directly. It is now known that more than half of RTKs can be cleaved and signal through this non-canonical mechanism in vitro. However, knowledge of the roles of non-canonical signaling by RTKs has primarily been uncovered through in vitro experiments and has not yet been confirmed in studies in an intact organism. An important step in investigating non-canonical signaling by RTKs is to directly assess their roles in vivo models.

ErbB4 is a well-characterized receptor tyrosine kinase (RTK) and a member of the epidermal growth factor receptor (EGFR) family. ErbB4 was the first RTK identified to be capable of cleavage-mediated non-canonical signaling due to alternative splicing generating the ErbB4-JMa with a cleavage site in the EJD. ErbB4 is hence the RTK with

the best understanding the roles of non-canonical signaling, particularly in neurodevelopment and organogenesis.

The broad aim of this dissertation was to study the roles of non-canonical ErbB4 signaling in vivo by creating mutant mice lacking the ability to form an ErbB4 ICD (E4ICD), and hence the ability to activate non-canonical signaling. Developing a novel ErbB4 mutant mouse that is incapable of non-canonical signaling could be used to validate or invalidate the putative roles of E4ICD signaling in an intact organism for the first time and further understanding of ErbB4 signaling mechanisms in vivo. I did this by using CRISPR/Cas9 gene editing to create an isoform-specific knock out mouse in which ErbB4-JMa expression is abrogated. Using this mouse, I showed that non-canonical signaling by ErbB4-JMa is required for regulation of GFAP expression during cortical development. I also showed that other developmental processes that require ErbB4 do not require ErbB4-JMa, and hence non-canonical signaling. These include development of the heart, mammary gland, neural ganglia and parvalbumin interneurons.

ErbB4 plays diverse roles in adult brain physiology in different cell types and brain regions. However, whether non-canonical ErbB4 signaling is important in these functions has scarcely been studied. A closer look at the mechanism of ErbB4 signaling and isoforms responsible for these functions may shed some light on why the functions of ErbB4 are so complex.

1.2: Receptor tyrosine kinases

RTKs are among the most important groups of receptors in biological systems. There are currently 58 known RTKs in humans, and they are classified into 20

subfamilies by homology of the extracellular domains and mechanism of activation (Lemmon & Schlessinger, 2010). RTKs have many functions, but they are primarily known to play key roles in cellular development, metabolism and apoptosis (Lemmon & Schlessinger, 2010). Reflecting their importance, dysfunction of RTK signaling can cause of numerous developmental disorders and oncogenesis (Du & Lovly, 2018; McDonell et al., 2015a),.

RTKs are generally monomeric, single pass transmembrane proteins with conserved structural regions that function by a similar mechanism. These regions are a ligand binding extracellular domain, a transmembrane domain, an intracellular tyrosine kinase domain, and a kinase activation site within the intracellular juxtamembrane region (Lemmon & Schlessinger, 2010).

1.3: Canonical RTK signaling

All RTKs signal through a well-described and well-understood signaling mechanism that results in indirect, phosphorylation-mediated activation of intracellular signaling cascades. This ‘canonical’ signaling is initiated by binding the receptor’s specific ligand, which is usually a growth factor, cytokine, or hormone. Ligand binding causes allosteric modulation of the extracellular domain and allows monomers of the receptor to dimerize. Dimerization leads to conformational changes within the intracellular domains that reciprocally activate the kinase site to trans-phosphorylate tyrosine residues. Phosphorylated tyrosine residues are then used as docking sites for many intracellular adaptor proteins that contain a phosphotyrosine binding (PTB) or Src homology 2 (SH2) domains. The docking of adaptor proteins is specific to the peptide motif surrounding the phosphor-tyrosine residues. The adaptor proteins are then

activated and phosphorylate downstream molecules to cause signaling cascades that mediate the RTK's effect, usually via the activation of transcription factors to regulate gene expression.

The most common downstream signaling pathways of RTKs are the extracellular signaling related kinases (Erk) and mitogen activated protein kinase (MAPK) pathways, which are initiated by the docking of the growth factor receptor binding protein 2 (Grb2) onto a phosphotyrosine site by its SH2 domain. This then activates a phosphorylation cascade: Ras activates MAPK kinase kinase (RAF), which activates MAPK kinase (MEK), which in turn activates MAPK (Fig. 1.1). The activation of MAPK by phosphorylation allows it to further phosphorylate and activate various transcription factors, thereby eliciting the cellular response to RTK activation. Another common example of RTK signaling is the phosphoinositide 3-kinase (PI3K) pathway that activates protein kinase B (AKT) by phosphorylation, which can then activate numerous other pathways, including mTOR and CREB to regulate the cellular response to RTK activation.

Each RTK has numerous tyrosine residues that can activate specific signaling pathways via different adaptor proteins. As such, the signaling networks of just one RTK can be highly complex with converging pathways and/or negative and positive feedback systems (Volinsky & Kholodenko, 2013). Such complexity is illustrated by the signaling through ErbB4. ErbB4 is phosphorylated at 19 of its 28 tyrosine residues within the intracellular domain and can recruit and phosphorylate at least 11 different signaling molecules (Kaushansky et al., 2008; Schulze et al., 2005). Furthermore, ErbB4 can generate opposing effects depending on its isoform composition (Lucas et al., 2022;

Muraoka-Cook et al., 2009). For example, ErbB4-JMa increases survival and proliferation, whereas ErbB4-JMb decreases survival and proliferation (Wali et al., 2014). Given the diversity of phosphorylation events and downstream effects, an RTK, like ErbB4, can have multiple, diverse, and sometimes counterintuitive roles, and these roles can vary depending on the biological system being investigated. The complexity of this intracellular signaling cascades by RTKs can be appreciated within the EGFR family of receptors in Fig. 1.1 (Schulze et al., 2005).

1.4: Non-canonical RTK signaling

Non-canonical signaling is a cleavage-mediated alternative pathway certain RTKs can utilize. As with canonical signaling, the RTK is activated by ligand binding, dimerization and trans-autophosphorylation. During non-canonical signaling, RTKs are cleaved in a process known as regulated intramembrane proteolysis (RIP) to release an intracellular domain (ICD) which can translocate directly to the nucleus and associate with transcription factors to bind promoter regions of chromatin and regulate transcription directly (Carpenter & Liao, 2009; Schlessinger & Lemmon, 2006; Song, et al., 2013). Most of the non-canonical signaling by RTKs has been shown in the context of sequential cleavage by metalloprotease and then the presenilin/gamma-secretase complex (PS1). However, there are also other mechanisms by which RTK ICDs can be created. Other enzymatic cleavage can also produce ICDs, such as in the case of EGFR, which can be cleaved by calpain (Liao & Carpenter, 2012), but not by presenilin/γ-secretase (PS1) (Merilahti et al., 2017). Splice variants of RTKs can also produce a truncated form of the receptor expressing only the ICD, as has been shown

to be the case for EGFR and ErbB3 (Andrique et al., 2012; Piccione et al., 2012), amongst others.

Non-canonical signaling occurs in almost half of all RTKs (Merilahti et al., 2017; Merilahti & Elenius, 2019), however the functional significance of non-canonical signaling by most RTKs has not been well studied and remains unclear. Other than ErbB4 (see below), the only other RTK with a well-defined in vivo role for ICD signaling is Ryk, which functions in neural progenitors during cortical development (Lyu et al., 2008). Stimulation of Ryk by Wnt3 can cleave Ryk to form an ICD that interacts with the chaperone proteins, Cdc37and Smek1/2, translocates to the nucleus and induces neuronal differentiation (Chang et al., 2017; Lyu et al., 2008, 2009). Non-canonical signaling by Ryk has also been shown to regulate the development of cortical interneurons (McKenzie et al., 2019). Aside from their roles in oncogenesis, there have been few other proposed roles for non-canonical signaling by RTKs in biological systems (Chen et al., 2020; Huang, 2021).

As more roles of non-canonical signaling by RTKs are discovered in vitro, it will be vital to develop animal model to investigate whether these roles are important in the intact organism. The most direct approach for this is to identify the cleavage sites of RTKs are develop mutant mice in which the cleavage site is mutated to abolish ICD formation. Mutant mice with cleavage-resistant proteins have been developed (discussed in Chapter 1.9), but this has not previously been done for RTKs that can be cleaved. The development of these mouse models will streamline the identification of bona fide, in vivo roles of non-canonical signaling by RTKs.

1.5: ErbB4 signaling

The epidermal growth factor receptor (EGFR) family of receptors is a group of four RTKs that share structural homology and the ability to be activated by growth factors with EGF-like domains. The EGFR was the first RTK to be shown to respond EGF activation by tyrosine kinase activity, and, therefore, the EGFR family is also known as Class I RTKs. The EGFR family consists of EGFR, ErbB2, ErbB3 and ErbB4 (known in humans as HER1, HER2, HER3 and HER4) (Fig. 1.1). ErbB4 was the last member of the EGFR family to be identified (Plowman et al., 1993).

The large extracellular structure of the EGFR family of receptors contains four domains and an extracellular juxtamembrane domain (EJD). Domains I and III are both lysine-rich domains and contain the ligand binding sites. Domains II and IV are both cysteine-rich. In their inactive states, EGFR, ErbB3 and ErbB4 have a "tethered" structural formation, where the dimerization arm of Domain II is sequestered by a beta-hairpin loop interaction with Domain IV. Upon ligand binding, the extracellular domains undergo conformational changes that unmask Domain II and dimerize with another activated receptor. This dimerization can occur in as homo- or hetero-dimerization for EGFR and ErbB4, both of which can dimerize with themselves or ErbB2. ErbB2 does not require ligand binding to dimerize, as ErbB2 exists in the “active” extracellular formation and can hence dimerize with each of the other activated receptors (Fig. 1.1).

ErbB4 is unique in this family of receptors in that alternative splicing of the *ERBB4* gene can generate different isoforms of ErbB4 (Fig. 1.2) that can utilize cleavage mediated non-canonical signaling via direct E4ICD nuclear function (Fig. 1.3). Exons 1-15 of the *ERBB4* gene encode the extracellular domain containing the cytosine rich domains (CRD), ligand binding site and the receptor dimerization domain (Fig. 1.2).

The alternative splice site at the EJD includes either *ERBB4* exon 16b or 16a, to generate ErbB4-JMb or ErbB4-JMa respectively (Fig. 1.2). Inclusion of exon 16a generates ErbB4-JMa with an elongated EJD containing the TACE cleavage site (Rio et al., 2000) allowing RIP-mediated non-canonical signaling (Fig. 1.2 and 1.3). In contrast, inclusion of exon 16b generates ErbB4-JMb that is not cleavable, and hence can only signal via a canonical mechanism (Fig. 1.3). All isoforms contain the PS1 cleavage site within the transmembrane (TM) domain, however without release of the extracellular domain (ECD) by cleavage, encoded by *ERBB4* exon 16a, the PS1 complex cannot access the cleavage site. Following the TM domain, the sequences encoding the intracellular domain (ICD) start at exon 18 (Fig. 1.2). There is a nuclear localization signal domain shortly after the TM domain that allows the E4ICD to be shuttled to the nucleus (Williams et al., 2004). There are 22 potential tyrosine phosphorylation sites within the TK domain which can facilitate interactions with numerous adaptor proteins to mediate various phosphorylation-mediated functions (Kaushansky et al., 2008). Within the sequences encoding the E4ICD there is an additional alternative splice site that can include *ERBB4* exon 26, containing an additional PI3K site to form CYT1, or exclude exon 26 to form CYT2 (Fig. 1.2).

Although non-canonical signaling is has been suggested to be possible for many RTKs, ErbB4 was the first receptor shown to be capable of cleavage-mediated signaling (Elenius et al., 1997; Rio et al., 2000; Vecchi et al., 1996). When the alternative splice isoforms ErbB4-JMa and ErbB4-JMb were identified, it was shown that only ErbB4-JMa contains a TACE cleavage site and can undergo RIP (Elenius et al., 1997; Vecchi et al., 1996). The membrane-bound E4ICD can then be cleaved by PS1 to release the soluble

E4ICD into the cytoplasm for direct nuclear signaling (Fig. 1.3). The secondary cleavage event by PS1 requires TACE cleavage first, as the ectodomain blocks access of the PS1 complex to the presenilin substrate cleavage site (Rio et al., 2000; Wolfe, 2020).

Since the identification of non-canonical signaling by ErbB4, there have been many roles attributed to E4ICD signaling. The first known role of E4ICD signaling was found in controlling the timing of cortical development (Sardi et al., 2006; Schlessinger & Lemmon, 2006). In addition, E4ICD signaling has been implicated in biological functions such development of the heart, mammary gland, lungs and immune cells (Hoeing et al., 2011; Iwamoto et al., 2017a; Muraoka-Cook et al., 2008; Schumacher et al., 2017). As ErbB4 signaling is known to play many roles in various biological systems, delineating which of the functions of ErbB4 are mediated by non-canonical and canonical signaling mechanisms will provide insights into the importance of this mechanism for ErbB4 and for RTKs in general. For these reasons, ErbB4 is an interesting target to study the role of non-canonical signaling.

1.6: The function of ErbB4 in the developing and adult brain

ErbB4 is known to play multiple roles in brain development and function. Non-canonical signaling by ErbB4 has been suggested to be important in transcriptional regulation of neural precursor cells during cortical development. However, the other roles ErbB4 in the brain, such as interneuron migration or synapse development and transmission, have not been studied in the context of non-canonical ErbB4 signaling. Identifying whether ErbB4 non-canonical signaling is important in the known roles of ErbB4 in the brain will improve our understanding of these biological processes.

1.6.1: ErbB4 in cortical development

The first role of non-canonical signaling by ErbB4-JMa discovered was in the regulation of cortical development. E4ICD was shown to repress the expression of astrocyte-associated genes to regulate the transition from neurogenesis to astrogenesis in the cortex (Sardi et al., 2006).

In development of the nervous system, the lateral ventricle is initially lined with neuroepithelial cells (NECs) that divide symmetrically around the ventricle. Cajal-Retzius cells are the first neural cell type to be produced by asymmetric division, which happens around E10.5 in mice (Fig. 1.4). NECs then become radial glial cells (RGCs), with basal and apical processes extending to the surface of the brain or the lateral ventricle, respectively. RGCs can expand the pool of progenitor cells by symmetrical division or produce intermediate progenitor cells (IPCs) or neurons by asymmetrical division. Neurons that are created by RGCs then migrate towards the pial surface along the RGC processes and form layers in an “inside out” organization in the cortical plate (CP) (Fig. 1.4).

As neurogenesis continues there are intrinsic in RGCs and extrinsic changes in the extracellular matrix and signaling factors from the cells in the developing cortex. These changes transcriptionally regulate the neurons being made to create different neuronal types in batches that form the cortical layers. Following neurogenesis, around E17.5 there is a transcriptional shift and RGCs and IPCs begin to differentiate into astrocytes (Fig. 1.4 Following the production of astrocytes by RGCs and IPCs continue into gliogenesis by producing oligodendrocyte precursor cells (OPCs) from around P0 to P7 (Fig. 1.4).

The transition from neurogenesis to astrogenesis is a tightly regulated process that depends on the interaction of many intrinsic and extrinsic factors. It involved a switch from RGCs repopulating upon asymmetrical division to differentiate directly into astrocytes. While this is known to be important, the actual programming events that mediate this transition are relatively unknown. EGFR signaling is also known to play important roles in the final stages of cortical development. EGFR signaling is known to promote the differentiation of progenitors in both astrogenesis (Fujimoto et al., 2016; Viti et al., 2003) and oligodendrogenesis (Yang et al., 2017). ErbB4, on the other hand, has been shown to repress the transition to astrogenesis. (Fig. 1.4) (Sardi et al., 2006). It was shown that loss of ErbB4 in vivo results in precocious astrogenesis by expression of GFAP and S100β in the developing cortex. Ectopic expression of ErbB4-JMa and E4ICD, but not ErbB4-JMb, can rescue the increased expression of GFAP (Sardi et al., 2006). It was found that this is due to direct binding of E4ICD to the promoter regions of GFAP and S100β in dissociated NPCs, confirming the role direct nuclear by E4ICD in this phenotype (Sardi et al., 2006). I will expand upon the role of ErbB4 in astrogenesis in Chapter 3.

1.6.2: ErbB4 in interneuron development and migration

ErbB4 is known to be involved development and migration of interneurons. Aberrant interneuron development due to the loss of ErbB4 is thought to be a partial cause of 'schizophrenia-like' behavioral endophenotypes observed in ErbB4$^{-/-}$ mice (H. Li et al., 2012; Neddens & Buonanno, 2010; Wang et al., 2018; Yin et al., 2013a). However, whether non-canonical signaling by ErbB4 is involved in interneuron development and function has not been assessed.

Cortical interneurons are derived from NPCs in the medial and lateral ganglionic eminences (MGE and LGE). These interneuron precursor cells express a variety of surface receptors that allow chemoattractant and repellant signals from the developing brain regions to direct their migration. ErbB4 is expressed in developing interneurons (Neddens et al., 2011; Neddens & Buonanno, 2010), and ErbB4-NRG1 chemoattractant signaling facilitates tangential migration of interneurons into the cortex (Flames et al., 2004; Raki et al., 2015.; Villar-Cerviño et al., 2015). Loss of ErbB4 hence results in fewer interneurons of various subtypes in the adult mouse cortex and hippocampus (Neddens & Buonanno, 2010; Wang et al., 2018).

While the role of ErbB4 on interneuron function is the best characterized, ErbB4 signaling has been shown to be important in synapse formation and physiology of other neuronal cell types in the brain (Li et al., 2007; Müller et al., 2018; Yin et al., 2013). Deficits in ErbB4 signaling in these different neurons has also been suggested to contribute to the behavioral endophenotypes observed in ErbB4$^{-/-}$ mice.

1.6.3: ErbB4 in adult brain function

ErbB4 is expressed in the brain from early development through to adulthood. Many studies have shown that ErbB4 is primarily expressed in inhibitory interneurons in the cortex, hippocampus, amygdala, and basal ganglia (Bean et al., 2014; Neddens et al., 2011; Neddens & Buonanno, 2010). ErbB4 expression in interneurons is known to regulate dendrite and synapse formation as well as long term potentiation and memory functions, among others (delPino et al., 2013a; Müller et al., 2018a; Seshadri et al., 2015; Wen et al., 2010; Yin et al., 2013b). ErbB4 is also expressed in the post-synaptic compartment of synapses and regulates excitability of interneurons and, thereby, plays

a crucial role in maintaining the excitatory-inhibitory balance in the brain. However, ErbB4 has also been shown to be expressed in other subsets of cells, including serotonergic cells in the dorsal raphe nucleus, noradrenergic neurons in the locus coeruleus and oxytocin neurons in the hypothalamus (Cao et al., 2018; Shamir et al., 2012; Zhang et al., 2017).

Both NRG1 and ERBB4 have been implicated in the development of schizophrenia (Banerjee et al., 2010; Corfas et al., 2004) and have also been identified as schizophrenia susceptibility genes (Greenwood et al., 2016) . The loss of ErbB4 in the entire brain results in a plethora of 'schizophrenia-like' behavioral endophenotypes (Mei & Xiong, 2008; Shamir et al., 2012a). ErbB4$^{-/-}$ mice have been shown to have decreased anxiety and/or manic behavior as indicated by increased time in the center of an open field test (OFT) and increased open arm time in the elevated plus maze (EPM) (Shamir et al., 2012a; Wang et al., 2018). ErbB4 deletion was also shown to result in memory deficits including impaired cued and contextual fear conditioning, consistent with observations of ErbB4 regulates hippocampal LTP (Shamir et al., 2012a). ErbB4$^{-/-}$ mice also have a deficit in pre pulse inhibition (PPI), a test that interrogates sensorimotor gating, and is suggested to be a biomarker for schizophrenia (Mena et al., 2016).

To assess the role of ErbB4 on interneuron function, the phenotypes of the ErbB4$^{-/-}$ mice have been compared to cell-type specific ErbB4 deletion in parvalbumin (PV) interneurons. Changes observed in the OFT and PPI in ErbB4$^{-/-}$ mice were recapitulated with PV-specific ErbB4 deletion. However, unlike the ErbB4$^{-/-}$ mice, EPM activity and fear conditioning was normal (Shamir et al., 2012a). A later study reported

tone-cued fear conditioning is impaired in these mice and can be rescued by ErbB4 expression in the amygdala (Lu et al., 2014). Elucidating the role of ErbB4 in PV interneurons is just part of the complex understanding of how the functions of ErbB4 in different brain regions cell types contribute to “schizophrenia-like” endophenotypes.

There have been many studies that have shown region-specific or cell type-specific ErbB4 deletion induces behavioral endophenotypes phenocopying the global loss of ErbB4$^{-/-}$. For example, ErbB4 deletion from noradrenergic neurons in the locus coeruleus results in “manic” behavior (Cao et al., 2018). Deletion of ErbB4 from medium spiny neurons in the nucleus accumbens causes similar behavioral deficits as those observed in ErbB4$^{-/-}$ mice (Geng et al., 2017a) as does ErbB4 deletion in fast-spiking interneurons (delPino et al., 2013). Deletion of ErbB4 from the dopaminergic neurons had no effect on most of the behaviors but maintained a deficit in spatial learning and memory (Skirzewski et al., 2018). Deletion of ErbB4 from serotonergic neurons resulted in increase in anxiety, unlike ErbB4$^{-/-}$ mice (S.-R. Zhang et al., 2020). Deletion of ErbB4 from somatostatin neurons in the central amygdala also resulted in increased anxiety (Ahrens et al., 2018). Deletion of ErbB4 from the prelimbic cortex increases toned-cue fear conditioning freezing (Chen et al., 2017), which is also opposite to ErbB4$^{-/-}$ mouse.

1.7 ErbB4 in cellular development and organogenesis

1.7.1: ErbB4 in cardiac development

When the first mutant mouse with germline deletion of ErbB4 was created, it was found to be embryonic lethal due to cardiac development deficits (Gassmann et al., 1995). This cardiac-associated lethality was rescued by cardiac-specific expression of ErbB4 under the control of the α-MHC promoter. For clarity, here and in subsequent

chapters of this dissertation, I will refer to the mouse without α-MHC ErbB4 expression as the “ErbB4-KO mouse” and the mouse that viable mouse with α-MHC ErbB4 expression as the “ErbB4$^{-/-}$ mouse”

The role of ErbB4 in cardiac development has not been thoroughly studied as the main ErbB4$^{-/-}$ mouse has expression of ErbB4 in the heart, making this procedurally difficult. However, one study has shown that non-canonical ErbB4 signaling may regulate cardiac valvulogenesis (Iwamoto et al., 2017). Loss of ErbB4 in mesenchymal cells was shown to result in hyperproliferation, and this was rescued by ectopic expression of ErbB4-JMa, but not by ErbB4-JMb (Iwamoto et al., 2017). These results suggest that non-canonical Erbb4 signaling may regulate cardiac development.

1.7.2: ErbB4 in neural ganglia development

In the first studies of the ErbB4-KO and ErbB4$^{-/-}$ mice, an aberrant neural bridge was also identified between the trigeminal (V) and geniculate (VII) ganglia during embryonic development as early as E11.5 (Martin Gassmann et al., 1995; Tidcombe et al., 2003). The potential consequences of this misguided neural development have not been found, if there are any, however, there have been indications of the underlying deficit that the results in the aberrant ganglia formation. ErbB4 is known to regulate development and migration of neurons and axonal development. It has been suggested that formation of the neural bridge between the ganglia in ErbB4$^{-/-}$ mice may be due to deficits in pathfinding by neural crest cells (Golding et al., 2000). This is consistent with previously identified roles of NRG1-ErbB4 signaling in neuronal migration (Rio et al., 1997). It is not currently known whether non-canonical signaling by ErbB4-JMa is involved in neural ganglia development.

1.7.3: ErbB4 in mammary gland development

ErbB4 signaling plays an intrinsic role in mammary gland development and lactation illustrated by the fact that ErbB4$^{-/-}$ mice fail to nurse their litter. Mammary development occurs in three stages throughout development. During embryogenesis, the mammary bud develops from the ectodermal epithelial cells. These buds form the directionality of the gland and contain the stem cells that will later proliferate into the fat pad. During puberty, terminal end buds proliferate, bifurcate and undergo lateral branching to fill the fat pad with mammary epithelium. ErbB4 signaling is not necessary for normal mammary development through puberty as nulliparous ErbB4$^{-/-}$ mice do not have any apparent deficits in mammary gland development prior to gestation (Tidcombe et al., 2003).

Other EGFR family receptors (EGFR, ErbB2 and ErbB3) are known to mediate the embryonic and pubertal mammary development and differentiation. Mammary expression of ErbB4, on the other hand, increases during pregnancy and post-partum lactation. The development of lactation requires extensive transcriptional changes for milk production and storage, and duct formation for milk secretion (Andrechek et al., 2008).

ErbB4 has been shown to be capable of regulating genes that are involved in lactation (Clark et al., 2005; Williams et al., 2004). The transcriptional regulation of these genes by ErbB4 was shown to require the interaction between E4ICD and STAT5 (Clark et al., 2005; Williams et al., 2004). It has further been shown that the E4ICD interacts with STAT5 to induce mammary cell proliferation in an in vitro model system (Han et al., 2016; Muraoka-Cook et al., 2006, 2008). Thus, there is a body of evidence

in vivo and in vitro that suggests that ErbB4-JMa E4ICD signaling is responsible for the role of ErbB4 in mammary gland development, giving good cause to presume that lacking direct E4ICD signaling in a mouse model might cause defects in mammary gland development and function. However, these investigations have been transfection-based overexpression cell models, in which interactions and effects can occur that are not necessary or functionally relevant in vivo.

1.7.4: ErbB4 in lung development

NRG1-ErbB4 signaling has long been known to play a role in lung development (Dammann et al., 2012). During lung development the epithelial cells differentiate into type I and type II alveolar cells. The type II alveolar cells secrete surfactants that are crucial for maintaining alveolus structure during development. Type II cells are known to express ErbB4, and NRG1, its ligand, is predominantly expressed in fibroblasts that promote surfactant production by activation of ErbB4.

Adult mice lacking ErbB4 have defects in pulmonary function and poor alveolar structure (Purevdorj et al., 2008). This is thought to be predominantly due to a delay in the timing of lung development and decreased surfactant production during late gestation (Liu et al., 2010). This observation is supported by evidence from experiments in epithelial pulmonary cell cultures showing that ErbB4 regulates surfactant production as well as alveolar cell differentiation (Liu et al., 2009; Zscheppang et al., 2007).

The mechanism by which ErbB4 regulates fetal lung development appears to be consistent with ErbB4-JMa intracellular domain signaling and recruiting transcription factors to regulate surfactant gene expression, specifically sftpb. It has been shown that ErbB4 regulates alveolar development by increasing the transcriptional expression of

sftpb1 is RIP-dependent (Fiaturi et al., 2014; Zscheppang et al., 2007). In separate studies, the control of sftpb1 expression by ErbB4 was shown to be dependent on interactions with YAP, STAT5 and thyroid transcription factor 1 (TTF1) to shuttle E4ICD to the nucleus and regulate the transcriptional expression of lung surfactant genes. (Hoeing et al., 2011; Marten et al., 2015; Zscheppang et al., 2013). Taken together, these findings strongly indicate that E4ICD signaling plays an important role in fetal lung development and the lack of the non-canonical ErbB4 signaling should affect adult pulmonary function.

1.7.5: ErbB4 in immune development and function

The immune system is an essential component of organisms to provide protection against pathogens. The immune system develops from hematopoietic stem cells in the bone marrow to create the innate and adaptive immune system, which is comprised of many different immune cell types. ErbB4 signaling has been implicated in both immune system development and in immune response by macrophages (M. A. Kinney et al., n.d.; Schumacher et al., 2017). Loss of ErbB4 has been shown to result in immature hematopoeisis, with decreased levels of splenic megakaryocytes (Kinney et al., 2019). It was also shown that there are fewer peripheral circulating lymphocytes and increased neutrophils and platelets in ErbB4$^{-/-}$ mice, indicating that ErbB4 plays a role in immune cell development (Kinney et al., 2019). While it is unknown whether non-canonical ErbB4 signaling is involved in immune cell development, it has been suggested to be involved in macrophage function. In colonic inflammation, cleavage of ErbB4 has been shown to be responsible for inducing apoptosis in proinflammatory macrophages (Schumacher et al., 2017). These studies indicate that E4ICD signaling is

involved in immune cell survival, and hence may also play a role in immune cell development.

Furthermore, ErbB4 is also known to regulate injury repair in the brain and other organs, which could be a result of aberrant immune development. NRG1-ErbB4 signaling has been shown to protect against brain injury from ischemic stroke and to promote functional recovery following traumatic brain injury (Deng et al., 2019; Guan et al., 2015; Navarro-González et al., 2019). Interestingly, mesenchymal stem cells have been used to treat ischemic stroke in pre-clinical models, and it was recently shown that ErbB4 activation by NRG1 treatment of the stem cells increases therapeutic efficacy (Ryu et al., 2019). The regulation of immune development by ErbB4 is also clinically relevant in the pathogenesis of multiple sclerosis (MS) as decreased expression of ErbB4 in immune cells is found in patients with severe relapsing remitting MS (Tynyakov-Samra et al., 2011). As ErbB4 is known to be clinically important, a better understanding of the signaling mechanisms of ErbB4 that are involved in immune cell development and function could provide improved therapeutic options for these disorders.

1.8: ErbB4 in cancer

The role of Erbb4 in cancer has been perplexing to clinicians and researchers. ErbB4 has been thoroughly described as both a tumor suppressor and an oncoprotein depending on the type of cancer, malignancy, or treatment among other variables (Lucas et al., 2022). The bulk of the research into the role of ErbB4 in cancer has shown that ErbB4 can have opposing effect depending on whether the CYT1 or CT2 isoform is expressed, and whether ErbB4 homo- or heterodimerizes (Lucas et al., 2022).

Surprisingly, there has been little research into the differential roles of ErbB4-JMa and ErbB4-JMb expression in cancer. However, ectopic expression of ErbB4 in fibroblasts has shown that ErbB4-JMa prevents apoptosis under stress conditions, whereas ErbB4-JMb activates apoptotic pathways (Sundvall et al., 2010). This indicates that non-canonical signaling by ErbB4-JMa can promote cellular growth whereas canonical by ErbB4-JMb signaling promote can cell death and could hence play opposing roles in oncogenesis.

Copy number variant analysis has shown that the expression of ErbB4 is generally downregulated in cancer (Jones et al., 2018). This aligns with the idea that ErbB4-JMb expression would activate cell death pathways and hence downregulation of this pathway would benefit oncogenesis. However, there are certain cases where increased ErbB4 expression and activation has been shown to lead to worse prognosis in cancer, contradicting the general knowledge that ErbB4 is a tumor suppressor (Donoghue et al., 2018). In a couple of the cases where ErbB4 expression is oncogenic and isoform expression analysis has been carried out, the cleavable ErbB4-JMa isoform is the only isoform expressed and upregulated, whereas ErbB4-JMb expression is entirely lost (Aldaregia et al., 2020). This indicates that ErbB4-JMa is an oncogenic isoform and expression leads to cancer development and worse prognosis. On the other hand, ErbB4-JMb appears to be a tumor suppressor and its downregulation leads to oncogenesis.

Interestingly, a known function of E4ICD signaling is the upregulation of PDGFRα expression via the transcription factor binding partner AP2 to promote cellular survival (Sundvall et al., 2010). PDGFRα amplification has been shown to result in poor

outcomes in pediatric brain tumors and is considered an important factor in therapeutic targeting (Koschmann et al., 2016). E4ICD upregulation of PDGFRα expression could be a mechanism by which increased ErbB4-JMa expression results in increased oncogenesis in certain tumor environments.

The implications of different ErbB4 isoform expression could be very important in cancer treatment, as only looking at ErbB4 expression could lead to misguided analysis and treatment depending on the relative expression levels of Erbb4-JMa or ErbB4-JMb. It is important to remember also that the interplay between ErbB4 function in oncogenesis and immunological response could also play a role in the outcomes of any treatments and a full understanding of both would be beneficial to the field.

1.9: Cleavage-resistant mouse models

Cleavage of many proteins is known to be an important step in intracellular signaling. However, there have been few mouse models that have shown that cleavage of the protein is required in the intact organism. Cleavage-resistant mutant mouse models can determine whether the cleavage event is responsible for effects caused by loss of the full receptor. There have not been any previous in vivo mouse models in which RTK cleavage has been abolished in this manner. However, there have been a handful of mutant mice created with cleavage-resistant receptors to investigate the role of cleavage-mediated signaling in vivo. These mouse models have shown that the creation of a cleavage-resistant mouse is a viable strategy to investigate the role of cleavage-mediated signaling in vivo.

The first cleavage-resistant mouse model was created to investigate whether the Notch cleavage and Notch ICD (NICD) signaling was critical in embryonic development.

Notch is a single pass transmembrane protein that is now best known for signaling through the NICD to regulate many aspects of early development (Brou et al., 2000). At the time, cleavage and NICD signaling had been proposed to be the main mechanism by which Notch functioned. However, as with ErbB4 at this point, that information was based primarily on in vitro observations. A knock-in mutant mouse ($N1^{VG}$) was created bearing a mutation in Notch (V1744G) that had been shown to vastly reduce its cleavage by PS1 in vitro (Huppert et al., 2000). As with the Notch-KO mouse, the mutant mouse with cleavage resistant Notch was embryonic lethal. At the early time points that could be studied it was shown that Notch function was primarily dependent on NICD formation as the $N1^{VG}$ mouse strongly phenocopied the Notch-KO mouse (Huppert et al., 2000). Thus, it was confirmed in vivo that cleavage of Notch and NICD signaling was the main signaling mechanism of Notch.

A mouse with cleavage-resistant type 1 collagen was made to study the role of collagen cleavage on ventricular remodeling (Lindsey et al., 2003). Matrix metalloprotease (MMP1) was expected to play a role in ventricular recovery and remodeling following injury via the cleavage of type 1 collagen. Blocking the cleavage of type 1 collagen by MMP1 was shown to impair left ventricular recovery from both transverse aortic constriction and myocardial infarction, confirming the importance of this cleavage on myocardial remodeling (Lindsey et al., 2003; Nong et al., 2011).

Two cleavage resistant mice were made to investigate the role of proteolysis of versican, an ECM chondroitin sulphate proteoglycan, in severe soft-tissue syndactyly. Cleavage of versican by the metalloprotease ADAMTS2 had been previously shown to be critical in versican signaling (Foulcer et al., 2014). A mouse with mutations at the

specific cleavage site and a mouse with deletion of the entire exon 8 bearing the cleavage site were created. Both of these mutant mice developed soft-tissue syndactyly, indicating that versican proteolysis is required for the normal digital development (Nandadasa et al., 2021).

A mouse was created with a cleavage-resistant mutation in the major cleavage site in Reelin, called the PA-DV KI mouse. Reelin is an extracellular matrix glycoprotein that is primarily secreted by Cajal-Retzius cells curing cortical development to regulate neuronal layering (Lossi et al., 2019). The Reelin-KO mouse, known as the Reeler mouse, has severe neurodevelopmental defects including severely disorganized cortical layering, aberrant hippocampal cellular organization, and cerebellar hypoplasia (Lossi et al., 2019). Proteolytic processing of Reelin at its multiple cleavage sites was thought to be important for its functional role in brain development, but this had not been tested in a mouse model in vivo (Jossin, 2020). Loss of Reelin cleavage in the PA-DV KI did not result in any of the more severe phenotypic hallmarks of Reelin function, such as disorganized cortical layering or cerebellar hypoplasia, indicating that cleavage of Reelin is not required for the main function of Reelin in vivo (Okugawa et al., 2020b). Cleavage of Reeelin was shown to be important in neuronal regulation of neuronal branching and of some cellular placement in the hippocampus, while the hippocampal organization was otherwise normal (Okugawa et al., 2020).

1.10: Dissertation outline

While there have not been any previous in vivo mouse models of RTK non canonical signaling, there have been a handful of KI mutant mice created with cleavage-resistant receptors to investigate the role of cleavage-mediated signaling in vivo. In this

dissertation, I will describe the development of an isoform-specific ErBb4-JMa$^{-/-}$ mouse line to study non-canonical ErbB4 signaling in an intact organism, marking a step forward in the study of RTK signaling mechanisms in vivo.

I will describe the use of the ErbB4-JMa$^{-/-}$ mouse to assess whether known phenotypes caused by the loss of ErbB4 reflect ErbB4-JMa non-canonical signaling. These studies provide confirmation of some roles of ErbB4 non-canonical signaling, such as regulation of astrogenesis during cortical development and immune cell development, as well as identifying novel targets of E4ICD signaling during cortical development. Further, I will use this mouse to clearly show that ErbB4-JMa is not required for normal cardiac, mammary, neural ganglia or interneuron development.

These studies validate the usefulness of the ErbB4-JMa$^{-/-}$ mouse as a tool to delineate the RTK signaling mechanisms involved in ErbB4 function. In the future this mouse can be used as a tool to study other roles of ErbB4 and will also be critical in understanding novel roles of ErbB4-JMa signaling. Not only that is provides the first in vivo model of RTK non-canonical signaling ablation, and hopefully others will follow to expand the understanding of this signaling mechanism that is now known to a broadly used by RTKs.

1.12: Figures

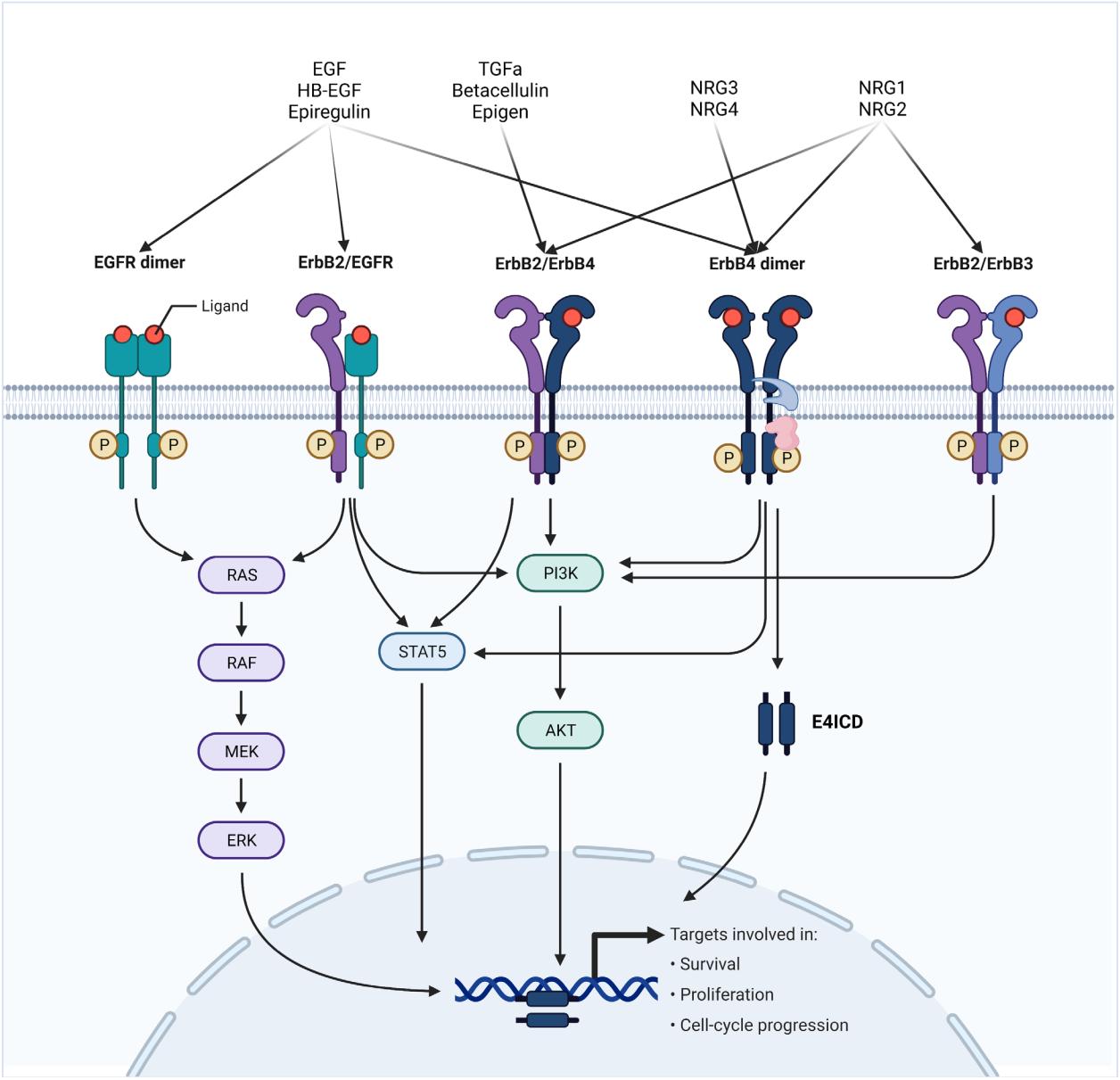

Figure 1.1: Schematic overview of signaling interactions by the EGFR family of RTKs. EGF family ligands are shown interacting with their known cognate receptors. Each receptor heterodimerizes with ErbB2 and shown, which can facilitate different mechanisms of intracellular signaling. Abbreviated versions of canonical RTK signaling pathways and interactions are shown. Figure created with BioRender.com.

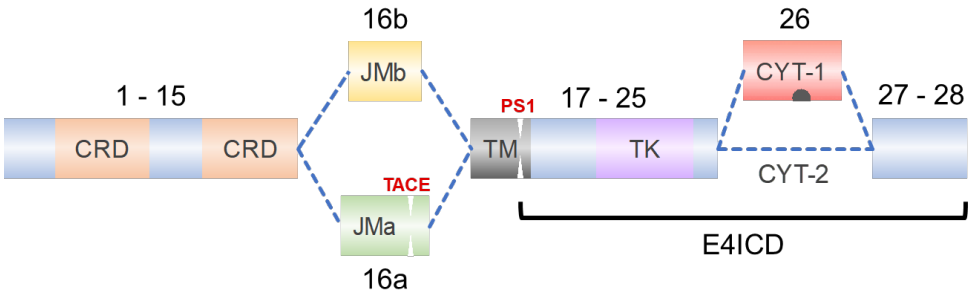

Figure 1.2: Schematic illustration of the ErbB4 isoform structure. Alternative splicing of ErbB4 generates different ErbB4 isoforms. Exons 1-15 contain the cytosine rich domains (CRD), ligand binding site and the receptor dimerization domain. Inclusion of exon 16b or 16a generates ErbB4-JMb or ErbB4-JMa respectively. Only ErbB4-JMa contains the TACE cleavage site is located within exon 16a, whereas all isoforms contain the presenilin/γ-secretase (PS1) cleavage site within the transmembrane (TM) domain. Following the TM domain, the intracellular domain (E4ICD) begins. There is a nuclear localization signal domain shortly after the TM domain. There are 22 potential tyrosine phosphorylation sites within the TK domain. Each of the isoforms can include exon 26, that contains an additional PI3K site, to form CYT1 or exclude exon 26 form CYT2.

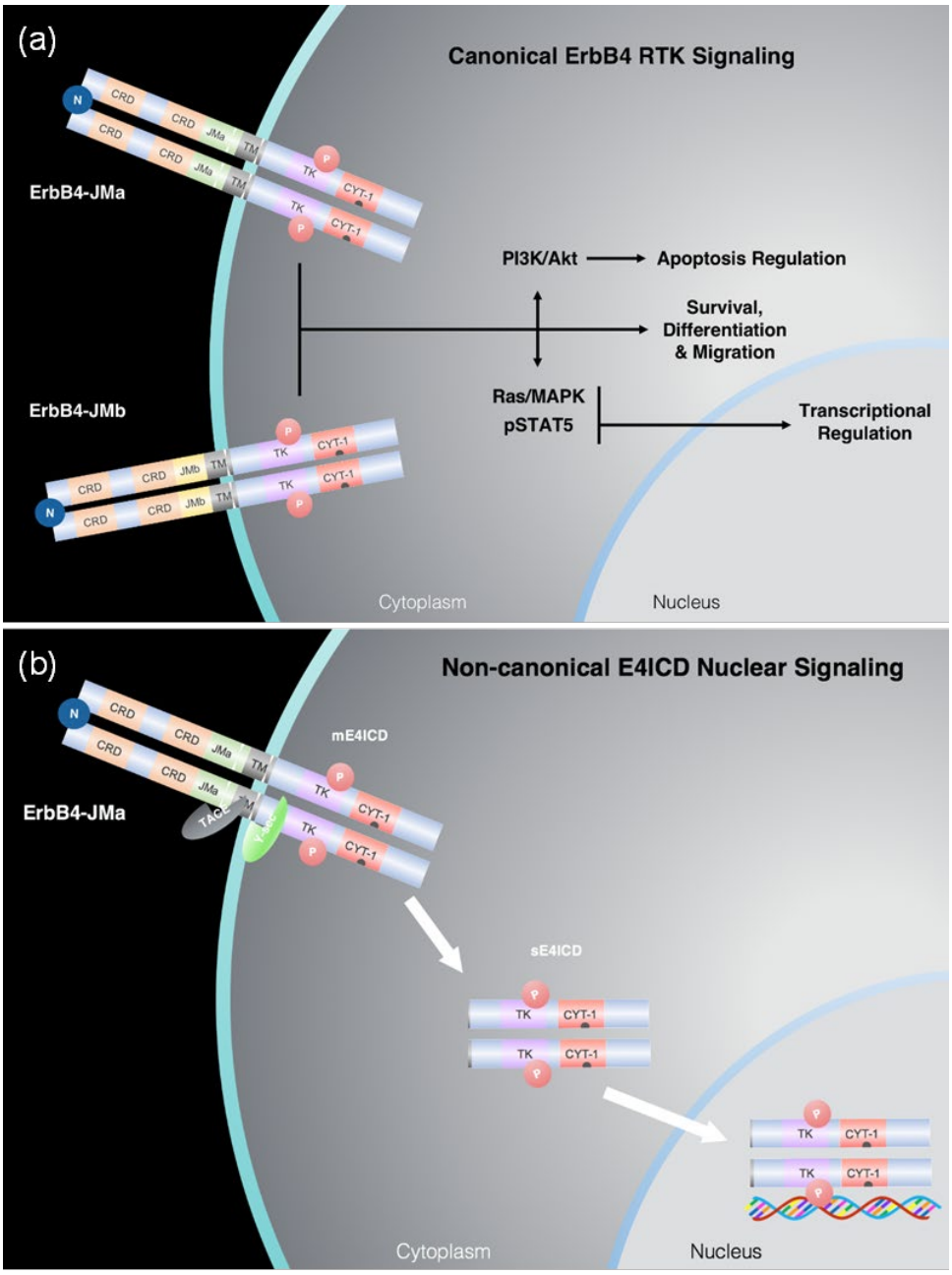

Figure 1.3: Schematic illustration of canonical and non-canonical ErbB4 signaling mechanisms. (a) ErbB4-JMa and ErbB4-JMb signal via canonical RTK pathways. ErbB4 is activated by ligand binding and dimerization followed by trans-autophosphorylation. Adaptor proteins can bind to phosphorylation sites in the intracellular domain to mediate downstream phosphorylation-mediated signaling cascades that can regulate various growth factor-mediated functions. (b) Only ErbB4-JMa can signal via non-canonical pathways. After activation ErbB4-JMa is cleaved in the juxtamembrane domain by TACE then in the transmembrane domain by PS1 to release the E4ICD. The E4ICD can shuttle directly to the nucleus and bind promoter regions to regulate transcription directly.

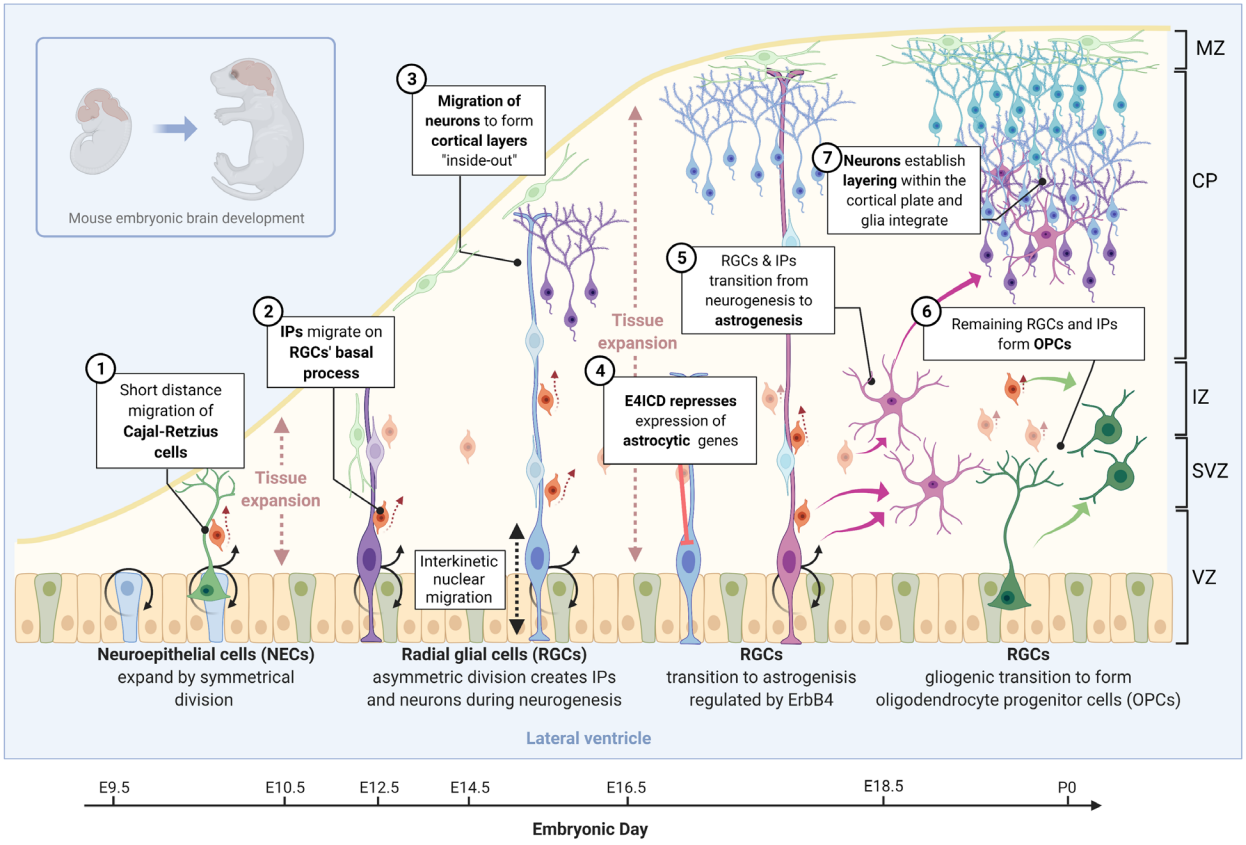

Figure 1.4: Overview timeline of mouse cortical development and the role of E4ICD. The lateral ventricle is initially lined with neuroepithelial cells (NECs) that divide symmetrically to expand the progenitor pool. Cajal-Retzius cells (born around E10.5), are the first neural cell type to be produced by asymmetric division. NECs become radial glial cells (RGCs), with basal and apical processes extending to the surface of the brain or the lateral ventricle, respectively. RGCs can expand the RGC pool by symmetrical division or produce intermediate progenitor cells (IPCs) or neurons by asymmetrical division. Neurons migrate towards the pial surface along the RGC processes and form layers in an “inside out” organization in the cortical plate (CP). During the final stages of neurogenesis, E4ICD represses astrocytic gene expression to regulate the timing of transition to astrogenesis. With ErbB4 signaling intact, RGCs and IPs begin to differentiate into astrocytes around E.17.5. This transition is precocious in ErbB4$^{-/-}$ mice. Following the production of astrocytes RGCs and IPs continue gliogenesis by producing oligodendrocyte precursor cells (OPCs) from around P0 to P7. Figure created with BioRender.com.

CHAPTER 2: Creation and Validation of an Isoform-Specific ErbB4-JMa$^{-/-}$ Mouse to Study the Role of Non-Canonical ErbB4 Signaling in Vivo [1]

2.1: Introduction

The receptor tyrosine kinase (RTK) family consists of 58 single-pass transmembrane receptors that mediate the actions of growth factors, hormones, and cytokines to regulate numerous biological processes (Lemmon & Schlessinger, 2010). These receptors play critical roles in many cellular developmental and physiological functions and their dysregulation is involved in numerous diseases and disorders (Lemmon & Schlessinger, 2010; McDonell et al., 2015). Initially, it was believed that RTKs signal through a single mechanism, now called the canonical RTK pathway. Briefly, ligand binding induces receptor dimerization resulting in RTK trans-phosphorylation, activation of adaptor proteins and phosphorylation of intracellular kinases to regulate cellular growth, survival, migration, and transcription (Lemmon & Schlessinger, 2010). It was later discovered that some RTKs are also capable of signaling through a non-canonical mechanism in which the intracellular domain (ICD) is released by regulated intramembrane proteolysis (RIP) and translocates to the nucleus where it regulates transcription (Sardi et al., 2006; Song, Rosen, et al., 2013). While

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[1] This chapter contains portions of the following manuscript partially written and edited by Gabriel Corfas:

Doherty R, MacLeod BL, Nelson MM, Ibrahim MMH, Borges BC, Jaradat N, Finneran M, Giger RJ, and Corfas G. Identification of in vivo roles of ErbB4-JMa and its direct nuclear signaling using a novel isoform-specific knock out mouse. In submission at *Scientific Reports.*

direct nuclear signaling by RTKs was initially thought to be rare, it is now apparent that over half RTKs can signal through this non-canonical mechanism (Merilahti et al., 2017; Merilahti & Elenius, 2019).

Non-canonical RTK signaling was first characterized in ErbB4, a member of the epidermal growth factor (EGF) receptor family. ErbB4 is activated by several cognate ligands, including NRG1, betacellulin and HB-EGF (Mei & Nave, 2014; Mei & Xiong, 2008). Alternative splicing generates two ErbB4 isoforms that differ in their ability to perform direct nuclear signaling due to different extracellular juxtamembrane domains (EJD) (Elenius et al., 1997). The EJD of ErbB4-JMa isoform, which is encoded by exon 16a, contains a cleavage site for tumor necrosis factor-α-converting enzyme (TACE) (Rio et al., 2000), whereas the EJD of the ErbB4-JMb isoform, encoded by exon 16b, is uncleavable (Elenius et al., 1997). Activation of ErbB4-JMa by its ligand neuregulin 1 (NRG1) (Zhou & Carpenter, 2000) or activation of protein kinase C (PKC) by 12-O-tetradecanoylphorbol-13-acetate (TPA) (Elenius et al., 1997; Rio et al., 2000; Vecchi et al., 1996) promotes TACE-dependent cleavage in the EJD, generating a soluble extracellular domain and a membrane-tethered 80-kD intracellular domain fragment (mE4ICD). Then, mE4ICD can be cleaved by presenilin/γ-secretase complex (PS1) in the intramembrane domain, releasing the soluble intracellular domain (E4ICD) (Rio et al., 2000; Sardi et al., 2006). Activated E4ICD can shuttle to the nucleus via a nuclear localization sequence where it interacts with other nuclear proteins and chromatin to induce transcriptional changes in gene expression (Sardi et al., 2006; Schlessinger & Lemmon, 2006).

ErbB4 is important for the development and function of several organs including the heart (Martin Gassmann et al., 1995), mammary glands (Tidcombe et al., 2003), lungs (Liu et al., 2010), and nervous system (Mei & Xiong, 2008). ErbB4 regulates several neurodevelopmental processes such as neuronal migration (Anton et al., 2004; Flames et al., 2004; H. Li et al., 2012), differentiation (Sardi et al., 2006), axon guidance (Golding et al., 2000), and synapse formation (Li et al., 2007; Müller et al., 2018; Yin et al., 2013). ErbB4 also regulates aspects of neuronal function and plasticity, including interneuron signaling (delPino et al., 2013; Müller et al., 2018), glutamatergic and dopaminergic neurotransmission (B. Li et al., 2007; Skirzewski et al., 2018), and hippocampal potentiation (Pitcher et al., 2008; Tian et al., 2017). This receptor has also been implicated in nervous system disorders, including schizophrenia (Mei & Xiong, 2008), neurological injury repair (Guan et al., 2015), and neurodegenerative diseases such as amyotrophic lateral sclerosis and Alzheimer's disease (Takahashi et al., 2013; H. Zhang et al., 2017).

However, the involvement of ErbB4 direct nuclear signaling has been tested for only a few of these biological processes. For example, our lab provided evidence that nuclear signaling by E4ICD is important for the timing of astrogenesis during cortical development (Sardi et al., 2006). Non-canonical E4ICD signaling has also been proposed to play roles in other developmental processes such as mammary gland (Muraoka-Cook et al., 2006), lung (Hoeing et al., 2011) and cardiac (Iwamoto et al., 2017) development. These studies were completed using cells in culture and/or mice with total ErbB4 loss of function. Therefore, without in vivo models, definitive proof that

non-canonical ErbB4 signaling is key for these events and identification of other processes that depend on E4ICD signaling have been difficult.

To fill this gap in knowledge, I aimed to create mutant mice using CRISPR/Cas9 gene editing to eliminate TACE-mediate ErbB4-JMa cleavage and block E4ICD formation and hence non-canonical signaling. Unexpectedly, mutations that eliminate ErbB4 cleavage in heterologous cells fail to prevent E4ICD formation in vivo. Fortunately, the CRISPR/Cas9-mediated mutagenesis also created mice carrying a premature stop codon within ErbB4 Exon 16a, thus abolishing expression of ErbB4-JMa without altering ErbB4-JMb.

2.2: Materials and methods

Animals

All animals were kept under a 12/12 h light/dark cycle and temperature (21-23°C) controlled environment and were fed *ad libitum* with a standard chow diet (5LOD, LabDiet, USA). Animal procedures were reviewed and approved by the University of Michigan Institutional Animal Care and Use Committee, in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility.

All mouse lines were maintained as homozygous, except for ErbB4$^{-/+}$, which were generated from mating of ErbB4$^{-/-}$ males and ErbB4$^{+/+}$ females. ErbB4$^{-/-}$ litters were cross-fostered to ErbB4$^{+/+}$ mothers for weaning. The genotypes of mice were confirmed by PCR detection of the transgenes in tail-derived DNA from the ErbB4$^{+/+}$, ErbB4$^{+/-}$,

ErbB4$^{-/-}$, ErbB4$^{TUC/TUC}$, ErbB4$^{JMa-/-}$ and each control mice at weaning and at the end of experiments.

For tissue harvesting, experiments were performed in 2–3 month-old mice. For embryonic harvesting, breeding cages of homozygous mouse lines (or ErbB4$^{-/-}$ males with ErbB4$^{+/+}$ females for ErbB4$^{-/+}$ embryos) were established, and confirmation of vaginal plugging was used for embryonic dating. Positive plug date has been denoted as embryonic day 0.5 (E0.5). Following vaginal plugging, females were euthanized at the indicated time points for embryonic tissue collection.

CRISPR/Cas9 gene editing to create mutant mice

Mouse lines were generated in collaboration with the Transgenic Animal Model Core, University of Michigan. Two single guide RNAs (sgRNA) (sequences: AAGTGGAATGGCCCGTCCAT and CGTGTTGTGGTAAAGTGGAA) were designed to create cut sites in the ErbB4-JMa sequence within Exon 16. These guides were expected to create a double strand break and introduce the ultramer oligonucleotide bearing the H641N;S642P mutations by homology directed repair, replacing the wild type sequence with the mutant sequence in exon 16. Alternatively, repair of the chromosome break by non-homologous end joining was expected to create other mutations in ErbB4 exon 16. The sgRNAs were co-injected into fertilized C57BL/6 eggs with the CRISPR/Cas9 components (px3330 plasmid, Addgene) and the ultramer oligonucleotide bearing the mutations. From more than 300 injections with both sgRNAs we received 39 putative founder mice. Sequencing analysis of ErbB4 Exon 16 identified 3 mice with the correct mutations (H641N;S642P) and 20 with other mutations. All founders with discernable H641N;S642P mutations and 12 other founders were crossed

with wild type mice to create obligatory heterozygotes and ErbB4 Exon 16 sequence of the progeny was analyzed. Of the founders, one mouse had only the H641N;S642P mutation and 2 had progeny carrying a single base pair deletion that creates a premature termination codon within *ERBB4* exon 16. Male and female heterozygote offspring from the founders were inbred to create homozygotes and back-crossed onto the C57Bl6 background. ErbB4-TUC (for the "TACE uncleavable" mice bearing the mutation H641N;S642P) and ErbB4-JMa^{-} (for the mice bearing a premature termination codon which will result in NMD of ErbB4-JMa transcripts) were the names given to the alleles.

Genotyping mutant mice

To determine mutations in *ERBB4* exon 16 of founder mice Sanger sequencing was carried out on PCR product of *ERBB4* exon 16. PCR amplification of crude genomic lysates from ear biopsies was done using the following primers: Forward: AGA ATG TGG CGC ATC CAG TA; and Reverse: TGC TCT CAT AAT TCC AAT ATG TGC T

To genotype ErbB4-JMa$^{-/-}$ mice, the primers above were used to amplify exon 16 by PCR. The PCR product was then subjected to restriction enzyme digest with NcoI to create discernible products.

To genotype ErbB4$^{TUC/TUC}$ mice, two PCRs were carried out on crude genomic lysates using forward primers that are specific to the wild type sequence or the TUC mutations as follows: WT Forward Primer: GAC GGG CCA TTC CAC TTT A; TUC Forward Primer: ACC GGC AAT CCA ACA CTG C; and Universal Reverse Primer: TGC TCT CAT AAT TCC AAT ATG TGC TTT AAT C

ErbB4$^{+/+}$, ErbB4$^{-/+}$ and ErbB4$^{-/+}$ mice were genotyped as described previously(Sardi et al., 2006; Tidcombe et al., 2003)

Cell culture and transfections

Human embryonic kidney (HEK-293) cells or mouse neuro 2A (N2A) cells were maintained in DMEM GlutaMAX (Gibco) with 10% fetal bovine serum (Atlanta) and 10 units/mL penicillin and 0.1 mg/mL streptomycin in a humidified 5% CO2/95% air incubator at 37°C. All transfections were carried out with Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol.

Site-directed mutagenesis

Site-directed mutagenesis was carried out on the ErbB4-JMa-CYT2 plasmid in pcDNA3.1 using the QuikChange Site-Directed Mutagenesis Kit (Aligent) according to the guidelines. The following primers were used to create H641N;S642P: Forward: TAC TAC CCA ATG GAC GGC AAT CCC ACT TTA CCA CA; and Reverse: TGT GGT AAA GTG GGA TTG CCG TCC ATT GGG TAG TA.

Western Blotting

Cells were treated as described and lysed with ice cold RIPA buffer (Sigma-Aldrich) or triton-based lysis buffer (Cell Signaling Technologies) with protease and phosphatase inhibitors (Invitrogen) for 10 min. Cerebellum tissue was incubated at 37°C for 45 mins prior to dounce homogenization in RIPA buffer. Lysates were vortexed and centrifuged at 14,000 rpm for 10 min and supernatant protein concentration was standardized via BCA assay (Pierce) and diluted in 4× Laemmli buffer (Bio-Rad) with 10% β-mercaptoethanol and run on 6% SDS polyacrylamide gels or 7.5% or 10% Mini-PROTEAN TGX Stain-Free Precast Gels (BioRad). Protein was transferred from the gel

onto 0.45 μm pore PVDF membrane for 1.5 hr at 15 V with a semi-dry transfer unit. Membranes were then incubated in 5% bovine serum albumin (Sigma-Aldrich) in 0.2% Tween-20 in TBS (TBS-T) blocking solution for 1 hr and then primary antibody (1:1000, ErbB4, phospho-ErbB4, Erk, phospho-ERK, Cell Signaling Technologies; 1:1000 GFAP, Dako; 1:5000 GAPDH, Abcam) in blocking solution overnight at 4°C. The next day, blots were washed in TBS-T, then incubated in HRP-conjugated secondary antibody (1:1000 goat anti-rabbit or 1:1000 goat anti-mouse, Cell Signaling Technology) in blocking solution for 45 mins. All blots were exposed with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific) and imaged on a Bio-Rad Chemidoc and analyzed using Bio-Rad Image Lab software.

Immunoprecipitation

Cerebellum samples were obtained by dissection of adult mouse brain and stored in ice cold DMEM. Once all samples were collected, they were incubated at 37°C for 45 mins, washed on PBS and lysed in ice cold 500ul RIPA buffer (Invitrogen) using a dounce homogenizer, vortexed and then centrifuged for 10 mins at 14,000 rpm and the supernatant was collected.

NPCs were plated in 10 cm dishes at 4 million cells per dish, with two dishes per condition in bFGF-containing NPC media. After 2 days of growth, replenishing half the media and bFGF each day, cells were treated with 100 ng/mL TPA (12-O-Tetradecanoylphorbol-13-Acetate, Cell Signaling Technologies) for 45 mins, washed with ice cold PBS and lysed with 500ul lysis buffer (Cell Signaling Technologies). Lysates were subjected to 3 x 5 sec sonication using a probe sonicator, then centrifuged at 14,000 rpm for 10 min and supernatants were collected.

An ErbB4 WB was carried out on the supernatant to normalize input levels of total ErbB4 using 40 µl of the sample, while the remaining lysate was frozen at -80°C. Lysates were defrosted on ice and diluted to equal total ErbB4 levels in RIPA buffer (ErbB4$^{-/-}$ samples were diluted to the same protein level as the highest concentration sample). ErbB4-conjugated agarose beads (Santa Cruz) were washed in RIPA buffer and 30 µl beads were added to 500 µl of each sample and incubated at 4°C overnight with rotation. Beads were washed four times in RIPA buffer and the sample was eluted in 1.5x Laemmli sample buffer (Bio-Rad) for 5 mins at 95°C. Western blot was carried out with ErbB4 antibody (1:1000, ErbB4, Cell Signaling Technologies). Samples from input, IP and supernatant were analyzed by anti-ErbB4 Western blot to measure the levels of the 80 kD E4ICD and full length 180 kD ErbB4.

Neural Precursor Cell Cultures

Timed pregnant females were euthanized via cervical dislocation at E14.5. Embryos were dissected in ice-cold phosphate-buffered saline (PBS) under sterile conditions, and the whole telencephalon without meninges was collected in a 15 mL conical tube containing PBS on ice. PBS was removed and cortices were dissociated into single cells with StemPro Accutase (ThermoFisher) for 5 min at RT, triturated then centrifuged at 2,000 rpm for 2 mins. Next, the pellets were resuspended in NPC media (DMEM GlutaMAX (Gibco) with 2% B27 without vitamin A (Invitrogen), 10 units/mL penicillin and 0.1 mg/mL streptomycin). NPCs were seeded as neurospheres in T75 flasks at 500,000 cells/mL and expanded for 2 days in NPC media supplemented with epidermal growth factor (EGF, 20 ng/mL) and basic fibroblast growth factor (bFGF, 20 ng/mL) in a humidified 5% CO2/95% air incubator at 37°C. Growth factors and B27

were replenished daily. On day 3, neurospheres were dissociated with Accutase (Invitrogen) into a single cell suspension and reseeded for NPC expansion or plated in NPC media supplemented with bFGF (20 ng/mL) onto plates coated with Poly-L-Lysine (Sigma) and Fibronectin (Corning).

RNA extraction and quantitative RT-PCR

Following RNA extraction with Qiagen RNeasy Kit with on-column DNase digestion (Qiagen 74004), RNA concentration and quality was assessed on Bio Tek plate reader. 1 µg of RNA per sample was transcribed into cDNA using an iScript cDNA Synthesis Kit (Bio-Rad) following manufacturer's instructions and diluted 1:4 in nuclease free water. Quantitative PCR was performed using iTaq SYBR Green Supermix (Bio-Rad) on a Bio-Rad CFX96 Thermocycler in 96 well format or an Applied Biosystems QuantStudio 5 Real-Time PCR System in 384 well format. Each well contained 5 µL iTaq SYBR Green Supermix, 3 pM of each forward and reverse primers and 2.5 µL diluted cDNA. The thermal cycler was run as follows as follows: 95°C for 30 s followed by 39 cycles of 95°C for 5 s and 60°C for 30 s. Normalized Gene Expression (NGE) was calculated using the efficiency of each primer with the following formula: $[\text{efficiency\_target}^{-CT_{target}}/\text{efficiency\_reference}^{-CT_{ref}}]$. The primers used for qRT-PCR are listed in Table 2.

2.3: Results

Mutation of the putative ErbB4-JMa TACE cleavage site prevents E4ICD formation in heterologous cells

To create a mouse mutant in which E4ICD direct nuclear signaling is missing, I decided to target the TACE cleavage site rather than the PS1 cleavage site. I took this

approach for two main reasons. First, targeting the TACE cleavage site in ErbB4 exon 16a would allow us to make specifically target ErbB4-JMa without having any effect on ErbB4-JMb as it includes exon 16b by alternative splicing (Fig. 1.2). Targeting the PS1 site would not be isoform-specific, as both isoforms include the exon 17 containing PS1 and mutation ErbB4-JMb, which may have unintended consequences. Second, I intended to validate the mutation by assessing the absence of the soluble E4ICD. Mutating the TACE cleavage site would prevent the primary cleavage event and prevent any formation of mE4ICD, allowing us to differentiate between the 180 kD full length ErbB4 and the 80 kD E4ICD (Fig. 2.1a). Blocking the PS1 cleavage site allows the primary TACE cleavage event to create mE4ICD. Differentiating between the mE4ICD and E4ICD would not be straightforward as both are around 80 kD, making it difficult to be certain the mutations had been effective (Fig. 2.1b).

I first sought to identify a mutation that eliminates TACE-dependent ErbB4-JMa cleavage. Previous studies pointed to H641 and S642 in exon 16a as key amino acids for this cleavage event (Cheng et al., 2003). Using site-directed mutagenesis on an ErbB4-JMa full-length clone, I found that mutations H641N;S642P prevent E4ICD formation in HEK-293 cells, i.e., eliminated the ligand-induced appearance of the 80 kD E4ICD band in Western blots (WB) (Fig. 2.2.a and 2.2.c). I also tested other mutations of the TACE cleavage site H641Q;S642Q and H641E;S642D, but found that they did not fully prevent formation of E4ICD (Fig. 2.2a). Importantly, this **T**ACE-**u**n**c**leavable ErbB4-JMa (ErbB4-TUC) retains its ability for ligand-induced auto-phosphorylation and down-stream signaling via induction of ERK phosphorylation (Fig. 2.2.c and 2.2.d). These results suggested that introducing the H641N;S642P mutations in exon 16a of

ErbB4 would create mice in which ErbB4-JMa retains the capacity for canonical signaling but not E4ICD-mediated direct unclear signaling.

Creating mice deficient in non-canonical ErbB4-JMa signaling using CRISPR/Cas9 gene editing

I therefore used CRISPR/Cas9 gene editing to create mutant mice bearing TUC mutations in ErbB4-JMa. Briefly, a guide RNA was used to create cut sites in exon 16a to replace the wild type sequences with an ultramer oligonucleotide bearing the H641N;S642P mutations by homology directed repair (Fig. 2.3) All 39 putative founder mice were assessed for genomic mutations in ErbB4 exon 16a by Sanger sequencing and chromatogram analysis of background mutations. I identified two mice with discernible TUC mutations (Allele A, H641N;S642P, Fig. 2.4), one of which had an extra deletion in exon 16a (Allele B, Fig. 2.4). Many other mutations were identified by “peaks on peaks” in exon 16a, one of which appeared to be a single base deletion that may result in a premature stop codon (Allele D, Fig. 2.4). Chromatogram analysis identified a total of 12 mice with mutations to be backcrossed, thereby creating obligate heterozygotes that could be assessed for mutations more clearly.

One mouse with only TUC mutations was identified as a heterozygote and was then inbred to homozygosity where only the TUC mutations were present. This indicated that CRISPR/Cas9 gene editing had replaced the TACE cleavage with the donor TUC oligonucleotide (Fig. 2.5).

As can be expected from CRISPR/Cas9 mutagenesis, non-homologous end joining also resulted in other founders bearing indels (Fig. 2.5). Assessment of heterozygotes found one mouse contained only a single base deletion that created a

premature stop codon within ErbB4 exon 16a (Fig. 2.5). mRNAs containing premature stop codons are degraded by nonsense-mediated mRNA decay (NMD) before translation, preventing expression (Arribere & Fire, 2018). As the mutation was in exon 16a, and not 16b, I anticipated ErbB4-JMb expression should not be affected in these mutants, creating an ErbB4-JMa isoform-specific knock out (KO) (as illustrated in Fig. 2.3). I named these alleles ErbB4^{TUC} for the "**T**ACE **u**n**c**leavable" mice bearing the mutation H641N;S642P, and ErbB4-JMa^{-} for the mice bearing the premature stop codon (Fig. 2.6). Back-crossing these mutants to homozygosity showed that all the ErbB4$^{TUC/TUC}$ and ErbB4-JMa$^{-/-}$ lines were viable, unlike in the ErbB4$^{-/-}$ mice (Tidcombe et al., 2003).

Validation of mutant ErbB4 mice

To assess the impact of the ErbB4$^{TUC/TUC}$ and ErbB4-JMa$^{-/-}$ mutations on the formation of E4ICD, I first used immunoprecipitation (IP) of NPC lysates with antibodies directed to the carboxy terminal end of ErbB4 and visualized the full-length receptor (180 kD) and E4ICD (80 kD) by WB. The E4ICD could be detected at 80 kD in ErbB4$^{+/+}$ NPCs that were treated with TPA to stimulate TACE cleavage of the receptor. While the initial assessment seemed to show that E4ICD formation was blocked in ErbB4$^{TUC/TUC}$ and ErbB4-JMa$^{-/-}$ NPCs (Fig. 2.6). However, longer exposure of the WB revealed that there appeared to be some E4ICD still detectable in ErbB4$^{TUC/TUC}$ NPCs.

I then assessed whether I could detect E4ICD in the adult cerebellum by IP, where both ErbB4-JMa and ErbB4-JMb are highly expressed (Elenius et al., 1997). By incubating the cerebella in DMEM at 37°C for 30 mins, the E4ICD band was clearly detectable in lysates from ErbB4$^{+/+}$ mice. Surprisingly, the E4ICD band was also clearly

detectable in ErbB4$^{TUC/TUC}$ mice, indicating that the in vivo TUC mutations in ErbB4 Exon 16a did not prevent cleavage of ErbB4-JMa (Fig. 2.6a). However, the ErbB4-JMa$^{-/-}$ lysates lacked E4ICD (Fig. 2.6a), indicating that the premature stop codon prevented the expression of ErbB4-JMa. To test if the loss of E4ICD in the ErbB4-JMa$^{-/-}$ tissues reflects the loss of ErbB4 cleavage, I used NPCs derived from the E14.5 telencephalon, which expresses both ErbB4-JMa and ErbB4-JMb (Sardi et al., 2006), and in which TPA induces E4ICD formation (Fig. 2.7). In concordance with the findings with cerebellar tissues, TPA treatment induced E4ICD formation in ErbB4$^{+/+}$ NPCs but not ErbB4-JMa$^{-/-}$ NPCs (Fig. 2.7a).

To determine if the lack of E4ICD formation in ErbB4-JMa$^{-/-}$ line was the result of the lack of ErbB4-JMa expression, I used quantitative WB (qWB) of ErbB4 from P0 cortex and NPC lysates. The total level of ErbB4 in ErbB4-JMa$^{-/-}$ mice was lower than ErbB4$^{+/+}$ mice, comparable to ErbB4 expression in heterozygous mice (ErbB4$^{+/-}$) (Fig. 2.7c & d), as would be expected if ErbB4-JMa expression was absent in the ErbB4-JMa$^{-/-}$.

As the single base mutation in ErbB4 exon 16a created a stop codon that was predicted to result in NMD of the ErbB4-JMa transcript, I performed quantitative RT-PCR (qPCR) on P0 cortex and NPCs to assess the levels of ErbB4 isoform expression. As expected, the ErbB4-JMa transcripts were almost entirely abolished in ErbB4-JMa$^{-/-}$ mice (Fig. 2.8), suggesting that it is degraded by NMD, while ErbB4-JMb expression was relatively normal.

Importantly the ErbB4-JMa$^{-/-}$ mice were viable and gave birth to and weaned regular size litters (7.87±0.39 vs 8.47±0.50, respectively; p=0.350, n=15

litters/genotype). The offspring of ErbB4-JMa$^{-/-}$ mice were normal weight at birth, unlike ErbB4$^{-/-}$ pups which weigh slightly less than ErbB4$^{+/+}$ pups (Fig. 2.9). ErbB4-JMa$^{-/-}$ pups also did not have any detectable changes in brain weight at birth, indicating that gross brain development was relatively normal, similar to the ErbB4$^{-/-}$ pups (Fig. 2.9). Together, these results show that the ErbB4-JMa$^{-/-}$ mouse is an ErbB4-JMa isoform-specific KO, predicted to be deficient in non-canonical E4ICD signaling, and this mouse line could be used to identify biological processes that require non-canonical ErbB4-JMa signaling in vivo for the first time.

2.4: Discussion

To address the challenges of investigating the roles of non-canonical ErbB4-JMa signaling in vivo, I created an ErbB4-JMa isoform-specific KO mouse. I was surprised to find that the TUC mutations (H641N;S642P) abolished E4ICD formation in vitro but not in the ErbB4$^{TUC/TUC}$ mouse in vivo. This could be due to a change in the preferred TACE cleavage site, that has been shown to shift from H641 to P637 upon receptor glycosylation (Goth et al., 2015). Thus, it is plausible that natural glycosylation of ErbB4 in vivo would cause the mutations that prevent TACE cleavage of ErbB4-JMa in heterologous cells to be ineffective. Alternatively, the existence of enzymes other than TACE that cleave ErbB4-JMa on a different site might exist in vivo. Whatever the reason for this result, the different effects of the TUC mutation in vitro and in vivo indicate that, without an animal model such as the ErbB4-JMa$^{-/-}$ mice, conclusions from studies on direct nuclear signal by other RTKs based solely on heterologous expression systems should be interpreted with caution.

Creating the ErbB4-JMa$^{-/-}$ mice will allow us to confirm, contradict, and expand the understanding of non-canonical ErbB4 signaling for the first time in the intact organism. The ErbB4-JMa$^{-/-}$ mouse line can be used to delineate whether other developmental and biological processes regulated by ErbB4 are dependent on non-canonical signaling in vivo.

2.5 Figures

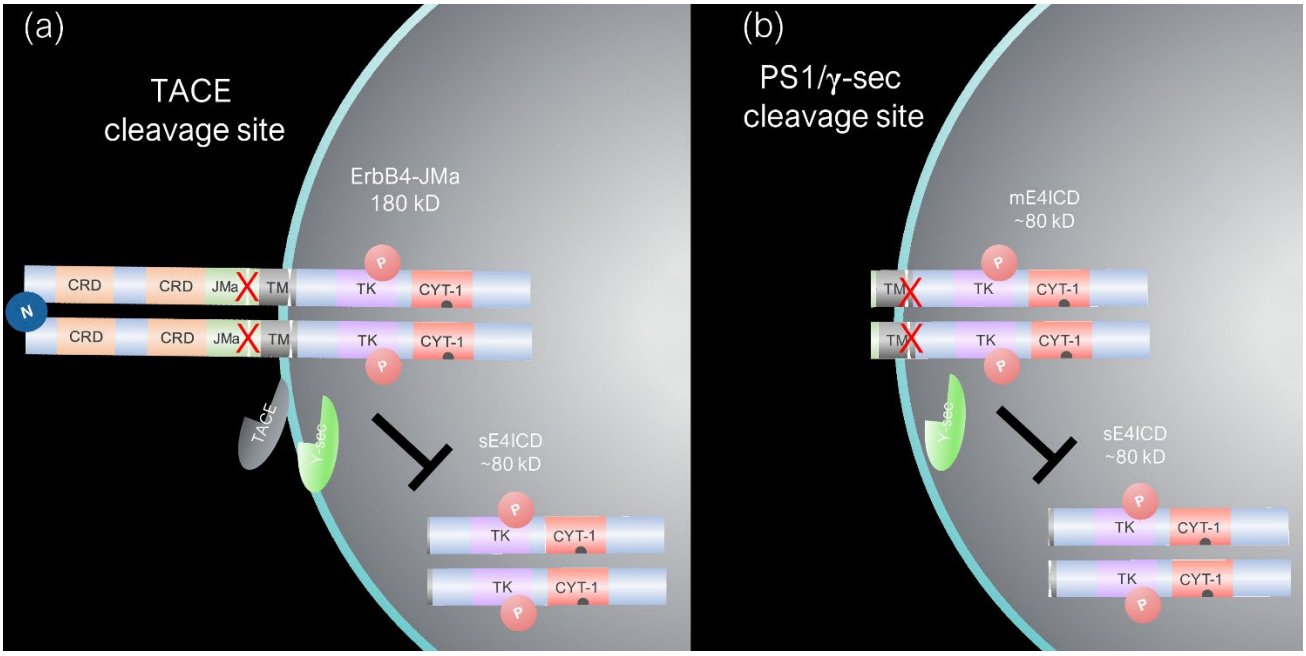


Figure 2.1: Approaches to block non-canonical E4ICD signaling. (a) Blocking the TACE cleavage site would prevent E4ICD formation and leave only the full length 180 kD ErbB4 intact. (b) Blocking the PS1 cleavage site would also prevent the formation of E4ICD but allow the full length 180 kD ErbB4 receptor to be cleaved by TACE leaving the 80 kD mE4ICD intact, which is the same size and the 80 kD soluble E4ICD.

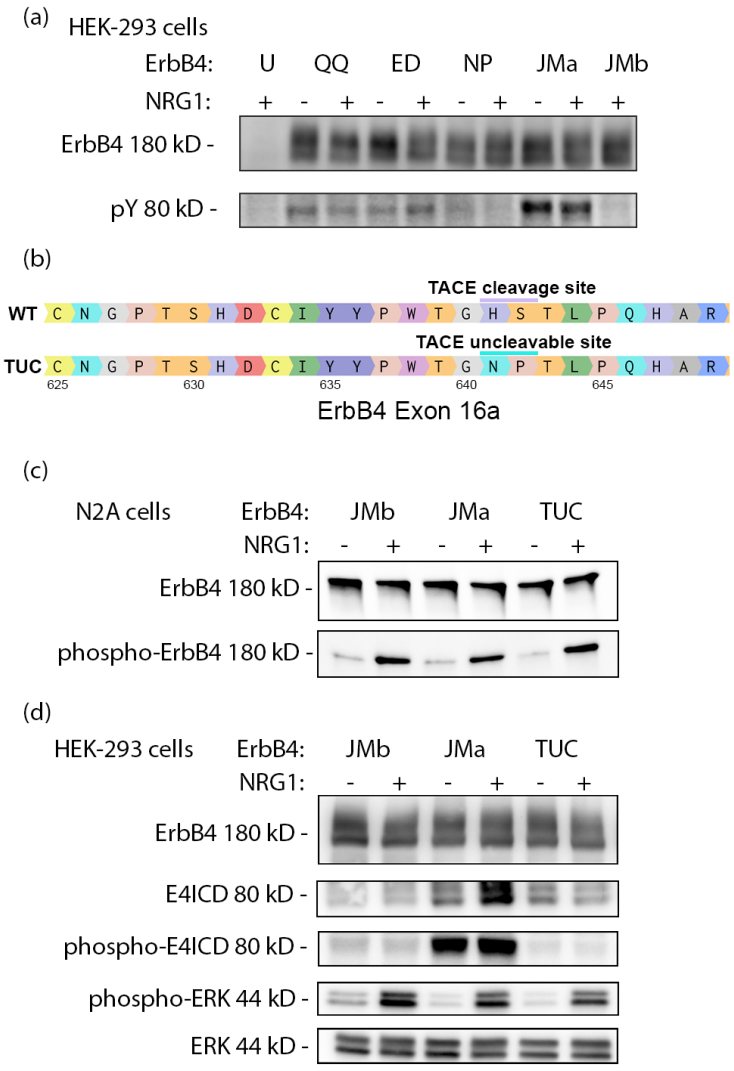
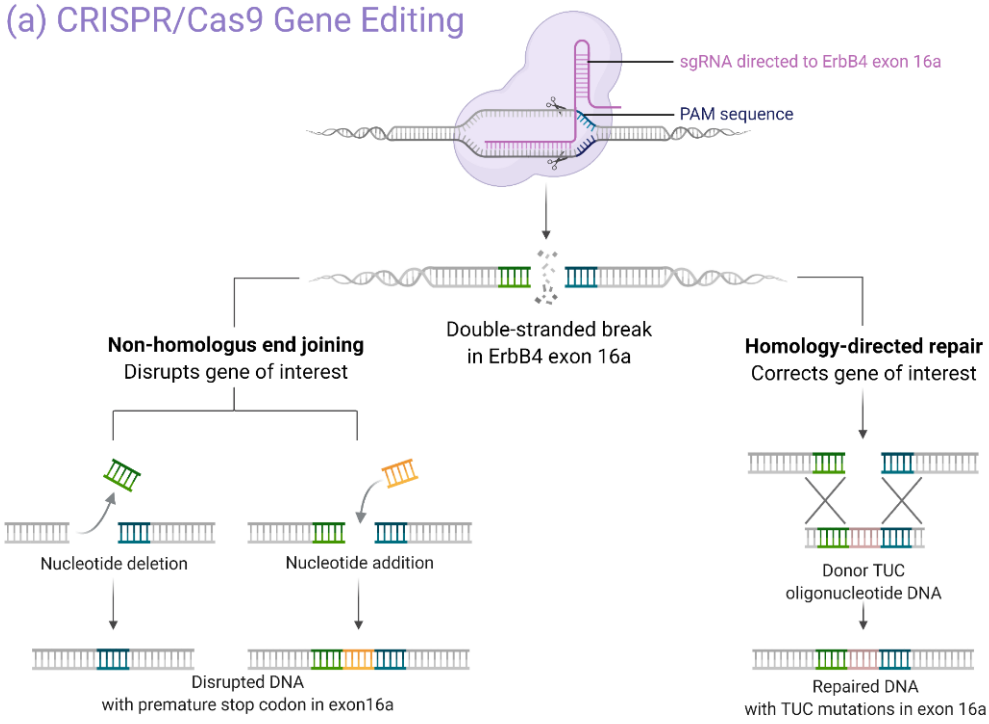
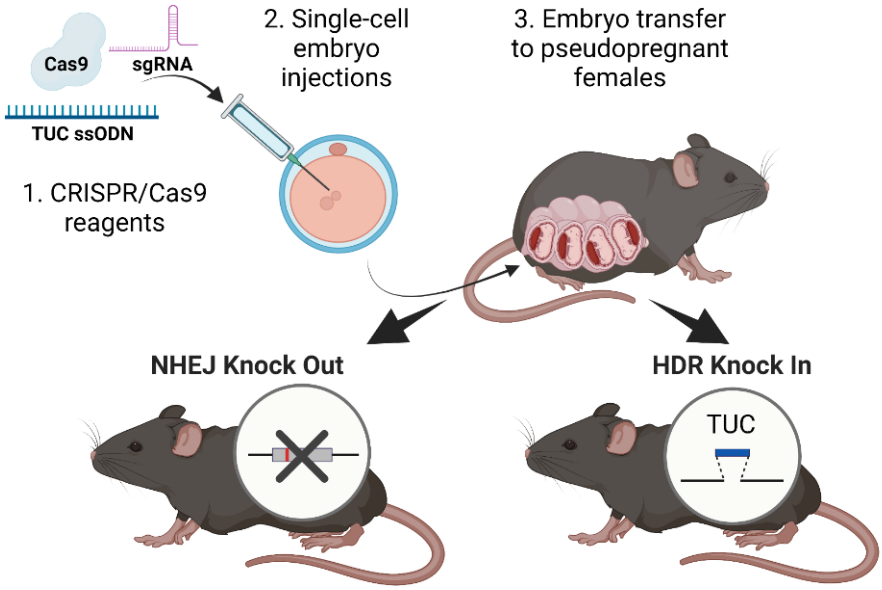


Figure 2.2: Site-directed mutagenesis of ErbB4-JMa prevents cleavage and E4ICD formation without altering RTK signaling. (a) Only H641N;S642P mutations completely prevent E4ICD formation by ErbB4-JMa. (b) Diagram of ErbB4 Exon 16a and the H641N;S642P mutation sites to create the TACE-uncleavable (TUC) plasmid. Western blots of cells transfected with ErbB4 plasmids show that (b) TUC mutations do not affect the phosphorylation of full length ErbB4, or (c) downstream signaling by ERK phosphorylation. (d) The TUC mutations prevent formation of E4ICD by blocking cleavage of ErbB4-JMa.

(a) CRISPR/Cas9 Gene Editing



(b) CRISPR/Cas9 NHEJ and HDR to create mutant mice



4. Identify potential mutant alleles by chromatogram analysis
5. Cross mutants with WT females to create obligate hetrozygotes
5. Inbreed desired mutant alleles to create homozygote KI/KO mice
6. Back-cross onto WT background to establish mutant mouse line

Figure 2.3: CRISPR/Cas9 gene editing was used to create ErbB4-JMa mutant mice by NHEJ or HDR of exon 16a. (a) Gene editing by CRISPR/Cas9 uses sgRNA to direct Cas9 enzyme to make a double strand break at a specific site, such as the TACE cleavage site in erbB4 exon 16a. This can be repaired by NHEJ, which can result in indels, or by HDR which can insert a donor oligonucleotide with mutations, such as the TUC mutations. (b) CRISPR/Cas9 gene editing can be used to create mutant mice by injecting the reagents into multiple mouse embryos at the single-cell stage and transferring them into pseudopregnant females. CRISPR/Cas9 gene editing can create KO mice via NHEJ indel mutations or KI mice by insertion of ODN into the gene target, in this case, TUC mutations at the TACE cleavage site of ErbB4 exon 16a. Figure created with BioRender.com.

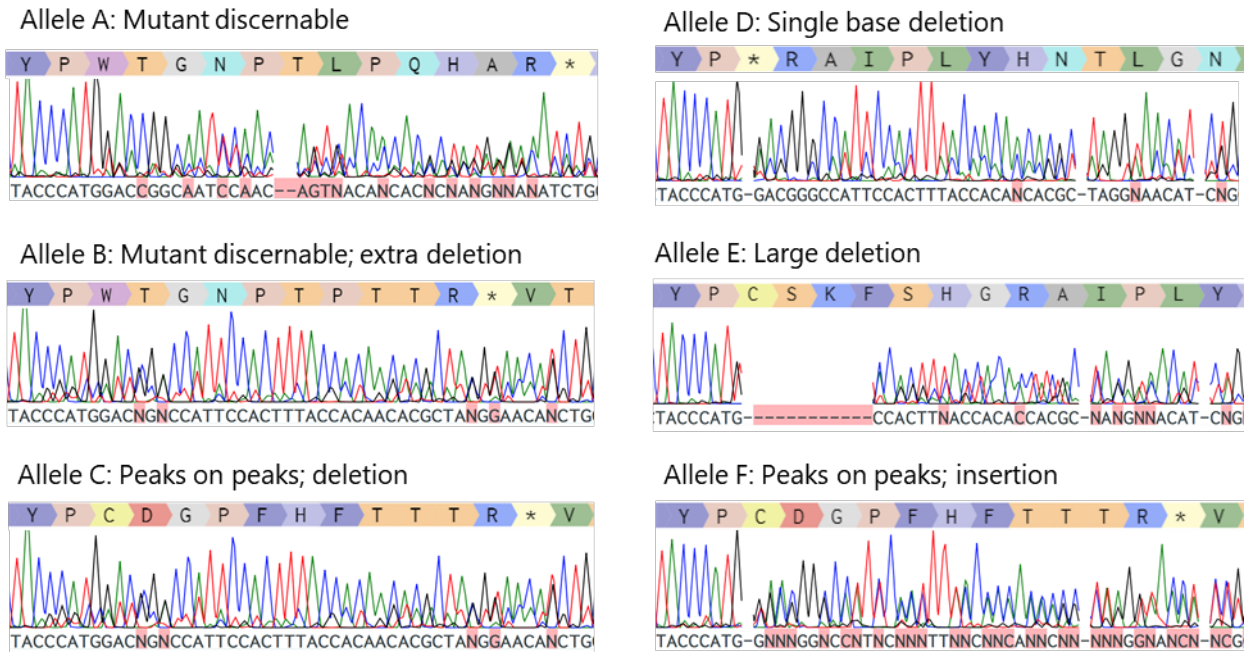


Figure 2.4: Representative chromatograms show multiple founder mice with background mutations in ErbB4 exon 16a. Chromatogram analysis of ErbB4 Exon 16 PCR Sanger sequencing of founder mice from CRISPR/Cas9 gene editing shows alleles with background mutations, known as “peaks on peaks”. Above chromatograms for each allele shows the predicted protein sequence around the sites of Cas9pinduced double strand break. TUC mutations (NP) were discernible in Allele A and Allele B. by analysis A single base deletion was identified in Allele D that appeared to result in a premature STOP codon. All other mice contained background mutations that were representative of various indels, as seen in Allele C, E and D.

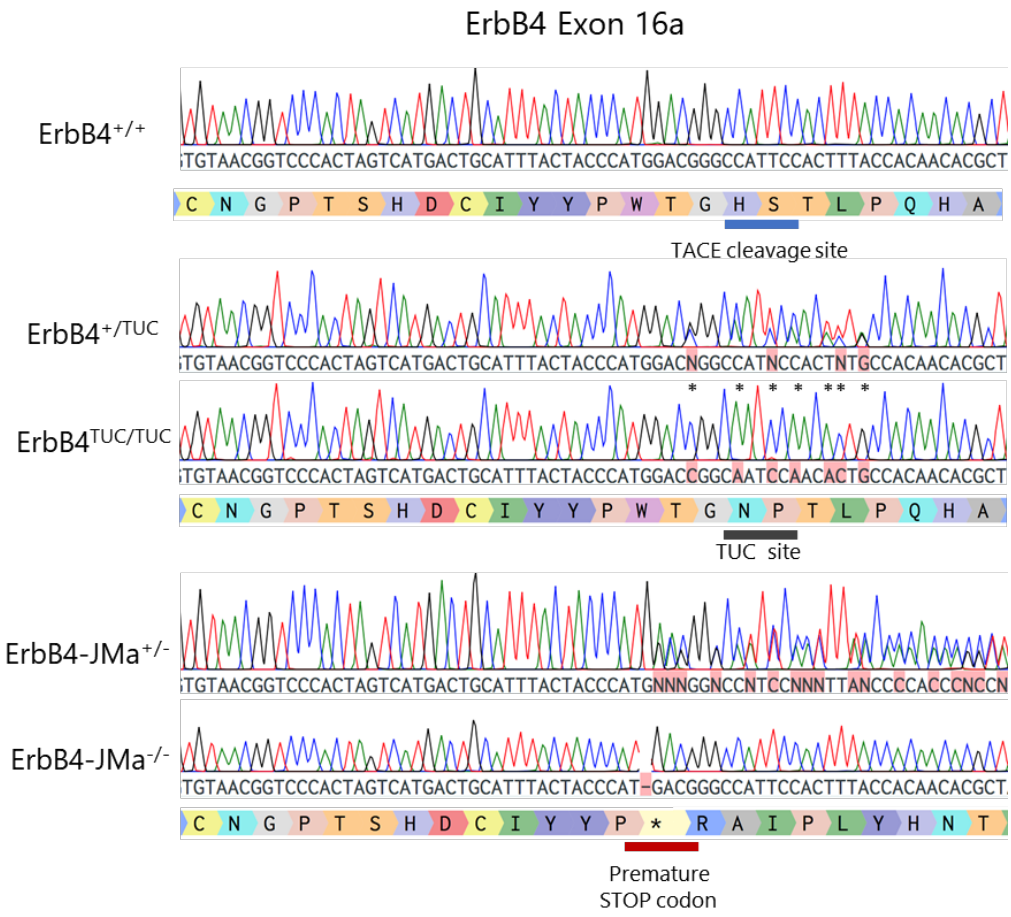


Figure 2.5: Mutant mice created using CRISPR/Cas9 gene editing to introduce ErbB4-TUC and ErbB4-JMa- alleles. Chromatogram analysis of ErbB4 Exon 16 PCR Sanger sequencing of homozygous WT, ErbB4$^{TUC/TUC}$ and ErbB4-JMa$^{-/-}$ mice showing the sequence mutations by CRISPR/Cas9 gene editing. (a) WT sequence shows the original sequence with the histidine-serine cleavage site. (b) Homology-directed repair resulted in inclusion of the mutated oligonucleotide sequence bearing the H641N;S642P TUC mutations. (c) Non-homologous end joining resulted in a premature stop codon due to a single base deletion, predicted to result in nonsense-mediated degradation of ErbB4-JMa RNA.

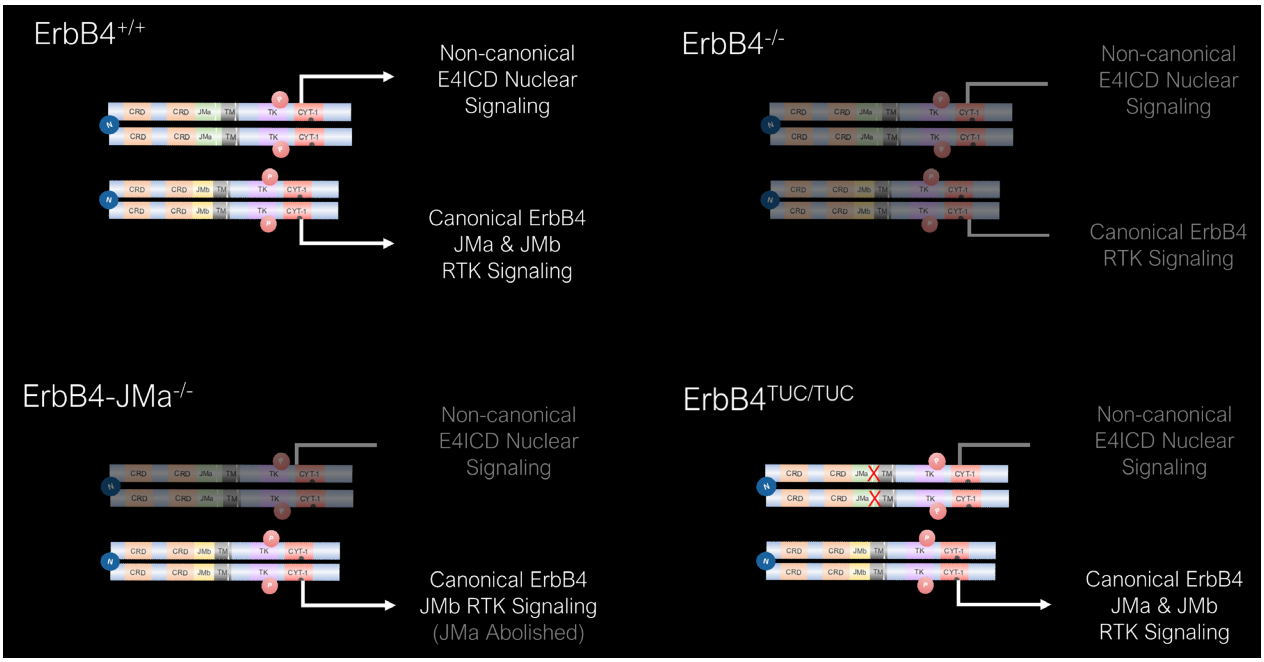
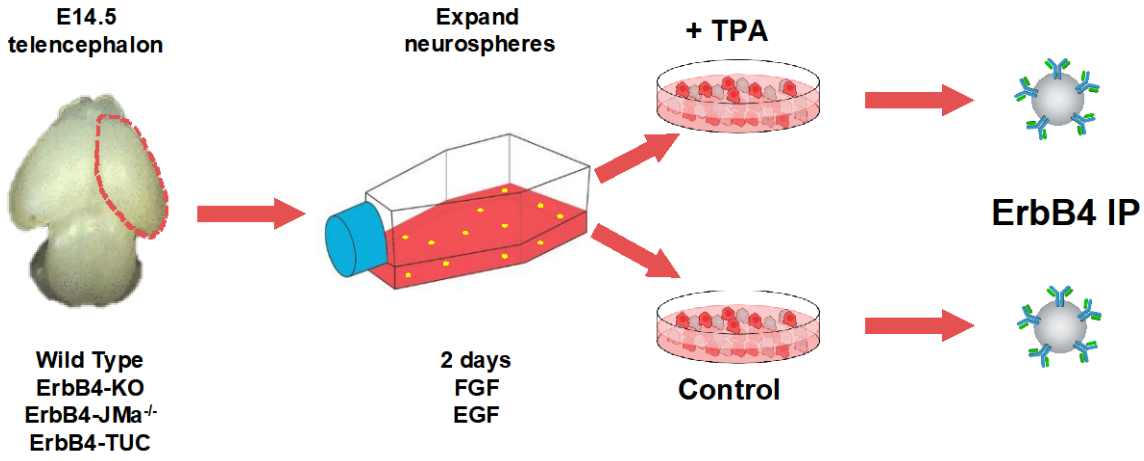


Figure 2.6: Overview putative effects of mutant mice created using CRISPR/Cas9 gene editing. ErbB4$^{+/+}$ mice have intact signaling of both canonical and non-canonical E4ICD nuclear signaling and ErbB4$^{-/-}$ mice lose all ErbB4 signaling, both canonical and non-canonical signaling. ErbB4-JMa$^{-/-}$ mice are predicted to lose all ErbB4-JMa signaling, both canonical and non-canonical, but leavencanonical siganling by ErbB4-JMa intact. ErbB4$^{TUC/TUC}$ mice are predicted to only lack E4ICD non-canonical signaling, but have canonical siganling by both ErbB4-JMa and ErbB4-JMb intact.

(a)



(b)

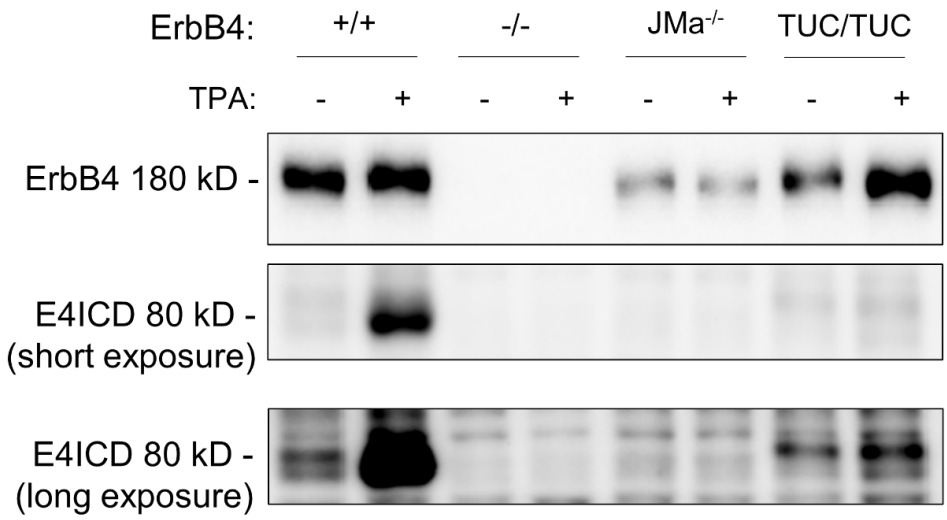


Figure 2.7: Mutant mice appear to lack E4ICD formation. (a) Experimental design to assess E4ICD formation by ErbB4 IP from TPA-stimulated NPCs. (b) Stimulation by TPA induces E4ICD formation in NPCs, but this appears to be prevented in, ErbB4$^{TUC/TUC}$ and ErbB4-JMa$^{-/-}$ NPCs by a short exposure. Longer exposure of the WB reveals some E4ICD formation in ErbB4$^{TUC/TUC}$, but not ErbB4-JMa$^{-/-}$ NPCs.

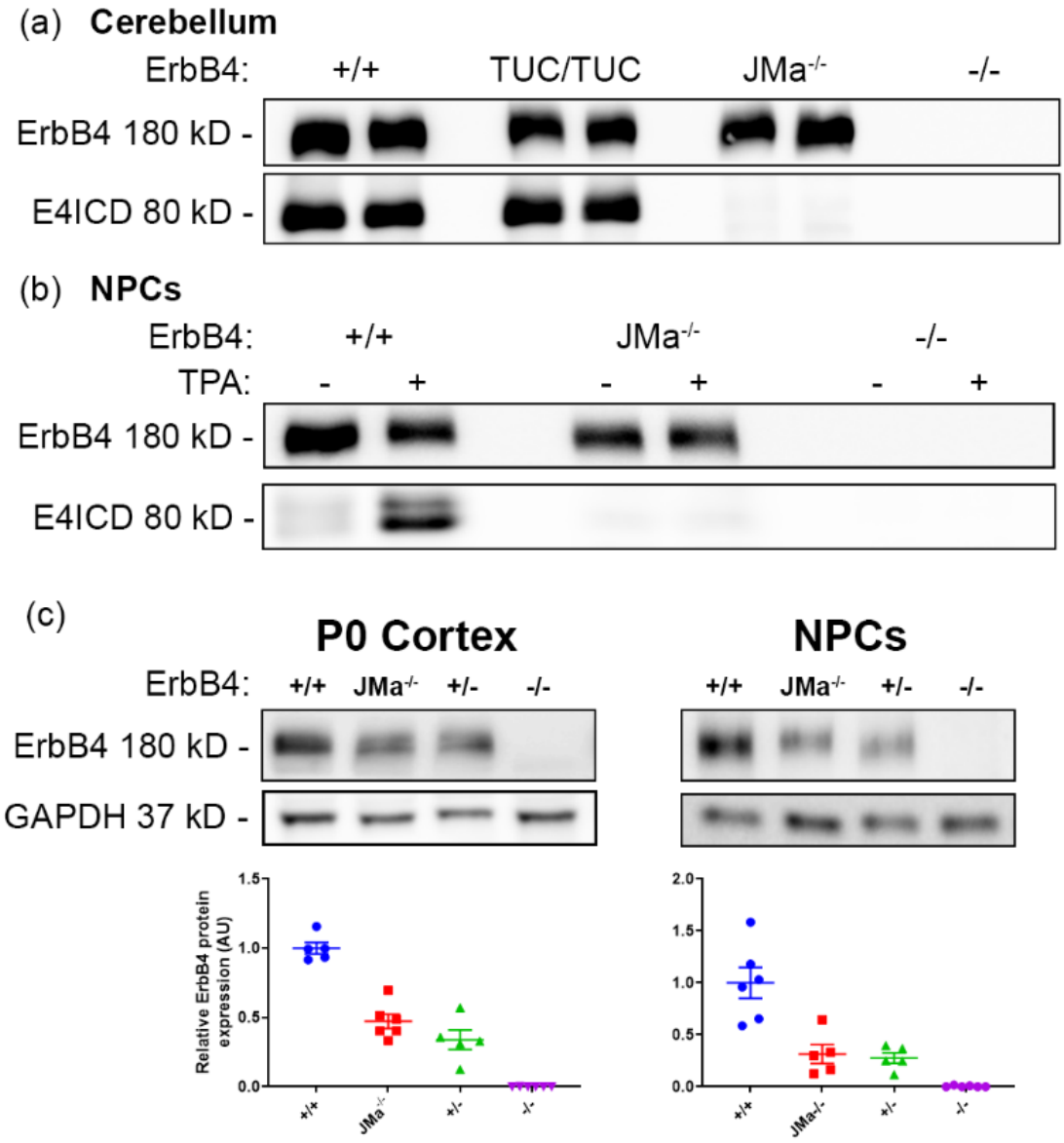


Figure 2.8: E4ICD formation is abolished in ErbB4-JMa$^{-/-}$ mice, but not ErbB4-TUC mice. (a) Immunoprecipitation of normalized quantities of ErbB4 from cerebellum lysates show that ErbB4-TUC mutations do not prevent formation of E4ICD by cleavage of ErbB4-JMa, whereas the mutation in ErbB4-JMa$^{-/-}$ mice completely prevents formation of E4ICD. (b) Stimulation of NPCs with TPA causes the cleavage of ErbB4 and formation of E4ICD in WT NPCs, but this formation is completely abrogated in NPCs from ErbB4-JMa$^{-/-}$ mice. (c) Western blot analysis of ErbB4 in NPCs and cortex shows that ErbB4 levels are decreased in ErbB4-JMa$^{-/-}$ mice to a similar level as ErbB4$^{+/-}$ mice; n = 5-7 per group.

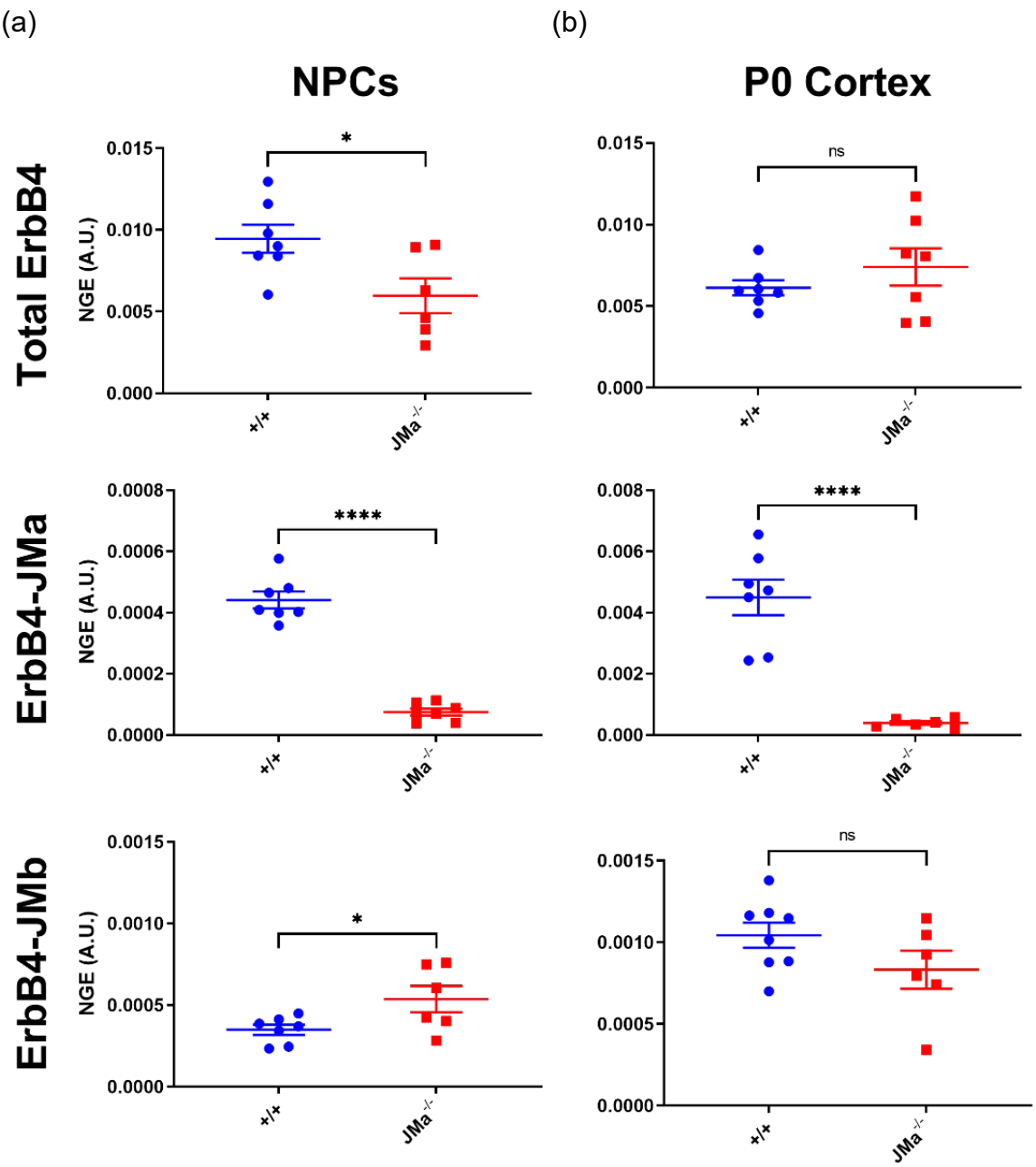


Figure 2.9: RNA expression analysis of ErbB4 isoforms of the ErbB4-JMa$^{-/-}$ mice. Erbb4-JMa isoform-specific qPCR in NPCs and cortex shows that ErbB4-JMa RNA expression is almost completely abolished in NPCs and cortex, whereas any alteration in ErbB4-JMb and total ErbB4 (by ErbB4 exon 2 qPCR) expression is minimal; n = 6-7 per group

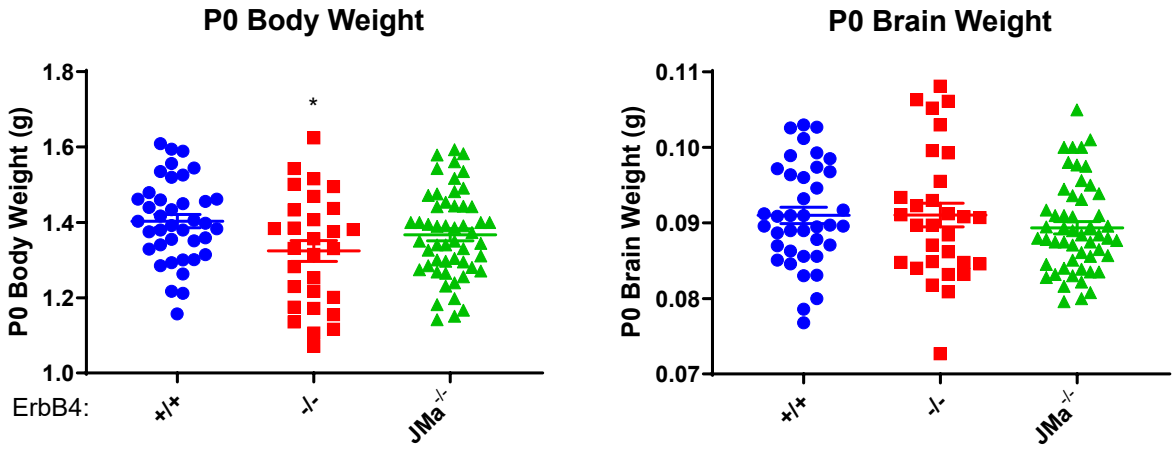


Figure 2.10: ErbB4-JMa does not play a role in the regulation of brain or body weight. Newborn pups were weighed, and their brains were dissected and weighed. Body and brain weight are unchanged in ErbB4-JMa$^{-/-}$ pups. There is a slight decrease in body weight of ErbB4$^{-/-}$ pups, but no change in brain weight n = 29-39

CHAPTER 3: Investigating the Role of Non-Canonical Signaling by ErbB4 in Cortical Development Using the ErbB4-JMa$^{-/-}$ Mouse[2]

3.1: Introduction

In Chapter 2, I validated that the novel ErbB4-JMa$^{-/-}$ mouse line abolishes E4ICD non-canonical signaling due to NMD of ErbB4-JMa transcripts, and that these mice are viable. It has yet to be determined if the ErbB4-JMa$^{-/-}$ mouse is a valid mouse model to determine if phenotypes found in ErbB4$^{-/-}$ mice are dependent on ErbB4-JMa. The regulation of the timing of astrogenesis had previously been identified as an E4ICD-dependent phenotype in ErbB4$^{-/-}$ mice and in NPCs (Sardi et al., 2006). Therefore, I investigated whether the regulation of astrogenesis is dependent on E4ICD signaling in the developing brain. To test this hypothesis, I used NPCs derived from ErbB4$^{+/+}$, ErbB4$^{-/-}$, ErbB4-JMa$^{-/-}$ and ErbB4$^{+/-}$ mice, where NPC differentiation could be controlled and regulated, to assess the effect of NRG1-ErbB4 signaling in NPCs from each of these lines. I further studied the regulation of GFAP expression in the developing brain using these mouse lines. The outcomes confirmed that non-canonical signaling by ErbB4-JMa regulates GFAP expression in NPCs and the ventricular zone (VZ) of the

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[2] This chapter contains portions of the following manuscript partially written and edited by Gabriel Corfas:

Doherty R, MacLeod BL, Nelson MM, Ibrahim MMH, Borges BC, Jaradat N, Finneran M, Giger RJ, and Corfas G. Identification of in vivo roles of ErbB4-JMa and its direct nuclear signaling using a novel isoform-specific knock out mouse. In submission at *Scientific Reports.*

cortex. These results also established that ErbB4-JMa$^{-/-}$ mice can be used to accurately study the role of non-canonical E4ICD signaling in vivo.

I then used the ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ mouse lines to screen for genes whose expression is regulated by the different ErbB4 signaling mechanisms during cortical development. A combination of bulk RNAseq and qRT-PCR identified 20 new ErbB4-regulated genes and allowed us to distinguish whether these changes in expression were dependent on ErbB4-JMa. These experiments display the usefulness of the ErbB4-JMa$^{-/-}$ mice to delineate whether ErbB4-regulated phenotypes are mediated by ErbB4-JMa non-canonical signaling.

3.2: Materials and Methods

Refer to Chapter 2.2 for previously described methods

GFAP assessment of NPCs

To assess of GFAP protein and RNA levels, NPCs were plated at 500,000 cells per well in 12-well plate format. Half of the media was replaced after one day, replenishing bFGF, then on the second day all the media was replaced with or without bFGF (to induce astrocytic differentiation and GFAP expression) and treated with 2 nM NRG1. After 24 hrs, cells were lysed in 100 µl RIPA buffer on ice to assess protein levels by western blotting or RNA was extracted according to RNeasy Mini Kit (Quiagen).

Luciferase Assay

To assess GFAP promoter activity by luciferase assay, NPCs were plated at 800,000 cells per well in 24-well plate format. Half of the media was replaced after one

day, replenishing bFGF and cells were co-transfected with a *GFAP*-promoter luciferase construct and *CMV*-Renilla (Sardi et al., 2006) at a 50:1 ratio using Lipofectamine 3000. After 24 hrs the media was replaced with or without bFGF and treated with 2 nM NRG1 for 2 days with bFGF and NRG1 replenishment after 1 day. GFAP-promoter driven luciferase activity was measured using the Dual-Luciferase Assay Kit (Promega), following the manufacturer's instructions on a BioTek plate reader. Relative values were obtained by normalizing firefly luciferase to Renilla luciferase in technical triplicate.

GFAP Immunofluorescence

Whole brains were dissected from P0 pups in the afternoon following birth and fixed in 4% PFA at 4°C overnight and then cryopreserved in 30% sucrose in PBS for 2 days at 4°C. Brains were embedded in OCT Compound (Fisher) and snap frozen in isopentane on dry ice. Frozen brains were cryosectioned at 50 µm into antifreeze solution and stored at -20°C. Sections were washed/permeabilized in TBS 0.2% Triton (TBS-T) for 20 mins and incubated in 5% normal goat serum in TBS-T for 1 hour. Sections were then incubated overnight at 4°C in primary antibody (1:1000. GFAP, Dako) then washed in TBS-T at room temperature. Sections were incubated in secondary antibody (1:1000, Alexa fluor 564 nm goat anti-rabbit, Invitrogen) for 1 hr at room temperature then washed in TBS-T, mounted on Superfrost plus slides (Fisher) and coverslipped with Fluoro-Gel II with DAPI (Electron Microscopy Services). For analysis, confocal images were taken at a magnification of 63x at the same location in the ventricular zone with a 12 µm and a 2 µm interval z-step, by a blinded investigator. The average GFAP intensity per ventricular zone area was calculated using Fiji software. Biological replicates were the average of the left and right side of the cortex.

RNA-Sequencing and analysis[3]

Pregnant females were euthanized via cervical dislocation. E15.5 embryos were placed in ice-cold PBS, brains were dissected, and the cortical hemispheres were isolated. The meninges and ganglionic eminences were removed from the cortex, and the tissue was stored in RNAlater (Invitrogen) until RNA extraction. RNA was extracted from wild-type (WT) and ErbB4 KO dorsal cortex using the Qiagen RNeasy Mini Kit with on-column DNase digestion (Qiagen), then analyzed with a BioAnalyzer to measure RNA quality. RNA with RNA integrity numbers (RINs) greater than 8 were sequenced (n = 4 of each genotype). Non-strand specific polyA-selected cDNA libraries were prepared. Single-end sequencing was then completed with read lengths of 50 nucleotides using an Illumina HiSeq-4000 Sequencing System. cDNA library preparation and sequencing were carried out by the University of Michigan DNA Sequencing Core. Sequences were mapped to the mouse genome (mm9) using HISAT, transcript counts obtained with HTseq-count, and differential gene expression analysis completed using DESeq2. All analysis was carried out with Galaxy. p-Values adjusted for multiple comparisons (q-value) < 0.05 indicated genes with statistically significant differences. Gene Ontology Analysis of dysregulated genes was performed using the Panther Classification system2. Ingenuity Pathway Analysis was performed using Qiagen IPA.

[3] Adapted from (Nelson et al., 2021)

3.3: Results

Regulation of GFAP expression by ErbB4 is mediated by ErbB4-JMa non-canonical signaling in NPCs

The Corfas lab previously showed that nuclear signaling by E4ICD regulates the timing of astrogenesis by repressing GFAP expression (Sardi et al., 2006). Specifically, they showed that NRG1-induced E4ICD signaling represses CNTF-induced GFAP expression in rat NPCs. We also showed that mice with complete loss of ErbB4 have increased GFAP expression in the ventricular zone of the developing brain, and that this phenotype can be rescued by expression of constitutively active E4ICD. Therefore, I tested if loss of ErbB4-JMa produces similar phenotypes. I found that a strong induction of GFAP expression can be triggered in mouse NPCs by removal of bFGF from the culture medium and that addition of NRG1 reduces the effect of bFGF removal on GFAP expression by more than 50% (Fig. 3.1a and b). The NRG1-induced repression of GFAP expression is absent in NPCs derived from both ErbB4-JMa$^{-/-}$ and ErbB4$^{-/-}$ embryos, indicating that ErbB4-JMa is responsible for repression of GFAP (Fig. 3.1b). Importantly, the effect of NRG1 on GFAP expression is normal in ErbB4$^{+/-}$ NPCs (Fig. 3.1b), indicating that the loss of the NRG1 response in the ErbB4-JMa$^{-/-}$ cells is not due to the reduction in full-length ErbB4 expression observed in ErbB4-JMa$^{-/-}$ NPCs. Similarly, NRG1 treatment of NPCs after bFGF removal repressed the activity of the *GFAP* promoter assessed by a luciferase reporter assay (Fig. 3.1c) and reduced the levels of GFAP protein in ErbB4$^{+/+}$ and ErbB4$^{+/-}$ NPCs, but not in ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ NPCs (Figs. 3.1d and e). These results corroborate that direct nuclear signaling by E4ICD represses astrocytic gene as previously identified by our lab. Since ErbB4$^{+/-}$

and ErbB4-JMa$^{-/-}$ have similar levels of full-length ErbB4 and therefore most likely similar levels of canonical signaling, these results indicate that the changes observed in ErbB4-JMa$^{-/-}$ tissues are due to loss of direct nuclear signaling. Furthermore, these results also show that NRG1/ErbB4-JMb signaling is not sufficient to regulate GFAP expression in NPCs.

Regulation of GFAP expression by ErbB4 is mediated by ErbB4-JMa non-canonical signaling in vivo

To determine if loss of ErbB4-JMa alters GFAP expression in vivo, I assessed GFAP levels in the ventricular zone in the neonatal brain using quantitative immuno-fluorescence (Fig. 3.2a). While ventricular zone (VZ) depth remained the same (Fig. 3.2b), we found increased GFAP levels in the VZ of ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ mice compared to ErbB4$^{+/+}$ and ErbB4$^{+/-}$ mice (Fig. 3.2c), indicating that the difference in GFAP expression is due to loss of ErbB4-JMa signaling. Together, these results provide further support to the notion that E4ICD signaling is necessary for the NRG1-mediated regulation of GFAP expression in NPCs in the developing brain. Furthermore, these results validate the ErbB4-JMa$^{-/-}$ mouse model as a tool to explore the roles of ErbB4-JMa and non-canonical ErbB4 nuclear signaling in vivo.

Identification of the signaling mechanism responsible for ErbB4-mediated gene expression during cortical development using ErbB4-JMa$^{-/-}$ mice

Having validated that the new mutant line specifically eliminates ErbB4-JMa and thus allows for the distinction between biological processes that depend on ErbB4-JMa or ErbB4-JMb, we set out to identify new genes whose expression is differentially regulated by the two isoforms during cortical development. We first identified genes

whose expression is altered by loss of ErbB4 by bulk RNA-seq on ErbB4$^{+/+}$ and ErbB4$^{-/-}$ E15.5 embryonic brain cortex samples. This identified 20 differentially expressed genes (DEGs), 12 upregulated and 8 downregulated in the KO (q value < 0.05, Table 1). Heat map analysis shows that gene expression differences were consistent between genotypes among different replicates (Fig. 3.3a). Ingenuity Pathway Analysis (IPA) shows that pathways known to be associated with ErbB4, such as outgrowth of cells, neurites and neurons, and growth and proliferation of neurons, are linked to DEGs (Fig. 3.4a). Pathway analysis of the top 200 upregulated and 200 downregulated genes shows that systems known to be regulated by ErbB4 were altered in ErbB4$^{-/-}$ samples, such as RTK phosphorylation (cAMP, CREB, PKA, GPCRs); synaptogenesis and synaptic plasticity; and breast cancer and tumor microenvironment (Fig. 3.4c). These analyses validated that the RNA-seq was reliable and consistent with expected roles of ErbB4 in the developing cortex.

I then used qRT-PCR to determine if some of the DEGs are specifically regulated by ErbB4-JMa, focusing on four genes that have been implicated in the regulation of NPCs or brain development (bold genes in Table 1). I found that WDFY1 (WD Repeat and FYVE Domain Containing 1) and PRSS12 (serine protease 12) are upregulated in the cortex of ErbB4$^{-/-}$ embryos ($p<0.0001$ and $p= 0.0174$, respectively) but not in ErbB4-JMa$^{-/-}$ tissue (Fig. 3.5a), showing that their expression is independent of ErbB4-JMa. Interestingly, expression of WDFY1 and PRSS12 was also increased in ErbB4$^{-/+}$ mice, indicating that loss of half of total ErbB4 was sufficient to phenocopy changes in gene expression in ErbB4$^{-/-}$ mice, as has been observed for other ErbB4-related phenotypes (Golub et al., 2004; Shamir et al., 2012b). In contrast, expression of CRYM (μ-crystallin)

and Dbi (diazepam binding inhibitor) is altered in the cortex of both ErbB4-JMa$^{-/-}$ ($p=0.0007$ for both) and ErbB4$^{-/-}$embryos ($p= 0.0015$ and $p<0.0001$, respectively) (Fig. 3.5b), showing that the JMa isoform and possibly ErbB4-JMa non-canonical signaling, are involved in their regulation. However, since these mRNA species were also altered in the ErbB4$^{-/+}$ mice, the results do not provide definite proof that these genes are regulated by non-canonical signaling.

3.4: Discussion

Here, I demonstrate that ErbB4-JMa$^{-/-}$ mice that lack E4ICD formation have defects in the regulation of GFAP expression, as predicted by prior studies (Elenius et al., 1997; Rio et al., 2000; Sardi et al., 2006). This provides proof of concept that the development of the ErbB4-JMa$^{-/-}$ mice is powerful tool to test the roles of non-canonical signaling of RTKs in vivo. This is a particularly important development as this signaling modality might be relevant to more than half the members of the RTK (Merilahti et al., 2017; Merilahti & Elenius, 2019).

As I had shown the ErbB4-JMa$^{-/-}$ mouse to be a reliable tool and that non-canonical signaling by ErbB4-JMa is responsible for repression of GFAP expression during cortical development in vivo, I was able to use this mouse to investigate whether non-canonical signaling was responsible for the regulation of other targets during brain development. Of the targets identified by bulk RNA-seq, CRYM and DBi were found to be regulated by ErbB4-JMa.

DBi inhibits benzodiazepine binding site of the GABAaRs (γ-aminobutyric acid type A receptors), blocking differentiation of NPCs to maintain and expand the progenitor pool (Alfonso et al., 2012; Dumitru et al., 2017). ErbB4 has also previously

been suggested to inhibit GABAaRs in the regulation of synaptic transmission (Geng et al., 2017; Mitchell et al., 2013), but not in cortical development, or in association with DBi. The decrease in DBi expression due to loss of ErbB4-JMa could mechanistically regulate this interaction and may play a role in the early transition from progenitor maintenance to astrogenesis.

CRYM encodes the protein μ-Crystallin, which was primarily known as a thyroid hormone binding protein (Kinney et al., 2021), but was recently identified as the most enriched marker of striatal astrocytes (Chai et al., 2017), indicating that ErbB4-JMa may also regulate the expression profile of cortical astrocytes. Combined with the in vivo confirmation that ErbB4-JMa signaling regulates the timing of astrogenesis, the identification of two more genes that have putative roles in the regulation of NPC differentiation and astrocyte development strongly suggests that non-canonical ErbB4 signaling during cortical development. Further studies defining the role of non-canonical ErbB4 signaling in NPC regulation and astrocyte development could reveal how its regulation of astrogenesis plays important roles in brain development and physiology.

Further experiments are required to delineate whether other genes are regulated by ErbB4 are dependent on ErbB4-JMa and to investigate how their interactions may affect cortical development. However, in this Chapter I have established that the ErbB4-JMa$^{-/-}$ mice can be used to quickly delineate whether known or novel functions of ErbB4 are regulated by ErbB4-JMa non-canonical signaling.

3.5 Figures

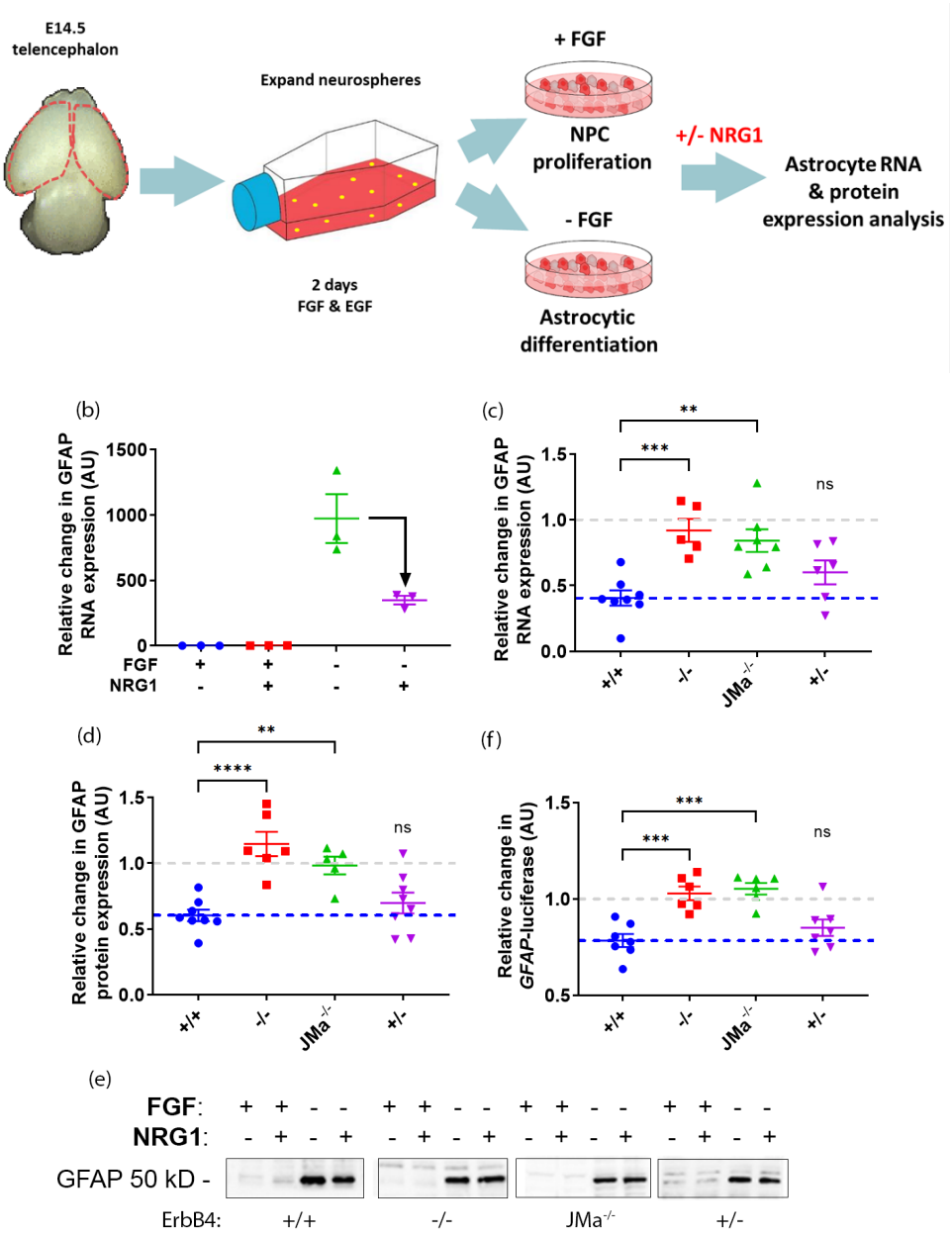


Figure 3.1. Repression of GFAP expression by ErbB4 is regulated by ErbB4-JMa via GFAP promoter inhibition. (a) schematic of experimental design to observe the effect of NRG1 treatment on GFAP expression. (b) Example of ErbB4$^{+/+}$ NPC GFAP RNA expression; n= 3 technical repeats. The increase in GFAP upon removal of FGF is repressed by treatment with NRG1 in ErbB4$^{+/+}$ and ErbB4$^{+/-}$ NPCs but does not have an effect both in ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ NPCs in (c) RNA levels by qPCR protein levels of GFAP (n = 5-8 per group) or (d) protein by qWB (e). (f) ErbB4$^{+/+}$ and ErbB4$^{+/-}$ NPCs expressing firefly luciferase upstream of the GFAP promoter shows that NRG1 treatment inhibits of the GFAP promoter expression, but NRG1 treatment of ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ NPCs does not repress GFAP promoter activity (n = 6-7 per group).

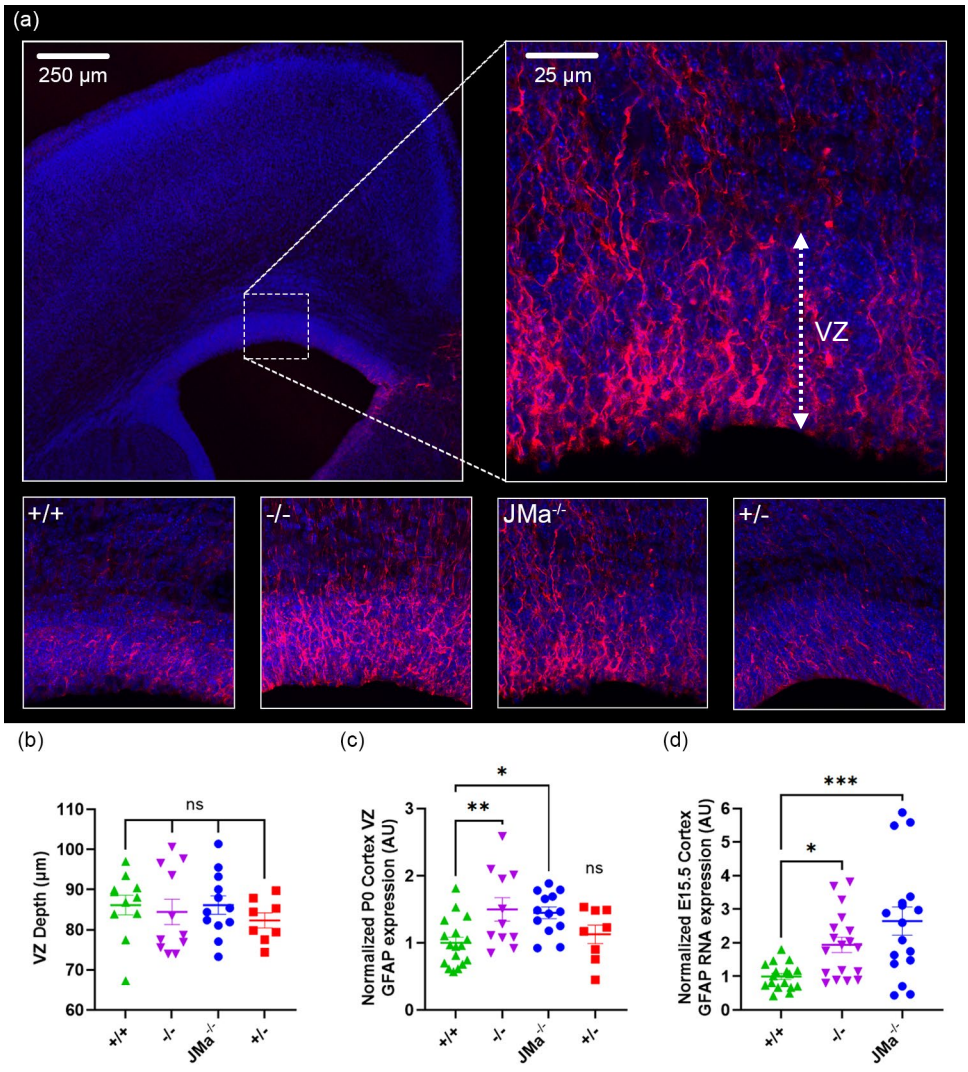


Figure 3.2. Repression of GFAP expression by ErbB4 is regulated by ErbB4-JMa in vivo.[4] (a) Example 10x and 63x images of the VZ brain area analyzed for GFAP immunofluorescence and representative 63x images of ErbB4$^{+/+}$, ErbB4$^{-/-}$, ErbB4-JMa$^{-/-}$ and ErbB4$^{+/-}$ mouse brains. (b) There was no effect on VZ depth; n= 8-13 per group. (c) Quantification of mean GFAP fluorescence intensity per area of VZ/SVZ shows an increase in GFAP expression in both ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ mice compared to ErbB4$^{+/+}$ mice; n = 8-17 pergroup. (d) Analysis of RNA expression in the E15.5 cortex by qPCR also shows an increase in GFAP expression in both ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ mice; n = 15-16 per group.

[4] Sectioning and GFAP immunofluorescence carried out by Brenna Macleod

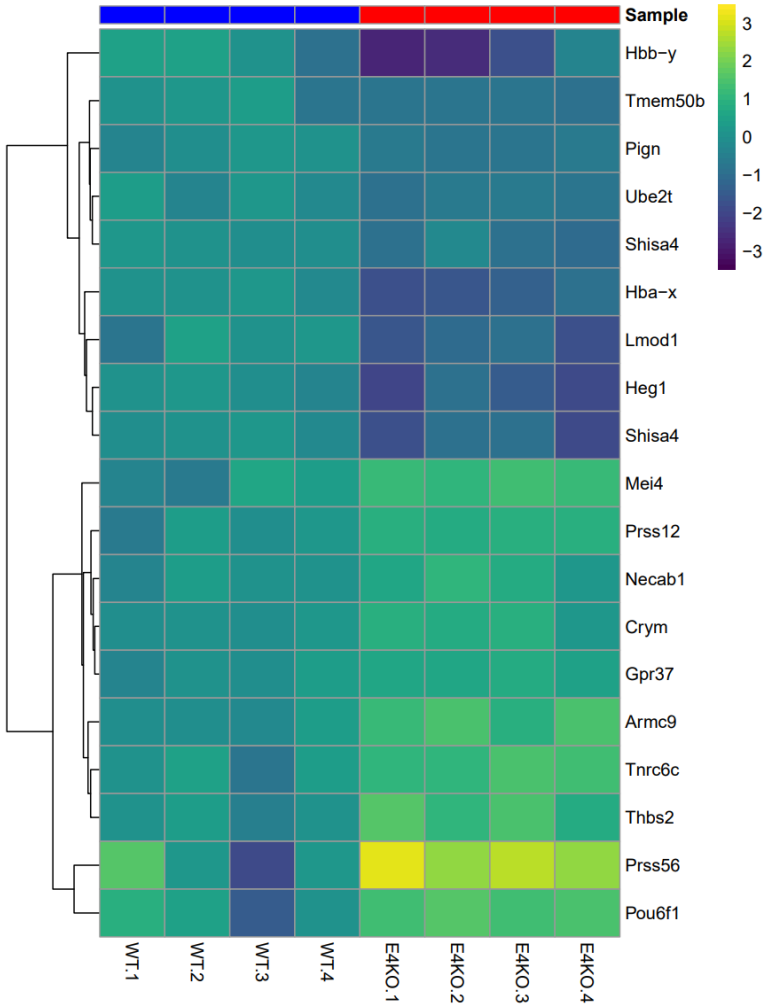
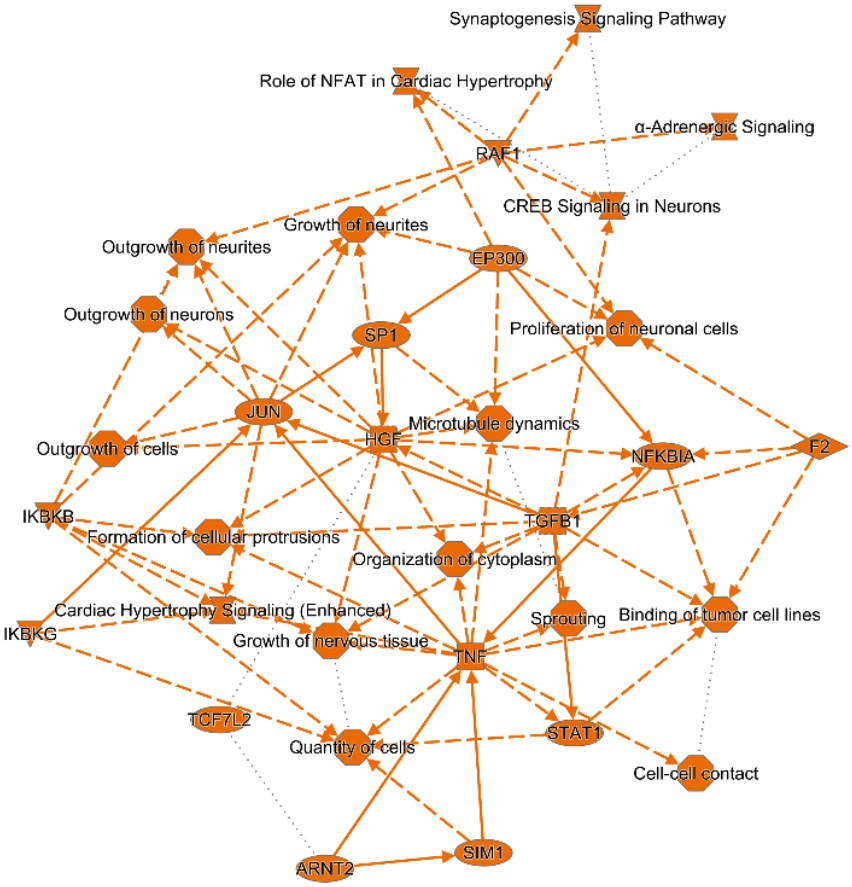


Figure 3.3: Heat map analysis of replicate samples for RNA-seq.[5] Heat map analysis of genes that had an adjusted P-value < 0.75 and lfc < .58 shows that genes that consistently upregulated in ErbB4$^{-/-}$ samples (E4KO) compared to ErbB4$^{+/+}$ samples (WT). With few exceptions, such as WT Prss56, there is little variation between replicates indicating that this data is reliable.

(a)


Synaptogenesis Signaling Pathway
Role of NFAT in Cardiac Hypertrophy
α-Adrenergic Signaling
RAF1
CREB Signaling in Neurons
Growth of neurites
Outgrowth of neurites
EP300
Outgrowth of neurons
SP1
Proliferation of neuronal cells
JUN
Microtubule dynamics
HGF
Outgrowth of cells
NFKBIA
F2
IKBKB
TGFB1
Formation of cellular protrusions
Organization of cytoplasm
Cardiac Hypertrophy Signaling (Enhanced)
Sprouting
Binding of tumor cell lines
IKBKG
Growth of nervous tissue
TNF
TCF7L2
STAT1
Quantity of cells
Cell-cell contact
SIM1
ARNT2

(b)

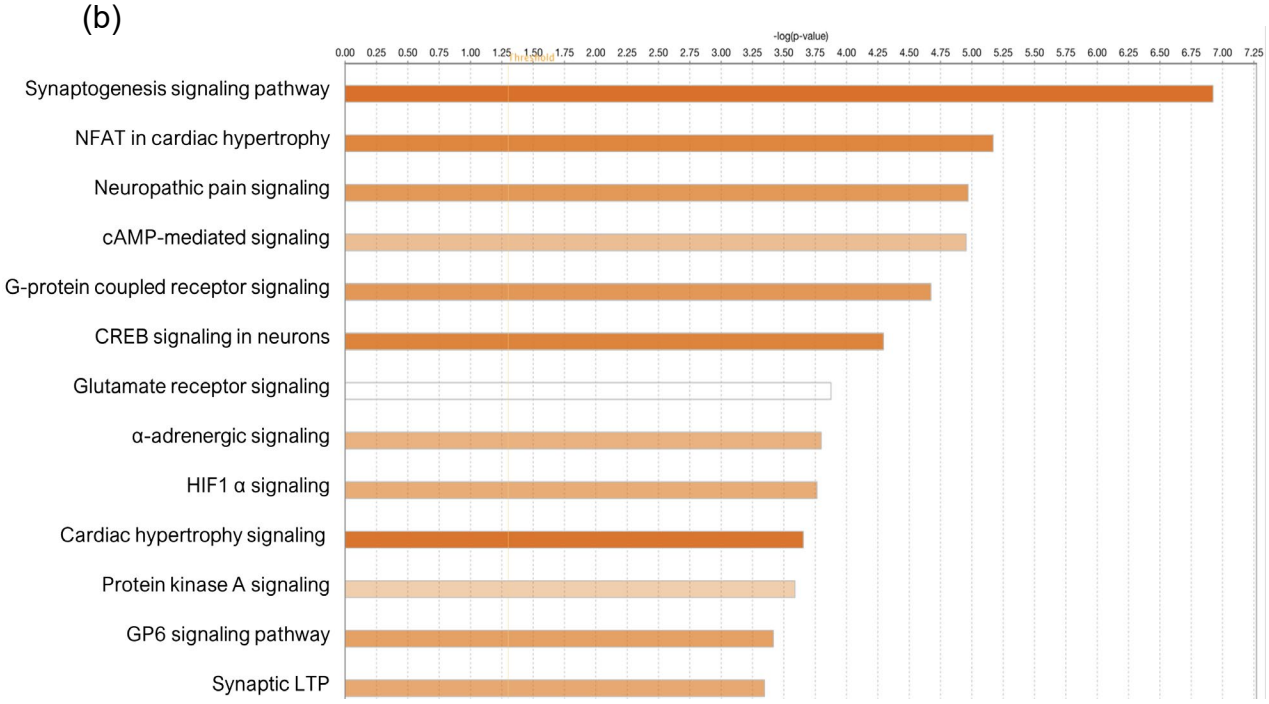

-log(p-value)
0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00 2.25 2.50 2.75 3.00 3.25 3.50 3.75 4.00 4.25 4.50 4.75 5.00 5.25 5.50 5.75 6.00 6.25 6.50 6.75 7.00 7.25
Threshold
Synaptogenesis signaling pathway
NFAT in cardiac hypertrophy
Neuropathic pain signaling
cAMP-mediated signaling
G-protein coupled receptor signaling
CREB signaling in neurons
Glutamate receptor signaling
α-adrenergic signaling
HIF1 α signaling
Cardiac hypertrophy signaling
Protein kinase A signaling
GP6 signaling pathway
Synaptic LTP

Figure 3.4: Ingenuity Pathway Analysis (IPA) of the top 200 up and downregulated genes identified by bulk RNA-seq of ErbB4$^{-/-}$ compared to ErbB4$^{+/+}$ E15.5 cortex[5].
(a) Summary interactome of IPA from the RNA-seq data showing hub genes and pathways that were changed by loss of ErbB4. (b) IPA identified numerous pathways that are regulated by ErbB4 in the E15.5 cortex, the top 13 pathways are shown ranked by significance.

[5] Heat map and pathway analysis carried out by Matthew Finneran and Roman Giger

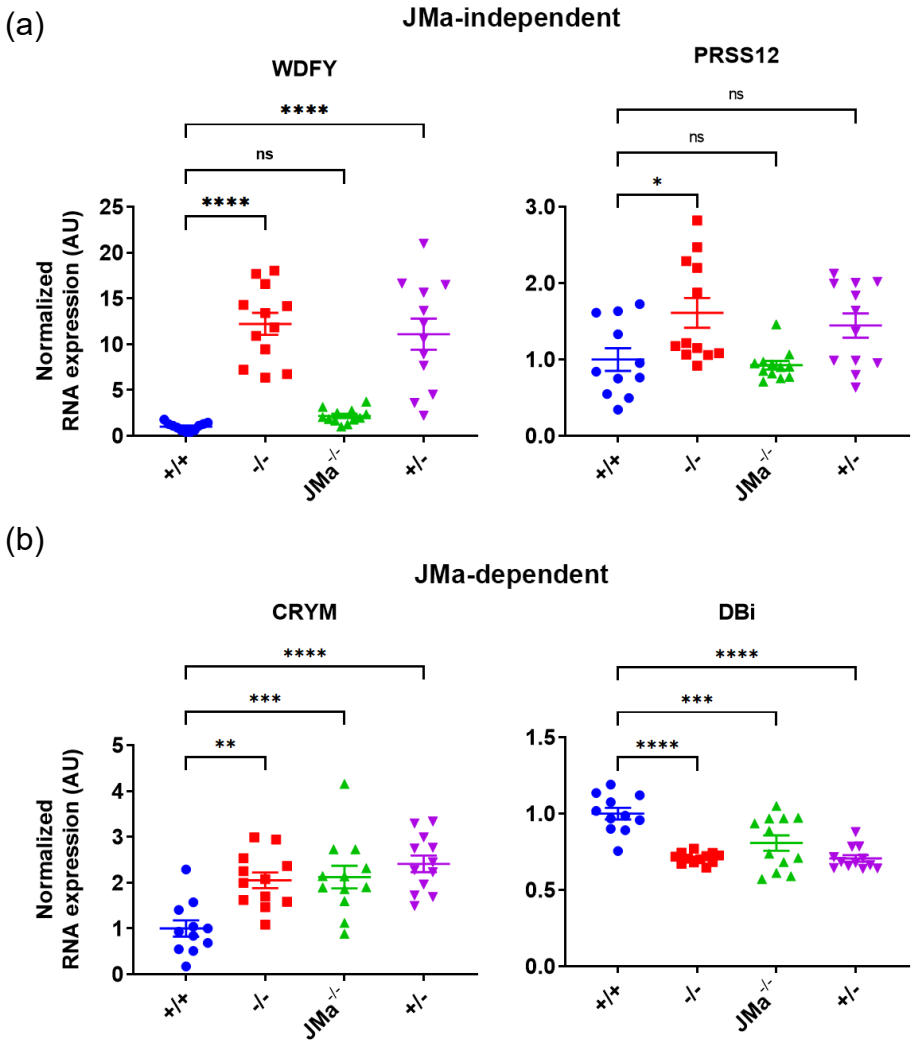


Figure 3.5: **Novel ErbB4-regulated targets identified by RNA-seq are regulated by ErbB4-JMa.** RNA-seq of E15.5 cortex identified 20 targets significantly regulated by ErbB4 during cortical development. Of the targets confirmed to be significantly different by qPCR in ErbB4$^{-/-}$ mice, (a) WDFY and PRSS12 were not changed in ErbB4-JMa$^{-/-}$ mice (n = 15-16 per group), whereas (b) Dbi and CRYM were significantly different in ErbB4-JMa$^{-/-}$ mice, as with ErbB4$^{-/-}$ mice (n = 15-16 per group).

3.5 Tables

Gene	q-value	Fold change	Name	Putative Roles
Wdfy1	**0**	**4.139**	**WD repeat and FYVE domain containing 1**	**TLR pathway, regulation of GFAP expression in NSCs**
Ndst3	3.23E-08	0.7058	N-deacetylase/N-sulfotransferase 3	Linked to schizophrenia and bipolar disorder
Pign	4.68E-08	0.7093	Phosphatidylinositol glycan anchor biosynthesis	GPI biosynthesis. Role in hypotonia, epilepsy, developmental delay
Lancl1	1.56E-07	0.7724	LanC like 1	Neural antioxidant, protects against MN loss in ALS & ischemia
Prss12	**0.00135**	**1.3310**	**Serine protease 12 (neurotrypsin, motopsin)**	**Linked to ID, modulates hippocampal function**
Corin	0.00145	1.1871	Corin, serine peptidase	Cardiac hormone, regulates salts
Shisa4	0.00215	0.7765	Shisa family member 4	WNT antagonist family
Mcm6	0.00297	0.7695	Minichromosome maintenance complex component 6	Possible role in cancer and glioma replication
Adora1	0.00371	1.2201	Adenosine A1 receptor	Adenosine receptor, PD/sleep/tumor progression
Crym	**0.00815**	**1.2860**	**μ-cyrstallin**	**Marker of striatal astrocytes. Thyroid hormone binding. Lens development**
Necab1	0.01059	1.2881	N-terminal EF-hand calcium binding protein 1	Ca-binding in CCK interneurons
Gpr37	0.01059	1.2865	G protein-coupled receptor 37	GPCR expressed on oligos with role in myelination/MS/PD/stroke
Gas5	0.01844	0.7869	Growth arrest specific 5	Inflammatory response, stroke & tumor progression. lncRNA.
Hrk	0.02897	1.2310	Harakiri	Bcl2 family apoptosis regulation
Prss56	0.02897	1.1548	Serine protease 56	Regulates neurogenesis, expressed in adult radial glia
Ube2t	0.02979	0.7901	Ubiquitin-conjugating enzyme E2T	Cancer proliferation, invasion, migration via H2AX
DBi	**0.03576**	**0.7966**	**Diazepam binding inhibitor**	**Maintains neural progenitor pool by promoting NSC proliferation**
A02Rik	0.03794	1.2232	6330403A02Rik mechanosensory transduction mediator	None known
Afap1l2	0.03960	1.2421	Actin filament associated protein 1 like 2	AFAP adaptor, may be involved in oligodendrocyte maturation
Opcml	0.04455	1.2299	Opioid-binding protein/cell adhesion molecule	GPI anchor, associated to schizophrenia.

Table 3.1: Genes identified as significantly different in ErbB4$^{-/-}$ E15.5 cortex by RNA-seq. A list of the genes in order of q-value significance is shown in the table along with the fold change, full name and known roles of those genes. In bold are genes of interest that were identified to study by qPCR analysis.

CHAPTER 4: Delineating the Roles of Non-Canonical Signaling by ErbB4 In Vivo Using the ErbB4-JMa$^{-/-}$ Mouse [6]

4.1: Introduction

In Chapter 2 and 3 I have shown that a novel mutant ErbB4-JMa$^{-/-}$ mouse that lacks non-canonical signaling is a valid and robust tool to investigate whether ErbB4 related phenotypes are dependent on ErbB4-JMa signaling in vivo**.** There are numerous roles of ErBb4 that could be investigated using ErbB4-JMa$^{-/-}$ mouse, overviewed in Chapter 1 and Table 4.1. However, as the ErbB4-JMa$^{-/-}$ mouse is a germline constituent knock out model, it is a tool that is most appropriate for the study of developmental phenotypes. Hence, in this chapter I will report results from investigations into the role of ErbB4-JMa in development of the heart, mammary glands, neural ganglia, interneurons, pulmonary function, and immune cells.

Phenotypic analysis of ErbB4-JMa$^{-/-}$ mouse has provided evidence that non-canonical signaling by ErbB4-JMa is not necessary for heart, mammary gland, neural ganglia development, and interneuron development, while immune cell development appears to be regulated by ErbB4-JMa. These findings emphasize how the ErbB4-JMa^{-}

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[6] This chapter contains portions of the following manuscript partially written and edited by Gabriel Corfas:

Doherty R, MacLeod BL, Nelson MM, Ibrahim MMH, Borges BC, Jaradat N, Finneran M, Giger RJ, and Corfas G. Identification of in vivo roles of ErbB4-JMa and its direct nuclear signaling using a novel isoform-specific knock out mouse. In submission at *Scientific Reports.*

$^{/-}$ mouse can be utilized to delineate whether known roles of ErbB4 require non-canonical signaling by ErbB4-JMa.

4.2: Materials & Methods

Mammary gland histology

At post-natal day 1 (P1) the number 4 linguinal mammary gland was dissected from the female mice. Mammary glands were spread onto Superfrost plus slides and fixed in 4% PFA overnight. Mammary glands were paraffin embedded and processed for haemotoxylin and eosin staining and then imaged at 4x and 40x magnification using bright field microscopy.

Neurofilament immunochemistry

Embryos were taken at E11.5 directly into 4% PFA and incubated overnight at 4°C, then kept at 4°C in 0.4% PFA and the rest of the steps were done at room temperature. Embryos were washed in TNT for 10 mins three times, then dehydrated in a methanol grade of 50%, 70% and 90 % for one hour and 100% overnight. Quench in Dents (3% hydrogen peroxide, 70% methanol, and 20% DMSO) for 5 hours or overnight. Embryos were washed twice and incubated for 2 days TNT and 2 days in 5% normal horse serum in TNT (HS-TNT). Embryos were incubated for 5 days in 0.75 ug/mL 2H3 antibody in HS-TNT then washed in TNT for 45 mins five times. Embryos were incubated for 3 days in horse anti-mouse IgG HRP-conjugated Ab at 1:250 in HS-TNT then washed in TNT for 45 mins five times. Signal was developed according to Vectastain DAB Kit (SK-4001) then washed in TNT for 45 mins five times and dehydrated in a methanol grade of 50%, 70% and 90 % for one hour and 100%

overnight. Embryo tissue was cleared in in BABB solution (1:2 benzyl alcohol/benzyl benzoate) and stored in BABB in glass containers. Images were taken on a dissecting microscope with camera at the magnifications indicated.

Parvalbumin interneuron immunofluorescence

Adult 3-month-old mice were transcardially perfused with PBS for 5 mins then 4% PFA for 8 mins. Brains were dissected and fixed in 4% PFA at 4°C overnight and then cryopreserved in 30% sucrose in PBS for 2 days at 4°C. Brains were embedded in OCT Compound (Fisher) and snap frozen in isopentane on dry ice. Frozen brains were cryosectioned at 50 µm into antifreeze solution and stored at -20°C. Sections were washed/permeabilized in TBS 0.2% Triton (TBS-Tx) for 20 mins and incubated in 5% normal goat serum in TBS-Tx (blocking buffer) for 1 hour. Sections were incubated overnight at 4°C in primary antibody (Swant, Rabbit anti-PV, 1:1000) in blocking buffer then washed for 10 mins in TBS-Tx at room temperature three times. Sections were incubated in secondary antibody (Invitrogen, Alexa fluor 564 nm goat anti-rabbit) for 1 hr at room temperature then washed for 10 mins in TBS-Tx three times, mounted on Superfrost plus slides (Fisher) and coverslipped with Fluoro-Gel II with DAPI (Electron Microscopy Services). For analysis confocal images were taken at a magnification of 10x in each area, blinded to the genotype. The number of PV+ cells per area were counted using Fiji sftware. Biological replicates were the average of the left and right side of the brain for each area. Hippocampal and cortical areas were identified by reference to the Allen Brain Atlas.

Pulmonary function test[7]

PFT analyses were performed in anesthetized mice following insertion of a tracheal tube for mechanical ventilation. Invasive lung function equipment and software employs a series of differential ventilation and pause techniques and Boyle's law functional residual capacity and quasistatic pressure volume maneuvers to accumulate data (The Data Systems International (DSI; Kansas City, MO)). Specialized software calculates a number of lung function parameters including inspiratory capacity (IC), vital capacity (VC), chord compliance, and expiration time, among others.

FACS analysis of splenocytes[8]

To analyze immune cell profiles in the spleen, mice were transcardially perfused for 5 min with ice-cold PBS to flush out all blood cells in circulation. The spleen was dissected, and splenocytes were passed through a 70-µm Falcon cell strainer (Corning, 352350). Red blood cells were lysed with Ammonium-Chloride-Potassium (ACK) lysing buffer. Samples were rinsed in DMEM with 10% FBS and spun down at 650 g for 5 min. This resulting pellet gently re-suspended in 1 mL of 27% Percoll (Sigma Aldrich, P4937) in PBS. Then 3 ml of 27% Percoll were added to bring the final volume to 4 ml. Samples were spun at 900 g for 20 min in a clinical centrifuge (Beckman Coulter Allegra 6R). The top layers (with myelin and other debris) were carefully aspirated. The final 100 µl were resuspended in 1 ml of PBS with 2% FBS and filtered through a pre-washed 40 µm Falcon filter (Corning, 352340). Cells were pelleted at 650 g for 5 min at 4°C. Cells were

[7] Adapted from (Hrycaj et al., 2018)
[8] Adapted from (Kalinski et al., 2020)

labeled with fixable viability dye (Thermo Fisher Scientific, 65086614), blocked with αCD16/32 (BD Pharmingen, 553141), and stained with fluorescent antibodies and isotype controls. Immune cells (CD45+) were further classified as neutrophils (CD45+CD11b+Ly6G+), monocytes (CD45+CD11b+Ly6G-CD11c-Ly6Ghi), B-cells (CD45+CD11b-CD19+) and T-cells (CD45+CD11b-CD19-CD3e+). Data were acquired using a FACSCanto II (BD Biosciences) flow cytometer and analyzed with FlowJo software (Treestar).

4.3: Results

ErbB4-JMa signaling is not required for heart, mammary gland, and neural ganglia development

ErbB4$^{-/-}$ mice have several phenotypes, some of which have been suggested to depend on non-canonical signaling based on in vitro assays (Iwamoto et al., 2017; Muraoka-Cook et al., 2006). We now found that some of these phenotypes are not present in ErbB4-JMa$^{-/-}$ mice, indicating that these biological processes do not depend on ErbB4-JMa or its direct nuclear signaling.

First, germline ErbB4-KO mice die during early embryonic development due to defects in heart formation (Gassmann et al., 1995), and therefore studies on ErbB4-KOs have been performed with mice rescued by cardiac-specific expression of ErbB4 under α-MHC promoter (Tidcombe et al., 2003). Unlike ErbB4$^{-/-}$ mice, ErbB4-JMa$^{-/-}$ mice are viable, and their heart is normal at the gross anatomical level (not shown), indicating that canonical signaling by ErbB4-JMb is sufficient for cardiac development.

Second, ErbB4$^{-/-}$ females have difficulty breastfeeding their pups due to a deficiency in post-natal mammary alveolar development (Tidcombe et al., 2003). The

initial studies of ErbB4$^{-/-}$ mice reported that 82% of pups from ErbB4$^{-/-}$ dams die before weaning (Tidcombe et al., 2003), whereas the size of litters weaned by ErbB4-JMa$^{-/-}$ dams is not different from those from wild type females (7.87±0.39 vs 8.47±0.50, respectively; p=0.350, n=15 litters/genotype). Furthermore, histological analysis of mammary glands from dams one day post-delivery showed that ErbB4$^{-/-}$ mice have abnormal, disorganized development of alveolar epithelial cells with lipid globules within the lumen (Fig. 4.1a), as previously shown (Tidcombe et al., 2003), whereas ErbB4-JMa$^{-/-}$ females develop normal post-delivery mammary alveolar structures (Fig. 4.1a).

Third, ErbB4$^{-/-}$ embryos form an aberrant neuronal bridge between the trigeminal ganglion (gV) and geniculate ganglion (gVII) due to aberrant migration and pathfinding by neural crest cells (Golding et al., 2000). This can be visualized by neurofilament immunostaining of whole mount E10.5 embryos (Tidcombe et al., 2003), which showed that the abnormal bridge is absent in ErbB4-JMa$^{-/-}$ embryos (Fig. 4.1b).

ErbB4-JMa signaling is not required for parvalbumin interneuron development and migration

Interneuron development is well known to be regulated by ErbB4. Interneuron precursors migrate tangentially from the MGE into the cortex and hippocampus to establish their position within the developing neurons. NRG1-ErbB4 signaling is known to regulate the migration of interneurons and loss of ErbB4 results in aberrant migration and decreased number of interneurons in the adult cortex and hippocampus. ErbB4 also regulates synaptic transmission onto interneurons, LTP and memory and the effect of ErbB4 on interneuron development and function is thought to play a part in the role of ErbB4 in schizophrenia and epilepsy. However, whether interneuron development and

function are regulated by ErbB4-JMa or ErbB4-JMb has not been studied. To address the role of ErbB4-JMa in interneuron development, I assessed the number of cortical and hippocampal parvalbumin (PV) interneurons in adult mice.

While others have previously shown decreased numbers of PV+ interneurons in the adult cortex of ErbB4$^{-/-}$ mice, I did not detect a difference in ErbB4$^{-/-}$ or ErbB4-JMa$^{-/-}$ mice in the somatosensory cortex (SC) or motor cortex (MC) (Fig. 4.2). There were fewer PV$_+$ interneurons in the CA3 region of the hippocampus of ErbB4$^{-/-}$ mice, the only region of the hippocampus in which a decrease in PV+ interneurons were detected (Fig. 4.2). There was no change in the number of interneurons in the CA3 region of the hippocampus ErbB4-JMa$^{-/-}$ mice, indicating that the regulation of PV interneuron development is not dependent of ErbB4-JMa signaling, but rather facilitated by ErbB4-JMb.

Pulmonary function testing of the role of ErbB4-JMa in pulmonary development was inconclusive

ErbB4 has been shown to play important roles in lung development (Dammann et al., 2012; Purevdorj et al., 2008). Pulmonary development is delayed in ErbB4$^{-/-}$ mice (Liu et al., 2010) and pulmonary function tests (PFT) reveal that they have changes that are phenotypically consistent with bronchopulmonary dysplasia (Purevdorj et al., 2008). It is thought that ErbB4-JMa non-canonical signaling is responsible for these outcomes as it has been shown that non-canonical E4ICD signaling transcriptionally regulates surfactant expression during development, and that aberrant surfactant production underlies the pulmonary development phenotype in the absence of ErbB4 (Hoeing et al., 2011; Zscheppang et al., 2011).

As E4ICD signaling had been shown to be involved in lung development we carried out PFT experiments to investigate whether lung development was dysregulated in ErbB4-JMa$^{-/-}$ mice. Our initial studies showed that inspiratory capacity (IC), vital capacity (VC), compliance and expiration time were all slightly increased in ErbB4-JMa$^{-/-}$ mice (Fig. 4.3a). Oddly, this was not the case for ErbB4$^{-/-}$ mice compared to ErbB4$^{+/+}$ mice. In this experiment, both ErbB4$^{+/+}$ and ErbB4$^{-/-}$ mice had αMHC-ErbB4 expression for cardiac rescue of ErbB4 lethality, whereas ErbB4-JMa$^{-/-}$ mice did not. We then tested whether αMHC-ErbB4 expression influenced PFT results. Interestingly, αMHC-ErbB4 expression (MHC+) in ErbB4$^{+/+}$ mice resulted in slightly lowered IC, VC, compliance, and expiration time compared to ErbB4$^{+/+}$ mice without αMHC-ErbB4 expression (MHC-) (Fig. 4.3b). This indicates that the differences observed in ErbB4-JMa$^{-/-}$ mice could have been due to the lack of αMHC-ErbB4 expression, rather than the role of ErbB4-JMa. Due to the confounding factor of αMHC-ErbB4 expression on PFT results and lack of any difference observed in ErbB4$^{-/-}$ mice, I did not pursue this line of investigation any further.

ErbB4-JMa signaling may be involved in immune cell development

Regulation of immune cell development by ErbB4 has been shown by peripheral erythroid profiles (Kinney et al., 2019) as well as in functional responses by macrophages (Schumacher et al., 2017) and M1/M2 phenotype of microglia (Ma et al., 2022). The stimulation of apoptosis in macrophages by ErbB4 was shown to be cleavage-dependent, indicating that E4ICD plays an important role in these immune cells during inflammation (Schumacher et al., 2017). To assess the role of E4ICD signaling on immune cell development we carried out a preliminary experiment

investigating whether the immune profile of splenocytes were altered in ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ mice compared to ErbB4$^{-/-}$ mice.

Flow cytometry was carried out on splenocytes to identify the proportion of CD45+ lymphocytes that were neutrophils, monocytes, B-cells or T-cells. While statistics are not very telling with just three biological replicates, there appeared to be a difference in ErbB4$^{-/-}$ mice that was phenocopied in ErbB4-JMa$^{-/-}$ mice. I observed a trend towards in increased proportion of neutrophils and monocytes in ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ mice (Fig. 4.4). There was also a trend towards lowered B-cells and T-cells in ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ mice (Fig. 4.4). While few of these differences were statistically significant, each immune cell type that was altered in ErbB4$^{-/-}$ and trended the same direction in ErbB4-JMa$^{-/-}$ mice. Combined with previous observations of an altered immune profile in ErbB4$^{-/-}$ mice, this preliminary experiment indicates that the regulation of immune cell development by ErbB4 may be dependent on ErbB4-JMa non-canonical signaling.

4.4: Discussion

Using the ErbB4-JMa$^{-/-}$ mice, I demonstrated that normal development of heart, mammary gland, neural ganglia, and interneurons are independent of non-canonical ErbB4-JMa signaling. These phenotypic results do not agree with in vitro studies that suggested that cardiac (Iwamoto et al., 2017) and mammary gland (Muraoka-Cook et al., 2008) development rely on non-canonical ErbB4 signaling. Along with the mechanistic results from ErbB4$^{TUC/TUC}$ mice, these findings further emphasize that in vitro observations of non-canonical ErbB4 can be unreliable, and that studies in the intact organism are required to draw conclusions about the non-canonical RTK signaling

system. These findings illustrate how critical it is to study the non-canonical RTK signaling in vivo and represent a step forward in our understanding of this system.

While repeating this experiment with a larger cohort is required, the results of splenocyte FACS analysis indicates that ErbB4-JMa non-canonical signaling plays an important role in immune cell development. The E4ICD has previously been shown to play a functional role in macrophage survival (Schumacher et al., 2017). Here I have provided further evidence that E4ICD signaling is important in lymphocyte biology. As the immune system plays a role many of the known functions of ErbB4, such as synaptic formation, dendrite morphology, injury repair, and oncogenesis it will be is crucial to be aware that the role of ErbB4-JMa may be playing a role in phenotypes studied with the ErbB4-JMa$^{-/-}$ mice.

In Table 4.1 the roles of ErbB4 that have been shown to be dependent on ErbB4-JMa in these studies are outlined, as well as a selection of other ErbB4-related phenotypes that are yet to be tested. Phenotypic analysis ErbB4-JMa$^{-/-}$ mice can be used to reveal whether these phenotypes are regulated by ErbB4 non-canonical signaling.

4.5 Figures

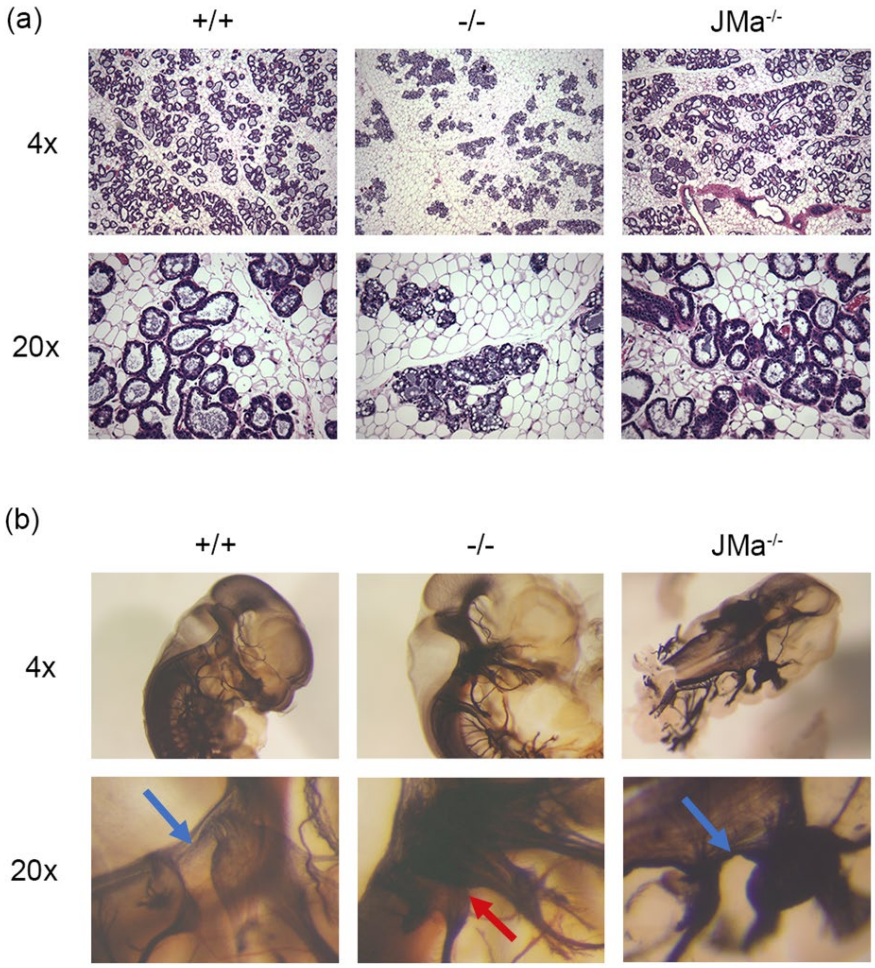

Figure 4.1: ErbB4-JMa is not required for regular development of mammary gland development and trigeminal ganglion development. (a) Hematoxylin and eosin staining of mammary gland from mice one day after giving birth show that ErbB4$^{-/-}$ mice form fewer ductal structures with vacuolar inclusions in the duct walls compared to ErbB4$^{+/+}$ mice. ErbB4-JMa$^{-/-}$ mice form regular mammary ducts and wean regular litters. (b) Neurofilament immunohistochemistry of E11.5 embryos shows an ectopic axonal bridge formation between the trigeminal and facial ganglion in ErbB4$^{-/-}$ mice (red arrow), but normal ganglionic development in ErbB4$^{+/+}$, and ErbB4-JMa$^{-/-}$ mice (blue arrows).

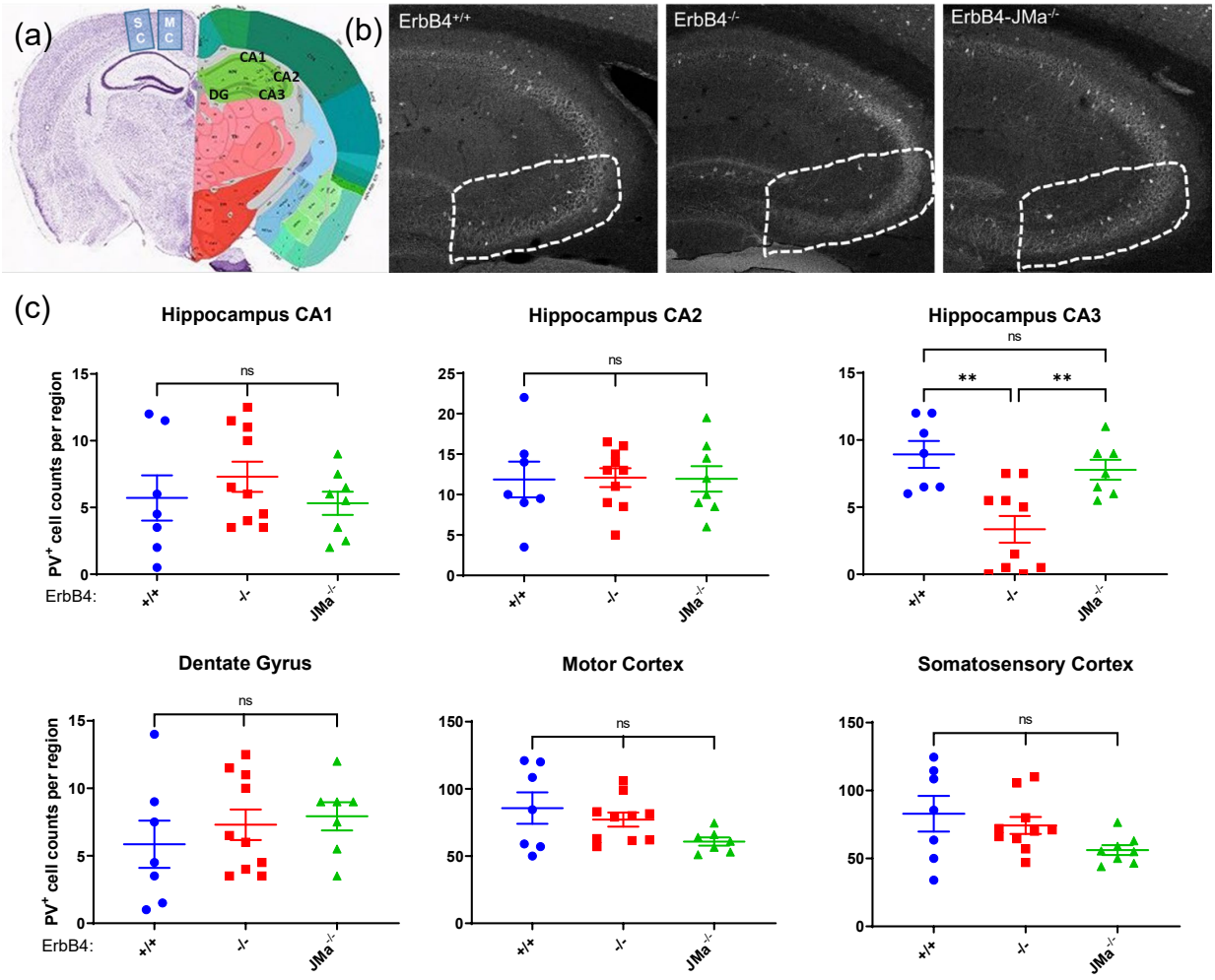

Figure 4.2: Interneuron development and migration is not regulated by ErbB4-JMa[9]. (a) Allen Brain Atlas at -2.0 mm posterior of bregma with representation of the brain areas that was assessed for PV+ interneuron development. Motor cortex = MC; somatosensory cortex = SC; dentate gyrus = DG; hippocampus CA1, CA2 and CA3. (b) Representative images of PV+ interneuron immunofluorescence in the hippocampus. Outlines regions represent CA3 region assessed for cell counts. (c) Quantification of each brain region investigated. The only significant difference found was a decrease in the number of PV+ interneurons in the CA3 of ErbB4$^{-/-}$ mice, compared to both ErbB4$^{+/+}$ and ErbB4-JMa$^{-/-}$ mice; n = 7-8 per group.

[9] Sectioning, parvalbumin immunofluorescence and cell counts carried out by Brenna L. MacLeod and Nada Jaradat

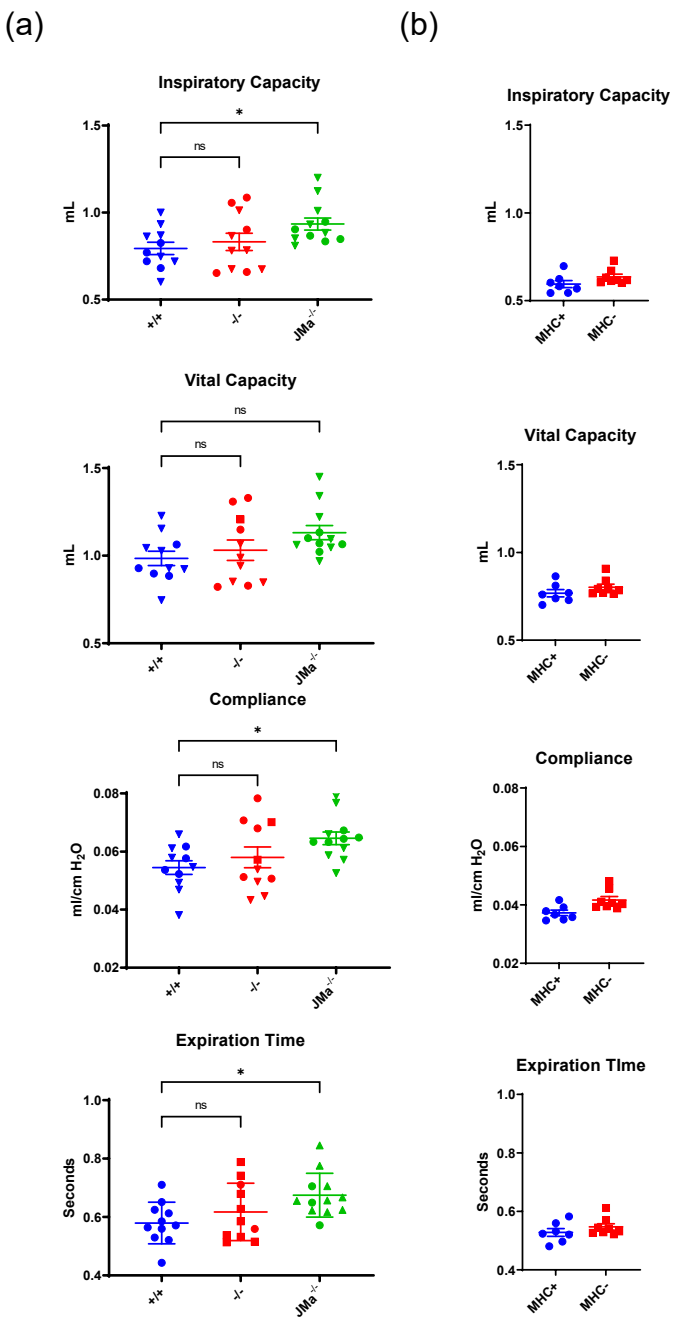

Figure 4.3: Pulmonary function testing of ErbB4 mutant mice was not revealing[10]**.** (a) PFT analysis of shown parameters IC, VC, chord compliance and expiration time of ErbB4$^{+/+}$, ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ mice; n = 10-12 per group. (b) Analysis of the effect of αMHC-ErbB4 expression on ErbB4$^{+/+}$ mice PFT shows a trend for increases in the MHC- group; n = 7 per group.

[10] Pulmonary function test carried out by Nikolas Lukacs

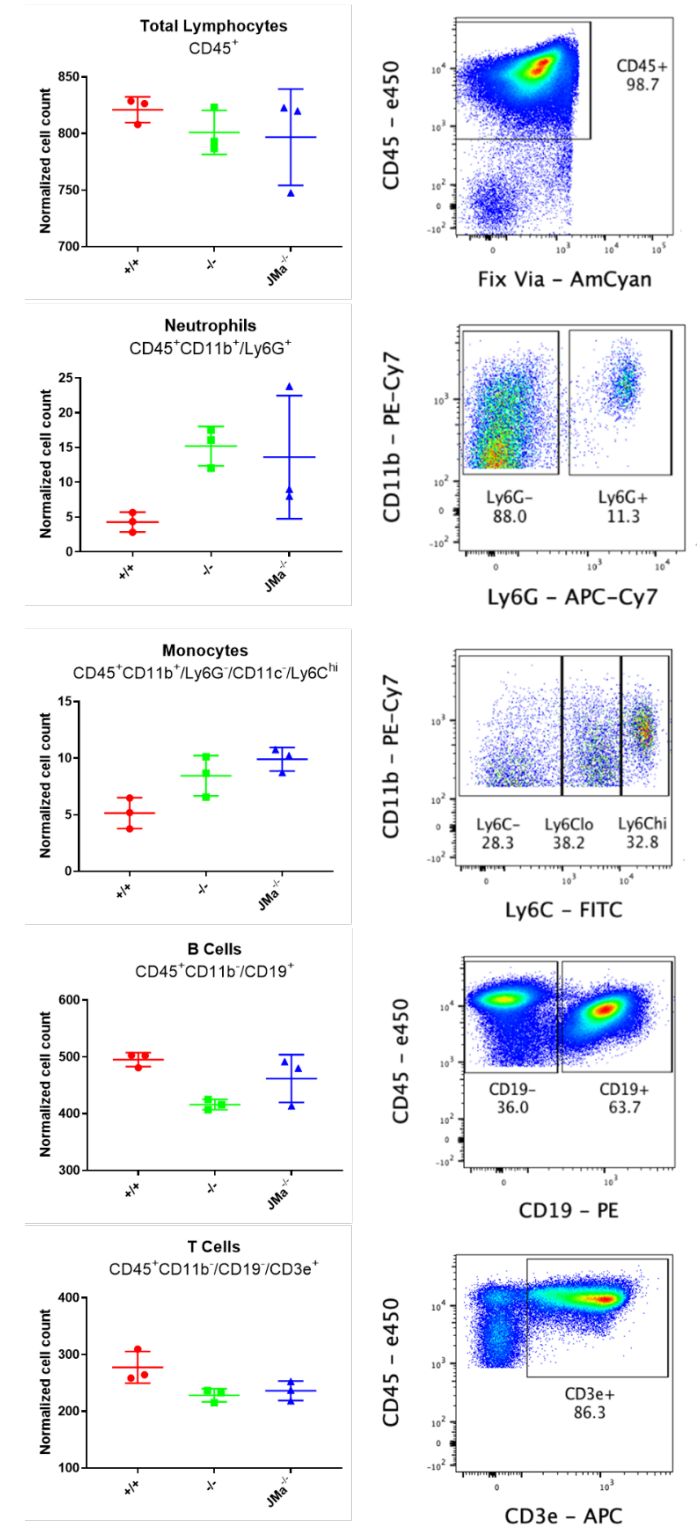
(a)
(b)
Total Lymphocytes
CD45+
Normalized cell count
850
800
750
700
+/+
-/-
JMa-/-
CD45 – e450
CD45+
98.7
Fix Via – AmCyan
Neutrophils
CD45+CD11b+/Ly6G+
Normalized cell count
25
20
15
10
5
0
CD11b – PE-Cy7
Ly6G–
88.0
Ly6G+
11.3
Ly6G – APC-Cy7
Monocytes
CD45+CD11b+/Ly6G-/CD11c-/Ly6Chi
Normalized cell count
15
10
5
0
CD11b – PE-Cy7
Ly6C–
28.3
Ly6Clo
38.2
Ly6Chi
32.8
Ly6C – FITC
B Cells
CD45+CD11b-/CD19+
Normalized cell count
600
500
400
300
CD45 – e450
CD19–
36.0
CD19+
63.7
CD19 – PE
T Cells
CD45+CD11b-/CD19-/CD3e+
Normalized cell count
400
300
200
100
CD45 – e450
CD3e+
86.3
CD3e – APC

Figure 4.4: FACS analysis of the role of ErbB4-JMa on immune cell development[11]. Flow cytometry carried out on splenocytes from ErbB4$^{+/+}$, ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ mice. (a) Analysis of normalized cell counts per 1000 cells of immune cell types shows trends for differences in both ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ mice neutrophils, monocytes, B-cells and T-Cells; n = 3 per group. (b) Examples of gating strategy for each cell type from ErbB4$^{+/+}$ mice.

[11] Flow cytometry carried out by Lucas Huffman

4.6 Tables

Role of ErbB4 signaling	E4ICD-dependent in vitro	JMa-dependent in vivo
Repression of astrogenesis	Yes	Yes
Cortical gene regulation	Unknown	Both
Cardiac development	Yes	No
Mammary gland development	Yes	No
Neural ganglia development	Unknown	No
PV interneuron development	Unknown	No
Pulmonary development	Yes	Unclear
Immune cell development	Yes	Yes
Cerebellar development	Unknown	Not tested
Oncogenesis	Yes	Not tested
Injury repair	Unknown	Not tested
Neurophysiology LTP/IPSCs	Unclear	Not tested
Synapse formation	Unknown	Not tested
Dendrite morphology	Unknown	Not tested
“Schizophrenia-like” behavior	Unclear	Not tested

Table 4.1: Delineation of ErbB4 phenotypes and evidence of regulation by E4ICD in vitro and in vivo. The various roles of ErbB4 signaling as identified by phenotypes in mice or cells are shown. Whether any of these roles have been shown to be dependent on ErbB4-JMa non-canonical signaling via E4ICD by in vitro experiments is shown in the next column. Whether these are dependent on ErbB4-JMa in vivo by experiments using the ErbB4-JMa$^{-/-}$ mice is shown in the last column.

CHAPTER 5: Research Synthesis

5.1 Summary of Findings

Cleavage-mediated non-canonical signaling by RTKs is an expanding field with significance in numerous different biological and pathological processes. A decade ago, there were only a handful of RTKs that were known be capable of signaling by this ICD-dependent mechanism, and fewer with known roles. The field has since expanded, and it is now clear that the majority of RTKs can use this non-canonical signaling mechanism, and as research advances, more roles of RTK signaling via ICDs are being revealed. However, most of the knowledge in this area is based upon what is known about non-canonical ErbB4 signaling, the first RTK identified to be capable of ICD signaling. Furthermore, the investigating signaling by ICDs has primarily been carried out in vitro, or in vivo with ectopic re-expression of ICDs. The next step forward in this field was to study non-canonical signaling by RTKs in the intact organism by creating mouse models in which formation of the ICD is abolished. A recent review highlighted the importance of this strategy as “most of our understanding about the biological relevance of gamma-secretase-mediated processing of RTKs is based on in vitro observations, there is also need for more thorough in vivo validation of the findings” (Merilahti & Elenius, 2019). In my thesis work, I created an isoform-specific ErbB4-JMa$^{-/-}$ mouse line to address this gap in knowledge and investigate the roles of non-canonical RTK signaling an intact organism for the first time.

In Chapter 1, I described creating the ErbB4-JMa$^{-/-}$ mouse using CRISPR/Cas9 gene editing and validating that E4ICD formation was abolished. I identified TUC mutations in the TACE cleavage site, which in vitro prevent E4ICD formation from ErbB4-JMa, while maintaining canonical phosphorylation-mediated ErbB4 signaling. Surprisingly, that formation of the E4ICD was intact in ErbB4$^{TUC/TUC}$ mice, that the TUC mutations were not penetrant in vivo. While this result was a set-back in the development of a mutant mouse cleavage-resistant ErbB4-JMa, it showed that mutations capable of preventing E4ICD formation in vitro are not necessarily capable of preventing cleavage in vivo, highlighting the importance of studying RTK systems and mechanisms in vivo. In the future, more thorough analysis of the alternative cleavage sites and mechanisms will be essential when developing RTK cleavage-resistant mutant mice. As the strategy to target the TACE cleavage site in ErbB4 exon 16a was specific to the ErbB4-JMa isoform, CRISPR/Cas9 gene editing also created the isoform-specific KO mouse ErbB4-JMa$^{-/-}$ by introducing a single base deletion via NHEJ repair. I validated that this mouse lacked expression of ErbB4-JMa due to NMD of its mRNA, and abrogated non-canonical ErbB4 signaling by abolishing expression of the isoform capable of creating E4ICD.

In Chapter 2, I used the ErbB4-JMa$^{-/-}$ mouse to show for the first time in vivo that non-canonical ErbB4 signaling via direct transcriptional regulation by E4ICD. The results provided in vivo corroboration of previous investigations by the Corfas lab showing that the E4ICD represses astrocytic gene expression by binding directly to promoter sites within the nucleus. I showed that the regulation of GFAP expression by NRG1 in NPCs was dependent on ErbB4-JMa non-canonical signaling and that

precocious astrogenesis observed in the developing mouse brain of ErbB4$^{-/-}$ is also seen in ErbB4-JMa$^{-/-}$ mice. This confirmed that the ErbB4-JMa$^{-/-}$ mice are a reliable tool to delineate whether ErbB4-dependent phenotypes are dependent on non-canonical ErbB4-JMa signaling or not. To further investigate the role of noncanonical ErbB4-JMa signaling in cortical development, I used RNA-seq data of E15.5 mouse cortex to identify genes that are regulated by ErbB4. From the 20 newly identified genes significantly different in ErbB4$^{-/-}$ mice, I used qPCR to investigate whether of four genes of interest were also changed in ErbB4-JMa$^{-/-}$ mice. Two genes, DBi and CRYM, were found to be changed ErbB4-JMa$^{-/-}$ mice, indicating that they are regulated by non-canonical ErbB4 signaling. Two other genes, WDFY and PRSS12 were not changed in ErbB4-JMa$^{-/-}$ mice, showing that they are regulated independent of ErbB4-JMa signaling, and that ErbB4-JMb is sufficient for their normal expression. These results displayed how readily the ErbB4-JMa$^{-/-}$ mice can be used to delineate whether developmental changes seen in ErbB4$^{-/-}$ mice are dependent on non-canonical signaling by ErbB4-JMa

Having confirmed that ErbB4-JMa$^{-/-}$ mice are a reliable tool to study the role of E4ICD in the intact organism, in Chapter 3 I carried out multiple phenotypic assessments to delineate whether ErbB4-JMa is required for the regulation of phenotypes known to be caused by the loss of ErbB4. I found that development of the heart, mammary gland, neural ganglia, hippocampal interneurons are not dependent on ErbB4-JMa signaling as they all develop normally in the ErbB4-JMa$^{-/-}$ mice. The regulation of *WDFY* and *PRSS12* during critical development were also shown not to be dependent on ErbB4-JMa in the Chapter 2. Technical difficulties prevented me from

being able to fully determine whether ErbB4-JMa is involved in lung development in vivo, as the PFT experiments did not show a difference seen in ErbB4$^{-/-}$ mice lung function. A preliminary experiment indicated that changes seen in immune cell development are dependent on ErbB4-JMa. While more experiments are required to confirm this, the implications of delineating whether ErbB4-JMa plays a role in the immune system are broad.

Of the roles of ErbB4 that have previously been identified, I was able to corroborate that ErbB4-JMa regulates the timing of astrogenesis and likely immune cells development, confirming previous in vitro assessments. Importantly, phenotypic assessment using the ErbB4-JMa$^{-/-}$ mice contradicted previous in vitro studies that have suggested that E4ICD signaling plays an important role in heart and mammary gland development. These findings show that while E4ICD can have these functions in vitro, it is not required in vivo. This reinforces the importance of in vivo models, such as the ErbB4-JMa$^{-/-}$ mice, to study roles of RTK signaling mechanisms.

5.2 Caveats of the research

In the ErbB4-JMa$^{-/-}$ mice, all ErbB4-JMa signaling is abolished, including canonical signaling by the full-length receptor and forward signaling by the ECD cleavage product. As with any mutant mouse there will always be potential unintended consequences, and the removal canonical ErbB4-JMa signaling is the most apparent potential pitfall with the ErbB4-JMa$^{-/-}$ mouse. The initial strategy was the use a combination of results from the ErbB4$^{TUC/TUC}$ and ErbB4-JMa$^{-/-}$ mice to circumnavigate the weaknesses in both lines. For example, the loss of canonical ErbB4 signaling is a weakness of the ErbB4-JMa$^{-/-}$ mice, it would have been intact in the ErbB4$^{TUC/TUC}$ mice.

On the other hand, the ErbB4$^{TUC/TUC}$ mice may have displayed increased canonical signaling due to the lack of processing of ErbB4-JMa, a weakness that is not present in the ErbB4-JMa$^{-/-}$ mice. As such these two mice would have compensated for the main weaknesses of one another when studied as a pair. Unfortunately, Erbb4-JMa cleavage was not abolished in the ErbB4$^{TUC/TUC}$ mice, and so I could not use this line in these studies.

I therefore used the ErbB4$^{+/-}$ mice as a control to observe whether loss of half of ErbB4 (both JMa and JMb) resulted in the same changes seen in ErbB4-JMa$^{-/-}$ mice, as ErbB4$^{+/-}$ mice have approximately the same levels of full length ErbB4 expression as ErbB4-JMa$^{-/-}$ mice. In certain scenarios, particularly in regulation of GFAP expression by ErbB4 in NPCS, there was no effect of losing half of total ErbB4 in ErbB4$^{+/-}$, which allowed us to confirm that the regulation of GFAP is due to the loss of ErbB4-JMa specifically and not due to any loss of canonical ErbB4 signaling. However, ErbB4$^{+/-}$ mice sometimes phenocopy ErbB4$^{-/-}$ mice, as seen with regulation of gene expression in the E15.5 cortex. In this case, the ErbB4$^{+/-}$ mice the result could be due to loss of full length ErbB4, or that loss of half of ErbB4-JMa is sufficient to induce the same changes. Fortunately, there were genes (WDFY1 and PRSS12) that were not changed in ErbB4-JMa$^{-/-}$ mice, indicating that the loss of full length ErbB4 in these mice does not have the same effect as loss of half of total ErbB4 in ErbB4$^{+/-}$ mice. Thus, the most reasonable conclusion isthat the changes in gene expression observed in ErbB4-JMa$^{-/-}$ mice (in CRYM and DBi) are specifically due to loss of non-canonical ErbB4-JMa signaling.

As the ErbB4-JMa$^{-/-}$ mouse is a constitutive germline mutant mouse (like the ErbB4$^{-/-}$ mice), studies using this mouse model beyond developmental studies are

confounded by possible developmental roles of ErbB4-JMa. For example, ErbB4 is important in the development and migration of interneurons as well as the physiological function of interneurons in adulthood. While the ErbB4-JMa$^{-/-}$ mouse could be used to study development of interneurons, it would be very difficult to separate whether changes in interneuron signaling physiology were due to the function of ErbB4-JMa in development (fewer or altered interneurons) or in the physiology (regulation of synaptic transmission on interneurons). For this reason, my phenotypic studies of the ErbB4-JMa$^{-/-}$ mice were limited to the role of ErbB4 in development.

Furthermore, some of the main physiological and/or neurodegenerative roles of ErbB4 are difficult to study in the adult ErbB4-JMa$^{-/-}$ mice without conflation of developmental and adult roles of ErbB4. NRG1-ErbB4 signaling is well known to be linked to schizophrenia pathogenesis and characterized in ErbB4$^{-/-}$ mice by “schizophrenia-like” behavior. While schizophrenia is thought of as a neuro-developmental disorder, cell specific ErbB4 deletion has suggested that there are specific regions in which ErbB4 is required for normal brain function in adulthood. One group addressed this issue to identify whether ErbB4 expression was important in development or adulthood. They developed mice with ErbB4 in which ErbB4 expression could be either induced or removed by tamoxifen administration in adulthood and were showed that re-expression of ErbB4 was sufficient to alleviate “schizophrenia-like” behavior (Wang et al., 2018). As such, it would be difficult to dissect the early developmental from physiological roles of ErbB4-JMa when studying the role of ErbB4-JMa using the ErbB4-JMa$^{-/-}$ mice in behavioral endophenotypes.

5.3 Future Directions

The findings outlined above have shown that I have been able to develop an isoform-specific ErbB4-JMa$^{-/-}$ mouse to study the role of non-canonical E4ICD signaling in the intact organism. I have validated that this mouse is reliable for the purpose that it was developed for and delineated certain ErbB4-related phenotypes that are dependent on non-canonical ErbB4 or not. The development of this novel tool opens many new avenues of research and provides a template for similar studies of other RTKs.

Whilst the ErbB4-JMa$^{-/-}$ mouse has been sufficient to determine whether ErbB4-JMa is required for certain phenotypes, there are caveats to these investigations as listed above. It would be beneficial to create two other mutant mice by CRISPR/Cas9 gene editing that would make this study more complete. First, while the development of the ErbB4$^{TUC/TUC}$ mouse failed to block E4ICD formation, a mouse expressing cleavage-resistant ErbB4-JMa would help solidify these studies. To do this one could further identify potential EJD cleavage sites in ErbB4-JMa for targeted mutation, although the outcome may be the same as the ErbB4$^{TUC/TUC}$ mouse. However, it would also be possible to create a mouse by CRISPR/Cas9 gene editing with the insertion of an oligonucleotide sequence by HDR that mimicked the majority of exon 16b. As this exon is known to be uncleavable, it would create an uncleavable ErbB4-JMa isoform that was very similar to ErbB4-JMb and would eliminate the chance of alternative cleavage sites in the EJD of ErbB4-JMa allowing E4ICD formation.

Another mouse that would be useful in these studies would be an ErbB4-JMb$^{-/-}$. Adding this mouse to these studies would allow us to allocate phenotypes as ErbB4-JMb independent. There may be increased expression of ErbB4-JMa in a mouse

lacking ErbB4-JMb signaling as ErbB4-JMa could compensate for lost canonical ErbB4 signaling. While this is an important caveat to keep in mind, one could assess whether this was the case by ErbB4 qWB and isoform-specific qPCR to assess any changes in expression.

Aside from creating new mice to include in these studies there are still a barrage of ErbB4-related phenotypes that can be assessed using the ErbB4-JMa$^{-/-}$ mice. A more thorough assessment of the role of ErbB4-JMa in lung development could be undertaken. There is a body of research indicating that E4ICD regulates surfactant production and lung development, and the ErbB4-JMa$^{-/-}$ mouse can be used to corroborate whether it is important in vivo. This is especially pertinent as there is also a body of research suggesting that E4ICD is important in the expression of lactation genes during mammary gland development, however this was not shown to be required for normal mammary gland development in vivo with the ErbB4-JMa$^{-/-}$ mice.

It would be prudent to follow up the preliminary experiments on immune cell development. The results from a small cohort of three mice per group were sufficient to indicate that ErbB4-JMa non-canonical signaling is involved in changes observed in ErbB4$^{-/-}$ mice. The role of ErbB4 in immune cells has been implicated in inflammation response, macrophage survival, cancer development, injury response in multiple organs, pathogenesis of MS, AD and ALS. Furthermore, microglia function is well known to play a role in synaptic development and function and well as dendritic branching, all of which are known to be regulated by ErbB4. Thus, if the role of ErbB4-JMa in immune development were to be confirmed by further experiments, there could

be a multitude of implications for how these systems intersect and are regulated by non-canonical RTK signaling.

As well as understanding how ErbB4 regulates immune development, the ErbB4-JMa$^{-/-}$ mouse could be used to study whether ErbB4-JMa and non-canonical signaling is importance in oncogenesis. As outlined in Chapter 1, ErbB4-JMa is upregulated in certain types of brain cancer and its activation results in poor prognosis. To study the intrinsic roles of ErbB4-JMa in brain cancer, oncogenic mutations could be introduced into the developing brains of these mice, such that the tumors lack ErbB4-JMa to investigate how this effects tumor development and survival. Cancer cells with or without ErbB4-JMa expression could also be cultured and implanted into either ErbB4$^{+/+}$ or ErbB4-JMa$^{-/-}$ mice to separate the intrinsic roles of ErbB4-JMa on cancer development from environmental factors, such as immune response. This would also be a reasonable paradigm to assess whether ErbB4-JMa plays a role in immune response in the tumor microenvironment.

To further understand the role of ErbB4-JMa signaling in cortical development, I would delineate whether the rest of genes identified by RNA-seq to be regulated by ErbB4 are JMa-dependent or JMa-independent. Assessment of the rest of the genes identified by qPCR as was done for WDFY1, PRSS12, CRYM and DBi would be the most straightforward way to do this. However, an alternative and possibly more thorough strategy could be to rerun the RNA-seq on both ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ mice compared to ErbB4$^{+/+}$ mice to observe which changes observed in the ErbB4$^{-/-}$ mice are also found in ErbB4-JMa$^{-/-}$ mice. It may provide a more specific assessment of the role of ErbB4-JMa as there could be changes in gene expression that are specific to

loss of ErbB4-JMa found in the ErbB4-JMa$^{-/-}$ mice. Furthermore, the use of single cell RNA-seq in the developing brain of ErbB4-JMa$^{-/-}$ mice could allow us to observe gene expression changes of specific cell populations.

It would also be interesting to use NPC cultures to investigate how ErbB4-JMa regulated the expression of the DBi, CRYM and any others that might be found by further experiments. As DBi has a known and characterized role in NSCs, that is to block GABAergic signaling and maintain proliferation of NPCs, rather than allowing differentiation. There is an obvious link to the known role of ErbB4 in NPCs, which is also to repress differentiation. Using NPCs, I would interrogate the specific ways in which might ErbB4-JMa and DBi interact to maintain the proliferation.

ErbB4-JMa was already known to regulate astrogenesis, however an intriguing outcome found in ErbB4-JMa$^{-/-}$ mice was the increased expression of CRYM during cortical development. CRYM was recently identified as the most prominent marker of striatal astrocyte heterogeneity compared to hippocampal GFAP expression (Chai et al., 2017). This indicates that loss of ErbB4-JMa may not only result in precocious astrogenesis, but also altered astrocyte type development. While still in its infancy, the role of astrocyte heterogeneity in brain development, function and disease is a burgeoning field (Matias et al., 2019). It would be relatively straightforward to assess whether these changes in CRYM expression are detectable in the adult cortex, hippocampus, and striatum by either qPCR of brain regions, or by GFAP and μ-crystallin immunofluorescence in the adult brain.

The cerebellum is a brain region where astrocyte heterogeneity has been well described. There are three subtypes of astrocyte, the Bergmann glia (BG) and granular

layer astrocytes and fibrous astrocytes in the cerebellar white matter. ErbB4 has been shown to regulate migration of cerebellar granule cells via Bergmann glia during cerebellar development (Rio et al., 1997) and cerebellar anatomy is abnormal in adult ErbB4$^{-/-}$ mice (Aldaregia et al., 2020; Tidcombe et al., 2003). It would first be worthwhile to investigate whether cerebellar anatomy is also abnormal in ErbB4-JMa$^{-/-}$ mice, but furthermore this could be a brain region with known astrocyte profiles in which I would investigate whether loss of ErbB4-JMa affects astrocyte development and heterogeneity.

As E4ICD has been shown to regulate the transition to astrogenesis by repressing astrocyte gene expression, it would stand to reason that it also plays a role in the development of late-born neurons. While preliminary experiments by myself and others have not identified any apparent differences in brain size or cortical layering, these have been relatively cursory studies. A thorough investigation into the effect of losing ErbB4 signaling on cortical neuronal development might be revealing.

The interactions of ErbB4 non-canonical signaling with other mechanisms that regulate astrogenesis could be explored more thoroughly. For example, the removal of FGF from NPCs causes an increase in ErbB4 expression (data not shown). Thus, it would be interesting to study how FGF and Erbb4 crosstalk during this transition. It would appear at initial assessment that decreased FGF signaling broadly initiates the transition to differentiation and gliogenesis while E4ICD more finely regulates this process by repressing the transition until the timing is appropriate (Miller & Gauthier, 2007). I also find it very intriguing that while ErbB4 represses the transition to gliogenesis, EGFR signaling is known to promote both astrogenesis (Viti et al.,

2003)and gliogenesis of OPCs (Yang et al., 2017) in late cortical development. In other systems ErbB4 and EGFR have been found to have opposite roles and cross-regulate during mesenchymal cell proliferation. It is possible that early ErbB4 non-canonical signaling also represses the expression of EGFR before the transition to astrogenesis. The loss of ErbB4 signaling could allow expression of EGFR and mechanistically induce gliogenesis. Alternatively, increasing expression of EGFR via other mechanisms could downregulate the expression of ErbB4, which would in turn remove its repressive effect on astrocytic genes and mechanistically mediate the transition to astrogenesis. A series of experiments using NPCs could interrogate which, if any of these scenarios is responsible for the regulation of gliogenesis.

As mentioned previously, the Mei lab developed two mouse lines in which ErbB4 expression could be either turned on or turned off in adulthood to investigate the effect of losing ErbB4 specifically during development or post-development (Wang et al., 2018). As the ErbB4-JMa$^{-/-}$ mice are germline constitutive isoform-specific KO mice, it is difficult to study phenotypes that are altered by developmental loss of ErbB4 in adulthood without conflation of pre- and post-development functions. Fortunately, this study has delineated a ErbB4-related phenotypes that are not affected by the loss of ErbB4 in development. Loss of ErbB4 only in adulthood was found to be sufficient to cause behavioral deficits and abnormal hippocampal IPSCs, whereas inhibitory and excitatory synapses onto interneurons were shown to be dependent on ErbB4 expression during development. Therefore, the ErbB4-JMa$^{-/-}$ mice would be a good tool to study behavior and IPSCs in adulthood, whereas synaptic development are less appropriate phenotypes to investigate using these mice.

To assess whether ErbB4-JMa non-canonical signaling is involved in the behavioral endophenotypes observed in ErbB4$^{-/-}$ mice would be relatively straightforward. Open field, elevated plus maze and pre-pulse inhibition tests of ErbB4-JMa$^{-/-}$ mice could be used to investigate whether ErbB4-JMa plays a role in the development of “schizophrenia-like” behavior. If ErbB4-JMa were to be found to play a role in these endophenotypes, it would then be important to dissect what roles of non-canonical ErbB4 signaling underlies this outcome, which would be significantly more difficult endeavor, as mentioned above.

Our lab has also been working on unpublished data investigating the interaction of ErbB4 and PARP1 and its role in brain development. It has been established that E4ICD and PARP1 are binding partners. PARP1 is well known to regulate DNA-damage repair and have been able to show that ErbB4-JMa signaling regulates genomic stability in the developing brain via E4ICD-PARP1 interactions within the nucleus. We have further shown that PARP1 is involved in the regulation of astrogenesis and that loss of PARP1 leads to similar changes in the regulation of GFAP expression as observed in ErbB4$^{-/-}$ and ErbB4-JMa$^{-/-}$ mice. The ErbB4-JMa$^{-/-}$ mice will be a critical tool in the exploration of the regulation of cortical development by the E4ICD-PARP1 interaction to make these studies more complete.

5.4 Conclusions and Final Thoughts

The work presented in this dissertation has shown the creation of a novel isoform-specific ErbB4-JMa$^{-/-}$ mouse and demonstrated the utility of this mouse to investigate the roles of non-canonical ErbB4 signaling in vivo. I have delineated certain developmental roles of ErbB4 as dependent on or independent of ErbB4-JMa non-

canonical signaling, reviewed in Table 4.1. Further studies will be needed to fully understand the functions and interactions of E4ICD signaling in various cellular environments. The development of the ErbB4-JMa$^{-/-}$ mouse provides a vital tool for better understanding of the roles of the E4ICD and will enhance studies of ErbB4 signaling mechanisms in the future, as outlined in the previous section. This work represents a significant development in the field of RTK signaling mechanisms and shows how vital it is to study these systems in mouse models, rather than relying solely on in vitro data to draw conclusions on the roles of non-canonical RTK signaling. The findings in this dissertation make it clear that mouse models abolishing cleavage-mediated non-canonical RTK signaling pathways are required to fully understand these systems. The creation of mutant mice like the ErbB4-JMa$^{-/-}$ mouse will be vital to study the roles non-canonical RTK signaling mechanisms in the future.

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