Hyperactive alpha2-chimaerin reveals the complexity of axon guidance signaling pathways in motor neuron development

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Hyperactive alpha2-chimaerin reveals the complexity of axon guidance signaling pathways in motor neuron development

A dissertation presented

by

Alicia Anne Nugent

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

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Hyperactive alpha2-chimaerin reveals the complexity of axon guidance signaling pathways in motor neuron development

Abstract

α2-chimaerin has striking importance for proper neural circuit formation. Individuals with gain-of-function mutations in α2-chimaerin have Duane retraction syndrome, a common neurogenic eye movement disorder, and α2-chimaerin knockout mice have a rabbit-like hopping gait, resulting from aberrant motor neuron wiring. While molecular mechanisms underlying altered gait circuitry in the loss-of-function mouse have been characterized, mechanisms underlying the etiology of DRS caused by mutant $CHN1$ remain unclear.

Here, we report the first $Chn1$ gain-of-function DRS mouse model ($Chn1^\text{KI}$), which harbors a knock-in point mutation identified in human patients. $Chn1^{\text{WT/KI}}$ and $Chn1^{\text{KIkI}}$ embryonic mice exhibit abducens nerve stalling, aberrant trochlear nerve branching, and first cervical spinal segment (C1) misrouting, which result from axon guidance defects. The $Chn1^\text{KI}$ mouse recapitulates the human DRS phenotype, thus providing a novel mouse model of DRS. $Chn1^{\text{KO/KO}}$ embryos display abducens nerve wandering and defasciculation, establishing that human DRS-causing mutations are indeed gain-of-function.

We combine detailed 3D whole embryo imaging with novel in vitro approaches to demonstrate that hyperactivated α2-chimaerin acts downstream of ephrin forward and reverse signaling selectively in abducens neurons to modulate nerve development, as C1 neurons use only ephrin forward signaling, and trochlear neurons do not significantly use ephrin signaling during nerve guidance. In vivo, we find that selectively removing ephrin forward or reverse
signaling via EphA4 dramatically impacts the development of the abducens nerve, distinct from bidirectionally removing EphA4 signaling.

Further experimentation reveals that alpha2-chimaerin and EphA4 can signal through other pathways. We find that hyperactivated alpha2-chimaerin modulates BDNF, GDNF, NGF, and HGF signaling *in vitro*, thus suggesting its role as a broad regulator of axon guidance pathways. Our studies lend insight into the complexity of axon guidance during development and highlight mechanisms that cause the abducens nerve to be selectively vulnerable to alpha2-chimaerin misregulation in DRS.
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CHAPTER 1

Introduction
**Principles of axon guidance for neural circuit formation**

During brain formation, neurons develop long axonal processes that culminate in a motile growth cone terminus that navigates through the developing environment to form stereotypic connections with distant synaptic partners. Proper axon navigation via growth cone steering is crucial for establishing neural circuits that elicit normal function. As the field of neurobiology turns increasingly toward the investigation of neural circuit composition and function, understanding the mechanistic building blocks that guide initial circuit formation is essential for generating a complete picture of brain function in normal health and during disease.

Cues expressed in the developing environment elicit neuronally-driven responses to guide axons to the appropriate target. Traditional axon guidance molecules such as semaphorin, ephrin, Netrin, Slit, Wnt, and neurotrophins exhibit tissue-specific expression patterns that are sensed by cognate receptors on the navigating growth cone (Brose et al., 1999; Cheng et al., 1995; Drescher et al., 1995; Huber et al., 2005; Luo et al., 1993; Lyuksyutova et al., 2003; Serafini et al., 1996; Tessier-Lavigne and Goodman, 1996; Tuttle and O'Leary, 1998). Additional factors as diverse as proteoglycans, secreted transcription factors, cell adhesion molecules, and neurotransmitters also guide growing axons (Lowery and Van Vactor, 2009; Tessier-Lavigne and Goodman, 1996). Guidance cues can be soluble, bound within the extracellular matrix, or expressed on the cell surface (Lowery and Van Vactor, 2009).

The growth cone senses environmental cues with cell surface receptors that communicate via intracellular signaling pathways to modulate axonal growth dynamics. The coordinated signaling of receptors and subsequent communication to underlying cytoskeletal machinery determines whether a growth cone will collapse, turn, or grow in response to cues expressed in the extracellular environment. Scaffolded and cytoskeletal regulatory proteins, such as Rho-
family GTPases, transduce and localize receptor signaling events to drive downstream cytoskeletal regulation and direct the growing axon (Lowery and Van Vactor, 2009).

Ephrin signaling exemplifies mechanisms of axon guidance signaling regulation

The effect of ephrin signaling on axon guidance has been extensively studied for over two decades in many neurobiological contexts, including motor neuron targeting, retinal topographic map formation, and olfactory and vomeronasal connectivity (Bonanomi et al., 2012; Cheng et al., 1995; Drescher et al., 1995; Knoll et al., 2001; Marquardt et al., 2005). The Eph/ephrin family exemplifies the complex regulation of axon guidance signaling pathways; appropriately, ephrin derives its name from the Greek *ephoros*, meaning overseer or controller (Flanagan and Vanderhaeghen, 1998). Ephrin ‘ligands’ are subdivided into an ephrin-A class (ephrin-A1 – ephrin-A5) that are GPI-anchored and lack an intracellular signaling domain, and an ephrin-B class (ephrin-B1 – ephrin-B3), which are transmembrane proteins (Flanagan and Vanderhaeghen, 1998). Ephrins bind to Eph receptor tyrosine kinases, which are also divided into two classes. EphA receptors (EphA1-EphA10) bind only ephrin-A molecules, and EphB receptors (EphB1-EphB6) to ephrin-Bs, with some exceptions (Pasquale, 2005). EphA4 can bind ephrin-A and –B molecules (Flanagan and Vanderhaeghen, 1998; Gale et al., 1996a; Gale et al., 1996b), and at high concentrations ephrin-A5 can bind to EphB2 in addition to EphA (Himanen et al., 2004). Ephs and ephrins require cell-cell contact and extensive receptor clustering to elicit effects on axon guidance (Davis et al., 1994; Pasquale, 2005).

Ephrin-As and -Bs are canonically known to induce axon repulsion via binding to cognate Eph receptors expressed on the growth cone, referred to as ephrin forward signaling (Figure 1.1). In limb motor neuron projections, ephrin-A is expressed in a gradient throughout
the ventral limb bud and repels lateral motor column axons originating from the lateral division (LMCl) that express EphA, pushing this neuronal population to innervate the dorsal limb bud (Kania and Jessell, 2003). In contrast, ephrin-B is expressed in a gradient in the dorsal limb bud, repelling LMC medial division (LMCm) axons that express EphB, such that LMCm innervates the ventral limb bud (Luria et al., 2008).

Ephrin molecules can also be expressed on growing neurons and respond to Ephs expressed in the surrounding environment, termed ephrin reverse signaling (Figure 1.1). Ephrin reverse signaling can generate either growth cone collapse or increased axon outgrowth, depending on the composition of co-receptors that ephrins use for signaling (Bonanomi and Pfaff, 2010; Pasquale, 2005). In limb motor neurons, EphA is expressed in the dorsal limb bud and attracts ephrin-A-expressing LMCl neurons toward the dorsal limb through the Ret co-receptor (Bonanomi et al., 2012; Marquardt et al., 2005). However, in retinal ganglion cells, ephrin-A uses p75NTR as a co-receptor to repel EphA-expressing growth cones (Lim et al., 2008).

Ephrin bidirectional signaling creates additional layers of complexity. Eph and ephrin molecules expressed on the same growth cone are segregated to different lipid domains, which likely prevents lateral attenuation of signaling (Marquardt et al., 2005), however bidirectional signaling can occur within the same cell; as explained above, LMCl neurons use both ephrin forward and reverse signaling to target dorsal limb innervation. Additionally, ephrin pathways signal in concert with other neurotrophic pathways during guidance. In ephrin reverse signaling, EphA4 and GDNF ligands can bind ephrin-A/Ret/GFRα1 co-receptors to induce potentiated attraction in limb motor neurons (Bonanomi et al., 2012). Whether ephrin attractant- or repellent-mediated co-receptors can be expressed within the same neuron to compete in a dynamic tug-of-war during ephrin reverse signaling remains unknown.
Figure 1.1: Models of bidirectional ephrin signaling. In ephrin forward signaling (left), ephrin expressed on an opposing cell (blue) binds to Eph expressed on the neuronal growth cone (green) to induce repulsion. In ephrin reverse signaling (middle and right), Eph expressed on an opposing cell bind to ephrin expressed on the neuronal growth cone. Ephrin-A lacks a transmembrane domain and uses co-receptors for signaling. Coincident signaling between EphA4 and GDNF ligands act through ephrin-A/Ret/GFRα1 receptors to further potentiate attraction in limb motor neurons (middle) (Bonanomi et al., 2012). Ephrin-A reverse signaling via the co-receptor Ret without coincident GDNF signaling generates less attraction (Bonanomi et al., 2012), whereas signaling through p75NTR causes repulsion (Lim et al., 2008) (right).
When ephrin molecules bind Eph receptors in forward signaling, intracellular phosphorylation events recruit scaffolding and signaling proteins. For example, Rho-family GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) act downstream to alter actin polymerization and influence the underlying cytoskeletal structure of the navigating growth cone (Cowan et al., 2005; Iwasato et al., 2007). Additionally, signaling can recruit translation machinery to alter mRNA translation (Tcherkezian et al., 2010) or activate ubiquitin ligases, proteases, and phosphatases to terminate signaling (Gatto et al., 2014; Hattori et al., 2000; Janes et al., 2005; Pasquale, 2005; Sharfe et al., 2003; Shintani et al., 2006). Ephrin signaling highlights the complex and dynamic molecular processes that regulate axon guidance.
Human disorders of axon guidance

Altering either intrinsic neuron-specific properties or extrinsic factors within the developing environment causes guidance abnormalities that have a striking consequence on neural circuit formation. While miswiring at the cortical level has been theorized to be involved in autism and other neurological disorders (Bakos et al., 2015; Nie et al., 2010), it is difficult to prove axon guidance defects underlie these disorders, given the complexity of brain circuitry and lack of sensitive techniques to identify aberrant connectivity in the cortex (Appendix I; Nugent et al., 2012).

Congenital Cranial Dysinnervation Disorders (CCDDs) serve as sensitive indicators of circuit miswiring in humans and provide insight into human disorders of axon guidance (Engle, 2010). Typically, CCDDs result from absence or aberrant innervation of cranial motor neurons onto extraocular muscles (EOM) or facial muscles, resulting in various types of strabismus (misalignment of the eyes or loss of full ocular motility) or facial paralysis that are easily identifiable in the clinic. In certain cases, these disorders are inherited in families, which permits genetic characterization. The Engle lab has a large cohort of families with CCDDs, and has identified mutations in many genes that alter the development or guidance of cranial motor neurons to cause specific disorders.

Ocular movements are controlled by three cranial nerves (Figure 1.2). The oculomotor nerve, or CNIII, is comprised of several midbrain subnuclei. During development, the oculomotor nerve divides into a superior and inferior division to innervate a majority of the eye muscles (Cheng et al., 2014; Fritzsch et al., 1995). The superior division of the oculomotor nerve contralaterally innervates the superior rectus (SR), responsible for elevated gaze. A midline subnucleus also feeds into the superior division and provides bilateral innervation to the levator
palpebrae superioris (LPS), which elevates the eyelid. The inferior division of the oculomotor nerve ipsilaterally innervates the medial rectus (MR) for medial gaze toward the nose (adduction), the inferior rectus (IR) that permits downward gaze, and the inferior oblique (IO), which controls torsional elevation and abduction of the eye (Leigh and Zee, 2006).

The trochlear nerve, CNIV, originates in the caudal midbrain. It is distinct from other cranial nerves because it exits dorsally at the border of the midbrain and hindbrain, then crosses to innervate the contralateral superior oblique (SO) to control torsional inward and downward eye rotation (Leigh and Zee, 2006).

The abducens nerve, CNVI, exits from the caudal pons to innervate the ipsilateral lateral rectus (LR) muscle, which is responsible for lateral gaze toward the ear (abduction) (Leigh and Zee, 2006). In mice, the abducens nerve also innervates the retractor bulbi (RB) muscle, which is absent in primates. The RB retracts the orbit to permit closure of the nictitating membrane.

Internuclear neural circuits connect the oculomotor, trochlear, and abducens nuclei for conjugate eye movements. The medial longitudinal fasciculus (MLF) is a network of ipsilaterally- and contralaterally-projecting excitatory and inhibitory interneurons that bidirectionally connect the abducens nucleus to the oculomotor nucleus, specifically the subnucleus controlling the medial rectus, for conjugate horizontal gaze. It also connects subnuclei of the oculomotor nucleus and the trochlear nucleus to control vertical gaze (Leigh and Zee, 2006).
**Figure 1.2: Ocular cranial nerve innervation of the extraocular muscles.** The oculomotor nucleus is composed of five subnuclei that exit the hindbrain in one nerve (blue), which divides into a superior division that innervates the levator palpebrae superioris (LPS) and superior rectus (SR) muscles, and an inferior division that innervates the medial rectus (MR), inferior rectus (IR), and inferior oblique (IO). The trochlear nerve (red) exits dorsally from the brainstem to innervate the superior oblique (SO). The abducens nerve (green) exits ventrally to innervate the lateral rectus (LR). Adapted from Miyake et al. (2008).
Human mutations alter ocular motor system development to cause strabismus

The Engle lab has identified mutations in *HOXA1* (Tischfield et al., 2005), *HOXB1* (Webb et al., 2012), *PHOX2A* (Nakano et al., 2001), *SALL4* (Al-Baradie et al., 2002), *ROBO3* (Jen et al., 2004), *CHN1* (Miyake et al., 2008), *TUBB3* (Tischfield et al., 2010), *TUBB2B* (Cederquist et al., 2012), and *KIF21A* (Yamada et al., 2003) that disrupt normal eye movement, mostly through alterations in ocular motor circuitry. These genes can be categorized as transcription factors important for neuron specification or proteins involved in neuronal growth and guidance (Figure 1.3). *HOXA1*, *HOXB1*, *PHOX2A*, and *SALL4* are transcription factors that specify neuronal identity (Pattyn et al., 1997; Rossel and Capecchi, 1999; Sakaki-Yumoto et al., 2006). Mutations identified in individuals with CCDDs are loss-of-function, resulting in failure of neuronal specification and, therefore, absence of muscle target innervation (Al-Baradie et al., 2002; Nakano et al., 2001; Tischfield et al., 2005; Webb et al., 2012). Mutations in *ROBO3*, *CHN1*, *TUBB3*, *TUBB2B*, and *KIF21A* instead alter axon guidance receptors (*ROBO3*), cytoskeletal regulatory proteins (*CHN1* and perhaps *KIF21A*), transport molecules (*KIF21A*), or the cytoskeleton itself (*TUBB3/2B*) (Brose et al., 1999; Cederquist et al., 2012; Cheng et al., 2014; Jen et al., 2004; Miyake et al., 2008; Renier et al., 2010; Tischfield et al., 2005; van der Vaart et al., 2013). Most mutations, with the exception of *ROBO3*, are dominant missense mutations that cause an altered gain in protein function to modify the axon guidance of ocular motor neurons, and in some cases (*TUBB3/2B*, *ROBO3*) the development of additional neuronal populations (Cederquist et al., 2012; Cheng et al., 2014; Chew et al., 2013; Jen et al., 2004; Miyake et al., 2008; Tischfield et al., 2010; Yamada et al., 2003).
Figure 1.3: CCDD-causing human mutations alter neuronal specification and guidance. The Engle lab has identified mutations in familial CCDDs. Identified mutations in transcription factors HOXB1, HOXA1, PHOX2A, and SALL4 are likely loss-of-function, and cause failure of ocular motor neuron specification. Recessive loss-of-function mutations in ROBO3 result in axon guidance defects. Dominant, missense mutations in CHN1, TUBB3, TUBB2B, and KIF21A cause ocular motor neuron axon growth and guidance defects by altering intracellular protein transport or cytoskeletal dynamics.
In 2008, the Engle lab identified that CHN1 mutations cause Duane retraction syndrome (DRS), the most common CCDD (Miyake et al., 2008). DRS is an eye movement disorder that manifests with limited abduction and eye retraction upon adduction (Figure 1.4A). Postmortem studies of sporadic DRS (Hotchkiss et al., 1980; Miller et al., 1982) and MRI of CHN1-DRS (Demer et al., 2007) revealed absence or hypoplasia of the abducens nerve, which normally innervates the LR to abduct the eye. Moreover, the LR can be aberrantly innervated by a branch of the oculomotor nerve (CNIII), resulting in co-contraction of the LR and MR and globe retraction (Huber, 1974). Individuals with DRS resulting from CHN1 mutations also have a high incidence of vertical eye movement abnormalities, and MRI revealed SO muscle hypoplasia and thinning of the oculomotor nerve (Demer et al., 2007), suggesting that additional ocular cranial nerves are also affected (Miyake et al., 2008). One family harboring CHN1 mutations only has vertical gaze abnormalities without DRS (Miyake et al., 2011). Ten families were found to inherit dominant, missense mutations in CHN1 (Figure 1.4B).

CHN1 encodes α-chimaerin, which has two isoforms: α1- and α2-chimaerin (Hall et al., 1993). α1-chimaerin has a unique N-terminal sequence that permits ubiquitination and protein degradation (Marland et al., 2011), followed by C1 and RacGAP domains. The longer isoform, α2-chimaerin, contains a unique N-terminal sequence encoding a SH2 domain in addition to the shared C1 and RacGAP domains. Both α1- and α2-chimaerin are selectively expressed in neurons, suggesting CHN1 mutations cause neurogenic phenotypes that result in DRS (Hall et al., 2001). α1-chimaerin is expressed in the nervous system during early postnatal stages and into adulthood in mouse (Hall et al., 2001) and regulates dendritic morphology (Buttery et al., 2006; Van de Ven et al., 2005). In mice and humans, α2-chimaerin is primarily expressed in the
nervous system during embryonic development, including cranial nerve nuclei (Hall et al., 2001; Miyake et al., 2008), and is reported to be involved in axon guidance and neuronal migration (Beg et al., 2007; Brown et al., 2004; Clark et al., 2013; Ferrario et al., 2012; Ip et al., 2012; Iwasato et al., 2007; Kao et al., 2015; Wegmeyer et al., 2007).

All DRS CHN1 mutations identified to date result in amino acid substitutions common to both α1 and α2 isoforms or unique to the α2-chimaerin N-terminal domain (Chan et al., 2011; Miyake et al., 2008; Miyake et al.); no mutations have been mapped to the small region unique to α1-chimaerin (Figure 1.4C). Clinical presentation is similar for all mutations, and together with expression data, supports the hypothesis that abnormal function of α2-chimaerin underlies DRS (Demer et al., 2007; Miyake et al., 2008).

Mapping mutations onto a 2-dimensional structure modeled from a close homolog, β2-chimaerin, revealed that most missense mutations in humans fall at or near residues that keep chimaerin in an autoinhibited form, thus suggesting that they could destabilize autoinhibition (Figure 1.4D). Indeed, mutations were found to increase α2-chimaerin activity and abnormally lower levels of active Rac-GTP, and a subset do so by destabilizing α2-chimaerin autoinhibition, causing the protein to assume an inappropriate open conformation (Miyake et al., 2008). These findings support a gain in α2-chimaerin function as underlying DRS. Moreover, overexpression of mutant CHN1 in chick embryos or zebrafish caused oculomotor nerve stalling and aberrant branching (Clark et al., 2013; Miyake et al., 2008).
Figure 1.4: Dominant missense mutations in CHN1 cause Duane retraction syndrome (DRS).

(A) Individual with DRS and missense CHN1 mutation. Upon leftward gaze (bottom), the patient’s left eye cannot abduct beyond the midline and the right eyelid narrows, indicating eye retraction. Image adapted from Miyake et al. (2008).

(B) Ocular cranial nerve innervation of the extraocular muscles in individuals with DRS. Autopsy, MRI, and electromyography of DRS patients with or without CHN1 mutations have shown unilateral or bilateral absence of abducens nucleus, absent abducens nerve innervation of the LR, and misinnervation of the LR by an aberrant branch from the oculomotor nerve. Specifically with CHN1 mutations, individuals can also have thinning of the oculomotor and trochlear nerves. Image adapted from Miyake et al. (2008).

(C) DRS-causing mutations mapped onto α1 and α2 isoforms of chimaerin. All identified mutations are missense and are not localized to a specific domain. Mutations are in areas that are unique to the α2 isoform or shared between both α1- and α2-chimaerin isoforms, suggesting mutations alter α2-chimaerin function to cause DRS. Image adapted from Miyake et al. (2011).

(D) Mutations mapped onto a putative 2D structure of α2-chimaerin, modeled from β2-chimaerin. White circles denote residues that are important for stabilizing the closed, autoinhibited form of chimaerin. Green circles indicate amino acids altered by human CHN1 mutations. White and green circles indicate human mutations that occur at autoinhibition-stabilizing residues, thus suggesting that some mutations destabilize autoinhibition. Further experiments showed all identified mutations are gain-of-function (Miyake et al., 2008). Image adapted from Miyake et al. (2011).

(E) Model of α2-chimaerin function in neurons. Receptor tyrosine kinase activation recruits the SH2 domain of α2-chimaerin, and the C1 domain tethers to the lipid membrane, activating the Rac-GAP domain to convert Rac-GTP to Rac-GDP and cause actin depolymerization. In certain circumstances, the SH2 domain can recruit proteins that generate inhibitory phosphorylation of CRMP2, which leads to destabilization of microtubules.
sponding heterozygous mutations. (†) Disruptions in these developmentally critical regions of 2-chimaerin have a critical developmental function in ocular motor axon pathfinding. 2-chimaerin, a Rac guanosine triphosphatase activating protein (RacGAP) signaling protein, binds to the lateral rectus muscle and translocates to the cell membrane. Mutations alter RacGAP activity in vitro and in vivo, with 2-chimaerin translocation to the cell membrane or enhancement of its ability to self-associate. Expression of mutant 2-chimaerin RacGAP partially suppresses the impaired motor pathway innervation by 1-chimaerin (bottom, 459 amino acids) protein.

Figure 1.4 (Continued)
α2-chimaerin is implicated in axon growth and guidance pathways

α2-chimaerin has been shown to act downstream of EphA4 (Beg et al., 2007; Iwasato et al., 2007; Shi et al., 2007; Wegmeyer et al., 2007) during axon guidance. Loss of α2-chimaerin in mouse results in aberrant midline re-crossing of the corticospinal tract (CST) and defects in spinal interneuron projections that establish central pattern generator (CPG) circuitry, resulting in a hopping gait (Beg et al., 2007; Iwasato et al., 2007; Wegmeyer et al., 2007), which phenocopies EphA4KO mice (Kullander et al., 2003; Kullander et al., 2001). Further investigation revealed that EphA4 activates α2-chimaerin, which decreases levels of Rac-GTP and causes growth cone collapse (Iwasato et al., 2007; Shi et al., 2007).

α2-chimaerin has also been reported to act downstream of Semaphorin3A/PlexinA/Neuropilin1 (Brown et al., 2004; Ferrario et al., 2012). Preventing phosphotyrosine interactions at the SH2 domain and inhibiting RacGAP function of α2-chimaerin in dorsal root ganglion (DRG) neurons prevented growth cone collapse in response to Sema3A (Brown et al., 2004). Further biochemical experiments identified an interaction between α2-chimaerin, PlexinA, and Neuropilin1 (Brown et al., 2004). Overexpressing DRS-causing mutant α2-chimaerin in the oculomotor nerve in chick caused nerve stalling and aberrant branching (Miyake et al., 2008). Knocking down semaphorin receptors PlexinA1 or A2 in the presence of hyperactivating α2-chimaerin mutations rescued oculomotor nerve phenotypes (Ferrario et al., 2012), suggesting a genetic interaction between semaphorin signaling and α2-chimaerin guides cranial motor neuron development.

Additional studies in non-axon guidance fields have implicated α2-chimaerin downstream of BDNF/TrkB (Ip et al., 2012; Mizuno et al., 2004), NGF (Mizuno et al., 2004), and EGF signaling (Colon-Gonzalez et al., 2008). Knockdown of α2-chimaerin in mouse cortex
by in utero electroporation was identified to perturb cortical neuron migration through a BDNF/TrkB-mediated pathway (Ip et al., 2012). Interestingly, the RacGAP domain of α2-chimaerin did not mediate the noted migration defect. Instead, it was found that the SH2 domain promoted inhibitory phosphorylation of CRMP2 to alter microtubule dynamics (Ip et al., 2012). Additionally, preliminary experiments indicated that α2-chimaerin expression was upregulated in cerebellar neurons upon exposure to BDNF and NGF (Mizuno et al., 2004) and that the RacGAP activity of α2-chimaerin was enhanced in transfected HeLa cells upon application of EGF (Colon-Gonzalez et al., 2008).

Thus, the strong implication of α2-chimaerin in axon guidance and neuronal development led us to hypothesize that gain-of-function mutations in α2-chimaerin alter axon growth or guidance of the abducens nerve to cause DRS. We hypothesized that hyperactivating mutations in α2-chimaerin enhance cytoskeletal depolymerization downstream of receptors involved in axon guidance to alter nerve growth and/or guidance (Figure 1.4E). It is intriguing that α2-chimaerin is broadly expressed in developing neurons, and yet hyperactivating mutations cause only strabismus. Thus, we anticipated that cell-type specific molecular signaling mechanisms, including but not limited to the selective activation of axon guidance receptors, generate selective vulnerability of the abducens nerve to hyperactivating mutations in α2-chimaerin.
References


CHAPTER 2

Methods
**Chn1<sup>KI</sup> mouse construct**

InGenious Targeting Laboratory, Inc. created the Chn1<sup>KI</sup> targeting vector and inserted it into a mouse 129 (RP22:77N16) BAC clone. The A60T basepair (L20F amino acid) substitution was inserted into exon 3 of Chn1 using two overlapping PCR fragments generated with forward primer PT5: GCTAGTCGCGAGTGTGTGAATTATGACATTTCTGC and mutation-containing reverse primer PT2: TGCTGCAGCTGGTAAACTACAAGAAGGGGAGAC (5’ of exon 3 and extending to the point mutation), plus forward primer PT3:

TGTCTCCCTTCTTGTAGTTACCAGCTGCACAG and reverse primer PT6:

GCTAGCTCGAGATTATGCTGTGACACCAGAG (from the point mutation to 3’ of exon 3; point mutation in bold italics; added restriction sites are underlined: PT5 = NruI, PT6 = XhoI).

The two primary PCR products were mixed and used as a template for a secondary PCR reaction, where PT5 and PT6 primers were used to amplify the entire sequence harboring the point mutation. NruI and XhoI were used to insert the PCR fragment into the wildtype BAC subclone. These restriction sites were engineered into the wildtype sequence using recombineering. The mutation-containing BAC was subcloned into a pSP72 ampicillin-resistant vector (Promega), then a pGK-gb2 loxP and FRT flanked neomycin selection cassette was inserted 5’ to exon 3. A single loxP site was inserted 3’ to exon 3. Presence of the mutation and proper insertion of the targeting vector were confirmed by restriction analysis and sequencing after each step.

The mutation-containing BAC construct was injected into mouse embryonic stem (ES) cells by the Boston Children’s Hospital IDDRC Mouse Gene Manipulation Core. Targeted ES cells were selected with the Neo cassette and screened using Southern blot, PCR, and DNA sequencing for proper insertion and correct sequence. Additionally, knock-in mice were
confirmed with genotyping primers Mut2F: GTAGGTGGAAATTCTAGCATCATCC and KIR: TGTGTGATGGGACTAAAGG. Subsequent offspring were crossed to FLPε mice (MGI: J:66893) to remove the Neo cassette.

**Generation of Chn1\(^{KO}\) mice**

*Chn1\(^{K/J/KI}\) mice were crossed with *EIIa:Cre* and *Prm:Cre* transgenic mice to create germline knockout *Chn1\(^{KO}\)* mice. Removing *Chn1* exon 3 preserves the promoter region and regulatory elements required for α1-chimerin expression (Dong et al., 1995). Knockout was confirmed with genotyping PCR primers Chn1 Common F: GGTTTTAAGCAGTCTCGGTGA and Chn1 Common R: TCCGAATAAGCAATACAACCTTT and with α2-chimaerin Western blot on P0-2 whole brain lysate. Additionally, *Chn1\(^{KO/KO}\)* mice had a hopping phenotype, as previously reported in other *Chn1* knockout lines (Beg et al., 2007; Iwasato et al., 2007; Wegmeyer et al., 2007).

**Additional mouse strains**

Littermate controls were used whenever possible; controls from the same genetic background were always used for each set of experiments. *Sema3A\(^{K108N}\)* (MGI: J:159834), *Bax\(^{KO}\)* (MGI: J:29253), *EphA4\(^{floX}\)* (MGI: J:157159), *Isl1\(^{Cre}\)* (MGI: J:107396), *Twist2\(^{Cre}\)* (MGI: J:81485), *Hb9:GFP* (MGI: J:88764), *Isl\(^{MN}:GFP\)* (MGI: J:132726), *Hb9\(^{Cre}\)* (MGI: J:57340), and *Ai14\(^{ROSA26:tdTomato}\)* (MGI: J:155793) mice are available from the Jackson Laboratory (Bar Harbor, ME), although at the time of experiments, *Isl\(^{MN}:GFP\)* mice were generously donated by Dr. Samuel L. Pfaff. *Ephexin5\(^{KO}\)* mice were generously provided by Dr. Michael E. Greenberg and *Pdgfrb\(^{Cre}\)* mice by Dr. Volkhard Lindner. See Table 2.1.
Table 2.1: Mouse strain information

<table>
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<tr>
<th>Mouse line</th>
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<tr>
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Whole mount embryo staining

$Chn1^{KI}, Chn1^{KO}, EphA4^{KO},$ and $EphA4^{floxflox}$ mice were crossed to $Hb9:GFP$ mice. Resulting E11.5 embryos were fixed in 4% PFA and prepared as described previously (Huber et al., 2005). Whole embryos were stained with primary 1:500 mouse anti-neurofilament (clone 2H3, Developmental Studies Hybridoma Bank), 1:500 rabbit anti-GFP (Invitrogen), 1:500 mouse anti-actin smooth muscle (Sigma), 1:500 chicken anti-GFP (Aves or Abcam), or 1:500 rabbit anti-tuj1 (Abcam) as indicated, then secondary 1:1000 goat anti-mouse Alexa Fluor 546 (Invitrogen), 1:1000 goat anti-rabbit Alexa Fluor 488 or 647 (Invitrogen), or 1:1000 goat anti-chicken Alexa Fluor 647 (Invitrogen). Littermate controls were used whenever possible, and all controls result from the same genetic background as mutants. Embryos were cleared with 1 part benzyl alcohol: 2 parts benzyl benzoate (Sigma; BABB), placed into a custom-designed, BABB-resistant holding chamber, and imaged using a Zeiss LSM710 laser scanning confocal microscope with Zen software (Zeiss).

Whole mount image processing and analysis

Images were processed in 3D using Imaris (Bitplane) to identify similar z-planes between embryos. Sagittal images represent compressed z-stacks of 12um step size and transverse images represent 8um step compressed z-stacks. Compressed z-stack images from Imaris were uniformly processed in Fiji/ImageJ. Whole mount measurements were made in Fiji/ImageJ using the 546/neurofilament channel to enable measurements from GFP-positive and -negative embryos. Number of exiting nerve bundles for abducens were counted in the sagittal view, from the point at which neurofilament first appeared immediately after nerve bundles exited the hindbrain. Abducens diameter was measured as previously described for oculomotor nerves.
(Cheng et al., 2014). Diameter was measured in the sagittal view after fasciculation in Area 2 and before re-branching at the orbit region in Area 3. Abducens length was measured in the sagittal view using Fiji Simple Neurite Tracer and accounted for x, y, and z-distances. Measurements were made from the hindbrain exit region, at the midpoint between the anterior and posterior nerve bundle exits, until the distal tip of abducens at the anterior region of the orbit. Using the Imaris Surface tool, abducens nerves from transverse confocal images were processed to create a surface rendering of each nerve/nerve fiber. Images were manually compared to the raw image to ensure all visible abducens fibers were rendered in Surfaces. Resulting images were used to quantify the number of nerve fibers that deviated medially or laterally from the normal abducens trajectory to obtain the wandering fiber measurement. Using the same transverse view, raw images were evaluated for trochlear branching. The number of exiting trochlear nerves on each individual side of the embryo was counted to obtain the trochlear branching measurement. Each experiment analyzed greater than three embryos from 3 or more independent litters.

**Orbital dissections**

E15.5 *ISL^MN::GFP*-positive mouse embryos from the indicated genetic crosses were fixed overnight in 4% PFA. Tissue around the orbit was removed, leaving the distal cranial nerves and EOMs intact. Orbits were incubated with 1:500 anti-actin α-smooth muscle-Cy3 antibody (Sigma-Aldrich) for 3 days at 4°C, washed 3X with PBS, then orbits were further dissected and flat mounted in 70% glycerol and 1% 1M KOH in PBS. Samples were imaged and processed as outlined for whole mount embryos. N ≥ 3 embryos from multiple litters were used for each gene condition.
Facial nucleus flat mount

E12.5 Isl<sup>MN</sup>:GFP embryos were fixed for 3 hours - overnight in 4% PFA at 4°C, then dissected in PBS using an open-book preparation. Samples were flattened on a slide and mounted in in 70% glycerol and 1% 1M KOH in PBS for clearing. Samples were images on the Zeiss LSM710 confocal.

Chn<sup>KI</sup>:Bax<sup>KO</sup> immunohistochemistry

E10.5 and E13.5 Chn<sup>KI</sup>:Bax<sup>KO</sup>:Hb9:GFP embryos were fixed overnight in 4% PFA, then dehydrated through a 25%, 50%, and 70% ethanol series. Embryos were embedded paracoronally in paraffin and serially sectioned at 10um. Every fifth section underwent deparaffinization, then was placed in pre-boiled 1:100 Antigen Unmasking Solution (Vector Labs) in water and incubated for 20 minutes in a vegetable steamer. Sections were blocked with 1XPBS/0.3% TritonX100/5% heat-inactivated goat serum, stained overnight at 4°C with 1:500 mouse anti-Isl1 (DSHB) and 1:500 chicken anti-GFP (Abcam), washed 3X PBS, incubated in secondary 1:1000 goat anti-mouse 546 (Invitrogen) and 1:500 goat anti-chicken FITC (Abcam) at room temperature for 1.5 hours, washed 3X PBS, stained with 1:1000 DAPI (Invitrogen), washed 2X PBS, and mounted in Fluoromount G (Southern Biotech). Sections were imaged using an Olympus BX51 fluorescence microscope. For abducens neuronal counts, Hb9:GFP- and Isl1- positive abducens neurons were located anatomically, adjacent to Hb9:GFP-negative and Isl-positive facial neurons, then counted manually using ImageJ. For oculomotor and trochlear counts, Isl-positive neurons were identified anatomically within the midbrain and rostral hindbrain and manually counted. N ≥ 3 embryos from multiple litters were used for each age and gene condition and experiments were blinded.
**Immunohistochemistry on cryosections**

For EphA4 staining, E11.5 *Hb9:GFP* embryos were fixed in 4% PFA overnight at \(4^\circ\)C. Embryos were incubated for 12 hours in 20% Sucrose/PBS, then 12 additional hours in 30% Sucrose/PBS before embedding for sagittal sectioning in O.C.T. (TissueTek). Samples were cryosectioned at 20\(\mu\)m. Sections were blocked in 5% Goat Serum/0.3% TritonX-100/PBS, and stained with 1:300 mouse anti-EphA4 (Santa Cruz) primary and 1:1000 goat anti-mouse Alexa Fluor 546, then mounted and images as above.

**Adult brain histology**

Chn1\(^{WT/WT}\) and Chn1\(^{KI/KI}\) age-matched adults (>P60) were anesthetized, then transcardially perfused with 1X PBS and 10% formalin. Mice were decapitated and whole heads were fixed in 10% formalin overnight. Brains were dissected from the skull and placed in PBS, then submitted to the Dana Farber/Harvard Cancer Center Rodent Histopathology Core for coronal paraffin embedding. Brains were microtome sectioned at 5-10\(\mu\)m. Matching anatomical levels between wildtype and mutant were stained with Luxol Fast Blue by the Rodent Histopathology Core.

**Affinity probe in situ**

Ephrin-A5-AP expression construct was generously shared by Dr. John G. Flanagan. HEK293-T cells were transfected with the expression construct, then lysate was applied to open book hindbrain samples as previously described (Feldheim et al., 1998).
DigiGait

Three cohorts of adult male and female littermate mice (>60 days) were tested on DigiGait (Mouse Specifics, Inc.): one cohort with $Chn1^{WT/WT}$, $Chn1^{WT/KI}$, and $Chn1^{KI/KI}$ mice; a second with $Chn1^{WT/WT}$, $Chn1^{WT/KO}$, and $Chn1^{KO/KO}$ mice, and a third with $Chn1^{KI:EphA4KO}$ mice with controls. Individual cohorts were housed on the same rack with up to 5 animals in the same cage. Mice were tested and analyzed blind to genotype. Mice were placed on a detergent-cleaned DigiGait treadmill platform, which was run at 10 centimeters per second. Videos were acquired with DigiGait Imaging Software (Mouse Specifics, Inc.) until 5-7 fluid steps of each limb were acquired. DigiGait videos were trimmed to these 5-7 steps posthoc using VirtualDub. Alternating steps and hops were manually counted for each video clip. Individual animals were characterized as “Alternates” if there was no synchronized hindlimb movement, “Alternates/occasional hop” if more than half of the steps did not have synchronized hindlimb movement, “Hops/occasionally alternates” if more than half of the steps exhibited synchronized hindlimb movement, and “Hops” if all steps had synchronized hindlimb movement. Trimmed videos were subsequently run through automated DigiGait Analysis software (Mouse Specifics, Inc.) to generate the DigiGait alternating gait percentage value.

Abducens, trochlear, and C1 explant culture

Acid washed and autoclaved German glass coverslips (VWR) were placed into 24-well plates and coated with 20ug/mL poly D-lysine (Millipore) in PBS overnight at 37°C. Wells were washed 3X with water, then coated with 10ug/mL laminin (Invitrogen) in PBS for 2 hours at 37°C. GFP-positive embryos were harvested in ice cold PBS and microdissected in ice cold HBSS. Abducens explants: The hindbrain was carefully separated from Hb9:GFP-positive
embryos using fine forceps and dissected along the dorsal surface in an open-book manner to reveal the abducens nucleus. Meningeal tissue was removed from the ventral hindbrain. GFP-negative tissue surrounding individual abducens nuclei was carefully trimmed away with a tungsten needle, leaving two intact nuclei explants per embryo. Both nuclei were placed into PDL/laminin coated wells with HBSS on ice during dissection of the remaining littermates.

Trochlear explants: \textit{Ist}^{MN}:GFP-positive midbrain tissue was dissected as outlined above. Trochlear nuclei were distinguished from oculomotor by their caudal anatomical location, carefully dissected from GFP-negative tissue, and placed into PDL/laminin coated wells with HBSS on ice. C1 explants: C1 was identified anatomically in \textit{Hb9}:GFP-positive embryos and removed with rostral and caudal cuts flanking the cervical segment. Dissection proceeded as outlined above. Due to the larger size of C1, dissected explants were divided into 2 equally sized pieces for each embryonic side using a tungsten needle, yielding 4 explants per embryo. 2 explants from the same unilateral side were placed in PDL/laminin-coated wells containing HBSS on ice. For each well, the two explants were positioned at opposite sides of the coverslip, HBSS was removed, and quickly replaced with Neurobasal (Invitrogen), 1X B27 (Invitrogen), 2mM L-glutamine (Invitrogen), and 100ug/mL Penicillin/Streptomycin (Invitrogen) containing indicated growth factors and/or proteins. Explants were cultured at 37\textdegree C in a cell culture incubator (5\% CO\textsubscript{2}/95\% air).

Recombinant proteins

ProSpec: GDNF, BDNF, CNTF, HGF, NGF, SDF-1, FGF8; concentration as indicated in figures. R&D Systems: 1ug/mL mouse EphA4, 200ng/mL mouse ephrin-B1, 200ng/mL mouse ephrin-B2, 300ng/mL mouse Sema3A, 300ng/mL mouse Sema 3C, and 300ng/mL mouse
Sema3F, 50ng/mL (Sholl) or 200ng/mL (collapse) human ephrin-A5. EMD Millipore: 50ng/mL (Sholl) or 300ng/mL (collapse) human IgG FC fragment. FC, ephrin-A5, and EphA4 were pre-clustered 1:5 (concentration ratio) with Goat Anti-Human IgG, Fcγ fragment specific Cy3 (Jackson ImmunoResearch) for 30 minutes at room temperature.

**Growth cone collapse experiments**

Abducens explants were grown in 25ng/mL GDNF, 25ng/mL BDNF, and 25ng/mL CNTF for 12-14 hours, then transferred to a Nikon Perfect Focus Eclipse Ti live cell fluorescence microscope using Elements software (Nikon), where they were maintained at 37°C in an environmental chamber with 5% CO2/95% air. Explants were imaged once a minute for 15 minutes to assess initial growth cone dynamics. 100ul of media was removed, indicated recombinant proteins were added, and the mixture was bath applied. Explants were reimaged once a minute for 30 minutes to assess growth cone dynamics with noted cues. Collapsed growth cones were quantified as any growth cone with fewer than 3 filopodia and no lamellipodia. Axon retraction was quantified as any axon that regressed more than the distance equal to one growth cone. For each condition, n≥30 or more axons from at least 4 independent experiments.

**Abducens growth characterization**

Images from the above abducens collapse experiments (before addition of cue) were used to compare growth characteristics of wildtype and mutant explants. Axon growth (distance and displacement) over the 15 minute imaging session was measured using the ImageJ MTrackJ Plugin. Growth cone size was measured using Elements software.
**Sholl analysis**

Abducens, trochlear, and C1 explants were grown in noted growth factors and proteins for 18 hours, then fixed for 1 hour at room temperature with 4% PFA and 4% sucrose in PBS. Explants were imaged on the Nikon Eclipse Ti fluorescence microscope. Images from each explant were tiled into a single image using a Fiji macro developed by the Harvard NeuroDiscovery Center Enhanced Neuroimaging Core (Figure 2.1, top). A second Fiji macro subtracted background fluorescence, applied a standard threshold to individual images (abducens and C1: triangle dark, trochlear: mean dark), and saved images as a mask (Figure 2.1, middle). The explant body was outlined manually in Fiji; a third macro drew concentric circles at 20um intervals and counted intersecting neuritis (Figure 2.1, bottom). The number of intersecting neurites per 20um interval was exported to Excel. N ≥ 7 explants were used from 3 or more independent experiments. Wildtype controls were present in each replicate within every condition (i.e. ephrin-A5+GDNF). Using SPSS, individual samples were graphed within each condition to assess distribution, and then were averaged across experiments within condition to obtain final outgrowth curves. To assess initial outgrowth, the number of intersections between 0-200um were summed and multiplied by 20um to yield an initial outgrowth integral value, then averaged across samples of a given condition. Averaging the micron distance of the last intersecting circle across all samples of a given condition generated the maximum outgrowth value. To assess total outgrowth, the number of intersections for each individual sample across all distances was summed and multiplied by 20um to yield a total outgrowth integral value, then averaged across samples of a given condition.
Figure 2.1: Sholl analysis workflow. (A) $\text{Chn1}^{\text{WT/WT}}$ (left) and $\text{Chn1}^{\text{WT/KI}}$ (right) abducens explants grown in 1ug/mL EphA4 and 25ng/mL GDNF. Raw images of $\text{Hb9:GFP}$ positive abducens explants (top), with standard threshold applied (middle), and after processing with Sholl analysis macro created on ImageJ (bottom).
Western blot

Postnatal day 0-2 whole brain was homogenized on ice in RIPA buffer (Thermo Scientific), 1mM EDTA (Gibco), and Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). E13.5 spinal cords were dissected and flash frozen in liquid nitrogen. After genotyping, 2-3 spinal cords from littermates of the same genotype were pooled and homogenized as above. Samples were incubated on ice for 30 minutes and clarified by spinning at 14000rpm for 15 minutes at 4°C. Supernatant protein concentration was measured using a BCA Protein Assay Kit (Pierce) on a NanoDrop 1000 (Thermo Scientific) and samples were adjusted to the same concentration within an experiment. After addition of NuPAGE LDS Sample Buffer (Invitrogen) and Sample Reducing Agent (Invitrogen), samples were incubated at 70°C for 10 minutes. Denatured protein was run on a Western blot as described previously (Cheng et al., 2014). Membranes were blocked with 5% milk in PBST, then incubated in 1:1000 rabbit anti-Chn1 (Abcam EPR9906), 1:500 rabbit anti-GAPDH (Santa Cruz), or 1:1000 mouse anti-EphA4 (Santa Cruz) overnight at 4°C. Blots were washed 3X with PBST, then incubated in secondary antibody 1:5000 Peroxidase AffiniPure donkey anti-rabbit and/or donkey anti-mouse IgG (Jackson ImmunoResearch). Protein was detected using an Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and imaged with a FujiFilm LAS-4000 with CCD camera (GE Healthcare) and MultiGauge software (GE Healthcare). Protein bands were quantified using the Gels tool in ImageJ. Experiments were performed with tissue from three different litters; n ≥ 3 samples per genotype.
Cortical neuron culture for Rac-GTP ELISA

12 well plates were coated with 50μg/mL poly-D-lysine for 2 hours to overnight at 37°C. Plates were washed 3 times with water before culture. Papain for cell dissociation was prepared as per manufacturer instructions (Worthington, Inc.). E16.5-17.5 mice were decapitated, then the brain was carefully removed and placed in ice-cold PBS. Genotyping was performed during dissections and dissociation. Each cortical hemisphere was microdissected in HBSS, avoiding lateral aspects. Both cortical hemispheres were cut into small pieces and placed into an individual eppendorf tube. HBSS was removed and 750μL pre-warmed Papain was added, then samples were incubated in a 37°C water bath for 30 minutes, flicking the tube every 5 minutes to resuspend the cells. Samples were triturated with a P1000 pipette and large pieces were allowed to settle. Supernatant was transferred to a new tube, then spun at 300g for 5 minutes. Supernatant was removed, and 750μL pre-warmed ovomucoid solution was added and mixed. Samples were re-spun at 300g for 5 minutes and supernatant was removed. Cells were resuspended in Neurobasal/B27/Penicillin/Streptomycin/Glutamine medium as outlined above. Cells from the same genotype within a litter were combined into one sample, counted using a ViCell Cell Viability Analyzer (Beckman Coulter), then diluted to 1x10^6 cells/mL. Experiments were always conducted simultaneously with wildtype and mutant littermates.

After 40-48 hours in culture, cells were serum starved in DMEM/Penicillin/Streptomycin for 2 hours. DMEM or 10μM PMA in DMEM was added for the amount of time specified (DMEM: 30 minutes, PMA: 15 or 30 minutes). Cells were washed 1X with cold PBS, then lysed in ice cold RIPA buffer with 1mM EDTA and 1X protease and phosphatase inhibitor (as outlined for Western blot) using a plastic cell scraper. After lysis, samples were rapidly processed to limit endogenous Rac-GTP to –GDP hydrolysis. Samples were immediately spun at 14000g for 10
minutes at $4^\circ$C and supernatant was stored at -80$^\circ$C. Samples were processed for Rac-GTP levels using a Rac1 G-LISA Activation (Colorimetric Based) Assay Kit (Cytoskeleton, Inc.) as recommended by the company’s protocols. When possible, two replicates per condition were run on the same plate and O.D. output values were averaged. Averaged values were normalized to the O.D. values obtained from wildtype DMEM within each experiment. N=4 experiments from 4 litters.

Statistics

All data was analyzed and graphed using SPSS software (IBM). Statistical methods are provided in each figure legend. Graphs represent mean ± standard error of the mean.

Animal Approval

All animal work was approved and performed in compliance with Boston Children’s Hospital Institutional Animal Care and Use Committee protocols.
References


CHAPTER 3

Bidirectional ephrin signaling alters abducens nerve development via α2-chimaerin
At the time of the submission of this dissertation, much of the work presented in this chapter has been submitted as a manuscript entitled:

Bidirectional ephrin signaling alters abducens nerve development via α2-chimaerin

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Author Contributions

Alicia A. Nugent performed most experiments and all formal analysis. Jong G. Park dissected and imaged orbital innervation presented in Figures 3.6 and 3.12. Yan Wei conducted Western blots for Figure 3.1, whole mount embryo staining for Figures 3.6 and 3.7, and affinity probe staining for Figure 3.9. Michelle M. DeLisle conducted DigiGait acquisition and A.A.N. analyzed DigiGait output for Figure 3.11. Wai Man Chan and Long Cheng confirmed correct insertion of Chn1KI construct in mouse ES cells. A.A.N. and Elizabeth C. Engle designed experiments and wrote the initial manuscript, to which A.A.N. added additional data for this thesis chapter.
Abstract

We report a knock-in α2-chimaerin mouse (Chn1\(^{KI}\)) that models Duane retraction syndrome (DRS), the most common form of congenital paralytic strabismus. Whole embryo imaging reveals stalled abducens nerve growth and selective trochlear and first cervical spinal nerve guidance abnormalities, thus identifying neuronal growth and guidance defects as a cause of DRS. Consistent with Chn1 mutations causing altered function, Chn1\(^{KO/KO}\) embryos display abducens nerve wandering distinct from the Chn1\(^{KI}\) phenotype. EphA4\(^{KO/KO}\) embryos exhibit similar abducens wandering, which parallels the known hopping gait shared by Chn1\(^{KO/KO}\) and EphA4\(^{KO/KO}\) adult mice. Combining Chn1\(^{KI}\) and EphA4\(^{KO/KO}\) alleles demonstrates that α2-chimaerin and EphA4 have different genetic interactions in distinct motor neuron pools; abducens neurons use bidirectional ephrin signaling via α2-chimaerin to direct growth, while cervical spinal neurons use only ephrin forward signaling, and trochlear neurons use neither. These findings uncover a novel role for ephrin bidirectional signaling upstream of hyperactivated α2-chimaerin, which may contribute to the selective vulnerability of abducens neurons in DRS.
Introduction

Despite the complexity of axon guidance during neurodevelopment and its importance for proper circuit formation and normal behavior, few human disorders have been established with certainty to result from abnormalities in this process. Human pathology and initial mechanistic studies indicate that Congenital Cranial Dysinnervation Disorders (CCDDs) can result from errors in axon guidance (Engle, 2010; Nugent et al., 2012). The precise alignment and coordinated movement of the eyes creates a sensitive system to identify pathologic innervation onto the extraocular muscles (EOM) that arises during development. Individuals with ocular CCDDs present with readily identifiable features, such as paralytic strabismus (loss of full ocular motility) or synkinesis (involuntary muscle movements concurrent with voluntary muscle movements), which are caused by dysinnervation of one or a few discrete muscle groups. Thus, CCDDs serve as models to investigate axon guidance mechanisms that are relevant to human development. Here, we present a novel mouse model of the most common CCDD, Duane retraction syndrome (DRS), and identify that the cranial nerve pathology seen in DRS can be caused by axon guidance defects. Additionally, we identify cellular and molecular mechanisms that underlie the etiology of the disorder.

DRS is a CCDD that affects approximately 1:1000 people (Engle, 2010). Affected individuals cannot move one or both eyes laterally toward the ear (limited abduction), and upon attempted medial eye movement toward the nose (adduction) the globe(s) retracts into the orbit. Autopsy and magnetic resonance imaging (MRI) studies of individuals with DRS have revealed specific loss of abducens motor neurons and their axons, which normally innervate the lateral rectus muscle (LR) to abduct the eye (Demer et al., 2007; Hotchkiss et al., 1980; Miller et al., 1982). Autopsy and electromyography studies support aberrant innervation of the LR by the
oculomotor nerve, which normally innervates the medial rectus muscle (MR) as well as four other EOM, typically causing co-contraction of MR and LR on attempted adduction and retraction of the globe into the orbit (Hotchkiss et al., 1980; Huber, 1974; Miller et al., 1982).

Genetic studies of rare pedigrees that segregate DRS as an autosomal dominant trait identified gain-of-function missense mutations in \textit{CHN1} that hyperactivate the encoded protein \(\alpha2\)-chimaerin (Chan et al., 2011; Miyake et al., 2008; Miyake et al., 2011). \(\alpha2\)-chimaerin-encoding mRNA is expressed in nearly all central and peripheral developing neurons in embryonic mice (Hall et al., 2001) and displays widespread neuronal expression at Carnegie Stage 15/16 in developing human embryos, including rhombomere 5 where abducens neurons are located (Miyake et al., 2008). Remarkably, despite broad neuronal expression of \(\alpha2\)-chimaerin, the phenotype of affected individuals with \textit{CHN1} mutations is limited to disordered eye movements. Almost all affected individuals have DRS and abducens nerve hypoplasia by MRI, with variable dysfunction of EOM innervated by the trochlear and/or oculomotor nerve (Demer et al., 2007; Miyake et al., 2008); one reported family has abnormal vertical gaze without DRS (Miyake et al., 2011).

While clinical exam findings, postmortem and MRI data, and \textit{CHN1} mutations all support a neurogenic rather than myogenic etiology for DRS, the precise cellular and molecular mechanisms underlying its neurogenic etiology remained unknown. The adult mouse abducens nucleus is comprised of only \(~\)100-130 neurons (Sturrock, 1989), thus presenting a challenge for neuronal subtype specific studies. Here, we use a combination of detailed \textit{ex vivo} imaging and abducens motor neuron culture experiments from \textit{Chn1} \textsuperscript{KI} embryonic mice to investigate how gain-of-function \textit{Chn1} mutations alter motor neuron development selectively within the ocular motor system to cause DRS.
α2-chimaerin is a Rac GTPase-activating protein (RacGAP) reported to regulate cytoskeletal dynamics (Ip et al., 2012; Iwasato et al., 2007; Kozma et al., 1996; Shi et al., 2007; Wegmeyer et al., 2007). It is comprised of a SH2 domain that binds to phosphorylated receptor tyrosine kinases (RTKs) (Beg et al., 2007; Shi et al., 2007), a C1 domain that binds phorbol esters and diacylglycerol (DAG) at the plasma membrane (Colon-Gonzalez et al., 2008), and a GAP domain that enhances the conversion of active GTP-bound Rac to inactive GDP-bound Rac (Kozma et al., 1996; Shi et al., 2007; Wegmeyer et al., 2007). α2-chimaerin exists in an autoinhibited form until upstream signaling releases it from inhibition (Brown et al., 2004; Colon-Gonzalez et al., 2008). Once activated, the GAP domain of α2-chimaerin reduces levels of Rac-GTP to alter actin dynamics and generate growth cone collapse (Brown et al., 2004; Iwasato et al., 2007; Kozma et al., 1996; Shi et al., 2007; Wegmeyer et al., 2007). Additionally, the SH2 domain can interact with CRMP2 to alter neurite development and neuronal migration, likely through a microtubule-mediated pathway (Brown et al., 2004; Ip et al., 2012). All reported human CHN1 mutations increase α2-chimaerin RacGAP activity to further dampen Rac-GTP levels, and a subset does so by destabilizing its closed, autoinhibited state and enhancing its recruitment to the plasma membrane (Miyake et al., 2008; Miyake et al., 2011).

α2-chimaerin is reported to act downstream of several receptors implicated in axon growth and guidance, including EphA4, TrkB, and Neuropilin1/PlexinA (Beg et al., 2007; Brown et al., 2004; Ferrario et al., 2012; Ip et al., 2012; Iwasato et al., 2007; Shi et al., 2007; Wegmeyer et al., 2007). EphA4<sup>KO/KO</sup> and Chn1<sup>KO/KO</sup> adult mice have a rabbit-like hopping gait, resulting from aberrant re-crossing of the corticospinal tract and miswiring of spinal interneurons that regulate central pattern generator (CPG) circuitry within the spinal cord (Asante et al., 2010; Beg et al., 2007; Dottori et al., 1998; Iwasato et al., 2007; Kullander et al., 2003; Serradj et al., 2010).
2014; Wegmeyer et al., 2007). Further biochemical experiments indicated that α2-chimaerin interacts with phosphorylated residues on EphA4 to alter cytoskeletal dynamics and elicit growth cone collapse (Beg et al., 2007; Iwasato et al., 2007; Shi et al., 2007). Additionally, α2-chimaerin is reported to signal downstream of BDNF/TrkB to regulate proper cortical neuron migration in the developing mouse brain (Ip et al., 2012), and downstream of Sema3A/PlexinA/Neuropilin1 in dorsal root ganglion (Brown et al., 2004) and oculomotor neurons (Ferrario et al., 2012). It is not known, however, if any of these receptors activate α2-chimaerin in developing abducens neurons. It has been suggested that hyperactivated α2-chimaerin may result in axon growth and guidance defects, as overexpression of mutant Chn1 in chick and zebrafish causes oculomotor nerve stalling and/or branching defects (Clark et al., 2013; Ferrario et al., 2012; Miyake et al., 2008).

Here, we establish the first Chn1 mutant knock-in mouse model of DRS and demonstrate that DRS-causing mutations in α2-chimaerin result in axon guidance defects in specific motor neuron populations. Our data provide insight into the selective vulnerability of the abducens nerve to gain-of-function α2-chimaerin mutations, as we find that different neuronal types harness α2-chimaerin in distinct ways to guide developing axons.
Results

DRS mutant α2-chimaerin alters developing abducens, trochlear, and first cervical spinal (C1) nerve projections

To investigate the etiology of DRS, we generated a Chn1 knock-in mouse model harboring the L20F amino acid substitution (Chn1\textsuperscript{KI/KI}), which is specific to the α2 isoform of chimaerin; this substitution segregated in a family with DRS, and enhanced α2-chimaerin membrane translocation and RacGAP activity \textit{in vitro}. We flanked exon 3 of the knock-in construct with LoxP sites to permit generation of a conditional α2-chimaerin knockout mouse upon exposure to Cre-recombinase (Chn1\textsuperscript{K0/K0}) (Figure 3.1A). There is a modest, but significant, reduction in α2-chimaerin protein level in Chn1\textsuperscript{K0/K0} postnatal day 0-2 brain and E13.5 spinal cord compared to Chn1\textsuperscript{WT/WT} levels (Figures 3.1B-3.1C). Despite a reduction in α2-chimaerin protein level, cultured cortical neurons from Chn1\textsuperscript{KI/KI} mice stimulated with PMA to activate α2-chimaerin show reduced levels of Rac-GTP compared to Chn1\textsuperscript{WT/WT} controls (Figure 3.1D). This confirms previous \textit{in vitro} experiments that found overexpression of L20F-mutant α2-chimaerin in heterologous cell lines enhances the RacGAP activity of α2-chimaerin to lower Rac-GTP levels (Miyake et al., 2008). Without PMA stimulation, Chn1\textsuperscript{K0/KI} cultures have similar Rac-GTP levels as Chn1\textsuperscript{WT/WT} cultures (Figure 3.1D), suggesting that mutant α2-chimaerin is not constitutively active at baseline.

Adult Chn1\textsuperscript{KI/KI} mice are born with the expected Mendelian frequency, are fertile, and have a normal lifespan. Chn1\textsuperscript{WT/KI} and Chn1\textsuperscript{KI/KI} mice exhibit unilateral or bilateral globe retraction of 61% and 72% penetrance that is not detected in Chn1\textsuperscript{WT/WT} or Chn1\textsuperscript{K0/K0} mice (Figures 3.2A-3.2C).
Figure 3.1: Chn1 knock-in and knockout mouse construct and initial characterization.

(A) Chn1 construct, with A60T point mutation (asterisk; L20F amino acid substitution) inserted into exon 3. Neomycin selection cassette (green) contains flanking FRT sites (purple chevron). Exon 3 is flanked with LoxP sites (yellow chevron) for α2-chimaerin specific knockout upon exposure to Cre-recombinase. Chn1WT/WT mice do not contain mutant construct, Chn1KI/KI mice contain floxed exon 3 with A60T mutation, and Chn1KO/KO mice lack exon 3 and downstream α2-chimaerin transcripts.

(B) Western blots of α2-chimaerin (top) and GAPDH (bottom) in postnatal day 0-2 brain of littermate Chn1WT/WT (n=3), Chn1WT/KI (n=3), and Chn1KI/KI (n=4) mice (left), Chn1WT/WT (n=3), Chn1WT/KO (n=3), and Chn1KO/KO (n=3) mice (middle), and E13.5 spinal cord in Chn1WT/WT (n=8) and Chn1KI/KI (n=8) embryos (right).

(C) Ratio quantification of α2-chimaerin to GAPDH in (B); left: P0-2 brain, Chn1KI/KI: p<0.001, Chn1WT/KO: p<0.001, Chn1KO/KO: p<0.001, one-way ANOVA with Tukey’s test; right: spinal cord, Chn1KI/KI: p=0.004, unpaired, two-tailed t-test.

(D) Rac-GTP ELISA of cultured cortical neurons from littermate E16.5-17.5 Chn1WT/WT (WT) and Chn1KI/KI (KI) mice, stimulated with DMEM (control), or PMA for 15 or 30 minutes. N = 4 litters; *p = 0.015, **p = 0.006 by unpaired two-tailed t-test, Bonferroni-corrected level of significance is p=0.016 for comparisons between WT DMEM and three KI conditions.
Figure 3.2: *Chn1* 

*Chn1* 

**Figure 3.2:** *Chn1* gain-of-function mutation alters development of abducens (VI), trochlear (IV), and first cervical spinal (C1) nerves. (A-C) Eyes of *Chn1* WT/WT (A), *Chn1* KI/KI (B), and *Chn1* KO/KO (C) adult mice (*Chn1* WT/WT: n=0/44 affected; *Chn1* WT/KI: 61.3% affected, n=144/186; *Chn1* KI/KI: 71.8% affected, n=28/39; *Chn1* KO/KO: n=0/9 affected). (D-F) Whole mount neurofilament staining of *Chn1* WT/WT (D), *Chn1* KI/KI (E), and *Chn1* KO/KO embryos (F), scale bar = 500um. White box: enlargement in (G-I), white bracket: transverse view in (M-O), yellow box: enlargement in (P-R). (G-I) Developing VI in E11.5 *Chn1* WT/WT (G; 20/20 nerves innervate), *Chn1* KI/KI (H; 3/10 nerves innervate; arrow: stalling region), and *Chn1* KO/KO (I; 7/10 nerves fasciculate with facial, 10/10 innervate; arrowhead: dorsally projecting fibers; arrow: aberrant fasciculation with VII). Area 1: VI hindbrain exit region; Area 2: VI fasciculation region; Area 3: VI EOM innervation region. (J) Number of VI fibers exiting the hindbrain within Area 1 (*Chn1* WT/KI: p=0.260, *Chn1* KI/KI: p<0.001, *Chn1* KO/KO: p<0.001). (K) VI diameter measured at line denoting Area 3 (*Chn1* WT/KI: p=0.005, *Chn1* KI/KI: p<0.001, *Chn1* KO/KO: p=0.003). (L) VI length measured from hindbrain exit to nerve terminus at the orbit (*Chn1* WT/KI: p=0.017, *Chn1* KI/KI: p<0.001, *Chn1* KO/KO: p=0.991). Data represent mean ± SEM; * p<0.05, **p<0.01, ***p<0.001, one-way ANOVA with Tukey’s test. (M-O) Transverse view of crossing IV in *Chn1* WT/WT (M), *Chn1* KI/KI (N), and *Chn1* KO/KO (O). P: posterior; A: anterior; T: tectum; IVR and IVL: right and left IV nerves, respectively; arrowheads: minor midline misprojections; arrows: aberrant IV branches. (P-R) XII and C1 projections in *Chn1* WT/WT (P), *Chn1* KI/KI (Q; arrow: direction of C1 dorsal turning toward axial musculature), and *Chn1* KO/KO embryos (R). Red: neurofilament, green: Hb9:GFP; III: oculomotor, IV: trochlear, VI: abducens, VII: branch of facial, XII: hypoglossal, C1: first cervical spinal segment; whole mount *Chn1* WT/WT: n=20 nerves, 10 embryos; *Chn1* WT/KI, KI/KI, KO/KO: n=10 nerves, 5 embryos; scale bar (G-R): 100um.
Figure 3.2 (Continued)

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VI nerve length (um)

Number of nerve fibers at exit

VI diameter at orbit (um)

Error Bars: +/- 1 SE
DRS is a congenital disorder and $\alpha_2$-chimaerin is expressed during embryonic and early postnatal development (Hall et al., 2001; Miyake et al., 2008). Thus, we crossed $Chn1^{KI}$ and $Chn1^{KO}$ mice to existing $Hb9:GFP$ transgenic mice to specifically visualize the abducens nerve distinct from other hindbrain motor neuron populations (Wichterle et al., 2002), conducted whole mount neurofilament staining at embryonic day 11.5 (E11.5), and used 3D whole embryo visualization to evaluate developing cranial nerves (Figures 3.2D-3.2F).

At E11.5, the $Chn1^{WT/WT}$ abducens nerve exits the hindbrain as multiple defasciculated nerve fibers (Figure 3.2G: Area 1, Figure 3.2J), which then fasciculate midway between the hindbrain and orbit (Figure 3.2G: Area 2) before defasciculating again upon reaching the primitive EOM anlage at the orbit (Figure 3.2G: Area 3, Movie 1). Compared to $Chn1^{WT/WT}$, following exit from the hindbrain, the $Chn1^{KI/KI}$ abducens nerve has fewer fascicles that appear to be over-fasciculated in Area 1 (Figures 3.2H, 3.2J), and stalls with variable penetrance in Area 2 (Figures 3.2H, 3.3A, and Movie 2). By contrast, the $Chn1^{KO/KO}$ abducens nerve exits with an increased number of defasciculated fibers within Area 1, some of which wander dorsally toward the hindbrain or ventrally to track along the developing buccal branch of the facial nerve, also with variable penetrance (Figures 3.2I, 3.2J, Movie 3). A subset of fibers in all $Chn1^{KO/KO}$ embryos, however, fasciculate within Area 2 and innervate the EOM anlage (Figures 3.2I, Movie 3).
Figure 3.3: Phenotype spectrum of Chn1^{KI/KI}, and Chn1^{WT/KI}, and Chn1^{KI/KO}.
(A) Thin Chn1^{KI/KI} abducens (VI) nerve, arrow: remaining nerve bundle projecting to orbit.
(B) Chn1^{WT/KI} VI nerve (5/10 nerves innervate), arrow: stalling region.
(C) Chn1^{KI/KO} VI nerve (3/10 nerves innervate).
(D) Transverse view of Chn1^{WT/KI} trochlear nerve, arrows: multiple nerve braches.
(E) Chn1^{WT/KI} C1 nerve, arrow: dorsal nerve turn.
Red: neurofilament; green: Hb9:GFP.
We measured abducens nerve length from the hindbrain exit to the orbit and found a 20.5% decrease across five Chn1\textsuperscript{WT/KI} embryos and 30.2% decrease across five Chn1\textsuperscript{KI/KI} embryos compared to wildtype, whereas the Chn1\textsuperscript{KO/KO} abducens nerve length does not significantly differ from wildtype (Figure 3.2K). Chn1\textsuperscript{WT/KI} and Chn1\textsuperscript{KI/KI} embryos display absence or thinning of the abducens nerve at Area 3, reflected by a significantly reduced abducens nerve diameter near the orbit (Figures 3.2L, 3.3A, 3.3B), whereas Chn1\textsuperscript{KO/KO} embryos have a significantly greater abducens nerve diameter compared to wildtype, which likely reflects nerve defasciculation (Figure 3.2L). Removing one mutant allele in Chn1\textsuperscript{KIIKO} embryos does not alleviate abducens stalling; instead embryos have the same stalling penetrance as seen in Chn1\textsuperscript{KI/KI} embryos (Figure 3.3C). Together, these data confirm the L20F amino acid substitution is not loss-of-function, as the Chn1 knock-in and knockout mice have different phenotypes, and demonstrate that both loss and gain of α2-chimaerin function cause defects in abducens development.

Because α2-chimaerin is broadly expressed in developing neurons (Hall et al., 2001; Miyake et al., 2008), we next examined other cranial and cervical nerves by whole mount and identified aberrant development of trochlear, hypoglossal, and first cervical spinal (C1) nerve projections at E11.5.

In E11.5 wildtype embryos, trochlear axons project dorsally, cross the midline in the tectum, and then exit the brainstem and fasciculate into a single nerve bundle, which projects anteriorly toward the contralateral superior oblique muscle (SO) (Figure 3.2M, Movie 1). In E11.5 Chn1\textsuperscript{WT/KI} and Chn1\textsuperscript{KI/KI} embryos, trochlear axons cross the midline and exit dorsally as in wildtype, but the axons form multiple fascicles instead of one nerve bundle, most of which extend appropriately toward the contralateral orbit, but some of which turn back inappropriately toward the ipsilateral orbit (Figures 3.2N, 3.3D, Movie 2). The trochlear nerve in Chn1\textsuperscript{KO/KO}
embryos does not differ significantly from wildtype (Figure 3.2O and Movie 3); occasionally there is a small branch at the midline tectum that projects caudally (Figure 3.2O), which is also seen with a lower frequency in wildtype embryos.

The $Chn1^{Kl/Kl}$ hypoglossal nerve exhibits varying levels of rostral hypoplasia and appears thin at its terminus near the developing mouth compared to $Chn1^{WT/WT}$ (Figures 3.2P-3.2Q, Movies 1 and 2). Caudal to the hypoglossal, the first cervical segment of the spinal cord (C1) in $Chn1^{WT/Kl}$ and $Chn1^{Kl/Kl}$ exits the brainstem appropriately, but turns dorsally toward the dermomyotome with variable penetrance and sidedness (Figures 3.2Q and 3.3E). Hypoglossal and C1 guidance defects were not found in $Chn1^{KO/KO}$ embryos (Figure 3.2R, Movie 3). We examined the trajectories of additional cranial nerve populations in $Chn1^{Kl/Kl}$ and $Chn1^{KO/KO}$ E11.5 embryos and found no differences in the migrating olfactory placode, oculomotor, trigeminal, glossopharyngeal, and vagus nerves compared to wildtype (Figures 3.2D-3.2F, Movies 1-3).

Arterial formations coincide with $Chn1^{Kl/Kl}$ aberrant abducens and C1 nerve development

Interestingly, we noted background blood vessel autofluorescence intersecting the points at which the abducens nerve stalled and C1 projection aberrantly turned in $Chn1^{WT/Kl}$ and $Chn1^{Kl/Kl}$ embryos. Using a smooth muscle actin antibody to stain developing arteries, we confirmed that the abducens stalls at the internal carotid artery (ICA), whereas normally the $Chn1^{WT/WT}$ abducens nerve navigates a trajectory to narrowly pass the ICA (Figure 3.4A, 3.4B). Some exiting abducens projections in $Chn1^{Kl/Kl}$ embryos appear to be attracted to the basilar artery (BA), which is also seen with a low frequency in $Chn1^{WT/WT}$ embryos, and is accentuated in $Chn1^{KO/KO}$ embryos (Figure 3.4A-3.4C).
Figure 3.4: Arterial formations coincide with Chn1\textsuperscript{KI/KI} aberrant abducens and C1 nerve development.

(A) Chn1\textsuperscript{WT/WT} abducens nerve (VI) exits from the nucleus (nVI) lateral to the basilar artery (BA) and passes the internal carotid artery (ICA) on its trajectory to the extraocular muscle anlage (EOM).

(B) Chn1\textsuperscript{KI/KI} VI stalls at the ICA.

(C) Chn1\textsuperscript{KO/KO} VI; arrowhead: projections to the BA; arrow: projections to the posterior ICA.

(D-F) At E11.5, the first cervical nerve (C1) exits near the vertebral artery (VA) and projects along the VA/anterior spinal artery (ASA) junction to meet with the hypoglossal nerve (XII) in Chn1\textsuperscript{WT/WT} embryos (D), but turns laterally toward the dermomyotome in Chn1\textsuperscript{KI/KI} embryos (E). arrow: dorsolateral C1 projection at the VA/ASA junction. (F) Smooth muscle actin from (E); arrow: VA/ASA junction from (E).

Red: smooth muscle actin; green Hb9:GFP; scale: 100um; n\geq3 embryos from \geq3 independent litters.
Aberrant $Chn1^{KI/KI}$ C1 projections turn dorsolaterally toward the body wall where the vertebral artery (VA) intersects with the anterior spinal artery, whereas $Chn1^{WT/WT}$ C1 continues along VA to connect with the developing hypoglossal nerve (Figure 3.4D-3.4F). Anatomically, this raises the interesting possibility that the arterial system creates a unique microenvironment that alters the development of selective nerves.

**Facial nucleus migration is abnormal in $Chn1^{KI/KI}$, but major white matter tracts in adult brain are normal.**

We identified abnormal migration of the facial nucleus and exiting facial nerve within the hindbrain. In $Chn1^{WT/WT}$ E12.5 embryos, the facial nucleus exhibits a stereotypic caudal and lateral migration between rhombomere 4 to 6 that appears like a “J” in an open-book flat mount preparation (Figure 3.5A) (Garel et al., 2000). In $Chn1^{KI/KI}$ E12.5 embryos, the nucleus prematurely migrates laterally within rhombomeres 4 and 5, which does not yield the same “J” appearance (Figure 3.5B). In addition, a subset of axons does not exit the hindbrain appropriately and instead turns to project caudally within the hindbrain (Figure 3.5B). The facial nucleus and nerve of $Chn1^{KO/KO}$ embryos is normal (data not shown). Overall brain morphology and crossing of corpus callosum, anterior commissure, and hippocampal commissure in adult $Chn1^{KI/KI}$ mice are unremarkable (Figure 3.5C-3.5F).
Figure 3.5: Facial nucleus migration is abnormal in Chn1<sup>KI/KI</sup>; major white matter tracts in adult cortex are normal.

(A-B) Open book facial nucleus (nVII) at E12.5 from Chn1<sup>WT/WT</sup> (A) and Chn1<sup>KI/KI</sup> (B) embryos. White: Isl<sup>MNGFP</sup>; arrowheads: abnormal cell body migration within nVII; arrows: facial nerve axons which fail to exit and posteriorly project within the hindbrain; Chn1<sup>WT/WT</sup>: 2/16 (12.5%) and Chn1<sup>KI/KI</sup>: n= 8/14 (57.1%) embryos have abnormal migration; scale bar = 100um.

(C-D) Luxol Fast Blue (LFB) stained coronal brain sections at the level of corpus callosum (CC) and anterior commissure (AC) in Chn1<sup>WT/WT</sup> (C) and Chn1<sup>KI/KI</sup> (D) adult mice.

(E-F) LFB stained coronal brain sections at the level of the hippocampal commissure in Chn1<sup>WT/WT</sup> (E) and Chn1<sup>KI/KI</sup> (F) adult mice.

(C-F) n=3 mice ≥P60; scale bar = 1mm.
The Chn1\textsuperscript{KI/KI} oculomotor nerve aberrantly innervates the lateral rectus muscle

Two autopsies of individuals with DRS have shown loss of abducens motor neurons and nerve and traced an aberrant oculomotor nerve branch to the LR (Hotchkiss et al., 1980; Miller et al., 1982). Moreover, electromyographic studies have recorded co-contraction of the LR with the medial rectus muscle (MR) and/or the inferior and superior recti, suggesting a subpopulation of oculomotor axons instead innervate the LR (Huber, 1974). Thus, we crossed Chn1\textsuperscript{KI} mice to Isl\textsuperscript{MN}:GFP reporter mice, which permits visualization of all cranial motor neuron populations (Lewcock et al., 2007), and dissected E15.5 mouse orbits to characterize EOM innervation and determine whether the mouse model recapitulates this feature of the human disorder.

In wildtype embryos, the abducens nerve crosses the oculomotor nerve in close proximity to the LR prior to its innervation (Figure 3.6A). The abducens nerve is absent within the orbit in E15.5 Chn1\textsuperscript{KO/KO} embryos, consistent with stalling rather than delayed outgrowth, and thus fails to innervate the LR (Figure 3.6B). While the oculomotor nerve has a normal trajectory to the orbit (Figure 3.6D, 3.6E, Movies 1 and 2), within the orbit the LR is innervated by one or two aberrant oculomotor nerve branches that originate from oculomotor growth cone decision regions (Cheng et al., 2014): the proximal branch arises from the same position as the superior oculomotor nerve division while the distal branch arises from the inferior oculomotor nerve division at the position where it itself branches to innervate the MR, inferior rectus muscle (IR), and inferior oblique muscle (IO) (Figure 3.6B). Thus, the oculomotor nerve provides innervation to the LR in the absence of the abducens nerve, confirming human autopsy and electromyographic data. In Chn1\textsuperscript{KO/KO} embryos, the LR receives normal innervation from the abducens nerve and there is no stalling or aberrant branching of the oculomotor nerve (Figure 3.6C), thus α2-chimaerin is not necessary for final target EOM innervation.
Figure 3.6: Oculomotor nerve misinnervates lateral rectus and abducens motor neurons undergo apoptosis following abducens nerve stalling in Chn1\textsuperscript{KI/KI} mice.

(A-C) Innervation at extraocular muscles (EOM) in Chn1\textsuperscript{WT/WT} (A), Chn1\textsuperscript{KI/KI} (B), and Chn1\textsuperscript{KO/KO} (C) E15.5 embryos. Dotted line: VI, arrow: aberrant III branch from the division between superior and inferior III, arrowhead: aberrant III branch from inferior branching region; red: smooth muscle actin (extraocular muscles), green: transgenic Isl\textsuperscript{MN}:GFP; III: oculomotor; IV: trochlear; VI: abducens; LR: lateral rectus. Scale bar = 100um. N\geq7 orbits for each genotype. (D) Number of abducens neurons at E10.5 in Chn1\textsuperscript{WT/WT} and Chn1\textsuperscript{KI/KI} embryos (n= 3 embryos, p=0.231, unpaired, two-tailed t-test).

(E) Number of abducens neurons at E13.5 in Chn1\textsuperscript{WT/WT}:Bax\textsuperscript{WT/WT}, Chn1\textsuperscript{KI/KI}:Bax\textsuperscript{WT/WT}, Chn1\textsuperscript{WT/WT}:Bax\textsuperscript{KO/KO}, and Chn1\textsuperscript{KI/KI}:Bax\textsuperscript{KO/KO} embryos (n= 3 embryos each; ***p<0.001, one-way ANOVA with Tukey’s test).

(F) Representative images of Hb9:GFP abducens motor neurons at E13.5 in indicated genotypes. Red: Isl1 (facial and abducens motor neurons), green: transgenic Hb9:GFP (abducens motor neurons); scale bar = 50um.

(G) Number of combined oculomotor (III) and trochlear (IV) neurons at E13.5 in Chn1\textsuperscript{WT/WT}:Bax\textsuperscript{WT/WT}, Chn1\textsuperscript{KI/KI}:Bax\textsuperscript{WT/WT}, Chn1\textsuperscript{WT/WT}:Bax\textsuperscript{KO/KO}, and Chn1\textsuperscript{KI/KI}:Bax\textsuperscript{KO/KO} embryos (n= 3 embryos; n.s., one-way ANOVA with Tukey’s test).

(H) Whole mount neurofilament staining of E11.5 Chn1\textsuperscript{WT/WT}:Bax\textsuperscript{KO/KO} and Chn1\textsuperscript{KI/KI}:Bax\textsuperscript{KO/KO} embryos. Red: neurofilament, green: transgenic Hb9:GFP; arrow: abducens stalling.

(I) Abducens length measurements in whole mount Chn1\textsuperscript{WT/WT}:Bax\textsuperscript{KO/KO} and Chn1\textsuperscript{KI/KI}:Bax\textsuperscript{KO/KO} embryos (Chn1\textsuperscript{WT/WT}:Bax\textsuperscript{KO/KO}: n=16 nerves, 8 embryos; Chn1\textsuperscript{KI/KI}:Bax\textsuperscript{KO/KO}: n=12 nerves, 6 embryos; ***p=0.001, unpaired, two-tailed t-test).
Figure 3.6 (Continued)

Chn1 WT/WT Chn1 KI/KI Bax KO/KO

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Dotted line: VI, arrow: aberrant III branch from the division between superior and inferior III, arrowhead: aberrant III branch from inferior branching region; red: smooth muscle actin (extraocular muscles), green: Isl1MN:GFP; III: oculomotor; IV: trocheal; VI: abducens; LR: lateral rectus. Scale bar = 100um. N ≥ 7 orbits for each genotype.

(D) Number of abducens neurons at E10.5 in Chn1 WT/WT and Chn1 KI/KI embryos (n = 3 embryos, p = 0.231, unpaired, two-tailed t-test).

(E) Number of abducens neurons at E13.5 in Chn1 WT/WT: Bax WT/WT, Chn1 KI/KI: Bax WT/WT, Chn1 WT/WT: Bax KO/KO, and Chn1 KI/KI: Bax KO/KO embryos (n = 3 embryos each; ***p = 0.000, one-way ANOVA with Tukey’s test).

(F) Representative images of Hb9:GFP abducens motor neurons at E13.5 in indicated genotypes. Red: Isl1 (facial and abducens motor neurons), green: Hb9:GFP (abducens motor neurons); scale bar = 50um.


(H) Abducens length in whole mount Chn1 WT/WT: Bax KO/KO and Chn1 KI/KI: Bax KO/KO embryos (Chn1 WT/WT: Bax KO/KO: n = 16 nerves, 8 embryos; Chn1 KI/KI: Bax KO/KO: n = 12 nerves, 6 embryos; ***p = 0.001, unpaired, two-tailed t-test).

(I) Sholl analysis of Chn1 WT/WT, Chn1 WT/KI, and Chn1 KI/KI abducens outgrowth from E11.5 explants grown for 12 hours in 25 ng/mL BDNF, GDNF, and CNTF. Data represent mean ± SEM.
**Chn1\textsuperscript{KI/KI} abducens motor neurons undergo apoptosis following abducens nerve stalling**

Since the abducens nerve stalls and fails to innervate the LR in \textit{Chn1\textsuperscript{KI/KI}} mice, we predicted that abducens motor neurons would undergo subsequent apoptosis. Indeed, while the number of \textit{Chn1\textsuperscript{KI/KI}} abducens motor neurons was not significantly different than wildtype at E10.5 immediately prior to abducens stalling (Figure 3.6D), the number was greatly reduced compared to wildtype by E13.5, two days after the noted abducens phenotype (Figures 3.6E, 3.6F). We also counted oculomotor and trochlear neurons at E13.5. At E13.5, the trochlear nucleus has migrated to a position immediately caudal to oculomotor. There are no known markers that distinguish between the two populations, making them difficult to distinguish. Thus, we pooled counts between oculomotor and trochlear, and did not find a significant change in motor number in \textit{Chn1\textsuperscript{KI/KI}} embryos (Figure 3.6G).

To confirm that abducens stalling was primary to the subsequent cell death, we crossed \textit{Chn1\textsuperscript{KI}} mice to \textit{Bax\textsuperscript{KO/KO}} mice, thus inhibiting apoptosis during embryogenesis. We confirmed that abducens and oculomotor/trochlear motor neuron number is comparable to wildtype in E13.5 \textit{Chn1\textsuperscript{KI/KI}:Bax\textsuperscript{KO/KO}} embryos (Figures 3.6E-3.6G), and observed similar abducens nerve stalling in \textit{Chn1\textsuperscript{KI/KI}:Bax\textsuperscript{KO/KO}} and \textit{Chn1\textsuperscript{KI/KI}} embryos (Figures 3.6H, 3.6I, 3.2H). These data support primary abducens nerve stalling at E11.5, failure of LR target innervation, and subsequent secondary abducens motor neuron apoptosis by E13.5.

Surprisingly, we noted C1 misprojections in E11.5 \textit{Chn1\textsuperscript{WT/WT}:Bax\textsuperscript{KO}} embryos, identical to the aberrant C1 turning toward the dermomyotome found in \textit{Chn1\textsuperscript{WT/KI}} and \textit{Chn1\textsuperscript{KI/KI}} (Figure 3.7A-3.7C). Previously, we had never seen this phenotype in \textit{Chn1\textsuperscript{WT/WT}} embryos and could reliably genotype \textit{Chn1\textsuperscript{WT/KI}} and \textit{Chn1\textsuperscript{KI/KI}} embryos visually based on its appearance. We hypothesized that the phenotype arose from the \textit{Bax\textsuperscript{KO}} pure C57Bl/6 genetic background. We
purchased C57Bl/6 mice from Jackson Laboratory for timed matings and indeed found that intercrossed pure C57Bl/6 mice have a C1 turning phenotype in 50% of offspring (Figure 3.7A-3.7B, n=10/20 embryos). The C1 phenotype was predominantly unilateral; only one embryo exhibited bilateral abnormalities. When accounting for sidedness, 35% of embryos were affected on the left and 20% on the right side (Figure 3.7C). Instead of investigating the genetic cause of the phenotype, we backcrossed $Chn1^{KI}/Bax^{KO}$ mice to the 129S1/6 $Chn1^{KI}$ background for at least 2 generations and analyzed several whole mounts to ensure there was no wildtype C1 misprojection before analysis of the data presented in Figure 3.6. Abducens nerve length in wildtype C57Bl/6 mice is slightly but significantly increased compared to wildtype embryos from a mixed C57Bl/6 and 129S1/6 genetic background, however the C57Bl/6 embryos were approximately a half-day older than mixed background embryos (Figure 3.7D). Regardless, the C57Bl/6 background does not hinder abducens nerve growth.
Figure 3.7: Wildtype mice from a pure C57Bl/6 genetic background exhibit unilateral first cervical spinal nerve (C1) misprojections

(A-B) Wildtype E11.5 embryos from a C57Bl/6 genetic background with normal (A) and misprojected (B) C1 nerves. Arrows: C1 nerve.

(C) Quantification of lateralized C1 misprojections. N= 10/20 embryos (50%) are affected; 9/20 embryos have unilateral projections and 1/20 embryo has bilateral misprojections. 7/20 embryos (35%) display misprojections on the left, 4/20 embryos (20%) on the right. See Figure 3.2P.

(D) Abducens length quantification from E11.5 mixed background C57Bl/6:129S1/6 (B6/129) mice compared to E12 pure background C57Bl6 mice; See Figures 3.2G, 3.2K. *p<0.05 by unpaired, two-tailed t-test.

Data are from three independent litters and represent mean ± SEM.
**Chn1\textsuperscript{KI/KI} abducens explants display normal outgrowth at baseline**

Next, we asked whether abducens nerve stalling resulted from an inherent problem in abducens outgrowth or from an inappropriate response to the surrounding environment. Using the *Hb9:GFP* transgenic mouse line to visualize abducens motor neurons distinct from other surrounding motor neurons, we dissected and cultured abducens explants from E11.5 *Chn1\textsuperscript{WT/WT}, Chn1\textsuperscript{WT/KI}, and Chn1\textsuperscript{KI/KI}* embryos. We developed a modified Sholl analysis to analyze outgrowth and detected no outgrowth differences between wildtype and mutant explants grown in a cocktail of BDNF, GDNF, and CNTF or in the absence of growth factors (Figure 3.8A-3.8C, 3.8G). We live-imaged *Chn1\textsuperscript{WT/WT}, Chn1\textsuperscript{WT/KI}, and Chn1\textsuperscript{KI/KI}* explants and analyzed growth cone size, axonal growth distance, and axon displacement (Figure 3.8D-3.8F and 3.8H-3.8J). Growth cone area was slightly reduced in *Chn1\textsuperscript{KI/KI}* abducens explants grown in BDNF/GDNF/CNTF (Figure 3.8D), and reduced to a similar degree in *Chn1\textsuperscript{WT/KI}* explants grown in the absence of growth factors (Figure 3.8H), which could suggest that hyperactive α2-chimaerin generates minor changes in cytoskeletal regulation at baseline to reduce growth cone area.

Axon growth distance was unaltered in mutant explants of either growth condition compared to wildtype, however growth displacement was reduced in *Chn1\textsuperscript{KI/KI}* explants grown in BDNF/GDNF/CNTF. This could result from mutant explants taking a more tortuous path than wildtype explants over the same amount of time. The noted differences were minor and did not broadly alter outgrowth (Figure 3.8C, 3.8G), suggesting that abducens nerve stalling *in vivo* does not result from differences in intrinsic growth ability. Moreover, because wildtype α2-chimaerin exists as an autoinhibited protein until upstream activation (Brown et al., 2004; Colon-Gonzalez et al., 2008), these data also indicate that DRS-mutant α2-chimaerin is not constitutively active in a manner that dramatically alters intrinsic outgrowth properties *in vitro.*
Figure 3.8: Baseline outgrowth properties of Chn1<sup>KI</sup> abducens explants. 

(A-F) Chn1<sup>WT/WT</sup>, Chn1<sup>WT/KI</sup>, and Chn1<sup>KI/KI</sup> abducens explants cultured in 25ng/mL BDNF, 25ng/mL GDNF, and 25ng/mL CNTF (Chn1<sup>WT/WT</sup>: n= 7 explants; Chn1<sup>WT/KI</sup>: n= 4 explants; Chn1<sup>KI/KI</sup>: n= 5 explants; 3 independent experiments). Sholl analysis of Chn1<sup>WT/WT</sup> (A) and Chn1<sup>KI/KI</sup> (B) with standard explant threshold of Hb9:GFP–positive abducens neurites (red), 20um Sholl ring intervals (green), and counts of intersecting neurites (white numbers). (C) Sholl analysis output curve of number of intersecting neurites versus micron distance from explant body. (D) Growth cone area of Chn1<sup>WT/WT</sup> (n=184), Chn1<sup>WT/KI</sup> (n=17), and Chn1<sup>KI/KI</sup> (n=109); *p<0.05 by one-way ANOVA with Tukey’s test. (E) Growth distance in 30 minutes for Chn1<sup>WT/WT</sup> (n=75) and Chn1<sup>KI/KI</sup> (n=47) neurites. (F) Growth displacement in 30 minutes for the same Chn1<sup>WT/WT</sup> (n=75) and Chn1<sup>KI/KI</sup> (n=47) neurites; **p<0.01 by unpaired, two-tailed t-test. (G-J) Chn1<sup>WT/WT</sup> and Chn1<sup>WT/KI</sup> abducens explants cultured without growth factors in 50ng/mL FC (Chn1<sup>WT/WT</sup>: n=12 explants; Chn1<sup>WT/KI</sup>: n=15 explants; 3 independent experiments). (G) Sholl analysis output curve of number of intersecting neurites versus micron distance from explant body. (H) Growth cone area from Chn1<sup>WT/WT</sup> (n=89) and Chn1<sup>WT/KI</sup> (n=120) explants, *p<0.05 by unpaired, two-tailed t-test. (I) Growth distance in 30 minutes for Chn1<sup>WT/WT</sup> (n=14) and Chn1<sup>WT/KI</sup> (n=9) neurites. (J) Growth displacement in 30 minutes for the same Chn1<sup>WT/WT</sup> (n=14) and Chn1<sup>WT/KI</sup> (n=9) neurites.
**EphA4**KO/KO and Chn1KO/KO mice exhibit a similar abducens phenotype

We postulated that abducens stalling in Chn1KI/KI and wandering in Chn1KO/KO embryos may result from misregulated axon growth and guidance in response to an appropriate balance of attractive and repellent guidance cues surrounding the developing nerve (Figure 3.9A). There is strong in vivo and in vitro evidence that α2-chimaerin acts downstream of EphA4 in developing CST and spinal neurons to elicit growth cone collapse (Beg et al., 2007; Iwasato et al., 2007; Kao et al., 2015; Shi et al., 2007; Wegmeyer et al., 2007). CST and CPG neuronal tracts in both Chn1KO/KO and EphA4KO/KO mice aberrantly cross the spinal cord midline, as their axons lack responsiveness to ephrin midline repellents (Beg et al., 2007; Iwasato et al., 2007; Kullander et al., 2003; Kullander et al., 2001; Wegmeyer et al., 2007). Thus, we hypothesized that gain-of-function Chn1KI mutations could act downstream of EphA4 to further enhance growth cone collapse and increase repulsion to ephrins.

*EphA4* is expressed throughout rhombomere 5, where the abducens nucleus is located, during the time of abducens development (Garel et al., 2000). Using an open book hindbrain preparation at E11.5, we found that AP-tagged ephrin-A5 bound to the abducens nucleus, suggesting that an ephrin-A5 binding partner is expressed at the nucleus (Figure 3.9B, 3.9C). Interestingly, we found that EphA4 lines the hindbrain exit at E11.5 and is notably expressed in the EOM anlage at the point of contact with the abducens nerve (Figure 3.9D, 3.9E). Thus, EphA4 is expressed in an appropriate spatiotemporal manner to influence abducens development and guidance.
Figure 3.9: Ephrin-A5 binds to abducens nucleus; EphA4 is expressed at hindbrain exit and EOM.

(A) Model of wildtype attractant and repellent guidance cues surrounding developing abducens (VI) nerve. Left: hindbrain and black VI nucleus, right: black orbit and red EOM, left and right connected by VI nerve in black; green triangles: attractant cues; orange triangles: repellent cues.

(B-C) Open book preparation of Chn1<sup>WT/WT</sup>:Hb9:GFP E11.5 embryo; images from the same sample. Hb9:GFP abducens nucleus before (B) and after binding with ephrin-A5-AP lysate (C).

(D-E) Sagittal sections of Chn1<sup>WT/WT</sup>:Hb9:GFP E11.5 embryo stained with mouse anti-EphA4 (red) and DAPI; scale: 100um. (D) arrow: EphA4 (red) at hindbrain exit; (E) arrow: abducens (green) contacts EOM anlage at EphA4-expressing region (red).
We assessed growth cone collapse and axon retraction in response to several isoforms of ephrin-A and –B molecules in wildtype abducens explants in vitro. Abducens growth cones exhibit significant collapse upon bath application of recombinant ephrin-A5 and ephrin-B1 and a trend toward collapse with ephrin-B2 (Figure 3.10A). Similarly, abducens axon shafts significantly retracted within 30 minutes in response to the addition of ephrin-A5 and ephrin-B1, with a trend toward increased retraction in response to ephrin-B2 (Figure 3.10B).

We focused specifically on the involvement of EphA4 in ephrin-mediated repulsion because α2-chimaerin is reported to act downstream of EphA4 and not EphB isoforms (Kao et al., 2015; Shi et al., 2007). We crossed EphA4<sup>lox/lox</sup> (Herrmann et al., 2010) to EIIa-Cre mice (Lakso et al., 1996) to create a germline knockout of EphA4 (EphA4<sup>KO/KO</sup>, Figure 3.10C). We reasoned that if ephrin/EphA4 signaling is the main regulator of abducens development upstream of α2-chimaerin, we could expect EphA4<sup>KO/KO</sup> and Chn1<sup>KO/KO</sup> embryos to have a similar abducens phenotype. Indeed, whole mount neurofilament imaging of EphA4<sup>KO/KO</sup> embryos revealed strikingly similar abducens nerve wandering as Chn1<sup>KO/KO</sup> embryos (Figures 3.10D, 3.10E, 3.2I, and Movie 4). There were subtle phenotype differences, however, as the EphA4<sup>KO/KO</sup> abducens appeared less defasciculated than the Chn1<sup>KO/KO</sup> and aberrant fascicles tended to track with the mandibular and cervical branches of the facial nerve, rather than the buccal branch. These results confirm that EphA4 signaling is important for proper abducens nerve development and suggest it may act within an α2-chimaerin-mediated pathway.
Figure 3.10: EphA4 is required for normal abducens nerve development.

(A-B) Percentage of wildtype axons in abducens explants exhibiting growth cone collapse (A) and axon shaft retraction (B) after addition of ephrin-A5, ephrin-B1, or ephrin-B2. N ≥ 30 axons from ≥ 4 experiments; *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA with Tukey’s test.

(C) Western blot of EphA4KO:EIIaCre whole brain lysate from P0-P2 mice for EphA4 (top) and GAPDH control (bottom).

(D-E) Whole mount neurofilament staining in E11.5 EphA4WT/WT (D) and EphA4KO/KO (E) embryos. N = 5 embryos from ≥ 3 litters; arrow: misprojection with facial nerve; arrowhead: dorsal projections to hindbrain; red: neurofilament, green: Hb9::GFP.
**Adult Chn1\textsuperscript{KI/KI}:EphA4\textsuperscript{KO/KO} mice exhibit a significant reduction in hopping gait**

Given the similarities between the Chn1\textsuperscript{KO/KO} and EphA4\textsuperscript{KO/KO} phenotypes, we asked if crossing Chn1\textsuperscript{KI/KI} to EphA4\textsuperscript{KO/KO} mice could partially or fully reverse the Chn1\textsuperscript{KI/KI} and/or the EphA4\textsuperscript{KO/KO} phenotypes. We first compared the gaits of Chn1\textsuperscript{KI}, Chn1\textsuperscript{KO}, EphA4\textsuperscript{KO}, and intercrossed Chn1\textsuperscript{KI}:EphA4\textsuperscript{KO} mice. Chn1\textsuperscript{WT/KI} and Chn1\textsuperscript{KI/KI} mice have a normal alternating gait (Figures 3.11A, 3.11B, and Movie 5). By contrast, both Chn1\textsuperscript{KO/KO} and EphA4\textsuperscript{KO/KO} mice predominantly hop, with infrequent alternating steps (Figures 3.11A, 3.11B, and Movie 6). Remarkably, Chn1\textsuperscript{KI/KI}:EphA4\textsuperscript{KO/KO} adult mice predominantly use an alternating gait, with an infrequent hop within a sequence of steps (Movie 7), while Chn1\textsuperscript{WT/KI}:EphA4\textsuperscript{KO/KO} mice have an intermediate phenotype (Figures 3.11A, 3.11B, and Movie 8). Thus, hyperactivated α2-chimaerin reverses the EphA4\textsuperscript{KO} hopping gait in a dose-dependent manner.

**The Chn1\textsuperscript{KI} C1 phenotype is rescued while the Chn1\textsuperscript{KI} trochlear phenotype is unaltered in Chn1\textsuperscript{KI}:EphA4\textsuperscript{KO/KO} embryos**

We next evaluated the C1 and trochlear axon projections in Chn1\textsuperscript{KI/KI}:EphA4\textsuperscript{KO/KO} mice to determine whether loss of EphA4 modified the Chn1\textsuperscript{KI/KI} phenotypes. The C1 projection in EphA4\textsuperscript{KO/KO} embryos is normal (Figure 3.11C). Chn1\textsuperscript{WT/KI or KI/KI}:EphA4\textsuperscript{KO/KO} embryos also had normal C1 projections (Figure 3.11D) compared to Chn1\textsuperscript{WT/KI or KI/KI}:EphA4\textsuperscript{WT/WT} embryos, which exhibit C1 dorsal turning (Figure 3.2Q). Thus, the EphA4\textsuperscript{KO/KO} allele reverses the Chn1\textsuperscript{KI} C1 guidance defects.
Figure 3.11: Chn1$^{KI}$ reverses EphA4$^{KO}$ hopping gait, and EphA4$^{KO}$ rescues Chn1$^{KI}$ C1 misprojections but does not alter Chn1$^{KI}$ trochlear phenotype.

(A) Manually scored walking behavior of Chn1$^{KI}$:EphA4$^{KO}$ adult mice

$\text{Chn1}^{WT/WT}$:$\text{EphA4}^{WT/WT}$: n=16/17 mice (94.1%) alternate, n=1/17 mice (5.9%)

alternating/occasionally hop; Chn1$^{WT/KI}$:$\text{EphA4}^{WT/WT}$: n=14/14 mice (100%) alternate;

Chn1$^{KI/KI}$:$\text{EphA4}^{WT/WT}$: n=10/10 mice (100%) alternate; Chn1$^{KO/KO}$: n= 10/11 mice (90.9%)

hop, n= 1/11 mice (9.1%) hop/occasionally alternate; Chn1$^{WT/WT}$:$\text{EphA4}^{KO/KO}$: n= 9/11 mice (81.8%)

hop, n= 2/11 mice (18.2%) hop/occasionally alternate; Chn1$^{WT/KI}$:$\text{EphA4}^{KO/KO}$: n= 1/6 mice (16.7%)

alternate, n= 2/6 mice (33.3%) alternate/occasionally hop, n= 3/6 mice (50.0%) hop/occasionally alternate; Chn1$^{KI/KI}$:$\text{EphA4}^{KO/KO}$: n= 5/9 mice (55.6%)

alternate, n= 4/9 mice (44.4%) alternate/occasionally hop; $\chi^2=114.08$, p=0.000).

(B) DigiGait-generated score of alternating gait percentage on same mice as (A). *p<0.05,

**p<0.01, ***p<0.001, one-way ANOVA with Tukey’s test.

(C-D) C1 projections in E11.5 Chn1$^{WT/WT}$:$\text{EphA4}^{KO/KO}$ (C) and Chn1$^{KI/KI}$:$\text{EphA4}^{KO/KO}$ (D)

embryos.

(E-F) Transverse view of bilateral trochlear nerve in E11.5 Chn1$^{WT/WT}$:$\text{EphA4}^{KO/KO}$ (E) and

Chn1$^{KI/KI}$:$\text{EphA4}^{KO/KO}$ (F) whole mount embryos. Arrows: multiple trochlear branches.

(G) Quantification of trochlear branching for noted genotypes; n= 10 nerves, **p<0.01, one-way ANOVA with Tukey’s test. Red: neurofilament; green: Hb9:GFP.
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**Page 1**
\textit{EphA4}^{K0/K0} embryos also display normal trochlear branching and trajectory (Figure 3.11E). By contrast, however, \textit{Chn1}^{WT/KI}:\textit{EphA4}^{K0/K0} and \textit{Chn1}^{K1/K1}:\textit{EphA4}^{K0/K0} embryos have similar trochlear abnormalities as \textit{Chn1}^{WT/KI or K1/K1}:\textit{EphA4}^{WT/WT} controls (Figures 3.11F, 3.11G). Thus, unlike the C1 phenotype, the \textit{Chn1}^{K1} trochlear phenotype was largely unaltered by the \textit{EphA4}^{K0} allele.

\textit{Chn1}^{K1}:\textit{EphA4}^{K0/K0} embryos have normalized abducens nerve exit but increased nerve stalling compared to \textit{Chn1}^{K1}, and have abducens nerve wandering similar to \textit{EphA4}^{K0/K0}

We next asked whether the \textit{Chn1}^{K1} abducens nerve phenotype could be re-balanced in \textit{Chn1}^{WT/KI or K1/K1}:\textit{EphA4}^{K0/K0} embryos. We found that \textit{Chn1}^{WT/KI or K1/K1}:\textit{EphA4}^{K0/K0} embryos display normalized abducens exit and fasciculation within Area 1 (Figures 3.12A-3.12E, 3.2G), supporting EphA4-mediated repulsion upstream of \alpha 2-chimaerin as responsible for the abducens exit and fasciculation phenotypes in \textit{Chn1}^{K1} mice.

By contrast, stalling at Area 2 is worsened in \textit{Chn1}^{WT/KI}:\textit{EphA4}^{K0/K0} compared to \textit{Chn1}^{WT/KI}:\textit{EphA4}^{WT/WT} embryos (Figures 3.12A, 3.12B), reflected both in shortened abducens nerve length (Figure 3.12F) and thinner nerve diameter near the orbit (Figure 3.12G). Moreover, stalled projections seen in \textit{Chn1}^{WT/KI and K1/K1}:\textit{EphA4}^{K0/K0} embryos do not stay within normal wildtype nerve boundaries (Movie 9), but instead wander to track with the mandibular and cervical branches of the facial nerve, similar to the \textit{EphA4}^{K0/K0} (Figure 3.12I-3.12L).
Figure 3.12: EphA4 and α2-chimaerin have a complex genetic interaction in abducens nerve development.

(A-D) Whole mount staining of E11.5 VI nerves in Chn1WT/KI:EphA4WT/WT (A), Chn1WT/KI:EphA4KO/KO (B), Chn1KI/KI:EphA4WT/WT (C), and Chn1KI/KI:EphA4KO/KO (D) embryos. Arrowhead: exited VI nerve fiber counted in (E); white arrow: location of VI stalling, measured in (F); yellow arrow: location of VI nerve diameter at orbit, measured in (G); n=10 nerves for all genotypes.

(E) Number of VI nerve fibers at hindbrain exit, as indicated by arrow in (A-B).

(F) VI length from hindbrain exit to most distal fiber projection.

(G) VI nerve diameter at orbit.

(H) Number of wandering VI nerve fibers as measured from transverse images in (M-P). (E-H) *p<0.05, *p<0.01, *p<0.001, one-way ANOVA with Tukey’s test; (E-G) n=10 nerves; (H) n=8 nerves.

(I-L) VI fasciculation with mandibular and cervical branches of the facial nerve in E11.5 Chn1WT/WT:EphA4KO/KO (J) and Chn1KI/KI:EphA4KO/KO (L) embryos. Arrows: Hb9:GFP VI axons tracking with red (neurofilament) facial nerve.

(M-P) Transverse view of VI in E11.5 Chn1WT/WT:EphA4WT/WT (M), Chn1WT/WT:EphA4KO/KO (N), Chn1KI/KI:EphA4WT/WT (O), Chn1KI/KI:EphA4KO/KO (P) embryos. nVI: VI nucleus, O: orbit, arrows: VI wandering or stalling fibers.

Figure 3.12 (Continued)
Figure 3.13: Transverse abducens images from \textit{Chn1}^{\text{KI}:EphA4^{\text{KO}}} \text{E11.5} embryos. (A-D) Raw images of E11.5 transverse bilateral abducens nerve shown in Figure 3.12 from \textit{Chn1}^{\text{WT/WT}:EphA4^{\text{WT/WT}}} (A), \textit{Chn1}^{\text{WT/WT}:EphA4^{\text{KO/KO}}} (B), \textit{Chn1}^{\text{KI/KI}:EphA4^{\text{WT/WT}}} (C), and \textit{Chn1}^{\text{KI/KI}:EphA4^{\text{KO/KO}}} (D) embryos. nVI: abducens nucleus; dashed white line: abducens nerve; O: orbit; arrows: wandering abducens projections; green: \textit{Hb9}:GFP; scale bar: 100um.
There is a trend toward fewer wandering abducens fibers in $Chn1^{WT/KI}$ and $KI/KI:EphA4^{KO/KO}$ embryos compared to $EphA4^{KO/KO}$ (Figure 3.12H), presumably because of the enhanced nerve stalling. A transverse (top-down) view of abducens whole mount projections reveals wandering abducens projections in more detail (Figure 3.12M-3.12P, 3.13B-3.13E), clearly demonstrating the abducens nerve stalling and wandering phenotype in $Chn1^{KI/KI}:EphA4^{KO/KO}$ embryos (Figure 3.12P, 3.13E) is distinct from, but has features of, $EphA4^{KO/KO}$ abducens nerve wandering (Figure 3.12N, 3.13C) and $Chn1^{KI/KI}$ nerve stalling (Figure 3.12O, 3.13D).

Within Area 3 at E15.5, $Chn1^{WT/WT}:EphA4^{KO/KO}$ orbits display normal innervation of the LR by abducens (Figure 3.12Q), although occasionally the nerve appears to be slightly thinner than in $Chn1^{WT/WT}:EphA4^{WT/WT}$ embryos, with minor aberrant innervation of the LR from oculomotor (data not shown). $Chn1^{KI/KI}:EphA4^{KO/KO}$ innervation is indistinguishable from $Chn1^{KI/KI}:EphA4^{WT/WT}$ embryos; the abducens nerve fails to innervate the LR, which instead is innervated by aberrant branches of the oculomotor nerve (Figures 3.12R and 3.6B).

Thus, there appears to be a complex genetic interaction between $\alpha_2$-chimaerin and EphA4 driving the growth of the abducens nerve; some features of the abducens phenotype are rescued, some are worsened, and others are unaltered. These data support direct or indirect interactions between $\alpha_2$-chimaerin and EphA4 signaling pathways that alter development of the abducens nerve differently than each individual allele. Moreover, in combination, these findings demonstrate that different motor neuron groups may use $\alpha_2$-chimaerin in divergent Eph/ephrin and non-ephrin mediated pathways to drive features of the $Chn1^{KI}$ phenotype.
Abducens axons respond to ephrin forward and reverse signaling in wildtype explant cultures, and both responses are altered in $Chn1^{KI}$ explant cultures.

The complex genetic interaction between $\alpha$2-chimaerin and EphA4 in the abducens nerve led us to ask whether abducens neurons use EphA4 signaling in a manner different from other motor neuron populations, potentially contributing to its specific developmental vulnerability to human DRS - causing $CHN1$ mutations. In addition to canonical EphA4 repulsive forward signaling, EphA4 can serve as a ligand for ephrin reverse signaling. In limb motor neurons, EphA4 is expressed on mesenchymal cells, binds to ephrin-A expressed on neuronal growth cones and, in concert with GDNF/Ret/GFR$\alpha$1 signaling, mediates attraction toward the target (Bonanomi et al., 2012; Dudanova et al., 2012; Marquardt et al., 2005).

We hypothesized that abducens neurons use ephrin forward signaling to mediate repulsion from inappropriate targets and ephrin reverse signaling to generate attraction to the orbit, while other motor neuron populations use only repulsive ephrin forward signaling (C1), or do not use ephrin signaling as a guidance mechanism (trochlear). Since we had found robust abducens growth cone collapse and retraction in response to various ephrin isoforms in vitro (Figures 3.10A, 3.10B), we used the modified Sholl analysis approach to measure neurite outgrowth of motor neuron explants. Sholl analysis outgrowth curves revealed differences in initial outgrowth (average area under the curve from 0-200um bin of neurite distance from the explant), maximal outgrowth (average maximum neurite distance), and total outgrowth (average area under the entire curve) in experimental conditions (Figures 3.14A-3.14J, 3.15A-3.15P).

We performed control experiments to confirm the modified Sholl analysis approach (Figure 3.8) was sensitive enough to detect changes in abducens explant outgrowth (Figures 3.14A-3.14G, 3.14J). Previous studies have found cultured neurons from $\alpha$2-chimaerin knockout
mice lack growth cone collapse in response to ephrins (Beg et al., 2007; Iwasato et al., 2007; Shi et al., 2007; Wegmeyer et al., 2007). Thus, we cultured abducens explants from E11.5 Chn1$^{KO/KO}$ and EphA4$^{KO/KO}$ embryos in ephrin-A5 with GDNF to determine whether explants exhibited enhanced outgrowth compared to wildtype using the Sholl analysis approach. We added GDNF to cultures because abducens explants cultured in ephrin-A5 alone have no quantifiable outgrowth (Figure 3.14J). We find that Chn1$^{KO/KO}$ and EphA4$^{KO/KO}$ explants indeed have increased outgrowth in ephrin-A5/GDNF compared to wildtype explants (Figures 3.14A-3.14F). Furthermore, Chn1$^{KO/KO}$ and EphA4$^{KO/KO}$ have similar outgrowth to one another and exhibit no change compared to GDNF alone (Figure 3.14G). Thus, EphA4 is likely the main regulator of α2-chimaerin-mediated repulsive signaling to ephrin-A5 in abducens neurons.

We next used the modified Sholl analysis approach to measure neurite outgrowth of wildtype and mutant embryonic abducens, C1, trochlear, and oculomotor explants maintained in culture with GDNF alone, ephrin-A5 with GDNF, or EphA4 with GDNF. Cultured Chn1$^{WT/WT}$ and Chn1$^{WT/KI}$ abducens explants have similar levels of outgrowth in GDNF (Figure 3.14A). Chn1$^{WT/WT}$ explants grown in ephrin-A5/GDNF display significantly decreased maximal outgrowth compared to GDNF alone (Figures 3.14A, 3.14B, 3.14D), confirming abducens explants are repelled by ephrin-A5. Moreover, Chn1$^{WT/KI}$ explants display significantly less maximal and total explant outgrowth in ephrin-A5/GDNF than Chn1$^{WT/WT}$ explants (Figures 3.14B, 3.14D), supporting our hypothesis that gain-of-function α2-chimaerin mutations increase ephrin-A5-mediated repulsion through canonical ephrin forward signaling.
Figure 3.14: Chn1KO/KO and EphA4KO/KO abducens explants similarly lack reduced outgrowth in ephrin-A5/GDNF.

(A) Sholl analysis of Chn1WT/WT (blue) and Chn1KO/KO (red) abducens explants grown in 50ng/mL ephrin-A5 and 25mg/mL GDNF (Chn1WT/WT: n=11; Chn1KO/KO: n= 10; 4 experiments).

(B-C) Quantification of maximum abducens outgrowth (B) and total outgrowth (AUC) (C) from (A).

(D) Sholl analysis of EphA4WT/WT (blue) and EphA4KO/KO (purple) abducens explants grown in 50ng/mL ephrin-A5 and 25mg/mL GDNF (EphA4WT/WT: n=11; EphA4KO/KO: n= 13; 4 experiments).

(E-F) Quantification of maximum abducens outgrowth (E) and total outgrowth (AUC) (F) from (D).

(G-J) Sholl analysis of E11.5 abducens explants. Averaged Chn1WT/WT and EphA4WT/WT (blue), Chn1KO/KO, and EphA4KO/KO abducens explants grown in 50ng/mL FC and 25ng/mL GDNF (G), Chn1WT/WT and Chn1KO/KO abducens explants in 1ug/mL EphA4 and 25ng/mL GDNF (H), Chn1WT/WT and Chn1WT/KI in 1ug/mL EphA4 (I), and Chn1WT/WT and Chn1WT/KI in 50ng/mL ephrin-A5 (J).

(B-C, E-F) *p<0.05, **p<0.01, unpaired two-tailed t-test.
Figure 3.15: Chn1<sup>KI</sup> mutations alter ephrin forward and reverse signaling specifically in abducens neurons.

(A-C) Sholl analysis of Chn1<sup>WT/WT</sup> (blue) and Chn1<sup>WT/KI</sup> (green) abducens explants cultured in 50ng/mL FC with 25ng/mL GDNF (A), 50ng/mL ephrin-A5 with 25ng/mL GDNF (B), and 1ug/mL EphA4 with 25ng/mL GDNF (C).

(D) Quantification of (A-C): initial abducens outgrowth, measured by area under the curve (AUC) between 0-200um (top), average maximum outgrowth for abducens explants (middle), and total explant outgrowth, measured by entire AUC (bottom).

(E-G) Sholl analysis of Chn1<sup>WT/WT</sup> and Chn1<sup>WT/KI</sup> C1 explants cultured in 50ng/mL FC with 1ng/mL GDNF (E), 50ng/mL ephrin-A5 with 1ng/mL GDNF (F), and 1ug/mL EphA4 with 1ng/mL GDNF (G).

(H) Quantification of (E-G): initial C1 outgrowth (top), average maximum outgrowth (middle), and total explant outgrowth (bottom).

(I-K) Sholl analysis of Chn1<sup>WT/WT</sup> and Chn1<sup>WT/KI</sup> trochlear explants cultured in 50ng/mL FC with 25ng/mL GDNF (I), 50ng/mL ephrin-A5 with 25ng/mL GDNF (J), and 1ug/mL EphA4 with 25ng/mL GDNF (K).

(L) Quantification of (I-K): initial trochlear outgrowth (top), average maximum outgrowth (middle), and total explant outgrowth (bottom).

(M-O) Sholl analysis of Chn1<sup>WT/WT</sup> and Chn1<sup>WT/KI</sup> oculomotor explants cultured in 50ng/mL FC with 25ng/mL GDNF (M), 50ng/mL ephrin-A5 with 25ng/mL GDNF (N; †: p=0.082 between 140-340um bin, unpaired two-tailed t-test), and 1ug/mL EphA4 with 25ng/mL GDNF (O).

(P) Quantification of (M-O): initial oculomotor outgrowth (top), average maximum outgrowth (middle), and total explant outgrowth (bottom).

† p≤0.08, * p<0.05, ** p<0.01, *** p<0.001. Black asterisks: significant comparisons within Chn1<sup>WT/WT</sup> explant between three cues, red asterisks: comparisons within Chn1<sup>WT/KI</sup> between cues, one-way ANOVA with Dunnett’s post-hoc test. Blue asterisks: significant comparisons between Chn1<sup>WT/WT</sup> and Chn1<sup>WT/KI</sup> within each cue by unpaired two-tailed t-test. N≥7 explants from ≥3 experiments for each condition.
Figure 3.15 (Continued)
To investigate coincident GDNF/ephrin reverse signaling, we cultured abducens explants in EphA4 with GDNF. Chn1WT/WT abducens explants display significantly increased initial and total outgrowth in EphA4/GDNF compared to GDNF alone (Figures 3.15A, 3.15C, 3.15D), indicating that abducens neurons are capable of using coincident GDNF/ephrin reverse signaling to modulate axon attraction in vitro. Remarkably, Chn1WT/KI explants do not have this increased outgrowth in EphA4/GDNF, and instead have similar outgrowth as in GDNF alone (Figures 3.15C, 3.15D). These results indicate that either directly or indirectly, hyperactive α2-chimaerin is recruited downstream of ephrin reverse signaling to attenuate the normal increase in abducens outgrowth that is seen upon addition of EphA4/GDNF in wildtype abducens cultures. It appears that the noted effects could be indirectly regulated through another receptor, as wildtype abducens explants cultured in EphA4 alone have slightly increased outgrowth compared to explants cultured in FC, but Chn1WT/KI abducens explants maintain baseline levels of outgrowth (Figures 3.14I, 3.8G). Additionally, Chn1KO/KO abducens explants cultures grown in EphA4/GDNF do not differ from wildtype, suggesting that α2-chimaerin is not necessary to mediate the increased outgrowth downstream of EphA4/GDNF stimulation (Figure 3.14H). Regardless of the direct or indirect involvement of α2-chimaerin in ephrin/GDNF reverse signaling pathways, it appears that Chn1KI hyperactivity not only decreases outgrowth to ephrin-A5 through canonical ephrin forward signaling, but also reduces the increased outgrowth of abducens neurons to ephrin reverse signaling in vitro.

We cultured C1 explants to determine whether the complete C1 rescue in Chn1KI:EphA4KO/KO embryos was through ephrin forward and/or reverse signaling. Chn1WT/WT and Chn1WT/KI C1 explants have similar outgrowth in GDNF alone (Figure 3.15E). Chn1WT/WT explants have significantly reduced initial outgrowth in ephrin-A5/GDNF (Figures 3.15E, 3.15F, and
3.15H), supporting use of ephrin forward signaling in C1 neurons. *Chn1*<sup>WT/KI</sup> C1 explants grown in ephrin-A5/GDNF display a further reduction in initial, maximal, and total outgrowth compared to wildtype (Figures 3.15F, 3.15H). By contrast, C1 explant outgrowth in EphA4/GDNF does not differ between wildtype and mutant, or from GDNF alone (Figures 3.15E, 3.15G, 3.15H). Together, these data support ephrin forward but not reverse signaling in the guidance of C1 projections, and that aberrant C1 turning in *Chn1*<sup>KI</sup> embryos is mediated through ephrin forward signaling.

*Ex vivo* whole mount neurofilament staining revealed that the *Chn1*<sup>KI/KI</sup> trochlear phenotype was unaltered by the *EphA4*<sup>KO</sup> allele, and so we hypothesized that trochlear explants would respond to neither ephrin forward or reverse signaling. *Chn1*<sup>WT/WT</sup> and *Chn1*<sup>WT/KI</sup> trochlear explants cultured in GDNF alone do not have significantly different outgrowth (Figure 3.15I). In ephrin-A5/GDNF, *Chn1*<sup>WT/WT</sup> trochlear explants do not have altered outgrowth compared to GDNF alone (Figures 3.15I, 3.15J, and 3.15L). *Chn1*<sup>WT/KI</sup> explants have a modest, but significant, reduction in maximal and total outgrowth in ephrin-A5/GDNF compared to wildtype (Figures 3.15J, 3.15L). Thus, in the correct circumstances, hyperactive α2-chimaerin can be recruited downstream of ephrin forward signaling to alter trochlear outgrowth. Wildtype trochlear explants grown in EphA4/GDNF do not significantly differ from GDNF alone (Figures 3.15I, 3.15K, and 3.15L), however there is a slight but significant reduction in maximal explant outgrowth (Figure 3.15L). There is also no difference in outgrowth between *Chn1*<sup>WT/WT</sup> and *Chn1*<sup>WT/KI</sup> explants in EphA4/GDNF (Figures 3.15K and 3.15L). Overall, it appears that trochlear neurons do not predominantly use ephrin forward or reverse signaling, suggesting that α2-chimaerin acts downstream of non-ephrin-mediated pathways to modulate trochlear nerve development.

Lastly, we cultured oculomotor explants to define the contribution of ephrin forward and
reverse signaling in the final ocular cranial nerve nucleus. \( Chn1^{KI/KI} \) embryos have normal oculomotor nerve targeting to the EOM anlage, but aberrant misinnervation of the lateral rectus, which secondarily occurs upon loss of the abducens nerve and is not likely caused by mutant \( \alpha_2 \)-chimaerin (Park et al, under review, Appendices II and III). Individuals with DRS harboring CHN1 mutations have oculomotor nerve thinning by MRI, however we did not detect noticeable thinning by embryonic whole mount staining. Thus, similar to trochlear explants, we did not expect to see alterations in \( Chn1^{WT/KI} \) oculomotor explant outgrowth compared to \( Chn1^{WT/WT} \).

\( Chn1^{WT/WT} \) and \( Chn1^{WT/KI} \) oculomotor explants have similar outgrowth in GDNF (Figures 3.15M, 3.15P), which has similar features to the Sholl analysis curves of trochlear explants. However, unlike trochlear explants, \( Chn1^{WT/WT} \) explants have greatly reduced maximal and total outgrowth in ephrin-A5/GDNF (Figures 3.15M, 3.15N, 3.15P). Initial, maximal, and total outgrowth of \( Chn1^{WT/KI} \) oculomotor explants grown in ephrin-A5/GDNF does not significantly differ from wildtype, but there is a trend toward reduced outgrowth between 140-340um (Figure 3.15N, 3.15P). This suggests that, like trochlear neurons, \( Chn1^{WT/KI} \) can also alter the growth of oculomotor neurons under certain circumstances in vitro. \( Chn1^{WT/WT} \) and \( Chn1^{WT/KI} \) explants do not have altered outgrowth in EphA4/GDNF (Figures 3.15M, 3.15O, 3.15P). Thus, oculomotor explants use ephrin forward signaling but not ephrin reverse signaling in vitro. It is unclear in vivo how responsiveness to ephrin forward signaling impacts the growth of the oculomotor nerve, as we have not detected changes in oculomotor nerve targeting or EOM innervation in \( EphA4^{KO/KO} \) embryos (Figures 3.10E, 3.12Q), except for secondary misinnervation of the LR in instances of abducens thinning.
Neuronal and mesenchymal EphA4 conditional knockouts reveal abducens phenotypes that differ from each other and from EphA4\(^{\text{KO/KO}}\).

Our in vitro explant data support our hypothesis that the complex genetic interaction between α2-chimaerin and EphA4 in abducens could result, at least in part, from the recruitment of α2-chimaerin in both ephrin forward and reverse signaling. Thus, we hypothesized that ephrin forward and reverse signaling have an important role in abducens development. To investigate, we conditionally removed EphA4 selectively in motor neurons or in the surrounding mesenchyme to evaluate the outgrowth of abducens axons with loss of ephrin forward or reverse signaling, respectively.

First, we generated EphA4\(^{\text{flox/flox}}\).Hb9\(^{\text{Cre/+}}\) mice to knockout EphA4 in Hb9-expressing motor neurons. There was not a significant abducens phenotype in E11.5 EphA4\(^{\text{CKO/CKO}}\).Hb9\(^{\text{Cre/+}}\) embryos (Figure 3.16A, 3.16B), but we were unsure whether this resulted from an ineffective Cre-mediated excision of EphA4. We generated Chn1\(^{\text{KI/KI}}\).Hb9\(^{\text{Cre/+}}\) mice, which should knockout Chn1 in Hb9-expressing motor neurons to yield a Chn1\(^{\text{KO/KO}}\) phenotype. Resulting whole mount neurofilament staining instead revealed the Chn1\(^{\text{KI/KI}}\) phenotype (Figure 3.16C), and thus we do not find this Cre effectively or completely excises genes in abducens motor neurons.
Figure 3.16: \(Hb9^{Cre}\) does not effectively excise genes in abducens nucleus, leading to minimal \(EphA4^{floxflox}:Hb9^{Cre/+}\) abducens nerve phenotype.

(A-C) Whole mount embryo neurofilament staining of E11.5 \(EphA4^{WT/WT}:Hb9^{Cre/+}\) (A), \(EphA4^{floxflox}:Hb9^{Cre/+}\) (B), and \(Chn1^{Kl/Kl}:Hb9^{Cre/+}\) embryos (C). Arrowhead: slight abducens wandering; arrow: abducens nerve stalling.
Next, we generated \( \text{Chn1}^{\text{KI/KI}}:\text{Isl}^{\text{Cre/+}} \) mice, in which Cre is present in \( \text{Is} \)-expressing motor neurons and not the surrounding mesenchyme (Figure 3.17A), to test Cre activity in abducens neurons. In whole mount neurofilament stained \( \text{Chn1}^{\text{KI/KI}}:\text{Isl}^{\text{Cre/+}} \) embryos, we saw the anticipated \( \text{Chn1}^{\text{KO/KO}} \) abducens phenotype, confirming the \( \text{Isl}^{\text{Cre}} \) mouse line effectively knocks out proteins in abducens neurons (Figure 3.17B).

Next, to determine the effect of removing ephrin forward signaling, we generated \( \text{EphA4}^{\text{flx/flx}}:\text{Isl}^{\text{Cre/+}} \) mice. Compared to \( \text{EphA4}^{\text{KO/KO}} \) embryos that only have a significant increase in number of wandering fibers (Figures 3.10D-3.10E, 3.12E-3.12H), \( \text{EphA4}^{\text{flx/flx}}:\text{Isl}^{\text{Cre/+}} \) embryos have, in addition to a significant increase in number of wandering fibers, a significant increase in abducens nerve fibers following exit from the hindbrain, and significant reductions both in abducens nerve length and in abducens nerve diameter at the orbit (Figure 3.17C-3.17M, 3.12E-3.12G, Movie 10). Compared to \( \text{EphA4}^{\text{KO/KO}} \) embryos, the \( \text{EphA4}^{\text{flx/flx}}:\text{Isl}^{\text{Cre/+}} \) embryos also appear to have larger bundles of wandering abducens nerve fibers and increased nerve pausing at the midpoint decision within Area 2. As a result, fewer abducens axons reach the orbital region in the ephrin forward signaling specific knockout compared to the complete knockout (Figure 3.17K-3.17L). Thus, selectively removing ephrin forward signaling by knocking out \( \text{EphA4} \) in motor neurons results in a different abducens phenotype than the \( \text{EphA4}^{\text{KO/KO}} \). These data alone support a role for both ephrin forward and reverse signaling in abducens development.
Figure 3.17: EphA4\textsuperscript{flox/flox}:Is\textsuperscript{Cre/+} embryos have fewer abducens projections to the orbit, but similar abducens nerve wandering as EphA4\textsuperscript{K0/K0}.

(A) Neuron-specific Is\textsuperscript{Cre} expression (red) in E11.5 EphA4\textsuperscript{WT/WT}:Is\textsuperscript{Cre/+}:Ai14\textsuperscript{TdTomato}:Hb9:GFP reporter embryo.

(B) Chn1\textsuperscript{K0/K0}-like abducens phenotype in Chn1\textsuperscript{KI/KI}:Is\textsuperscript{Cre/+} E11.5 embryo.

(C-D) Sagittal view of E11.5 abducens nerve in EphA4\textsuperscript{WT/WT}:Is\textsuperscript{Cre/+} (C) and EphA4\textsuperscript{flox/flox}:Is\textsuperscript{Cre/+} (D) embryos. Arrow: wandering region within Area 2.

(E-H) Imaris surface-rendered (E and G) and raw (F and H) transverse view of bilateral E11.5 abducens nerves in EphA4\textsuperscript{WT/WT}:Is\textsuperscript{Cre/+} (E and F) and EphA4\textsuperscript{flox/flox}:Is\textsuperscript{Cre/+} (G and H) embryos. Arrow: wandering region within Area 2.

(I) Abducens fasciculation with mandibular and cervical facial nerve branches in EphA4\textsuperscript{flox/flox}:Is\textsuperscript{Cre/+} E11.5 embryo (arrow).

(J-M) Abducens number of exiting nerve fibers (J), length (K), diameter at orbit (L), and wandering nerve fibers (M).

**p<0.01, ***p<0.001, unpaired two-tailed t-test, data represent mean ± SEM; red: neurofilament, green: Hb9:GFP; scale bar = 100um; N≥5 embryos from ≥3 independent litters.
Figure 3.17 (Continued)

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Error Bars: +/- 1 SE

I. Number of nerve fibers at exit

J. Number of nerve fibers at exit

K. VI nerve length (um)

L. VI diameter at orbit (um)

M. Number of wandering fibers

** p < 0.01

*** p < 0.001
To directly investigate the role of ephrin reverse signaling in abducens development, we removed EphA4 from the mesenchyme by generating $EphA4^{flox/flox}:Pdgfrb^{Cre}$ mice. $Pdgfrb^{Cre}:Ai14^{TdTomato}$ reporter crosses revealed that $Pdgfrb^{Cre}$ is specifically expressed in the mesenchyme, however it covers approximately 60% of mesenchymal cells (Figure 3.18A, 3.18B). When we evaluated $EphA4^{flox/flox}:Pdgfrb^{Cre}$ abducens nerve development, we saw a partially penetrant nerve thinning and stalling phenotype compared to $EphA4^{WT/WT}:Pdgfrb^{Cre}$ controls (Figure 3.18C-E). Thus, we sought a more highly expressing mesenchyme-specific Cre line.

$Twist2^{Cre}$ is very broadly expressed in the mesenchyme through which the abducens grows at E11.5, but is not expressed in the brain, hindbrain, or spinal cord (Figure 3.19A). However, unbeknownst to us upon starting these experiments, the knock-in Cre allele at the $Twist2$ locus is approximately 14Mb from the $EphA4$ gene locus on Chromosome 1. Thus, it required 456 embryos to obtain 4 $EphA4^{flox/flox}:Twist2^{Cre+}$ embryos (one embryo was E10 and thus unable to be used for analysis).
Figure 3.18: $Pdgfrb^{Cre}$ has sparse mesenchymal-specific expression and leads to partially penetrant abducens stalling phenotype in $EphA4^{flox/flox} : Pdgfrb^{Cre}$ embryos.

(A-B) Wildtype $Pdgfrb^{Cre}:Ai14^{TdTomato}:Hb9:GFP$ reporter mice with red Cre expression, blue DAPI stain, and green $Hb9:GFP$-positive abducens nucleus (A) and nerve (B); arrow: abducens nerve.

(C-E) E11.5 whole mount staining of $EphA4^{WT/WT} : Pdgfrb^{Cre}$ (C), affected $EphA4^{CKO/CKO} : Pdgfrb^{Cre}$ (D), and unaffected $EphA4^{CKO/CKO} : Pdgfrb^{Cre}$ (E) embryos. Red and white: neurofilament; green: $Hb9:GFP$; scale bar: 100um.
In contrast to EphA4\(^{KO/KO}\) and EphA4\(^{flox/flox:Isl^{Cre/+}}\) embryos, EphA4\(^{flox/flox:Twist2^{Cre/+}}\) embryos exhibit complete abducens nerve stalling compared to littermate controls (Figure 3.18E-3.18H, 3.19C-3.19D, Movie 11). The abducens nerve in EphA4\(^{flox/flox:Twist2^{Cre/+}}\) embryos exits with significantly fewer nerve fibers (Figure 3.18I), which stall within the mesenchyme (Figure 3.18G-3.18H, 3.19C-3.19D). There is a dramatic reduction in abducens length and complete lack of abducens innervation near the orbit compared to EphA4\(^{KO/KO}\) or EphA4\(^{flox/flox:Isl^{Cre/+}}\) embryos (Figure 3.18J-3.18K, 3.12F-3.12G). While initial abducens nerve wandering within the mesenchyme in EphA4\(^{flox/flox:Twist2^{Cre/+}}\) embryos is similar to EphA4\(^{KO/KO}\) and EphA4\(^{flox/flox:Isl^{Cre/+}}\), axons stall and do not track with the facial nerve (Figure 3.18L, 3.12H, 3.19F). Thus, selective loss of ephrin reverse signaling results in an abducens phenotype that is very severe and has distinguishing features from EphA4\(^{KO/KO}\) and EphA4\(^{flox/flox:Isl^{Cre/+}}\) abducens nerve phenotypes.
Figure 3.19: EphA4\textsuperscript{floxflox}:Twist2\textsuperscript{Cre+} embryos display abducens stalling.
(A) Mesenchyme-specific Twist2\textsuperscript{Cre} expression (red) in E11.5 EphA4\textsuperscript{WT/WT}:Twist2\textsuperscript{Cre/+}:Ai14\textsuperscript{TdTomato}:Hb9:GFP reporter embryo.
(B-C) Sagittal view of abducens nerve in EphA4\textsuperscript{WT/WT}:Twist2\textsuperscript{Cre/+} (B) and EphA4\textsuperscript{floxflox}:Twist2\textsuperscript{Cre/+} (C) embryos. Arrows: stalling within Area 2.
(D-G) Imaris surface-rendered (D and F) and raw (E and G) transverse view of bilateral abducens nerves in EphA4\textsuperscript{WT/WT}:Isl\textsuperscript{Cre+} (D and E) and EphA4\textsuperscript{floxflox}:Isl\textsuperscript{Cre+} (F and G) embryos. Arrows: stalling within Area 2.
(H) Lack of abducens fasciculation with mandibular and cervical facial nerve branches in EphA4\textsuperscript{floxflox}:Twist2\textsuperscript{Cre+} E11.5 embryo.
(I-L) Abducens number of exiting nerve fibers (I), length (J), diameter at orbit (K), and wandering nerve fibers (L).

**p<0.01, ***p<0.001, unpaired two-tailed t-test, data represent mean ± SEM; red: neurofilament, green: Hb9:GFP; scale bar = 100um; N≥3 embryos from ≥3 independent litters.
Figure 3.19 (Continued)

<table>
<thead>
<tr>
<th>Treatment</th>
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<tr>
<td>EphA4^{WT/WT}:Twist2^{Cre/+}:Ai14^{tdTomato}</td>
<td>A</td>
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<td>EphA4^{WT/WT}:Twist2^{Cre/+}</td>
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<td>EphA4^{fl/fl}:Twist2^{Cre/+}</td>
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**Figure Legends:**

- **A**: Representative image of EphA4^{WT/WT}:Twist2^{Cre/+}:Ai14^{tdTomato}.
- **B**: EphA4^{WT/WT}:Twist2^{Cre/+}.
- **C**: EphA4^{fl/fl}:Twist2^{Cre/+}.
- **D**: EphA4^{WT/WT}:Twist2^{Cre/+}.
- **E**: EphA4^{fl/fl}:Twist2^{Cre/+}.
- **F**: EphA4^{WT/WT}:Twist2^{Cre/+}.
- **G**: EphA4^{fl/fl}:Twist2^{Cre/+}.

Additional panels include bar graphs showing:

- Number of nerve fibers at exit
- VI nerve length (µm)
- VI diameter at orbit (µm)
- Number of wandering fibers

Error Bars: +/- 1 SE
Discussion:

**Chn1^KI** mice reveal abducens guidance defect as a primary etiology of DRS

Neuronal pathfinding is essential for establishing functional circuits to elicit normal behavior. We report that *CHN1* mutations identified in human patients, when modeled in mouse, cause primary abducens nerve stalling within the mesenchyme, preventing the abducens nerve from contacting the LR. The oculomotor nerve sends aberrant branches to innervate the LR. Thus, axon guidance defects during early development can cause the lack of or aberrant innervation phenotypes seen in CCDDs. The *Chn1^KI* mouse provides a tool with which to further investigate cellular and circuit mechanisms underlying DRS and elucidate general principles of axon guidance that are relevant to human neurodevelopment.

**α2-chimaerin is critical for proper abducens guidance during embryonic development**

We identify that loss and gain of α2-chimaerin function alters abducens nerve guidance, proving that α2-chimaerin is essential for normal abducens development. It is interesting that removing or enhancing α2-chimaerin signaling in abducens neurons causes different nerve guidance phenotypes, as well as distinct phenotypes in different populations of motor neurons. *Chn1^KI* mice have altered abducens, C1, and trochlear projections, whereas *Chn1^KO* mice display a different abducens nerve guidance phenotype, normal C1 and trochlear projections, but altered gait circuitry and limb innervation (Beg et al., 2007; Iwasato et al., 2007; Kao et al., 2015; Wegmeyer et al., 2007). Thus, an increase or decrease in α2-chimaerin activity has unique consequences on motor neuron development, which may lend insight into its specific functions in different motor neuron populations. We also identify a novel and important role of EphA4 in abducens nerve guidance, and find a complex genetic interaction between *EphA4* and *Chn1* in
abducens nerve development.

**EphA4 forward and reverse signaling are both critical for proper abducens guidance during embryonic development**

Ephrin forward and reverse signaling has been characterized to guide projecting limb motor neurons (Bonanomi et al., 2012; Dudanova et al., 2012; Marquardt et al., 2005), and α2-chimaerin is known to act downstream of ephrin forward signaling in several neuronal types (Beg et al., 2007; Iwasato et al., 2007; Shi et al., 2007; Wegmeyer et al., 2007), including limb motor neurons (Kao et al., 2015). We expand upon these findings to define the involvement of ephrin forward and reverse signaling in abducens nerve development. We find that selectively removing ephrin forward signaling by knocking out EphA4 in motor neurons reduces successful LR targeting of the abducens nerve, as axons lose the appropriate level of repulsion from incorrect targets. Selectively removing ephrin reverse signaling by knocking out EphA4 in the mesenchyme causes severe abducens stalling, likely because the nerve is not properly recruited through the mesenchyme toward its target. Both of these abducens phenotypes are more severe than the complete EphA4^{K/O}; therefore disrupting the balance of attraction or repulsion alone during axon navigation can be more detrimental than interfering with both simultaneously.

**α2-chimaerin and EphA4 exhibit varied genetic interactions in different motor neuron populations**

We provide evidence that it is possible to restore the balance between hyperactive α2-chimaerin and upstream receptors to normalize gait behavior in EphA4^{K/O} mice. Removing EphA4 in the presence of hyperactivating α2-chimaerin mutations ameliorated the hopping gait
seen in EphA4KO/KO mice. It remains unclear whether corrected CST and/or CPG projections account for restored gait.

Intriguingly, abducens, trochlear, and C1 nerve phenotypes were reversed to varying degrees in Chn1KI:EphA4KO/KO mice, which our in vitro experiments demonstrate can be partially accounted for by the different recruitment of α2-chimaerin in ephrin forward and reverse signaling in these motor neuron types. Our data lends insight into potential mechanisms that generate selective vulnerability of abducens neurons to hyperactivating α2-chimaerin mutations (Figure 3.20). We propose three possibilities to account for the varying degrees of EphA4/α2-chimaerin genetic interaction in abducens, C1, trochlear, and CST/CPG neurons:

First, Chn1KI:EphA4KO/KO may rescue a Chn1KI phenotype when EphA is used only as a neuronal receptor for ephrin forward signaling to mediate repulsion, and not when it is used as a ligand in ephrin reverse signaling. CST and CPG neurons have been characterized to use ephrin forward signaling upstream of α2-chimaerin to mediate guidance (Asante et al., 2010; Beg et al., 2007; Iwasato et al., 2007; Shi et al., 2007; Wegmeyer et al., 2007). If future experiments identify that these neurons do not use ephrin reverse signaling to modulate guidance, similar to our findings in C1 neurons, Chn1KI:EphA4KO/KO phenotype rescue may occur specifically in neuronal types that re-balance EphA receptor signaling through α2-chimaerin, but not in cell types that simultaneously use both ephrin forward and reverse signaling.
Figure 3.20: Models of α2-chimaerin signaling downstream of ephrin forward and reverse signaling in abducens neurons. In ephrin forward signaling (left), hyperactive α2-chimaerin acts downstream of EphA4 to enhance repulsion in response to ephrin ligands. In ephrin reverse signaling with GDNF (middle), hyperactive α2-chimaerin may act directly or indirectly downstream of ephrin/Ret/GFRα1 to attenuate the normal increase in attraction in response to GDNF and EphA4 ligands. Alternatively, a competing ephrin reverse signaling pathway could exist (right), in which α2-chimaerin acts downstream of a p75NTR or another co-receptor to induce axon repulsion. Hyperactivated α2-chimaerin may act within this potential pathway to increase repulsion in response to EphA4 ligands.
Second, receptor composition in different neuronal types may mediate integration of different ligands that act through α2-chimaerin to yield different phenotypes. We show that abducens neurons use ephrin reverse signaling in vitro to mediate increased outgrowth in concert with GDNF, similar to reports in limb motor neurons (Bonanomi et al., 2012). If ephrin-A signals via Ret in concert with GDNF/GFRα1/Ret in abducens neurons to mediate potentiated outgrowth, hyperactive α2-chimaerin could misregulate downstream signaling pathways to dampen increased outgrowth; further experiments would be necessary to investigate such co-receptor signaling mechanisms. In retinal ganglion cells, ephrin-A uses p75NTR as a co-receptor to mediate axon repulsion (Lim et al., 2008). If abducens neurons use both p75NTR and GFRα1/Ret as co-receptors to modulate ephrin reverse signaling, thus combining elements of RGC and limb motor neuron axon guidance, this could account for an additional layer of phenotype specificity.

Third, expression of different cytoskeletal regulatory pathways (i.e. RacGAPs/GEFs) in specific motor neuron populations may modulate the signaling contribution of α2-chimaerin. Our finding that Chn1KO/KO and EphA4KO/KO abducens nerve wandering and fasciculation phenotypes are slightly different supports the possibility that not only could α2-chimaerin act downstream of another receptor(s), but EphA4 could signal through other cytoskeletal regulatory proteins in addition to α2-chimaerin. The varied expression and recruitment of diverse RacGAP/GEF pathways in different motor neuron populations may further influence phenotype rescue and selective vulnerability.

**Mutant α2-chimaerin informs the complexity of axon guidance signaling pathways in neuronal development**

Our data highlight the importance of investigating molecular mechanisms in a cell-type
specific manner. Despite the technical challenges posed by the few hundred abducens motor neurons that project to a discrete muscle target, we were able to combine in vivo genetic interaction studies and motor neuron subtype specific in vitro cultures to assess the implications of altered signaling pathways on their development. Moreover, we have found that even this simple neuronal circuit uses complex guidance pathways to establish connectivity, which can serve as a model from which to investigate the breadth of signaling complexity that exists between various guidance receptors during development. Particularly, by using a gain-of-function DRS-causing mutation in α2-chimaerin, we have identified the importance of signaling balance in neuronal circuit formation. In our experimental paradigm, α2-chimaerin loss-of-function does not appear to perturb ocular motor circuit formation nearly as much as altered function, highlighting the sensitivity of neuronal guidance mechanisms and the importance of investigating physiologic gain-of-function mutations in neurological disorders. Thus, our investigation into the effects of hyperactivated α2-chimaerin on abducens and motor neuron development not only provides insight into DRS etiology and selective vulnerability, but also informs broad principles that guide neuronal circuit formation.
Acknowledgements

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References


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CHAPTER 4

Neurotrophic factors regulate abducens outgrowth through hyperactive α2-chimaerin-mediated pathways
Contributions

Alicia A. Nugent and Elizabeth C. Engle designed experiments. A.A.N. performed most experiments and all formal analysis. Yan Wei conducted whole mount embryo staining for Figures 4.3 and 4.4 and A.A.N. analyzed resulting data. Jong G. Park dissected and imaged orbital innervation presented in Figure 4.4.
Abstract

Ephrin bidirectional signaling acts upstream of α2-chimaerin to regulate abducens nerve development. However, it remains unclear whether additional pathways also impact abducens nerve development via α2-chimaerin mediated pathways. We find that E11.5 Chn1KO/KO:EphA4KO/KO whole mount embryos exhibit a more severe abducens nerve phenotype than either Chn1KO/KO or EphA4KO/KO embryos, demonstrating that α2-chimaerin and/or EphA4 also act through other pathways to affect abducens development. We use a combination of in vivo mouse crosses and in vitro abducens Sholl analyses to investigate whether non-ephrin guidance pathways and downstream effectors of ephrin signaling interact with hyperactive α2-chimaerin to modulate abducens development. Candidate-based in vivo screens indicate that Sema3A loss-of-function mutations and knockout of Ephexin5 does not alter abducens nerve guidance and does not modify the Chn1KI abducens stalling phenotype. However, in vitro abducens explant Sholl analyses identify mutant α2-chimaerin as a broad regulator of abducens outgrowth downstream of several growth factor pathways, including BDNF, GDNF, HGF, and NGF signaling.
Introduction

α2-chimaerin is widely expressed in many neuronal cell types during development and remains expressed in cortical areas with high levels of plasticity (Hall et al., 2001). Thus, it is remarkable that individuals with hyperactivating mutations in α2-chimaerin do not have additional neurological deficits beyond abnormalities in eye movement. In Chapter 3, we implicated ephrin bidirectional signaling in the activation of α2-chimaerin in abducens neurons. Although Chn1KO/KO and EphA4KO/KO abducens nerve wandering is very similar, the mutant mice do have slightly different phenotypes. This led us to ask if additional growth and guidance pathways both upstream and downstream of α2-chimaerin might affect its activity in abducens neurons; these in turn may generate an additional layer of signaling specificity to permit cell-type specific phenotypes.

Features of the α2-chimaerin structure support a more widespread role in signaling. The C1 domain permits activation through DAG-mediated signaling pathways, creating a mechanism for α2-chimaerin to regulate cytoskeletal dynamics (Colon-Gonzalez et al., 2008). The SH2 domain enables binding to phosphorylated receptor tyrosine kinases and other interacting proteins such as CRMP2 and Cdk5 (Beg et al., 2007; Ip et al., 2012; Qi et al., 2004; Shi et al., 2007). Upon release from autoinhibition via either C1 or SH2 domain-mediated mechanisms, the RacGAP domain is available to covert Rac-GTP to Rac-GDP to alter actin dynamics (Colon-Gonzalez et al., 2008; Ip et al., 2012; Iwasato et al., 2007; Kozma et al., 1996; Shi et al., 2007; Wegmeyer et al., 2007). Thus, α2-chimaerin could potentially interact with many different upstream receptors and downstream pathways involved in axon growth and guidance.

These structural features may also help regulate α2-chimaerin specificity. DAG-mediated pathways localize the C1 domain next to active Rac, thus restricting the activity of α2-chimaerin...
spatially within the cell (Colon-Gonzalez et al., 2008). Either α2-chimaerin itself or other interacting proteins may restrict the signaling implications of SH2 or RacGAP domain-mediated pathways, as altering residues on the SH2 domain, but not removing the RacGAP domain, affected cortical neuronal migration (Ip et al., 2012). Additionally, the selective expression of other Rho-GTPases could attenuate the downstream signaling implications of α2-chimaerin’s RacGAP domain.

In addition to EphA4, several other receptor-ligand pairs have been implicated upstream of α2-chimaerin. As mentioned in Chapter 1, overexpressing DRS-causing Chn1 mutations in the developing chick oculomotor nucleus causes nerve stalling and branching defects, which can be rescued by simultaneously knocking down PlexinA1 or A2 (Ferrario et al., 2012). In developing mouse cortex, knock-down of α2-chimaerin causes cortical neuron migration defects through a BDNF/TrkB mediated pathway (Ip et al., 2012). In addition, studies have implicated NGF and EGF signaling upstream of α2-chimaerin (Colon-Gonzalez et al., 2008; Mizuno et al., 2004).

In this chapter, we first determine that \(Chn1^{KO/KO}:EphA4^{KO/KO}\) embryos have a modified, and worsened, abducens nerve phenotype compared to embryos from either independent knockout, thus confirming that other pathways interact with α2-chimaerin and/or EphA4 to alter abducens nerve development. Next, we investigate several candidate signaling pathways to determine if they, too, regulate the activity or downstream effects of α2-chimaerin in abducens development. Similar to the approaches from Chapter 3, we use a combination of \textit{ex vivo} whole mount embryo staining and \textit{in vitro} Sholl analyses to investigate potential interactions of additional pathways with hyperactive α2-chimaerin. We find that Sema3A and Ephexin5 do not alter abducens nerve growth or modify the \(Chn1^{KI/KI}\) abducens nerve phenotype \textit{in vivo}. \textit{In vitro},
we find that certain concentrations of GDNF, BDNF, HGF, and NGF alter \(Chn1^{WT/KI}\) abducens explant outgrowth compared to wildtype explants, suggesting these are appropriate candidates for further \textit{in vivo} studies. Furthermore, we establish that there is specificity of \(\alpha_2\)-chimaerin signaling in abducens neurons, as several candidate pathways do not modify the effects of hyperactive \(\alpha_2\)-chimaerin \textit{in vivo}. We provide \textit{in vitro} data to support the interaction of hyperactivated \(\alpha_2\)-chimaerin with pathways beyond ephrin bidirectional signaling to impact \(Chn1^{KI}\) abducens nerve development.
Results

*Chn1\(^{KO/KO}\):EphA4\(^{KO/KO}\)* double mutant embryos have reduced abducens nerve diameter compared to *Chn1\(^{KO/KO}\)* and *EphA4\(^{KO/KO}\)* single mutants.

In Chapter 3, we found *Chn1\(^{KO/KO}\)* and *EphA4\(^{KO/KO}\)* embryos exhibited similar abducens phenotypes (Figures 3.2I, 3.10E), however, there were a few notable differences. *Chn1\(^{KO/KO}\)* embryos exhibited an increase in abducens nerve defasciculation compared to *EphA4\(^{KO/KO}\)* embryos, as measured by the number of abducens fibers at the hindbrain exit and nerve diameter at the orbit (Figures 3.2J, 3.2L, 3.12E, 3.12G). Wandering *Chn1\(^{KO/KO}\)* projections tended to track with the buccal branch of the facial nerve (Figure 3.2I), whereas wandering *EphA4\(^{KO/KO}\)* projections tended to track with the mandibular and cervical branches of the facial nerve (Figure 3.12E). In both mutants, a subset of abducens nerve fibers always reached the EOM anlage, resulting in a normal abducens nerve length (Figure 3.2K, 3.12F).

We intercrossed *Chn1\(^{KO/KO}\)* and *EphA4\(^{KO/KO}\)* mice and find that *Chn1\(^{KO/KO}\):EphA4\(^{KO/KO}\)* embryos display reduced nerve diameter at the orbit, but not reduced length, compared to littermate control *Chn1\(^{WT/KO}\):EphA4\(^{WT/KO}\)*, *Chn1\(^{KO/KO}\):EphA4\(^{WT/KO}\)*, and *Chn1\(^{WT/KO}\):EphA4\(^{KO/KO}\)* embryos (Figure 4.1A-4.1F), as well as compared to *Chn1\(^{KO/KO}\)* and *EphA4\(^{KO/KO}\)* embryos (Figure 3.2L, 3.12G). Haploinsufficiency of the *Chn1* or *EphA4* allele on a complete knockout background of the opposing allele (i.e. *Chn1\(^{KO/KO}\):EphA4\(^{WT/KO}\) versus *Chn1\(^{KO/KO}\)* and *Chn1\(^{WT/KO}\):EphA4\(^{KO/KO}\) versus *EphA4\(^{KO/KO}\)*) does not alter the abducens nerve length or nerve diameter at the orbit (Figures 4.1A-4.1C, 4.1E-4.1F, 3.2K-3.2L, 3.12F-3.12G).
Figure 4.1: *Chn1<sup>KO/KO</sup>:EphA4<sup>KO/KO</sup>* embryos exhibit a reduction in abducens nerve fiber projections to the orbit compared to *Chn1<sup>KO/KO</sup>* and *EphA4<sup>KO/KO</sup>* embryos.

(A-D) Sagittal view of abducens nerve in E11.5 *Chn1<sup>WT/KO</sup>:EphA4<sup>WT/KO</sup>* (A), *Chn1<sup>KO/KO</sup>:EphA4<sup>WT/KO</sup>* (B), *Chn1<sup>WT/KO</sup>:EphA4<sup>KO/KO</sup>* (C), and *Chn1<sup>KO/KO</sup>:EphA4<sup>KO/KO</sup>* (D) whole mount embryos. Arrows: wandering abducens nerve fibers; arrowhead: pausing and exploration at the internal carotid artery.

(E-G) Quantification of abducens nerve length (E), diameter at the orbit (F), and wandering (G). N≥3 embryos from ≥3 independent litters; data represent mean ± SEM; *p<0.05, ** p<0.01, ***p<0.001, n.s.: not significant by one-way ANOVA with Dunnett’s test.

(H-K) Transverse Imaris surface-rendered images of bilateral abducens nerve in E11.5 *Chn1<sup>WT/KO</sup>:EphA4<sup>WT/KO</sup>* (H), *Chn1<sup>KO/KO</sup>:EphA4<sup>WT/KO</sup>* (I), *Chn1<sup>WT/KO</sup>:EphA4<sup>KO/KO</sup>* (J), and *Chn1<sup>KO/KO</sup>:EphA4<sup>KO/KO</sup>* (K) embryos. O: orbit, nVI: abducens nucleus; arrows: wandering abducens nerve fibers.

Scale bar: 100um; red: neurofilament, green: *Hb9:GFP*. 
Figure 4.1 (Continued)

- **A, C**: Images of EphA4<sup>WT/WT</sup> and EphA4<sup>KO/KO</sup>
- **B, D**: Images of Chn1<sup>WT/WT</sup> and Chn1<sup>KO/KO</sup>

**E**: Bar graph showing Abducens length (um) for different conditions:
- **WT/WT**: Green bars
- **KO/KO**: Black bars
- Error Bars: +/- 1 SE

**F**: Bar graph showing Abducens diameter (um) for different conditions:
- **WT/WT**: Green bars
- **KO/KO**: Black bars
- Error Bars: +/- 1 SE

**G**: Bar graph showing Number of wandering fibers for different conditions:
- **WT/WT**: Green bars
- **KO/KO**: Black bars
- Error Bars: +/- 1 SE

**H-J**: Additional images illustrating nVI and nVI for EphA4<sup>WT/WT</sup> and EphA4<sup>KO/KO</sup> conditions.
Figure 4.2: *Chn1^{KO/KO}:EphA4^{KO/KO}* embryos exhibit a slight increase in nerve wandering and exploration around the internal carotid artery compared to *Chn1^{KO/KO}* and *EphA4^{KO/KO}* embryos.

(A-D) Transverse raw images of bilateral abducens nerve in E11.5 *Chn1^{WT/KO}:EphA4^{WT/KO}* (A), *Chn1^{KO/KO}:EphA4^{WT/KO}* (B), *Chn1^{WT/KO}:EphA4^{KO/KO}* (C), and *Chn1^{KO/KO}:EphA4^{KO/KO}* (D) embryos. O: orbit, nVI: abducens nucleus; arrows: wandering abducens nerve fibers. Scale bar: 100um; red: neurofilament, green: *Hb9:GFP*. 
Abducens nerve wandering is significantly enhanced in $Chn1^{KO/KO}:EphA4^{KO/KO}$ embryos compared to $Chn1^{WT/KO}:EphA4^{WT/KO}$, and is slightly but not significantly enhanced compared to $Chn1^{KO/KO}:EphA4^{WT/KO}$ and $Chn1^{WT/KO}:EphA4^{KO/KO}$ embryos (Figure 4.1G-4.1K, 4.2A-4.2D). The abducens nerve in $Chn1^{KO/KO}:EphA4^{KO/KO}$ displays increased wandering, pausing, and exploration around the internal carotid artery and in the Z-direction, which may account for the similar nerve length, despite reduced targeting to the EOM anlage (Figures 4.1D, 4.1K, 4.2D). Thus, combining $Chn1^{KO/KO}$ and $EphA4^{KO/KO}$ alleles does not yield a dominant pathway; rather the resulting $Chn1^{KO/KO}:EphA4^{KO/KO}$ abducens nerve phenotype is different than either independent knockout allele, supporting additional pathways downstream of $\alpha 2$-chimaerin and/or EphA4 that modulate abducens nerve development.

*Sema3A loss-of-function does not alter abducens development, nor modify the Chn1$^{KI}$ phenotype.*

$\alpha 2$-chimaerin has been characterized to act downstream of semaphorin signaling in chick oculomotor development (Ferrario et al., 2012) and in dorsal root ganglion neurons (Brown et al., 2004). Therefore, we postulated that misregulated abducens growth and guidance in response to Sema3A surrounding the developing nerve could contribute to the $Chn1^{KI/KI}$ and $Chn1^{KO/KO}$ phenotypes. We first tested if wildtype abducens motor neuron explants responded to several isoforms of semaphorin3 *in vitro*. We cultured abducens explants using the approach outlined in Chapter 3 (Figures 3.10A-B), then live imaged growth cone dynamics after addition of recombinant Sema3A, 3C, or 3F. We found abducens growth cones transiently collapsed to Sema3A, but rebounded within 30 minutes such that there was no significant axon retraction or growth cone collapse (Figures 4.3A, 4.3B). This is similar to the response previously reported in
mouse embryonic stem cell-derived Hb9:GFP spinal motor neurons (Nedelec et al., 2012).
Abducens axons did not significantly collapse or display axon retraction in response to Sema3C or 3F (Figures 4.3A, 4.3B).

Transient collapse to Sema3A in vitro could have a meaningful guidance impact in vivo. Thus, we crossed Chn1KI mice to Sema3A^K108N loss of function mutant mice (Merte et al., 2010) to examine the genetic interaction between these pathways in mouse abducens nerve development. Sema3A^K108N/K108N mice did not exhibit altered abducens nerve development alone, nor did the mutant modify the Chn1KI/KI phenotype (Figures 4.3C-4.3F). Interestingly, the genetic background of the Sema3A^K108N line did alter penetrance of the Chn1KI phenotype. Chn1KI/KI mice crossed to the Sema3A background, whether harboring wildtype Sema3A or the K108N substitution, do not display abducens nerve stalling, as all genotypes have similar abducens length measurements (Figures 4.3E, 4.3F, 4.3L). Chn1WT/KI and KI/KI:SemaWT/WT and Chn1WT/KI and KI/KI:SemaK108N/K108N embryos did, however, have a significant reduction in abducens nerve diameter at the orbit compared to Chn1WT/WT:SemaWT/WT and Chn1WT/WT:SemaK108N/K108N controls (Figures 4.3E, 4.3F, 4.3K) comparable to the original, more penetrant, Chn1KI genetic background (Figure 3.L).
Figure 4.3: Lack of significant genetic interaction between hyperactive α2-chimaerin and Sema3A in mouse abducens and trigeminal cranial nerve development.

(A-B) Percentage of wildtype abducens (VI) axons exhibiting growth cone collapse (A) and axon shaft retraction (B) after addition of Sema3A, Sema3C, or Sema3F. N≥30 axons from ≥4 experiments.

(C-F) Representative images of VI in whole mount embryos from noted genotypes. The genetic background of the Sema3A\textsuperscript{K108N} line alters the penetrance of the Chn1\textsuperscript{KI} abducens nerve stalling phenotype, but not the penetrance of the reduced number of fibers at exit from the hindbrain or reduced diameter at area 3. Arrow: VI nerve thinning in Chn1\textsuperscript{KI/KI}:Sema\textsuperscript{WT/WT} (D) and Chn1\textsuperscript{KI/KI}:Sema\textsuperscript{K108N/K108N} (F).

(G-J) Trigeminal nerve whole mount staining in noted genotypes. Sema3A\textsuperscript{K108N/K108N} embryonic mice (I) are confirmed to have exuberant branching of the trigeminal nerve and DRG projections (Merte et al., 2010). The trigeminal phenotype is unaltered in Chn1\textsuperscript{KI/KI}:Sema3A\textsuperscript{K108N/K108N} double mutants (J); red arrowhead: ophthalmic V exuberant branching.

(K-L) E11.5 VI nerve diameter (K) and length (L) in noted Chn1\textsuperscript{KI}:Sema\textsuperscript{K108N} genotypes. Chn1\textsuperscript{WT/KI} and KI/KI:Sema3A\textsuperscript{WT/WT} and Chn1\textsuperscript{WT/KI} and KI/KI:Sema3A\textsuperscript{K108N/K108N} embryos have a significant reduction in abducens nerve diameter at the orbit compared to Chn1\textsuperscript{WT/WT}:Sema3A\textsuperscript{WT/WT} and Chn1\textsuperscript{WT/WT}:Sema3A\textsuperscript{K108N/K108N} controls (K). Chn1\textsuperscript{KI/KI} mice, whether harboring wildtype Sema3A or the K108N substitution, do not display abducens nerve stalling (L). N≥4 embryos from ≥3 independent litters. Data represent mean ± SEM; *p<0.05, ***p<0.001, n.s.: not significant by one-way ANOVA with Tukey’s test. Scale bar: 100um; red, black and white images: neurofilament, green: Hb9::GFP.
Figure 4.3 (Continued)

**A** Percent growth cone collapse

**B** Percent axon retraction

**C** Sema3A

**D** Sema3C

**E** Sema3F

**F** I

**G** J

**H** K

**I** L

**n.s.**

**Abducens nerve diameter (um)**

**Abducens nerve length (um)**
Embryonic mice were reported to have exuberant branching of the trigeminal nerve and DRG projections, identical to the phenotype in Sema3AKO/KO mice (Merte et al., 2010). Indeed, we confirm exuberant trigeminal branching in Chn1WT/WT:Sema3AK108N/K108N embryos compared to Chn1WT/WT:Sema3AWT/WT and Chn1KI/KI:Sema3AWT/WT embryos (Figures 4.3G-4.3I), and find that the phenotype is unaltered in Chn1KI/KI:Sema3AK108N/K108N double mutants (Figure 4.3J). Together, these data support the lack of significant genetic interaction between hyperactive α2-chimaerin and Sema3A in mouse abducens and trigeminal cranial nerve development.

**Ephexin5, RhoA-GEF, does not alter Chn1KI phenotype**

Ephexin family proteins are RhoA-GEF molecules that convert RhoA-GDP to RhoA-GTP to cause growth cone collapse (Kozma et al., 1996; Sahin et al., 2005; Shamah et al., 2001). Ephexin5 is characterized to act downstream of EphB2 to restrict excitatory synapse formation (Margolis et al., 2010). Interestingly, in collaboration with members in the lab of Dr. Michael Greenberg, we noted that adult ephexin5 knockout mice (E5KO/KO) exhibited narrowing of the palpebral fissure (squinted eyes) compared to wildtype and heterozygous littermates, which phenocopied Chn1KI mice (Figures 3.2A-C, data not shown). Thus, we obtained E5KO/KO mice from the Greenberg lab and conducted whole mount staining in E11.5 embryos to determine whether E5KO/KO embryos displayed abnormal ocular motor nerve development, and whether it altered the abducens phenotype in Chn1KI/KI embryos.

We find that the abducens nerve development is normal in E11.5 E5KO/KO embryos and nerve length does not differ from wildtype (Figures 4.4A-4.4B, 4.4E). However, abducens nerve diameter at the orbit is significantly increased in E5KO/KO embryos, resembling the increase seen
in Chn1\(^{KO/KO}\) embryos (Figure 3.2L), suggesting that perhaps ephexin5 has a contribution to nerve fasciculation or innervation of the orbital region. We crossed Chn1\(^{KI/KI}\) mice with the E5\(^{KO/KO}\) line to determine whether E5 modified the Chn1\(^{KI/KI}\) abducens nerve phenotype. Chn1\(^{KI/KI}\):E5\(^{KO/KO}\) embryos display similarly reduced abducens nerve length and nerve diameter at the orbit as Chn1\(^{KI/KI}\):E5\(^{WT/WT}\) embryos (Figures 4.4C-F). Thus, there is no genetic interaction between Ephexin5 and α2-chimaerin in abducens nerve development.

We wondered whether the increase in abducens nerve diameter in E5\(^{KO/KO}\) embryos resulted in altered EOM innervation at later ages, so we conducted orbital dissections of E15.5-16.5 E5\(^{KO/KO}\) embryos. Initial EOM innervation appears normal in E5\(^{KO/KO}\) embryos compared to wildtype (Figures 4.3G-4.3H), and thus we do not find that Ephexin5 alters ocular cranial nerve targeting. Ephexin5 is implicated in synapse formation, so perhaps later neuromuscular junction abnormalities or visual defects in cortical pathways account for the eye squint in E5\(^{KO/KO}\) mice. It would be interesting to evaluate the contribution of Ephexin1, a homologous RhoA GEF family member of Ephexin5, in abducens nerve development, as it has been shown to act downstream of EphA4 in axon guidance and synapse formation (Fu et al., 2007; Sahin et al., 2005; Shamah et al., 2001). However, we were unable to obtain E1\(^{KO/KO}\) mice.
Figure 4.4: Ephexin5\textsuperscript{ko} does not alter embryonic ocular motor nerve development or modify Chn1\textsuperscript{ko} abducens nerve stalling.

(A-D) Representative images of the abducens nerve in E11.5 Chn1\textsuperscript{WT/WT}:E5\textsuperscript{WT/WT} (A), Chn1\textsuperscript{WT/WT}:E5\textsuperscript{KO/KO} (B), Chn1\textsuperscript{KI/KI}:E5\textsuperscript{WT/WT} (C), and Chn1\textsuperscript{KI/KI}:E5\textsuperscript{KO/KO} (D) whole mount embryos. Arrow: abducens nerve thinning and stalling. Red, black and white images: neurofilament, green: Hb9:GFP; scale bar: 100um.

(E-F) E11.5 abducens nerve length (E) and diameter at the orbit (F) in noted Chn1\textsuperscript{KI}:E5\textsuperscript{-} genotypes. N≥3 embryos from ≥3 independent litters; data represent mean ± SEM; *p< 0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Tukey’s test.

(G-H) Innervation at extraocular muscles (EOM) in E5\textsuperscript{+/+} (G) and E5\textsuperscript{KO/KO} E15.5 embryos. Dotted line: VI; arrow: abducens innervation of LR; red: smooth muscle actin (EOM), green: transgenic Is\textsuperscript{myo}:GFP; III: oculomotor; IV: trochlear; VI: abducens; LR: lateral rectus. Scale bar:100um. N≥3 orbits.
Sholl analysis of candidate neurotrophic factors reveal altered abducens outgrowth in $Chn1^{KI}$ explants.

We used the modified Sholl analysis approach (Figures 3.8A-3.8C, 3.8G, 3.14A-3.14J, 3.15A-3.15P) to screen for additional candidates that regulate normal abducens outgrowth and alter outgrowth upstream of hyperactive $\alpha_2$-chimaerin. High and low concentrations of GDNF, BDNF, HGF, NGF, CNTF, SDF-1, and FGF8 were used to assess changes in outgrowth of $Chn1^{WT/WT}$ and $Chn1^{WT/KI}$ abducens explants compared to explants grown in 50ng/mL FC without additional growth factors (referred to as baseline outgrowth; Figure 3.8G).

We find that certain concentrations of GDNF, BDNF, NGF, and HGF elicit different outgrowth responses in $Chn1^{WT/WT}$ abducens explants compared to $Chn1^{WT/KI}$ explants (Figure 4.5A-4.5L), whereas CNTF, SDF-1, and FGF8 do not alter $Chn1^{WT/KI}$ outgrowth compared to $Chn1^{WT/WT}$ (Figures 4.6A-4.6H).

As reported in Chapter 3 (Figure 3.15A), 25ng/mL GDNF similarly and significantly increases the outgrowth of $Chn1^{WT/WT}$ and $Chn1^{WT/KI}$ abducens explants compared to baseline (Figure 4.5A, 4.5C, 4.5J-4.5L). Interestingly, while a concentration of 1ng/mL GDNF significantly increases outgrowth in $Chn1^{WT/WT}$ abducens explants, it does not increase outgrowth in $Chn1^{WT/KI}$ explants (Figures 4.5A-4.5B). As a result, $Chn1^{WT/KI}$ explants do not significantly differ from baseline and have significantly reduced outgrowth compared to $Chn1^{WT/WT}$ abducens explants (Figures 4.5J-4.5L).
Figure 4.5: GDNF, BDNF, HGF, and NGF regulate \textit{Chn1^{WT/WT}} and \textit{Chn1^{WT/KI}} abducens explant outgrowth differently at specific protein concentrations.

(A-I) Sholl analysis of \textit{Chn1^{WT/WT}} (blue) and \textit{Chn1^{WT/KI}} (green) abducens explants cultured in 50ng/mL FC (A; same data as from Figure 3.8G), 1ng/mL GDNF (B), 25ng/mL GDNF (C; experiments performed independently of data from Figure 3.15A), 5ng/mL BDNF (D), 25ng/mL BDNF (E), 5ng/mL HGF (F), 25ng/mL HGF (G), 5ng/mL NGF (H), and 25ng/mL NGF (I).

(J-L) Quantification of initial outgrowth measured by the area under the curve (AUC) between 0-200um bin (J), average maximum outgrowth (K), and total outgrowth measured by total AUC (L) for abducens explants. † p<0.1, * p<0.05, ** p<0.01, *** p<0.001. Black asterisks: significant comparisons within \textit{Chn1^{WT/WT}} explant between three cues, red asterisks: comparisons within \textit{Chn1^{WT/KI}} between cues, one-way ANOVA with Dunnett’s test. Blue asterisks: significant comparisons between \textit{Chn1^{WT/WT}} and \textit{Chn1^{WT/KI}} within each cue by unpaired two-tailed t-test. N≥8 explants from ≥3 experiments for each condition.
Figure 4.5 (Continued)

![Graphs showing the number of intersections and their relationship to various factors and genotypes.](Image)
5ng/mL or 25ng/mL BDNF does not significantly modify the outgrowth of *Chn1*\(^{WT/WT}\) abducens explants compared to baseline (Figures 4.5A, 4.5D-4.5E, 4.5J-4.5L). However, 5ng/mL BDNF slightly and significantly reduces the outgrowth of *Chn1*\(^{WT/KI}\) explants, such that there is a significant difference in outgrowth compared to *Chn1*\(^{WT/WT}\) explants (Figures 4.5D, 4.5J-4.5L). Interestingly, 25ng/mL BDNF restores the outgrowth of *Chn1*\(^{WT/KI}\) explants such that they are similar to *Chn1*\(^{WT/WT}\) explants (Figures 4.5E, 4.5J-4.5L). Although not significant, increasing concentrations of HGF from 5ng/mL to 25ng/mL trend toward increasing *Chn1*\(^{WT/WT}\) abducens explant outgrowth (Figures 4.5F-4.5G, 4.5J-4.5L). However, *Chn1*\(^{WT/KI}\) explants do not display an increase in outgrowth in either concentration of HGF and as a result, mutant outgrowth significantly differs from wildtype outgrowth in 25ng/mL HGF (Figures 4.5F-4.5G, 4.5J-4.5L). Conversely, low concentrations of NGF (5ng/mL) increase the outgrowth of *Chn1*\(^{WT/WT}\) abducens explants, but at higher concentrations (25ng/mL) wildtype explants do not differ from baseline (Figures 45.H-4.5L). *Chn1*\(^{WT/KI}\) abducens explants do not have increased outgrowth in either concentration and remain at baseline, thus mutant outgrowth is significantly reduced compared to wildtype outgrowth in 5ng/ml NGF (Figures 4.5H-4.5L).

We find that CNTF, SDF-1, and FGF8 do not alter *Chn1*\(^{WT/KI}\) outgrowth compared to *Chn1*\(^{WT/WT}\) abducens explants. Whereas increasing concentrations of CNTF do not alter the outgrowth of *Chn1*\(^{WT/WT}\) abducens explants (Figures 4.6A-4.6B, 4.6F-4.6H), 25ng/mL CNTF significantly reduces the total outgrowth of *Chn1*\(^{WT/KI}\) explants compared to its own baseline outgrowth (Figure 4.6B, 4.6H). However, there is not a significant difference between wildtype and mutant explants in 25ng/mL CNTF (Figure 4.6B, 4.6F-4.6H).
Figure 4.6: CNTF, SDF-1, and FGF8 do not cause different outgrowth effects on Chn1<sup>WT/WT</sup> and Chn1<sup>WT/KI</sup> abducens explant outgrowth.

(A-E) Sholl analysis of Chn1<sup>WT/WT</sup> (blue) and Chn1<sup>WT/KI</sup> (green) abducens explants cultured in 1.5ng/mL CNTF (A), 25ng/mL CNTF (B), 5ng/mL SDF-1 (C), 25ng/mL SDF-1 (D), and 5ng/mL FGF8 (E).

(F-H) Quantification of initial outgrowth measured by the area under the curve (AUC) between 0-200um bin (F), average maximum outgrowth (G), and total outgrowth measured by total AUC (H) for abducens explants. † p<0.1, * p<0.05, ** p<0.01, *** p<0.001. Black asterisks: significant comparisons within Chn1<sup>WT/WT</sup> explant between three cues, red asterisks: comparisons within Chn1<sup>WT/KI</sup> between cues, one-way ANOVA with Dunnett’s test. Blue asterisks: significant comparisons between Chn1<sup>WT/WT</sup> and Chn1<sup>WT/KI</sup> within each cue by unpaired two-tailed t-test. 

N≥7 explants from ≥3 experiments for each condition. FC data in bar graphs is the same data as presented in Figures 3.8G, 4.5A, 4.5J-4.5L.
SDF-1 does not alter the outgrowth of Chn1\textsuperscript{WT/WT} or Chn1\textsuperscript{WT/KI} abducens explants (Figures 4.6C-4.6D, 4.6F-4.6H). 5ng/mL FGF8 increases the outgrowth of wildtype explants, and there is no significant difference between the outgrowth of Chn1\textsuperscript{WT/WT} and Chn1\textsuperscript{WT/KI} abducens explants (Figures 4.6E-4.6H).

These results demonstrate that certain concentrations of GDNF, NGF, and FGF8 significantly increase the outgrowth of Chn1\textsuperscript{WT/WT} abducens explants in vitro; HGF trends toward increasing Chn1\textsuperscript{WT/WT} outgrowth (Figures 4.5B-4.5C, 4.5G-4.5H, 4.5J-4.5L, 4.6E-4.6H). BDNF, CNTF, and SDF-1 do not alter Chn1\textsuperscript{WT/WT} abducens explant outgrowth compared to baseline and no tested cues significantly reduce Chn1\textsuperscript{WT/WT} outgrowth (Figures 4.5D-4.5E, 4.5J-4.5L, 4.6A-4.6D, 4.6F-4.6H). Hyperactive α2-chimaerin reduces abducens outgrowth compared to wildtype outgrowth in certain concentrations of GDNF, BDNF, HGF, and NGF (Figures 4.5B-4.5L). CNTF, SDF-1, and FGF8 do not modulate Chn1\textsuperscript{WT/KI} abducens explant outgrowth differently than Chn1\textsuperscript{WT/WT} outgrowth (Figures 4.6A-4.6H). Chn1\textsuperscript{WT/KI} abducens explants only have increased outgrowth in 25ng/mL GDNF (Figures 4.5C, 4.5J-4.5L).
Discussion

Enhanced $\text{Chn}1^{\text{KOKO}}:\text{EphA}4^{\text{KOKO}}$ abducens nerve phenotype compared to $\text{Chn}1^{\text{KOKO}}$ and $\text{EphA}4^{\text{KOKO}}$ suggests involvement of other pathways in abducens development

Despite the similar phenotype of $\text{Chn}1^{\text{KOKO}}$ and $\text{EphA}4^{\text{KOKO}}$ embryos reported in Chapter 3, we find that $\text{Chn}1^{\text{KOKO}}:\text{EphA}4^{\text{KOKO}}$ embryonic mice have reduced targeting of the abducens nerve to the EOM anlage during development compared to individual knockout alleles. This suggests that $\alpha_2$-chimaerin and/or EphA4 act downstream of other pathways during embryonic development to guide the abducens nerve.

$\text{Sema3A}^{\text{K108N}}$ loss-of-function does not alter abducens nerve growth or genetically interact to modify $\text{Chn}1^{\text{KI}}$ abducens phenotype

Upon investigation of other candidate pathways, we find that loss of Sema3A signaling does not alter abducens nerve development or modify the $\text{Chn}1^{\text{KI/KI}}$ abducens nerve phenotype. This is notable, given prior studies that reported overexpression of hyperactivated $\alpha_2$-chimaerin or knock-down of semaphorin receptors PlexinA1 and A2 in chick oculomotor nerve caused oculomotor nerve stalling and branching defects, yet simultaneous knock-down of PlexinA1 or A2 with overexpression of $\alpha_2$-chimaerin rescued oculomotor nerve stalling defects (Ferrario et al., 2012; Miyake et al., 2008). There are several differences between the prior studies and our findings that may lend insight into the noted discrepancies.

First, studies in chick were performed in the oculomotor nerve rather than abducens for technical reasons. We show that oculomotor neurons have very different outgrowth properties compared to abducens neurons in vitro (Figures 3.15A-3.15P), thus PlexinA1/A2 may not have a crucial role upstream of $\alpha_2$-chimaerin in abducens development. That said, we also do not see
modification of oculomotor nerve trajectory in \textit{Sema3A}^{K108N/K108N} embryos at E11.5, although there may be differences with final EOM innervation at later ages. These differences in oculomotor phenotype may reflect germline knock-in with endogenous expression levels of mutant \(\alpha_2\)-chimaerin in our mouse model versus overexpression of mutant \(\alpha_2\)-chimaerin in chick.

Second, PlexinA1 and A2 bind many different semaphorin isoforms (Sharma et al., 2012; Worzfeld and Offermanns, 2014); thus loss of function from isoforms such as Sema3C or Sema3F, rather than Sema3A, may cause the noted oculomotor nerve phenotypes in chick. Loss of Sema3F was reported to cause oculomotor nerve defasciculation in mouse (Sahay et al., 2003). Thus, hyperactive \(\alpha_2\)-chimaerin may act within a PlexinA/Neuropilin2 mediated pathway to increase repulsion downstream of Sema3F. Therefore, while loss of Sema3A function did not alter abducens development, removing receptors involved in semaphorin signaling may do so.

\textit{Ephxin5} loss-of-function also does not alter abducens nerve growth or genetically interact to modify \textit{Chn1}\textsuperscript{KI} abducens phenotype

We find that Ephxin5 does not alter EOM targeting of ocular cranial nerves and does not modify the \textit{Chn1}\textsuperscript{KI/KI} abducens nerve phenotype. Thus, it is unclear what causes eye squint in \textit{E5}\textsuperscript{K0/K0} mice. Although initial EOM targeting is normal for all ocular cranial nerves, neuromuscular junction formation could be abnormal since studies have implicated Ephxin5 in synaptogenesis (Margolis et al., 2010). Ephxin5 could also influence retinal development, photosensitivity, higher-order cortical visual processing, or perhaps even non-neuronal processes to alter the appearance of the eyes.
Sholl analysis reveals hyperactive α2-chimaerin alters abducens outgrowth downstream of GDNF, BDNF, NGF, and HGF

We find that three of the seven candidate growth factors in our experiments, GDNF, NGF, and FGF8, significantly increase the outgrowth of wildtype abducens explants. NGF increases outgrowth at only low concentrations, suggesting that the in vivo concentration is important for determining the overall effect on abducens nerve development. High concentrations of HGF also trend toward increasing wildtype abducens outgrowth, requiring further investigation. Given these findings, it would be interesting to investigate knockout mice of the cognate receptors Ret (GDNF receptor), p75/TrkA (NGF receptors), FGFR (FGF8 receptor), and Met (HGF receptor) to determine whether loss of these axon growth and guidance signaling pathways alters abducens nerve development.

Additionally, we find that different concentrations of GDNF, BDNF, HGF, and NGF alter the outgrowth of Chn1\textsuperscript{WT/KI} abducens explants compared to Chn1\textsuperscript{WT/WT} explants. BDNF did not alter wildtype growth, suggesting that mutant α2-chimaerin may disrupt balance to elicit a phenotype, even if a given factor does not have a role in normal development. In future experiments, it would be interesting to cross Ret, TrkB, p75/TrkA, or Met knockout mice to Chn1\textsuperscript{KI} mice to determine whether any of these candidate pathways are able to modify Chn1\textsuperscript{KI/KI} abducens nerve stalling.

Interestingly, while we find that higher concentrations of GDNF generate similar outgrowth in Chn1\textsuperscript{WT/WT} and Chn1\textsuperscript{WT/KI} abducens explants, we find that low concentrations of GDNF increase Chn1\textsuperscript{WT/WT} outgrowth but fail to increase Chn1\textsuperscript{WT/KI} outgrowth. This resembles the outgrowth differences reported in Chapter 3 whereby Chn1\textsuperscript{WT/KI} abducens explants that were
grown in EphA4 with higher concentrations of GDNF did not have the increased outgrowth seen in wildtype explants.

These findings lead us to hypothesize that Ret is an important regulator of abducens growth in vivo. Ret is highly expressed in abducens motor neurons at E11.5 (Garel et al., 2000), thus making it a good candidate for further studies. Based on the canonical understanding of Ret signaling, however, it is difficult to place α2-chimaerin directly downstream of its activation. Ret is known as a growth-promoting receptor (Manie et al., 2001), and it seems unlikely that it would directly recruit α2-chimaerin, which causes growth cone collapse. Instead, α2-chimaerin could be a component of a feedback loop that limits the downstream activity of Ret. Perhaps hyperactivation of α2-chimaerin perturbs the balanced feedback loop to enhance repulsion. In conclusion, we find that hyperactive α2-chimaerin has broad implications downstream of neurotrophic signaling pathways in abducens development, which informs future in vivo experiments.
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References


CHAPTER 5

Discussion
Summary of findings

We inserted a DRS-causing knock-in point mutation identified in individuals with DRS into mouse to characterize the etiology and cellular mechanisms underlying this common eye movement disorder. We find that $Chn1^{WT/KI}$ and $Chn1^{KI/KI}$ mice recapitulate the human DRS phenotype. We identify that the abducens nerve stalls during development in $Chn1^{WT/KI}$ and $Chn1^{KI/KI}$ embryonic mice, which results in failure of the abducens nerve to reach the LR, subsequent abducens motor neuron apoptosis, and misinnervation of the LR by the oculomotor nerve. Thus, $CHN1$ mutations cause abducens nerve growth and guidance abnormalities that lead to DRS. Additionally, $Chn1^{WT/KI}$ and $Chn1^{KI/KI}$ embryonic mice have aberrant trochlear nerve branching and first cervical spinal segment (C1) misrouting. We confirm that DRS-causing mutations found in human patients are gain-of-function, as $Chn1^{KO/KO}$ embryos display an abducens nerve wandering and defasciculation phenotype that is distinct from $Chn1^{WT/KI}$ and $Chn1^{KI/KI}$ abducens nerve stalling.

We also demonstrate that ephrin forward and reverse signaling are essential for proper abducens nerve development and act upstream of hyperactivated $\alpha_2$-chimaerin in abducens neurons to guide developing axons. Conditional knockout of EphA4 in motor neurons and mesenchyme reveals that ephrin forward and reverse signaling impact the development of the abducens nerve, as abducens phenotypes are distinct from the complete $EphA4^{KO/KO}$ embryo. Furthermore, bidirectional ephrin signaling is harnessed differently in specific motor neuron populations, as C1 and oculomotor neurons use only ephrin forward signaling and trochlear neurons do not significantly use ephrin signaling for guidance.
While we identify that ephrin signaling upstream of mutant α2-chimaerin is likely a main contributor to altered abducens development in Chn1KI mice, we find α2-chimaerin can signal through other guidance pathways during abducens development. Sema3A and Ephexin5 do not alter abducens development alone or modify the Chn1KI abducens phenotype. However, hyperactivated α2-chimaerin alters abducens neuron outgrowth in response to BDNF, GDNF, NGF, and HGF signaling in vitro, which suggests that α2-chimaerin has a broader role in axon growth and guidance pathways than is currently established. Through the use of a human disease-causing mutation, we have identified novel signaling pathways that act upstream of α2-chimaerin in a cell-type specific manner to guide developing motor neurons. This work highlights the complexity of axon guidance during neuronal development and lends insight into mechanisms that generate selective vulnerability of the abducens nerve to α2-chimaerin misregulation in DRS.
Hyperactive α2-chimaerin alters axon growth and guidance to cause DRS

Previously, the Engle Lab identified that dominant missense human mutations in CHN1 can cause DRS through a gain-of-function mechanism (Miyake et al., 2008). Inserting one of the identified human mutations into a mouse model reveals that altered function of the encoded protein, α2-chimaerin, causes selective abducens nerve stalling, trochlear branching defects, and C1 nerve misprojections, as well as abnormal facial nucleus migration and nerve exit abnormalities within the hindbrain. We confirm CHN1 mutations are gain-of-function, as Chn1KO/KO mice have abducens nerve wandering and defasciculation that is different than the Chn1KI phenotype.

It is interesting that α2-chimaerin is broadly expressed in developing neurons (Hall et al., 2001), and yet we find overall adult Chn1KI/KI brain morphology is normal. In addition, CHN1-DRS patients have normal cognition and no other reported neurological abnormalities beyond strabismus (Chan et al., 2011; Demer et al., 2007; Miyake et al., 2008; Miyake et al., 2011). Several possibilities may account for the selective vulnerability of the ocular motor system to hyperactivating CHN1 mutations. First, there could be another protein or set of proteins expressed in unaffected neuronal types that balances the activity of hyperactive α2-chimaerin. Secondly, receptor composition in different neuronal types may lead to the varied recruitment of α2-chimaerin in a cell-type specific manner to elicit a phenotype. We find that baseline abducens outgrowth properties are similar between Chn1WT/WT, Chn1WT/KI, and Chn1KI/KI explants in culture, suggesting that α2-chimaerin needs to be activated to generate a phenotype. Our Rac-GTP ELISA assays further indicate that mutant α2-chimaerin must be activated to alter Rac-GTP levels. As explained in more detail below, our work provides evidence to support that receptor composition is important for the selective vulnerability of the abducens nerve to hyperactivating
mutations in $\alpha_2$-chimaerin. However, it would be of interest in future experiments to determine whether the neuronal expression of other cytoskeletal regulatory proteins modulates the activity of $\alpha_2$-chimaerin in a cell-type specific manner.

**Chn1$^{KI}$ mice recapitulate features of human DRS and inform underlying etiology**

We identify that the $Chn1^{KI}$ mouse model recapitulates human DRS, as there is loss of the abducens nerve and motor neurons, in addition to aberrant innervation of the LR by branches of the oculomotor nerve. We wondered whether misinnervation is caused by hyperactivate $\alpha_2$-chimaerin altering growth and branching of the oculomotor nerve, thus permitting misinnervation, or whether a wildtype oculomotor nerve would generally misinnervate the LR in the absence of the abducens nerve. Previous studies have shown that $Mafb$ is expressed in rhombomeres 5 and 6, where the abducens nerve develops, and is not expressed in developing midbrain oculomotor neurons (Cordes and Barsh, 1994; Giudicelli et al., 2003; Kim et al., 2005; Sadl et al., 2003). Thus, in collaboration with Jong G. Park, a medical student in the Engle Lab, we obtained $MafB$ mutant mice (Appendix II) (Yu et al., 2013), that have been demonstrated to lack abducens nerves (Cordes and Barsh, 1994; McKay et al., 1994). We find that in the absence of the abducens nerve in $MafB^{KO/KO}$ embryos, the oculomotor nerve also sends branches to misinnervate the LR, which appear indistinguishable from the misinnervation seen in $Chn1^{KI/KI}$ mice. Thus, hyperactive $\alpha_2$-chimaerin is not essential for oculomotor nerve misinnervation of the LR. Therefore, despite our finding that mutant oculomotor neurons in vitro have reduced outgrowth in ephrin-A5/GDNF, we do not find notable oculomotor nerve developmental abnormalities that are directly caused by $Chn1$ mutations.
Bidirectional ephrin signaling via EphA4 is essential for proper abducens nerve development

We find that normal abducens development is not perturbed by loss of Sema3A or Ephexin5 function. However, bidirectional EphA4 signaling is essential for normal abducens development. Selectively removing ephrin forward signaling through EphA4 results in loss of abducens projections to the orbit that is more severe than the complete EphA4 knockout.

Conditional knockout of ephrin reverse signaling, using EphA4 as a ligand, causes abducens nerve stalling with complete penetrance, demonstrating that both forward and reverse signaling through EphA4 are essential for proper abducens nerve guidance.

It is possible that additional EphA and EphB isoforms are also important for abducens development. In future experiments, it would be interesting to use the IslCre and Twist2Cre conditional knockout systems we establish here to characterize the contribution of other bidirectional Eph/ephrin signaling isoforms in the development of the abducens nerve. While the chromosomal positioning of Twist2Cre in close proximity to EphA4 makes it extremely difficult to obtain mesenchyme-specific EphA4 knockout mice, we anticipate that Twist2Cre can be used successfully for conditional knockout of other Eph/ephrin molecules in the mesenchyme.

α2-chimaerin and EphA4 display a complex genetic interaction selectively in abducens neurons, which is mediated by bidirectional ephrin signaling

We find that α2-chimaerin and EphA4 have varied genetic interactions in different motor neuron populations. Intercrossing Chn1KI/KI:EphA4KO/KO mice restores normal alternating gait and C1 projections, does not alter aberrant Chn1KI/KI trochlear branching, and results in a complex altered phenotype in the abducens nerve. Our in vitro experiments suggest the varied phenotype
recovery in different motor neuron populations can be explained, in part, by ephrin forward and reverse signaling. Abducens explants have reduced outgrowth in ephrin-A5/GDNF compared to wildtype, indicating that mutant Chn1 enhances ephrin-A forward signaling. In addition, abducens explants fail to exhibit potentiated outgrowth in EphA4/GDNF as seen in wildtype explants, demonstrating that mutations also reduce attraction downstream of ephrin reverse signaling mediated by EphA4/GDNF ligands.

By contrast, we find that C1 explants have reduced outgrowth only in response to ephrin-A5/GDNF, and have no change in response to ephrin reverse signaling with EphA4/GDNF. Additionally, ephrin forward signaling upstream of α2-chimaerin has been implicated in CST/CPG circuit formation (Beg et al., 2007; Iwasato et al., 2007; Wegmeyer et al., 2007), but it remains to be determined whether ephrin reverse signaling impacts CST/CPG projections. Trochlear neurons do not have dramatic alterations in outgrowth in response to either ephrin forward or reverse signaling. These results suggest that motor neuron populations which use only ephrin forward signaling via EphA4, and not ephrin reverse signaling, upstream of α2-chimaerin have restored guidance (CST/CPG and C1). Motor neurons that do not use ephrin signaling will not have altered guidance (trochlear), and motor neuron populations that use both ephrin forward and reverse signaling upstream of α2-chimaerin have a complex genetic interaction (abducens).

It is challenging to assess the exact contribution of ephrin forward and reverse signaling upstream of α2-chimaerin in vivo. Chn1KI is flanked by LoxP sites in our mutant line, and thus we cannot cross Chn1KI/KI mice to EphA4lox/flox:Isl1Cre/+ or EphA4lox/flox:Twist2Cre/+ mice because Chn1 will be excised. Importantly, however, even if we modified the Chn1 construct to permit these experiments, we still do not anticipate that either genetic cross will rescue the Chn1KI/KI
abducens stalling phenotype. We find that *Chn1* mutations increase repulsion downstream of ephrin forward signaling, and thus *Chn1<sup>KI/KI</sup>:EphA4<sup>fl/fo</sup>:Isl<sup>Cre/+</sup> embryos may have a partial rescue of abducens nerve stalling. However, we also identified that *Chn1<sup>KI/KI</sup>* mutations attenuate attraction downstream of ephrin reverse signaling through EphA4/GDNF ligands, thus *Chn1<sup>KI/KI</sup>:EphA4<sup>fl/fo</sup>:Isl<sup>Cre/+</sup> embryos may still have an abducens nerve phenotype that could be as severe as seen in EphA4<sup>fl/fo</sup>:Twist2<sup>Cre/+</sup> embryos.

We find that *Chn1<sup>KI/KI</sup>* and EphA4<sup>fl/fo</sup>:Twist2<sup>Cre/+</sup> embryos have similar abducens phenotypes. Our *in vitro* data support this finding, as *Chn1<sup>WT/KI</sup>* explants do not display increased outgrowth in response to ephrin reverse signaling. Therefore, we anticipate that crossing the *Chn1<sup>KI/KI</sup>* and EphA4<sup>fl/fo</sup>:Twist2<sup>Cre/+</sup> alleles to generate *Chn1<sup>KI/KI</sup>:EphA4<sup>fl/fo</sup>:Twist2<sup>Cre/+</sup>* embryos would not greatly modify the abducens nerve stalling phenotype. To improve *Chn1<sup>KI/KI</sup>* abducens nerve development, we hypothesize that α2-chimaerin-mediated repulsion downstream of ephrin forward signaling would need to be attenuated while simultaneously enhancing attraction through ephrin reverse signaling. Another possible approach to alleviate nerve stalling in *Chn1<sup>KI</sup>* mice would be to alter the regulation of another Rho-GTPase molecule or other downstream pathway in abducens neurons.

Upon conceiving of the *Chn1<sup>KI</sup>::EphA4<sup>KO</sup>* experiments, we anticipated that intercrosses would yield either phenotype rescue (or phenotype modification in the direction of rescue) or an EphA4<sup>KO/KO</sup> phenotype. As mentioned earlier in Chapter 5, we do not find that mutant α2-chimaerin is constitutively active in cultured cortical neurons or abducens explants. Thus, if α2-chimaerin were to act only downstream of EphA4 as a receptor, we might expect to see the EphA4<sup>KO/KO</sup> phenotype in *Chn1<sup>KI/KI</sup>::EphA4<sup>KO/KO</sup>* mice. The fact that we see a variable phenotype rescue implies other pathways activate α2-chimaerin. These could be ephrin or non-ephrin
mediated pathways, although we hypothesize that additional EphA pathways are involved because activation of another EphA receptor may permit proper spatiotemporal regulation of α2-chimaerin signaling. Thus, in the subtypes where we see restored function (CST/CPG and C1), we anticipate that other EphA pathways are activating α2-chimaerin at a lower level than that elicited by EphA4 activation, such that the phenotype is normalized. In abducens neurons, it is less clear how to distinguish whether additional isoforms of EphA/ephrin are recruiting α2-chimaerin, or whether α2-chimaerin is additionally activated via non-ephrin mediated pathways. We find that α2-chimaerin can be activated downstream of other neurotrophic pathways in vitro, which may contribute to the complex phenotype seen in Chn1\(^{KI/KI}\), EphA4\(^{KO/KO}\) embryos, in addition to bidirectional EphA4 signaling.

**Internal carotid artery likely creates a microenvironment to elicit abducens nerve stalling in Chn1\(^{KI}\) mice**

We find that the abducens nerve normally deftly navigates around the internal carotid artery (ICA) to reach the developing EOM anlage. In Chn1\(^{KI/KI}\) embryos, the abducens stalls at the ICA, and in Chn1\(^{KO/KO}\) embryos, the abducens begins its aberrant fasciculation with the facial nerve and its turning toward the basilar artery at the ICA. This suggests that the ICA creates a microenvironment that alters the development of the abducens nerve in Chn1\(^{KU/KI}\) mice, and thus may lend insight into pathways that regulate abducens development upstream of α2-chimaerin. VEGF is an attractive candidate, as it is known to guide both developing vasculature and peripheral nerves (Gu et al., 2003). Interestingly, loss of the VEGF164 isoform results in similar facial nucleus migration abnormalities as seen in Chn1\(^{KU/KI}\) mice (Schwarz et al., 2004). Future experiments could determine whether abducens neurons express receptors implicated in VEGF
signaling, whether $Chn1^{KI}$ abducens explants have different outgrowth in VEGF compared to wildtype explants, and if VEGF loss-of-function mice have an abducens phenotype. Additionally, ephrins are highly expressed in embryonic arteries (McBride and Ruiz, 1998; Wang et al., 1998) and may regulate the interaction between nervous system and vascular development.

In Chapter 3, we successfully used Sholl analysis to inform and direct \textit{in vivo} experiments, and anticipate that it will continue to be a reliable system to assess the implications of putative growth and guidance cues before intercrossing mutant mouse lines. Therefore, the Sholl analysis method would be a useful tool to first assess \textit{in vitro} whether a given arterial candidate pathway is important for abducens development.

**Interactions between guidance pathways may be regulated by $\alpha_2$-chimaerin**

As mentioned in Chapter 3, it remains unclear whether hyperactive $\alpha_2$-chimaerin is recruited directly downstream of ephrin reverse signaling via EphA4/GDNF ligands. If so, this would suggest that $\alpha_2$-chimaerin acts downstream of the growth-promoting receptor Ret (Manie et al., 2001), which seems unlikely. Instead, $\alpha_2$-chimaerin could be activated in a feedback loop from coincident EphA4/GDNF ligand activation through Ret, and perhaps its hyperactivity overcompensates to shut down increased outgrowth from coincident signaling.

Another possibility is that ephrin competes for binding of different co-receptor pathways during abducens development. This phenomenon has been shown to occur in limb motor neuron guidance with GPI-anchored protein GFR$\alpha_1$ (Bonanomi et al., 2012). Low expression of GFR$\alpha_1$ permits its co-receptor, Ret, to interact with ephrin-A and relay coincident GDNF/EphA4 signaling through ephrin-A/Ret and GFR$\alpha_1$/Ret co-receptor systems (Bonanomi et al., 2012).
However, high expression of GRFa1 competes for Ret binding, and thus GDNF signaling through the GFRα1/Ret pathway dominates over EphA4 signaling through ephrin-A/Ret coreceptors, leading to less outgrowth than with coincident EphA4/GDNF ligand-mediated signaling (Bonanomi et al., 2012). A similar receptor competition mechanism could apply to ephrin reverse signaling pathways. In retinal ganglion cells, ephrin-A uses p75NTR as a coreceptor to inhibit outgrowth (Lim et al., 2008), thus perhaps these pathways are active in abducens neurons and have misregulated activity through hyperactive α2-chimaerin. This is an intriguing possibility because we find that BDNF and NGF both reduce outgrowth of mutant Chn1 abducens explants compared to wildtype explants, and both neurotrophic factors can signal through Trk/p75NTR-mediated pathways (Dechant and Barde, 2002). Thus, investigating signaling pathway interactions could lead to interesting findings that not only inform molecular mechanism of DRS, but also general principles of axon guidance.

Conclusion

The complexity of axon guidance is remarkable, as would be predicted given the countless diverse, yet stereotypic circuits that precisely form in the nervous system during development. Using a mutation identified to alter eye movement circuitry in human patients, we have identified axon guidance mechanisms that are crucial for proper development of ocular motor neuron circuitry. This work not only informs the etiology and developmental mechanisms underlying Duane retraction syndrome, but also provides insight into the complexity of nervous system development and mechanisms that generate selective vulnerability in distinct neuronal populations.
References


APPENDIX

Appendix I:

Human disorders of axon guidance
At the time of the submission of this dissertation, Appendix Section 1 has been published as a literature review entitled:

**Human Disorders of Axon Guidance**

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Alicia A. Nugent wrote sections on HGPPS and CMM, Adrianne L. Kolpak wrote sections on TUBB3 Syndromes, Elizabeth C. Engle edited the review.
Abstract

Axon pathfinding is essential for the establishment of proper neuronal connections during development. Advances in neuroimaging and genomic technologies, coupled with animal modeling, are leading to the identification of an increasing number of human disorders that result from aberrant axonal wiring. In this review, we summarize the recent clinical, genetic and molecular advances with regard to three human disorders of axon guidance: Horizontal gaze palsy with progressive scoliosis, Congenital mirror movements, and Congenital fibrosis of the extraocular muscles, Type III.
Introduction

The field of axon guidance has expanded dramatically over the past few decades. Animal models and in vitro neuronal culture techniques have fostered the discovery of many molecules that are essential for the correct guidance and target innervation of exploring axonal processes during neurodevelopment. Surprisingly, there are still relatively few identified human disorders resulting from axon guidance defects, and most of the identified disorders are rare. This does not necessarily mean that aberrant axon guidance does not cause common human disorders, but rather that, until recently, technology has hindered the identification of subtle white matter tract miswiring in the human nervous system. The advent of high-resolution magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI), along with advances in genetic technologies, have now helped to define the first series of human genetic disorders of axon guidance (Engle, 2010; Izzi and Charron, 2011), and will likely contribute to the discovery of many additional axon guidance disorders in the future. This article is an update to the Engle 2010 review (Engle, 2010) and predominantly highlights recent work that contributes to the further understanding of Horizontal gaze palsy with progressive scoliosis (HGPPS), Congenital mirror movements (CMM), and Congenital fibrosis of the extraocular muscles, Type III (CFEOM3).

Horizontal Gaze Palsy with Progressive Scoliosis

Horizontal gaze palsy with progressive scoliosis (HGPPS) is a rare disorder that results from axonal midline crossing defects of specific populations of neurons in the hindbrain and possibly spinal cord. The disorder presents with congenital bilateral absence of horizontal eye movement. During childhood, patients develop debilitating scoliosis. There are reported delays in motor development, but otherwise patients are intellectually and physically normal, and mirror
movements have not been reported (Amoiridis et al., 2006; Chan et al., 2006). This autosomal recessive disorder was described as early as 1970 (Dretakis, 1970), but it was not until 2004 that loss of function mutations in the axon guidance receptor ROBO3 were found to underlie HGPPS (Abu-Amero et al., 2009; Abu-Amir et al., 2011; Amouri et al., 2009; Chan et al., 2006; Jen et al., 2004; Ng et al., 2011; Volk et al., 2011).

MRI, functional MRI, DTI, and evoked potential studies of HGPPS patients reveal a normal corpus callosum (Avadhani et al., 2010; Haller et al., 2008; Otaduy et al., 2009; Sicotte et al., 2006), but a virtually pathognomonic hindbrain malformation that correlates with failure of the corticospinal tracts (CST) and dorsal column tracts to decussate in the hindbrain (Figure AI.1) (Amoiridis et al., 2006; Avadhani et al., 2010; Haller et al., 2008; Jen et al., 2004; Ng et al., 2011; Otaduy et al., 2009; Sicotte et al., 2006). A HGPPS patient harboring ROBO3 mutations who suffered a stroke in the motor cortex resulting in ipsilateral limb weakness and facial palsy further confirmed uncrossed CST in this disorder (Ng et al., 2011). Sensorimotor evoked potential studies in a second patient revealed ipsilateral CST but partial crossing of the dorsal column tracts, suggesting there may be some variability in midline crossing defects within and between patients (Amoiridis et al., 2006). DTI of HGPPS patients revealed that cerebellar tracts are also uncrossed or abnormally located, which may contribute to progressive scoliosis (Avadhani et al., 2010; Otaduy et al., 2009; Sicotte et al., 2006).
Figure A1.1: Schematic of axon guidance defects in HGPPS, CMM, and CFEOM3. With normal guidance (purple): corticospinal tract (CST) axons navigate from the motor cortex to the spinal cord, decussating in the medulla (cell bodies labeled ‘1’); corpus callosum (CC) and anterior commissure axons cross from one hemisphere to the other (cell bodies labeled ‘2’); interneuron populations decussate in the hindbrain (cell bodies labeled ‘3’); and oculomotor axons extend to their target extraocular muscles (EOM, cell bodies labeled ‘4’). In HGPPS (blue), CST and hindbrain interneuron axons fail to decussate, resulting in ipsilateral projections. In CMM (green), it is proposed but not proven that while some CST axons decussate appropriately, others fail to do so and project ipsilaterally. In CFEOM3 (brown), there is variable thinning of the CC and anterior commissure axons, supporting abnormal guidance of these axonal tracts and the oculomotor nerve (CNIII) has abnormal guidance, resulting in the dysinnervation of EOM. AR = autosomal recessive; AD = autosomal dominant.
FMRI and brainstem auditory evoked potential (BAEP) studies indicate that HGPPS patients also have abnormal activation of auditory pathways (Amoiridis et al., 2006; Haller et al., 2008). Two patients exhibited high-frequency hearing loss in the right ear, which may contribute to abnormal BAEPs (Amoiridis et al., 2006). However, if these findings are not a result of unilateral sensorineural hearing loss, they may reflect auditory circuit rewiring in HGPPS patients. Future advances in imaging technology should help continue to define axon guidance abnormalities in this disorder.

Robo3 (Rig1) is a transmembrane protein predominantly expressed in hindbrain and spinal cord commissural axons during embryonic development, and exists in two splice variants, Robo3.1 and Robo3.2 (Camurri et al., 2004; Chen et al., 2008; Jen et al., 2004; Sabatier et al., 2004). Slit is expressed by the floorplate and normally transmits a repellent response upon binding to Robo1 and Robo2 receptors expressed on crossing commissural axons (Long et al., 2004). As commissural axons approach the floorplate, Robo3.1 is thought to interfere with Slit repulsion to allow midline crossing (Chen et al., 2008; Sabatier et al., 2004). Once axons cross the floorplate, Robo3.1 is quickly downregulated and Robo1, Robo2, and Robo3.2 transmit responses that propel growing axons away from the midline to prevent recrossing (Chen et al., 2008; Sabatier et al., 2004). ROBO3 mutations underlying HGPPS include nonsense, frameshift, splice site, and missense changes that are spread across the gene (Abu-Amero et al., 2009; Abu-Amero et al., 2011; Amouri et al., 2009; Chan et al., 2006; Jen et al., 2004; Ng et al., 2011; Volk et al., 2011). To date, none map to residues that would alter expression of one splice variant over the other, and thus HGPPS mutations are predicted to result in loss of both ROBO3.1 and ROBO3.2 function.
Studies of $Robo3^{-/-}$ mice have provided extensive insights into the molecular mechanisms and anatomical miswiring underlying HGPPS. In $Robo3^{-/-}$ mice, commissural axons fail to cross both the spinal cord and hindbrain midline at E11.5 (Sabatier et al., 2004). $Robo3^{-/-}$ die at birth, however, and it has not been determined whether the CST, which decussates just prior to birth, is also uncrossed. Interestingly, Robo3 does not appear to be expressed in developing upper motor neurons, which form the CST (Barber et al., 2009; Camurri et al., 2004), although a more detailed spatiotemporal expression analysis is needed to formally conclude this. Thus, the lack of CST decussation in HGPPS may be secondary to the failure of earlier populations of commissural neurons to form a midline bridge for CST axons to cross later in development (Marillat et al., 2004; Sabatier et al., 2004).

Subsequent studies of $Robo3^{-/-}$ mice have identified additional defects in the crossing of cerebellar, auditory, and breathing circuitry (Bouvier et al., 2010; Di Meglio et al., 2008; Marillat et al., 2004; Renier et al., 2010). Cerebellar neuronal migration, axonal and neuronal midline crossing, and nucleus cytoarchitecture are abnormal in $Robo3^{-/-}$ mice, which further substantiates abnormal cerebellar tracts noted in DTI of HGPPS patients (Avadhani et al., 2010; Otaduy et al., 2009; Sicotte et al., 2006; Tamada et al., 2008). Interestingly, mouse precerebellar neurons require a translational regulatory protein, Musashi1, for protein expression of Robo3.1 and 3.2 in order to permit proper cerebellar neuron and axonal midline crossing events (Kuwako et al., 2010). If Musashi1 proves to have a similar regulatory role in human hindbrain horizontal gaze circuitry, its corresponding gene, $MSI1$, would be an interesting candidate disease gene for patients with horizontal gaze palsy with or without scoliosis who do not harbor mutations in $ROBO3$. It is particularly relevant for patients with additional clinical or imaging evidence of
central nervous system guidance errors, as the msil<sup>−/−</sup> mouse develops hydrocephalus and has variable agenesis of the corpus callosum with Probst’s bundles (Sakakibara et al., 2002).

To directly investigate the etiology of horizontal gaze palsy in HGPPS, Renier <i>et al</i> (Renier et al., 2010) used a conditional knockout approach in mice to selectively remove Robo3 expression in rhombomeres 3 and 5 of the hindbrain (Robo3:Krox20Cre). These animals survive, and consistent with human MRI data (Bosley et al., 2005; Jen et al., 2004), the abducens motor neurons in Robo3:Krox20Cre mice form normally and axons appear to correctly innervate the lateral rectus muscle (Renier et al., 2010). However, there is reduced axonal midline crossing at the level of the abducens nucleus and a paucity of projections between the abducens nucleus and contralateral oculomotor nucleus, with occasional sparse ipsilateral projections. This suggests that the medial longitudinal fasciculus and the paramedian pontine reticular formation interneuron populations, which cross the midline of the hindbrain to control oculomotor and abducens motor nuclei and permit horizontal gaze, are unable to cross in Robo3:Krox20Cre mice, resulting in disrupted lateral and medial gaze. Indeed, horizontal eye movements are limited in Robo3:Krox20Cre mice, while vertical gaze is preserved (Renier et al., 2010), recapitulating eye motility defects seen in HGPPS patients.

Similar to human studies, BAEPs were also found to be abnormal in Robo3<sup>−/−</sup> mice. Closer anatomical investigation revealed that axons from the anteroventral cochlear nucleus, the main auditory projections that decussate in the hindbrain, do not cross in Robo3<sup>−/−</sup> mice, but rather form ipsilateral Calyx of Held synapses (Renier et al., 2010). In addition, Bouvier <i>et al</i> (Bouvier et al., 2010) identified asynchronous breathing in P0 Robo3<sup>−/−</sup> mice, resulting from an absence of interneuron crossing in the hindbrain. The group hypothesizes that scoliosis in
patients could result from asynchronous muscle contraction, however it remains to be determined whether humans with HGPPS breathe asynchronously.

It is remarkable that HGPPS patients have relatively normal gross motor, sensory, and proprioceptive behavior considering human patient imaging and mouse anatomical studies have identified ROBO3/Robo3 as a critical regulator of midline crossing in the hindbrain and spinal cord. This suggests that axons innervate targets correctly, but on the ipsilateral rather than contralateral side of the body, as demonstrated in studies of Robo3−/− mice (Bouvier et al., 2010; Renier et al., 2010). There is mounting evidence that horizontal gaze palsy results from uncrossed and/or absent oculomotor and abducens internuclear tracts. The etiology of scoliosis remains more elusive, and may arise from the combined absence of CST, reticulospinal, and/or cerebellar tract crossing events, or perhaps asynchronous breathing.

**Congenital mirror movements**

Congenital mirror movements (CMM) are a synkinesis in which pathological involuntary movements on one side of the body occur simultaneously with intentional movements on the contralateral side. Mirror movements typically occur in the fingers, hands, and forearms and can be observed in young children, but normally disappear by 7 years of age unless there is underlying pathology (Gallea et al., 2011; Koerte et al., 2010). There are two primary hypotheses as to the anatomic correlate of pathologic CMM. First, fMRI data has supported aberrant signaling across the corpus callosum, resulting in decreased interhemispheric inhibition of the contralateral motor cortex (Leinsinger et al., 1997). Second, transcranial magnetic stimulation (TMS) studies have supported either incomplete decussation of the CST, or abnormal distal branching of axons within the contralateral CST, such that projections from the primary
motor cortex synapse simultaneously on both ipsilateral and contralateral interneurons or motor neurons within the spinal cord (Cincotta et al., 2003; Farmer et al., 1990; Gallea et al., 2011; Srour et al., 2010).

CMM occurs as a component of various syndromes, including X-linked Kallmann syndrome and some forms of Joubert syndrome (Ferland et al., 2004; Mayston et al., 1997; Vulliemoz et al., 2005). Non-syndromic, isolated CMM also occurs, and can be sporadic or segregate as an autosomal dominant trait with incomplete penetrance. Recently, heterozygous mutations in the \textit{DCC} gene were reported to underlie isolated CMM in 3 unrelated families of French-Canadian, Iranian and Italian descent (Depienne et al.; Srour et al., 2010). Affected family members have MM primarily in the hands and fingers, with only a few reporting lower extremity involvement or disability as a result of the MM. Several individuals harboring heterozygous \textit{DCC} mutations are reported to have normal brain MRI scans without corpus callosal or hindbrain abnormalities (Srour et al., 2010). TMS studies of affected individuals harboring \textit{DCC} mutations reveal simultaneous bilateral muscle contractions, with no latency in muscle response from either side, supporting the presence of both ipsilateral and contralateral CST, or aberrant axonal branching of CST axons, rather than abnormal signaling across the corpus callosum (Figure AI.1) (Cincotta et al., 2003; Depienne et al.; Srour et al., 2010). Notably, 3 autosomal dominant families and 2 sporadic individuals with CMM do not harbor \textit{DCC} mutations or deletions, one of which is a large pedigree that does not map to the \textit{DCC} locus (Depienne et al.; Djarmati-Westenberger et al., 2011; Srour et al., 2010). Thus, CMM is a genetically heterogeneous disorder, and future studies will likely identify additional causative genes.
The DCC gene encodes an axon guidance receptor that responds to netrin, a well-characterized axon guidance cue involved in attractive midline guidance decisions in cortex, brainstem, and spinal cord (Izzi and Charron, 2011). DCC mutations identified in all 3 dominant CMM families produce frameshifts and introduce premature stop codons. Two mutations are predicted to truncate the DCC protein near the N-terminus, producing a very short transcript that would encode only a few extracellular immunoglobulin-like domains and would lack the netrin binding, transmembrane, and cytoplasmic domains. As predicted, in vitro data confirms that one mutation significantly decreases netrin binding when expressed in COS-7 cells (Srour et al., 2010). It is not known, however, whether the truncated DCC proteins are secreted, sequestered intracellularly, or degraded, or whether the mutant transcripts may be subject to nonsense-mediated mRNA decay. The third reported mutation, in exon 26, is predicted to truncate only the last two intracellular domains (P2 and P3) while retaining the netrin-binding and transmembrane domains (Depienne et al.). If translated, it would produce a DCC protein lacking the portion of the cytoplasmic domain required for DCC dimerization and netrin-1-induced attractive axonal turning (Stein et al., 2001). Given that all CMM patients have indistinguishable phenotypes regardless of their specific mutation, and 2 of the mutations would produce short, truncated proteins lacking most functional domains and unlikely to dimerize with full-length DCC, it can be argued that the mutations likely result in DCC haploinsufficiency.

The importance of netrin/DCC signaling in axon guidance is highlighted by studies of Dcc−/− mice, which die at P0 and exhibit agenesis of the corpus callosum, absence of CST pyramidal decussation, and misprojection of the anterior commissure and spinal commissural axons (Fazeli et al., 1997; Finger et al., 2002). At P0, the CST has only reached the upper cervical spinal cord, and thus it is not known if the CST axons would follow the correct
trajectory and synapse on the appropriate motor neurons, albeit ipsilaterally. Notably however, a spontaneous mouse mutant, $Dcc^{Kanga}$, harbors a deletion of $Dcc$ exon 29, truncating the protein prior to the last cytoplasmic functional domain (P3), similar to the third heterozygous human mutation that truncates both P2 and P3 (Depienne et al.; Finger et al., 2002). Unlike $Dcc^{-/-}$ mice, $Dcc^{Kanga/Kanga}$ mice are viable, possibly because they were studied on a different genetic background. The $Dcc^{Kanga/Kanga}$ exhibit axon guidance defects within the corpus callosum, as well as failed decussation of the CST resulting in aberrant ipsilateral projections (Finger et al., 2002). Phenotypically, the mice cannot alternate movement of their hindlimbs and exhibit a hopping gait. While a hopping gait has been offered as the mouse equivalent of human mirror movements, the $Dcc^{Kanga/Kanga}$ mice have fully uncrossed, ipsilateral CST projections. Thus, for the $Dcc^{Kanga/Kanga}$ mice to phenocopy CMM in humans, in which TMS studies reveal simultaneous contralateral and ipsilateral CST innervation, one would need to hypothesize that the ipsilateral CST axons branch within the spinal cord to innervate both ipsilateral and contralateral interneurons and motor neurons. However, it is possible that the mouse hopping gait and human mirror movements are not equivalent. Of note, $Dcc^{Kanga/Kanga}$ mice with hopping gait harbor homozygous, while humans with CMM harbor heterozygous $Dcc$ mutations, and perhaps this dosage effect results in partial CST decussation in humans. If this were the case, analyzing CST decussation in heterozygous $Dcc$ mutant mice may prove informative.

An alternative explanation for the $Dcc^{Kanga/Kanga}$ hopping phenotype is that it results from alterations in the central pattern generator circuitry caused by aberrant commissural interneuron (CIN) migration across the spinal cord midline. *In vitro* electrophysiological studies show that loss of netrin-1 induces synchronous, rather than alternating, firing in equivalent left and right regions of isolated netrin-1$^{-/-}$ spinal cords (Rabe et al., 2009). A similar synchronous firing
phenomenon is observed in spinal cords harvested from mice lacking ephrinB3, EphA4 or alpha2-chimaerin and, in contrast to the netrin-1−/− mouse which dies at P0, these mice survive and have hopping gaits (Dottori et al., 1998; Iwasato et al., 2007; Kullander et al., 2003).

Additionally, in ephrinB3−/−, EphA4−/−, and Chn1−/− mice, both CST and CIN axons aberrantly cross the spinal midline (Dottori et al., 1998; Iwasato et al., 2007; Kullander et al., 2003; Kullander et al., 2001), though it is not clear whether aberrant CST or CIN, or both, contribute to the synchronous limb movement and hopping gait. It remains to be determined whether CIN and central pattern generator abnormalities exist in Dcc mutant mice, and if these pathways exist in humans and are disrupted in CMM. Finally, DCC is also expressed in corpus callosum axons as they cross the midline, and Dcc−/− mice are acollosal (Fazeli et al., 1997). Thus it remains possible that humans with CMM have additional abnormalities in interhemispheric connectivity. It will be interesting to review DTI scans from individuals with CMM who harbor DCC mutations, as this may help to localize the anatomic pathway underlying their mirror movements.
Congenital fibrosis of the extraocular muscles type 3 (CFEOM3) and the TUBB3 syndromes

CFEOM3 is an autosomal dominant congenital eye movement disorder with variable penetrance. Affected individuals are born with variable unilateral or bilateral ophthalmoplegia, limited vertical ductions, and blepharoptosis (drooping eyelids) (Figure AI.2A) (Mackey et al., 2002; Tischfield et al., 2010). MRI shows hypoplasia of the oculomotor nerve and the muscles innervated by its superior branch, the levator palpebrae superioris and superior rectus (Figure AI.2B) (Demer et al., 2010; Tischfield et al., 2010). The oculomotor nerve can also aberrantly innervate the lateral rectus muscle, which is normally innervated by the abducens nerve (Demer et al., 2010). CFEOM3 can occur in isolation, or in association with facial weakness, progressive axonal sensorimotor polyneuropathy, and/or social and intellectual disabilities. In patients with social and intellectual disabilities, MRI reveals agenesis or hypoplasia of the anterior commissure and corpus callosum, as well as malformed basal ganglia (Figure AI.2C-AI.2G) (Tischfield et al., 2010). Both isolated and syndromic CFEOM3 result from specific, repetitive heterozygous missense mutations in TUBB3, which encodes the neuronal-specific beta-tubulin isotype III, a subunit of microtubules (Tischfield et al., 2010).
Figure A1.2: TUBB3 mutation-specific abnormalities in white matter tracts and basal ganglia.

(A) Photograph of a child harboring a TUBB3 R262H amino acid substitution who exhibits severe CFEOM3 and facial weakness.
(B) Brainstem MRI from a patient with predominantly left-sided CFEOM3 reveals unilateral hypoplasia of the left oculomotor nerve (arrow; D417N substitution).
(C-H) Sagittal and axial MR images from individuals harboring R62Q, R262C, and R262H TUBB3 substitutions as indicated. (C,D) R62Q results in normal appearance of the corpus callosum (CC) and anterior commissure (AC); (E,F) R262C causes CC posterior thinning (E) and AC hypoplasia (F); (G,H) while R262H causes CC hypoplasia (G), AC agenesis and dysmorphic basal ganglia (H). (Reprinted, with permission, from (Tischfield et al., 2010)).
Tubb3 is expressed in all post-mitotic neurons and is upregulated during the period of axon growth and guidance (Jiang and Oblinger, 1992). A knock-in mouse model harboring the most common TUBB3 mutation, and resulting in the Tubb3 R262C amino acid substitution, recapitulates the human phenotype and confirms misguidance of the oculomotor nerve, corpus callosum, and anterior commissure without alterations in cortical architecture, suggesting a primary defect in axon pathfinding. When modeled in yeast, these mutations increase microtubule stability, alter microtubule dynamics, and a subset also decrease kinesin plus-end movement (Tischfield et al., 2010). Given the pan-neuronal expression of TUBB3, it is not yet clear why certain populations of axons are selectively affected by these mutations. These data suggest, however, that this tubulin isoform may be downstream of a subset of axon guidance receptors, and critical for the cytoskeleton to respond correctly to upstream signaling. Future molecular and functional studies will help to further elucidate the etiology of CFEOM3, and the additional TUBB3 mutation-specific neurologic disorders, referred to as the TUBB3 syndromes.
Conclusion

HGPPS, CMM, and CFEOM3 are examples of the contribution that human disease can provide for the elaboration of basic scientific principles, and the insight that mouse models provide toward the understanding of human disorders. These disorders arise primarily from axonal midline crossing defects or peripheral nerve misprojections, which are easier to characterize using current research techniques and technologies. Certainly advances in human genetics and sequencing methodologies, technological developments in human neuroimaging, and continued animal model research will foster the characterization of additional human disorders that arise from subtler axon guidance defects. Preliminary research has hinted that axon guidance abnormalities may contribute to schizophrenia, and autism spectrum disorders such as tuberous sclerosis (Chen et al., 2011; Geschwind and Levitt, 2007; Nie et al., 2010). Perhaps high resolution DTI of humans will help to clarify whether axonal targeting abnormalities contribute to these, and other, disorders.

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Appendix II:

Loss of MAFB function in human and mouse causes
Duane syndrome, aberrant extraocular muscle innervation, and inner ear defects
At the time of the submission of this dissertation, the work presented is this appendix has been accepted for publication in *The American Journal of Human Genetics*, entitled:

**Loss of MAFB function in human and mouse causes**

**Duane syndrome, aberrant extraocular muscle innervation, and inner ear defects**

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Author Contributions

Jong G. Park, Max A. Tischfield, Alicia A. Nugent, and Elizabeth C. Engle conceptualized the project and planned experiments. A.A.N. conducted whole mount embryo staining, assisted with initial orbital dissection technique, and assisted with analysis method for Figure AII.2. J.G.P. conducted most experiments and analyzed data. J.G.P and E.C.E. wrote the manuscript. Long Cheng, Silvio Alessandro Di Gioia, and Wai-Man Chan assisted with experiments. Gail Maconachie, Thomas M. Bosley, C. Gail Summers, David G. Hunter, Caroline D. Robson, and Irene Gottlob assisted with clinical ascertainment and findings.
Abstract

Duane retraction syndrome (DRS) is a congenital eye movement disorder defined by limited outward gaze and retraction of the eye on attempted inward gaze. Here, we report three loss-of-function $MAFB$ mutations causing DRS and a dominant-negative $MAFB$ mutation causing DRS and deafness. Using genotype-phenotype correlations in humans and $Mafb$ knockout mice, we propose a threshold model for variable loss of MAFB function. Postmortem studies of DRS have reported abducens nerve hypoplasia and aberrant innervation of the lateral rectus muscle by the oculomotor nerve. Our studies in mouse confirm this human DRS pathology. Moreover, we demonstrate that selectively disrupting abducens nerve development is sufficient to cause secondary innervation of the lateral rectus muscle by aberrant branches of the oculomotor nerve, which form at developmental decision regions close to target extraocular muscles. Thus, we present evidence that the primary cause of DRS is failure of the abducens nerve to fully innervate the lateral rectus muscle in early development.
Duane retraction syndrome (DRS [MIM: 126800 and MIM: 604356]) was first described clinically in 1905 (Duane, 1905), and is the most common of the congenital cranial dysinnervation disorders with a prevalence of 1:1000 (Engle, 2010). Affected individuals have unilaterally or bilaterally limited horizontal eye movement accompanied by globe retraction and palpebral fissure narrowing on attempted adduction (movement of the eye inward toward the nose). DRS can be classified into three types: type 1 in which abduction is limited (movement of the eye outward toward the ear); type 2 in which adduction is limited; and type 3 in which both abduction and adduction are limited. Type 1 is the most common and type 2 is the least common form. Postmortem brainstem and orbital examinations of two adults (Hotchkiss et al., 1980; Miller et al., 1982) and magnetic resonance imaging of four individuals (Demer et al., 2007) with type 1 and type 3 DRS revealed absence or hypoplasia of the abducens nerve, which normally innervates the lateral rectus (LR) extraocular muscle to abduct the eye. These studies also showed aberrant LR muscle innervation by axons of the oculomotor nerve, which normally innervates the medial rectus (MR), inferior rectus (IR), superior rectus (SR), and inferior oblique (IO) extraocular muscles. Electromyographic recordings of the extraocular muscles in individuals with DRS demonstrated co-contraction of the LR muscle with the MR muscle, and less frequently co-contraction of the LR muscle with the IR or SR muscles (Huber, 1974; Strachan and Brown, 1972). Thus, while clinical studies support aberrant peripheral innervation in DRS, the developmental underpinnings of the aberrant innervation, and whether it arises from primary pathology in the oculomotor nerve or secondary to abducens nerve hypoplasia, is unknown.
In this study, we report mutations in MAFB (MIM: 608968; GenBank: NG_023378, chromosome 20q12) that cause human DRS and model DRS pathogenesis in Mafb knockout mice. The mouse Mafb gene (Kreisler/Krmll) encodes a transcription factor of the basic leucine zipper family (Kataoka et al., 1994a). Previous studies of mice with the hypomorphic Mafb allele kreieler (kr) (Eichmann et al., 1997; Hertwig, 1942; Sing et al., 2009) have shown that Mafb is expressed in rhombomeres 5 and 6, where the abducens nerve develops, and is not expressed in developing midbrain oculomotor neurons (Cordes and Barsh, 1994; Giudicelli et al., 2003; Kim et al., 2005; Sadl et al., 2003). Mafb is required for proper hindbrain segmentation (Cordes and Barsh, 1994; McKay et al., 1994), and regulates other transcription factors involved in hindbrain patterning (Manzanares et al., 1997). Here, we identify haploinsufficient and dominant negative MAFB mutations in humans with DRS and propose a threshold model for loss of MAFB function causing DRS and deafness. We demonstrate that Mafb knockout mice recapitulate human DRS pathology and can serve as an animal model for studies of aberrant innervation. Furthermore, our findings establish that the aberrant branching of the oculomotor nerve in DRS is not primary, but arises secondary to disruptions of abducens nerve development.

All participants were enrolled with informed consent according to the guidelines of the Declaration of Helsinki and with approval of the Institutional Review Board at Boston Children’s Hospital. We enrolled pedigree FA that segregated DRS together with congenital sensorineural hearing loss as a dominant trait (Figure AII.1A). One of the members with DRS (Figure AII.1B) underwent CT imaging for evaluation of deafness, and was found to have inner ear common cavity anomalies (Figure AII.1C). The combination of DRS with inner ear defects suggested a disruption of early hindbrain development similar to that previously reported in individuals with HOXAI mutations and in MafbKO/KO mice (Tischfield et al., 2005; Yu et al.,
Affected family members did not harbor mutations in HOXA1 (data not shown). Thus, we screened MAFB in this pedigree and, subsequently, in an additional 400 probands with DRS and 10 probands with both DRS and hearing loss for exonic mutations with targeted Sanger sequencing (Genewiz), and for copy number variations with droplet digital PCR (Bio-Rad) (Table S1). We identified heterozygous single base pair frameshift deletions in pedigrees FA (c.803delA), 0819 (c.440delG), and PM (c.644delA), and a heterozygous full gene deletion in pedigree N (Figure AII.1A, S1, and S2). The mutations in pedigrees FA and 0819 arose de novo (Figure S1), and none of the MAFB variants were present in the ExAC database. Unlike pedigree FA, affected members of pedigrees 0819, PM, and N had isolated DRS without hearing loss (Table AII.1).

To confirm that loss of MAFB causes DRS in vivo, we studied Mafb complete knockout mice (Yu et al., 2013), which we crossed to ISL<sup>MN</sup>:GFP reporter mice that contain a farnesylated GFP which localizes to the membrane of motor neurons, allowing us to visualize the developing cranial motor axons (Lewcock et al., 2007). At E11.5, Mafb<sup>WT/WT</sup> embryos have normal hindbrain and cranial nerve development by wholemount examination (Figures AII.2A and AII.2D) (Huber et al., 2005). Mafb<sup>WT/KO</sup> embryos have hypoplastic abducens nerves (Figures AII.2B and AII.2E). Mafb<sup>KO/KO</sup> embryos have severe malformations of the hindbrain, and as a result have absent abducens nerves and fusion of the glossopharyngeal and vagus nerves (Figures AII.2C and AII.2F).
Figure AII.1: Mutations in *MAFB* cause DRS.

(A) Pedigrees FA, PM, 0819, and N segregate mutations in *MAFB* as noted. Three of the four affected FA pedigree members also have unilateral or bilateral congenital hearing loss (indicated by *).

(B) FA IV:1 has bilateral DRS, characterized by bilaterally limited eye abduction and narrowing of the palpebral fissures with globe retraction during attempted eye adduction.

(C) Axial CT images of the right temporal bone of a healthy control with normal cochlea and vestibule (arrows), and of individual FA IV:1 who has a cystic common cavity anomaly (arrow).
Table AII.1: Clinical summary of individuals with mutations in *MAFB*. All affected individuals have DRS, which is bilateral in eight, right-sided in four, and unknown if bilateral or unilateral in two. Three of four affected members of pedigree FA have hearing loss, with sidedness corresponding to sidedness of their DRS. FA III:1 did not report hearing loss but was not formally tested, thus hearing loss may demonstrate incomplete penetrance. All four pedigrees are Caucasian.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Mutation</th>
<th>DRS</th>
<th>Hearing Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>N II:2</td>
<td><em>MAFB</em> deletion</td>
<td>Bilateral</td>
<td>No</td>
</tr>
<tr>
<td>N II:5</td>
<td><em>MAFB</em> deletion</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>N II:6</td>
<td><em>MAFB</em> deletion</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>N III:3</td>
<td><em>MAFB</em> deletion</td>
<td>Bilateral</td>
<td>No</td>
</tr>
<tr>
<td>N III:5</td>
<td><em>MAFB</em> deletion</td>
<td>Unilateral (right side)</td>
<td>No</td>
</tr>
<tr>
<td>N III:6</td>
<td><em>MAFB</em> deletion</td>
<td>Bilateral</td>
<td>No</td>
</tr>
<tr>
<td>FA II:2</td>
<td>c.802delA</td>
<td>Unilateral (right side)</td>
<td>Unilateral (right side)</td>
</tr>
<tr>
<td>FA III:1</td>
<td>c.802delA</td>
<td>Unilateral (right side)</td>
<td>Not reported, but not formally tested</td>
</tr>
<tr>
<td>FA III:3</td>
<td>c.802delA</td>
<td>Unilateral (right side)</td>
<td>Unilateral (right side)</td>
</tr>
<tr>
<td>FA IV:1</td>
<td>c.802delA</td>
<td>Bilateral</td>
<td>Bilateral</td>
</tr>
<tr>
<td>0819 II:2</td>
<td>c.439delG</td>
<td>Bilateral</td>
<td>No</td>
</tr>
<tr>
<td>PM II:4</td>
<td>c.644delA</td>
<td>Bilateral</td>
<td>No</td>
</tr>
<tr>
<td>PM III:5</td>
<td>c.644delA</td>
<td>Bilateral</td>
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</tr>
<tr>
<td>PM III:6</td>
<td>c.644delA</td>
<td>Bilateral</td>
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</tr>
</tbody>
</table>
Figure AII.2: Mafb knockout mice embryos demonstrate DRS pathology. (A-F) Whole mount sagittal confocal images at E11.5. (A) Mafb\textsuperscript{WT/WT} embryos have normal hindbrain cranial nerve development. White line indicates region of developing rhombomerres 5 and 6. (B) Mafb\textsuperscript{WT/KO} embryos have abducens nerve hypoplasia but no other major abnormalities of hindbrain cranial nerve development. (C) Mafb\textsuperscript{K/O KO} embryos have loss of rhombomerres 5 and 6 resulting in loss of hindbrain area (white line), absent abducens nerve, and fusion of the glossopharyngeal nerve with the vagus nerve (arrow). (D-F) Medial sagittal sections highlighting the developing oculomotor and abducens nerves. (D) In Mafb\textsuperscript{WT/WT} embryos, the abducens nerve is present (short arrow) and reaches the developing eye. (E) Mafb\textsuperscript{WT/KO} embryos have a hypoplastic abducens nerve (short arrow). (F) Mafb\textsuperscript{K/O KO} embryos have an absent abducens nerve (short arrow). (G-O) Confocal images of the right orbit in mouse embryos from the inferior view. (G) At E12.5 in Mafb\textsuperscript{WT/WT} embryos, the abducens nerve (arrowhead) innervates the LR muscle, while the oculomotor nerve innervates the IR muscle and developing IO muscle. (H) In E12.5 Mafb\textsuperscript{WT/KO} embryos, the abducens nerve is hypoplastic (arrowhead) and innervates the LR muscle, while the oculomotor nerve begins to send aberrant branches towards the LR muscle (arrow) and the RB muscle. (I) In E12.5 Mafb\textsuperscript{K/O KO} embryos, the abducens nerve is absent (arrowhead), and the oculomotor nerve sends aberrant branches towards the LR muscle (arrow) and the RB muscle. (J) At E13.5 in Mafb\textsuperscript{WT/WT} embryos, the abducens nerve (arrowhead), oculomotor nerve, and extraocular muscles continue to develop normally. (K) In E13.5 Mafb\textsuperscript{WT/KO} embryos, the hypoplastic abducens nerve (arrowhead) innervates the LR muscle, while the oculomotor nerve forms a second more distal aberrant branch towards the LR muscle (yellow arrow) in addition to the proximal aberrant branch formed earlier (white arrow). (L) In E13.5 Mafb\textsuperscript{K/O KO} embryos, the abducens nerve is absent (arrowhead), and the oculomotor nerve sends a distinct distal aberrant branch towards the LR muscle (yellow arrow) in addition to the proximal aberrant branch formed earlier (white arrow) and the other aberrant branches contacting the RB muscle. (M) Mafb\textsuperscript{WT/WT} embryos at E16.5 have the final developmental pattern of the orbit, with the abducens nerve (arrowhead) innervating the LR muscle. (N) In E16.5 Mafb\textsuperscript{WT/KO} embryos the abducens nerve remains hypoplastic (arrowhead), and a proximal aberrant branch (white arrow) and a distal aberrant branch (yellow arrow) of the oculomotor nerve also innervate the LR muscle. (O) In E16.5 Mafb\textsuperscript{K/O KO} embryos the abducens nerve remains absent (arrowhead), and a proximal aberrant branch (white arrow) and a distal aberrant branch (yellow arrow) of the oculomotor nerve innervate the LR muscle. (P) At E16.5, the diameter of the abducens nerve is significantly reduced in Mafb\textsuperscript{WT/KO} embryos compared to Mafb\textsuperscript{WT/WT} embryos, and absent in Mafb\textsuperscript{K/O KO} embryos. (Q) At E16.5, the diameter of the oculomotor distal aberrant branch is significantly increased compared to the oculomotor proximal aberrant branch in both Mafb\textsuperscript{WT/KO} and Mafb\textsuperscript{K/O KO} embryos. The diameter of the distal aberrant branch is significantly greater in Mafb\textsuperscript{K/O KO} compared to Mafb\textsuperscript{WT/KO} embryos. These aberrant branches are not present in Mafb\textsuperscript{WT/WT} embryos. III, oculomotor nerve; IV, trochlear nerve; V, trigeminal nerve; VI, abducens nerve; VII, facial nerve; IX, glossopharyngeal nerve; X, vagus nerve; IO, inferior oblique; IR, inferior rectus; LR, lateral rectus; RB, retractor bulbi; scale bar = 100 µm. *, p<0.05; ****, p<0.0001; differences were measured using Tukey’s multiple comparisons test, and error bars report standard error of the mean (GraphPad Prism). N>10 for each genotype.
Figure AII.2 (Continued)
We used a novel orbital dissection technique to visualize the developing cranial nerves and extraocular muscles in mouse embryos. We dissected the fixed brain tissue around the orbits of ISL<sup>MN</sup>:GFP positive mouse embryos, leaving the distal cranial nerves and extraocular muscles intact. The orbits were then incubated with anti-actin α-smooth muscle-Cy3 antibody (Sigma-Aldrich) for 3 days at 4°C. The orbits were further dissected and flat mounted in the inferior orientation in 70% glycerol and 1% 1M KOH in PBS. We observed that by E12.5 in Mafb<sup>WT/WT</sup> embryos, the abducens nerve is present in the orbit and contacts the developing LR muscle (Figure AII.2G). In Mafb<sup>WT/KO</sup> embryos a hypoplastic abducens nerve contacts the developing LR muscle, and aberrant branches of the oculomotor nerve begin to form in the direction of the LR muscle and retractor bulbi (RB) muscle (Figure AII.2H). In Mafb<sup>KO/KO</sup> embryos the abducens nerve is absent, and an aberrant branch of the oculomotor nerve forms and contacts the developing LR muscle along a similar trajectory as the wildtype abducens nerve, while other aberrant branches develop and contact the RB muscle (Figure AII.2I). These aberrant branches all arise from the developmental decision region of the oculomotor nerve where it normally divides into its superior and inferior divisions (Cheng et al., 2014).

In E13.5 Mafb<sup>WT/WT</sup> embryos, the abducens nerve and LR muscle continue to develop, with no visible aberrant branches from the oculomotor nerve (Figure AII.2J). In Mafb<sup>WT/KO</sup> embryos, the hypoplastic abducens nerve remains in contact with the LR muscle (Figure AII.2K). In both Mafb<sup>WT/KO</sup> and Mafb<sup>KO/KO</sup> embryos, the oculomotor nerve forms a second more distal aberrant branch that extends towards the LR muscle, in addition to the more proximal aberrant branches contacting the LR and RB muscles (Figures AII.2K and AII.2L). The distal aberrant branch arises from a second decision region where the oculomotor nerve’s inferior division normally branches to innervate the MR, IR, and IO muscles (Cheng et al., 2014).
By E16.5, $Mafb^{WT/WT}$ embryos have developed the adult configuration of extraocular muscles and cranial nerves (Figure AII.2M) (Fritzsch et al., 1995). In $Mafb^{WT/KO}$ embryos the abducens nerve remains hypoplastic compared to $Mafb^{WT/WT}$ embryos, but provides some innervation to the LR muscle (Figures AII.2N and AII.2P). In $Mafb^{KO/KO}$ embryos, the abducens nerve is absent and provides no innervation to the LR muscle (Figures AII.2O and AII.2P). In $Mafb^{WT/KO}$ and $Mafb^{KO/KO}$ embryos, the LR muscle receives innervation from the distinct proximal and distal aberrant oculomotor nerve branches (Figures AII.2N and AII.2O). The diameter of the distal aberrant branch is significantly greater than the proximal aberrant branch in both $Mafb^{WT/KO}$ and $Mafb^{KO/KO}$ embryos, and the distal branch itself is significantly larger in $Mafb^{KO/KO}$ compared to $Mafb^{WT/KO}$ embryos (Figure AII.2Q).

We next investigated whether DRS and hearing loss in pedigree FA shared a common etiology. We hypothesized that pedigree FA harbored a heterozygous dominant-negative $MAFB$ mutation that caused the more severe phenotype of DRS with hearing loss, while the pedigrees with isolated DRS had heterozygous loss-of-function mutations that resulted in haploinsufficiency. Our hypothesis that $MAFB$ function at 50% would cause isolated DRS and below 50% would also cause inner ear defects in humans was based on previous reports that $Mafb^{KO/KO}$ embryos have inner ear defects (Yu et al., 2013), homozygous $kr/kr$ mice have both abducens nerve and inner ear defects (Deol, 1964), $Mafb^{WT/KO}$ embryos have normal inner ear development (Yu et al., 2013), and hypomorphic $kr/+\phantom{x}$ mice have normal inner ear and abducens nerve development (Deol, 1964; McKay et al., 1994).

$MAFB$ consists of three critical functional domains: an extended homology region (EHR) and a basic region (BR) required for DNA binding, and a leucine zipper (LZ) required for dimerization (Kataoka et al., 1994a; Kerppola and Curran, 1994). The frameshift mutation in
pedigree 0819 (p.Gly147Alafs*78) occurs between the N-terminal polyhistidine regions, and is predicted to result in a mutant MAFB protein that retains the first polyhistidine region followed by 78 altered amino acids, truncating at a new stop codon (Figure AII.3A). The frameshift mutation in pedigree PM (p.Gln215Argfs*10) occurs at the beginning of the EHR, and is predicted to result in a mutant MAFB protein that retains the first 3 amino acids of the EHR followed by 10 altered amino acids, truncating at the same stop codon as the 0819-mutant MAFB (Figure AII.3A). The frameshift mutation in pedigree FA (p.Asn268Metfs*125) occurs in the LZ, and is predicted to result in a mutant MAFB protein that retains the EHR, BR, and the first leucine of the LZ, followed by 125 altered amino acids (Figure AII.3A). Thus, we further investigated the functional consequences of the truncation and altered amino acids in the 0819-mutant protein, as well as the retained functional domains and altered amino acids in the FA-mutant protein.
Figure AII.3: Less than 50% MAFB function causes DRS and inner ear defects.

(A) N has a full gene deletion and therefore no mutant MAFB. 0819 and PM are predicted to have truncated MAFB proteins that lack the EHR, BR, and LZ domains followed by 77 and 8 altered amino acids, respectively. FA is predicted to have a MAFB protein that retains the wildtype EHR, BR, and beginning of the LZ, followed by 125 altered amino acids. 

(B) Luciferase assay shows wildtype MAFB increases transcription by approximately 150 fold. The 0819- or FA-mutant protein alone does not have any transcriptional activity. Co-expression of wildtype MAFB with FA-mutant, but not 0819-mutant MAFB reduces the transcriptional activity of the wildtype protein compared to wildtype alone. **, p<0.01; differences were measured using Tukey’s multiple comparisons test, and error bars report standard error of the mean (GraphPad Prism). Triplicates were performed for each experiment.

(C) Threshold model for loss of MAFB function. At greater than 50% MAFB function, Mafo_{WT/WT} and kr/+ mice have no phenotypic alterations. At 50% MAFB function, Mafo_{WT/KO} mice and members of pedigrees N, 0819, and PM with heterozygous loss-of-function mutations have isolated DRS. At some level below 50% MAFB function, kr/kr mice, members of pedigree FA with a dominant-negative mutation, and Mafo_{KO/KO} mice have both DRS and inner ear defects.
MAFB is a single exon gene, and we found expression of the FA-mutant MAFB mRNA in FA subject-derived lymphoblasts by Sanger sequencing (Genewiz) and allelic discrimination (Thermo Fisher Scientific) of reverse transcribed lymphoblast cDNA (Qiagen) (Table S2, Figure S3). We successfully overexpressed FA- and 0819-mutant MAFB constructs (Genewiz) in HEK293T cells by subcloning into a pCMV N-Myc vector (Thermo Fisher Scientific) and transfecting with Lipofectamine 2000 (Invitrogen) (Figure S4). We measured the transcriptional activity of wildtype, FA-mutant, and 0819-mutant MAFB proteins by luciferase assay (Promega) using 25 ng of caCE2 luciferase vector that contains 6x Maf recognition elements in the promoter (Kajihara et al., 2001), or Renilla luciferase control reporter vector (Promega), and 2 ng of MAFB and/or control constructs for each experiment. We found no activity in either mutant alone (Figure AII.3B). We then co-expressed wildtype MAFB with each mutant and found that FA-mutant MAFB, but not 0819-mutant MAFB, reduced the transcriptional activity of the wildtype protein (Figure AII.3B). These data support a heterozygous dominant-negative mechanism for FA-mutant MAFB, and a heterozygous loss-of-function mechanism for 0819-mutant MAFB.

Combining our human and mouse data, we propose a threshold model for variable loss of MAFB function (Figure AII.3C). The heterozygous loss-of-function MAFB alleles in pedigrees N, 0819, and PM result in 50% protein function and cause isolated DRS, consistent with DRS pathology and normal inner ear development in Mafb^{WT/KO} mice. The heterozygous dominant-negative MAFB allele in pedigree FA results in less than 50% protein function and causes both DRS and deafness, consistent with absent abducens nerves and inner ear defects in living kr/kr mice, as well as DRS pathology and inner ear defects in Mafb^{KO/KO} embryos.
In this study, we establish that mutations in MAFB cause DRS, and find that MAFB mutations are present in ~1% of DRS probands in our cohort, making it a more common cause of DRS than the previously identified dominant mutations in CHN1 (Miyake et al., 2008) (DURS2 locus, MIM: 118423) and SALL4 (Al-Baradie et al., 2002) (DRRS locus, MIM: 607343), and recessive mutations in HOXA1 (Tischfield et al., 2005) (ABDS/BSAS locus, MIM: 142955). Our findings also demonstrate allelic diversity in this gene (Walsh and Engle, 2010). Hotspot missense mutations in the N-terminal transactivation domain were previously reported to cause multicentric carpotarsal osteolysis (MIM: 166300) (Zankl et al., 2012), and mutations in noncoding regions have been associated with cleft lip/cleft palate (Beaty et al., 2010). These previously reported mutations are most likely gain-of-function since they do not disrupt the EHR, BR, or LZ domains of MAFB, and therefore act through different pathogenic mechanisms than the loss-of-function and dominant-negative mutations we have identified.

We also present a quantitative threshold model for loss of MAFB function causing DRS and inner ear defects using genotype-phenotype correlations in humans and mice. The 0819-mutant MAFB protein lacks the EHR, BR, and LZ domains, and the PM-mutant MAFB protein lacks most of the EHR and all of the BR and LZ domains, so both are predicted to have no dimerization or DNA binding function as previously demonstrated in vitro (Kataoka et al., 1994b; Sadl et al., 2003). In contrast, the FA-mutant protein retains the EHR, BR, and part of the LZ domain, and these residual functional domains could interfere with the DNA binding or dimerization of the wildtype protein. Our in vitro data are consistent with these predictions, and show that FA-mutant MAFB reduces the transcriptional activity of wildtype MAFB.

Identifying mutations in MAFB that cause human DRS also provides an instructive mouse model to confirm human DRS pathology and to further our understanding of cranial nerve
development and aberrant extraocular muscle innervation. Because *Mafb* is not expressed in developing oculomotor neurons (Cordes and Barsh, 1994; Giudicelli et al., 2003; Kim et al., 2005; Sadl et al., 2003), the orbital pathology of *Mafb* knockout mice establishes that the aberrant innervation of the LR muscle by the oculomotor nerve in DRS arises secondarily to absent or reduced LR muscle innervation by the abducens nerve. This secondary mechanism of aberrant innervation suggests that DRS could also arise following non-genetic disruptions to abducens nerve development prior to its innervation of the LR muscle, including *in utero* exposure to thalidomide or misoprostol (Miller et al., 2009), and possibly following infectious or vascular embryonic injuries ( Parsa and Robert, 2013). Moreover, these finding likely account for why DRS is far more common than congenital isolated sixth nerve palsies (Brodsky, 2010); congenital sixth nerve palsies without retraction could only result from destructive insults to the abducens nerve that occur after the abducens nerve has fully innervated the LR muscle, and thus has prevented secondary aberrant innervation by the developing oculomotor nerve.

The stereotypic aberrant branching of the *Mafb*KO/KO oculomotor nerve at its two decision regions (Cheng et al., 2014) may explain aspects of the highly stereotypic co-contraction of the LR with the MR muscle, and the less frequent co-contraction of the LR with the SR and IR muscles, as reported by human electromyography (Huber, 1974; Strachan and Brown, 1972). At its proximal decision region, the oculomotor nerve divides into superior and inferior divisions, and axons destined for any of its innervated muscles could potentially be rerouted to form the small branches that aberrantly innervate the LR muscle in DRS. In particular, misdirection of a subset of superior division axons normally destined for the SR could account for the rare reports of LR and SR muscle co-contraction. The aberrant branch from the oculomotor distal decision region is significantly larger than the aberrant branch from the proximal decision region; greater
axon rerouting from the distal region could therefore account for the more frequent co-contraction of the LR with the MR or IR muscles. Since co-contraction of the LR with the MR muscle is the most common aberrant innervation pattern in DRS, extraocular muscle innervation may be further programmed along separate horizontal and vertical axes that have yet to be defined. Finally, in the absence of the abducens nerve, we observe aberrant branching of the oculomotor nerve to the RB muscle as well as the LR muscle at its proximal decision region. The RB muscle is normally innervated by the abducens nerve in mouse and develops adjacent to the proximal decision region of the oculomotor nerve. Thus, it is likely that all of the extraocular muscles ensure their innervation in early development by secreting shared factors that induce nerve branching and growth, resulting in competition between oculomotor and abducens axons for these factors at two specific decision regions.

is required for proper hindbrain segmentation (Cordes and Barsh, 1994; McKay et al., 1994), and regulates other transcription factors involved in hindbrain patterning (Manzanares et al., 1997). Here, we identify haploinsufficient and dominant negative MAFB mutations in human patients and propose a threshold model for loss of MAFB function causing DRS and deafness. We demonstrate that Mafb knockout mice recapitulate human DRS pathology and can serve as an animal model for studies of aberrant innervation. Furthermore, our findings establish that the aberrant branching of the oculomotor nerve in DRS is not primary, but arises secondary to disruptions of abducens nerve development.
Supplemental data are Appendix 3

Supplemental Data include four figures and two tables.

Acknowledgements

We thank W.-M. Yu and L.V. Goodrich for MafB\textsuperscript{flax} mice; S.L. Pfaff for Isl\textsuperscript{MN}:GFP mice; H. Ogino and S. Takahashi for \textit{cα}CE2 construct; R.J. McLean, A. Mellors, M.G. Thomas, and A.-U. Haq for clinical exams. Funding support included R01EY12498 (E.C.E), HD018655 (E.C.E), and Research to Prevent Blindness, Inc. (C.G.S.). J.G.P. is a Howard Hughes Medical Institute Medical Research Fellow, and E.C.E. is a Howard Hughes Medical Institute Investigator.

Web Resources

ExAC Browser, http://exac.broadinstitute.org/


UCSC Genome Browser, https://genome.ucsc.edu/


References


Appendix III:

Supplemental Figures:

Loss of MAFB function in human and mouse causes

Duane syndrome, aberrant extraocular muscle innervation, and inner ear defects

At the time of the submission of this dissertation, the work presented in this appendix has been accepted for publication in The American Journal of Human Genetics, entitled: 

Loss of MAFB function in human and mouse causes Duane syndrome, aberrant extraocular muscle innervation, and inner ear defects
Table S1: Sequences of primers and probes used for targeted screening of *MAFB*. Related to Figure AII.1. C859 refers to ddPCR probe at position 859 of the *MAFB* coding sequence. P-935 refers to ddPCR probe at position -935 upstream of the *MAFB* coding sequence in the *MAFB* promoter region. *MAFB* primers 1, 2, and 3 were used for Sanger sequencing across the *MAFB* coding sequence. All sequences are listed 5’→3’. F, forward; R, reverse.

<table>
<thead>
<tr>
<th>C859 Forward</th>
<th>GCAGGTGGAGCGAGCTTAAGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>C859 Probe</td>
<td>CTGGCCCGCGAGAG</td>
</tr>
<tr>
<td>C859 Reverse</td>
<td>GAGTTTTCTCGCACTTGACCTTGT</td>
</tr>
<tr>
<td>P-935 Forward</td>
<td>GGAGAGGGCTCAAACAGGCATA</td>
</tr>
<tr>
<td>P-935 Probe</td>
<td>AGTGCGACCCCAAGTG</td>
</tr>
<tr>
<td>P-935 Reverse</td>
<td>GCGCCTTTGCGCACAGT</td>
</tr>
<tr>
<td>MAFB 1 F</td>
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</tr>
<tr>
<td>MAFB 1 R</td>
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<tr>
<td>MAFB 2 F</td>
<td>ACTGGATGGCGAGCAACTAC</td>
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<tr>
<td>MAFB 3 R</td>
<td>GTGCGCTACTCTCGCCTTAG</td>
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</tbody>
</table>
Figure S1: MAFB mutations in pedigrees 0819, PM, and FA. Related to Figure AII.1.
(A) In pedigree 0819 there is a heterozygous deletion of the G at position 439 (orange arrow) in the coding sequence causing a frameshift by 1 bp.
(B) In pedigree PM there is a heterozygous deletion of the A at position 644 (orange arrow) in the coding sequence causing a frameshift by 1 bp.
(C) In pedigree FA there is a heterozygous deletion of the A at position 802 (orange arrow) in the coding sequence causing a frameshift by 1 bp.
(D) In pedigree 0819 the c.439delG mutation arose de novo in individual II:2.
(E) In pedigree FA, segregation analysis places the c.802delA mutation on the black allele of individual II:2, as both this allele and the mutation are inherited by her affected and not by her unaffected offspring (data not shown). Although DNA was not available from parents I:1 and I:2, the de novo status of the mutation in II:2 can be concluded indirectly based on the inheritance of the black allele by II:4 and II:5, both of whom have wildtype MAFB sequence. Parentheses denote assumed haplotype.
(D-E) It is unknown whether the de novo mutations in pedigrees 0819 and FA arose on the paternal or maternal allele, but they are shown on the paternal allele for illustrative purposes.
Figure S2: Deletion of \textit{MAFB} in pedigree N. Related to Figure AII.1.

(A) Custom Droplet Digital PCR (ddPCR) probes were designed in the promoter and coding regions of \textit{MAFB} (left side). ddPCR assay revealed a copy number of 2 for unaffected members of pedigree N and a copy number of 1 for affected members, indicating a deletion in \textit{MAFB} (right side).

(B) Omni2.5Exome BeadChip kit (Illumina) assay of an affected member of pedigree N showed a loss of the 0.5 B allele frequency and reduction of the log R ratio to -1.0 in a ~600 kb region of chromosome 20, consistent with a heterozygous deletion. Using Human Genome Browser GRCh37/hg19, we find that the only gene within the deleted region is \textit{MAFB}. 
Table S2: Sequences of primers used for *Mafb* mouse genotyping, lymphoblast cDNA and cloning of *MAFB* constructs. Related to Figure AII.3. The *Mafb* WT F primer is located upstream of the 5’ LoxP site, the *Mafb* WT R primer is located between the flanking LoxP sites, and the *Mafb* KO R primer is downstream of the 3’ LoxP site. After Cre-mediated LoxP excision, the WT R primer can no longer bind and the only PCR product is the KO band. The cDNA F and R primers were used to reverse transcribe FA lymphoblast mRNA into cDNA to detect the FA mutant *MAFB* transcript. The *MAFB* cloning primers included restriction enzyme sites for Ascl (F) and NotI (R) to facilitate subcloning into the N-Myc expression vector. All sequences are listed 5’→3’. F, forward; R, reverse.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td><em>Mafb</em> WT F</td>
<td>CCTCAACGGCTTCGGGGGCTCCTC</td>
</tr>
<tr>
<td><em>Mafb</em> WT R</td>
<td>CGCTCTCCGAAGTCTTGGCTCTA</td>
</tr>
<tr>
<td><em>Mafb</em> KO R</td>
<td>ATGCAGACACGGAGTCCTCT</td>
</tr>
<tr>
<td>cDNA F</td>
<td>ACCAGCTCGTGTCATGTC</td>
</tr>
<tr>
<td>cDNA R</td>
<td>CCTGGGCTAAGGCAGAGTAG</td>
</tr>
<tr>
<td>Cloning F</td>
<td>TATAGGCACGCATGGCCGAGAGCATGAGCATG</td>
</tr>
<tr>
<td>Cloning R</td>
<td>CCTTGCGCTAAGGCAGAGTAGGCAGGCGCCGC</td>
</tr>
</tbody>
</table>
Figure S3: The mutant MAFB transcript in pedigree FA is expressed in affected individuals. Related to Figure AII.3.

(A) Sanger sequencing of cDNA reverse transcribed from FA lymphoblast mRNA shows the heterozygous single base pair frameshift deletion c.802delA.

(B) Taqman assay for allelic discrimination shows the presence of both the mutant and wildtype alleles in cDNA from FA lymphoblasts, the presence of only the wildtype allele in cDNA from control lymphoblasts, and no signal of either probe from water.
Figure S4: Expression of the wildtype, 0819-mutant (c.439delG), and FA-mutant (c.802delA) MAFB constructs in HEK293T cells. Related to Figure AII.3.  
(A-B) Site-directed mutagenesis was performed to delete the G at position 439 (orange arrow) and the A at position 802 (orange arrow) in the MAFB coding sequence.  
(C) Overexpression of the Myc-tagged wildtype, 0819-mutant, and FA-mutant MAFB constructs in HEK 293T cells detected by western blot. The 0819 mutation results in the expected truncated protein product. The FA mutation results in the expected larger protein product (upper band) and a smaller modified or cleaved product (lower band). Ladder of molecular weights in kDa on the left.
Appendix IV:

Movies from Chapter 3:

Bidirectional ephrin signaling alters abducens nerve development via α2-chimaerin
Movie 1: \(Chn1^{WT/WT}\) E11.5 whole mount embryo. For all whole mount embryo movies, red neurofilament staining highlights all developing cranial nerves and green staining for endogenous \(Hb9:GFP\) specifically highlights VI, XII, and spinal motor neurons. Movie begins with a sagittal view, nose to the right and brainstem to the left. Abbreviations for all whole mount embryo movies: III: oculomotor, IV: trochlear, V: trigeminal, VI: abducens, VII: facial, XII: hypoglossal, C1: first cervical spinal segment.

Movie 2: \(Chn1^{KI/KI}\) E11.5 whole mount embryo exhibits abducens nerve stalling, aberrant trochlear branching, and C1 misprojection. First arrow at 6 seconds: C1 misprojection; second arrow at 19 seconds: abducens stalling, third arrow at 33 seconds: multiple trochlear branches on the left side.

Movie 3: \(Chn1^{KO/KO}\) E11.5 whole mount embryo exhibits abducens nerve wandering. Arrow at 20 seconds: abducens misfasciculation with the buccal branch of the facial nerve. Trochlear and C1 nerves appear normal.

Movie 4: \(EphA4^{KO/KO}\) E11.5 whole mount embryo exhibits abducens nerve wandering. First arrow at 12 seconds: abducens misfasciculation with the mandibular/cervical branch of the facial nerve; second arrow at 15 seconds: wandering abducens projection. Trochlear and C1 nerves appear normal.

Movie 5: \(Chn1^{KI/KI}\) mice have normal alternating gait. For all gait movies, DigiGait video was acquired using a camera below a clear treadmill. Note normal alternation of limbs during walking.
**Movie 6: EphA4$^{KO/KO}$ mice hop.** Note synchronous movement of forelimbs and hindlimbs during walking. Occasionally, mice use alternating forelimb movement during exploration.

**Movie 7: Chn1$^{KI/KI}$:EphA4$^{KO/KO}$ mice have restored alternating gait.** Mice mostly use alternating gait for locomotion. Hindlimb movements appear slightly more restricted and less fluid than wildtype. Occasionally within a series of alternating steps, mice use synchronous hindlimb movement.

**Movie 8: Chn1$^{WT/KI}$:EphA4$^{KO/KO}$ mice use both alternating and hopping gait.** Mice use a combination of alternating gait and synchronous forelimb and hindlimb movement during locomotion.

**Movie 9: Chn1$^{WT/KI}$:EphA4$^{KO/KO}$ E11.5 whole mount embryo exhibits abducens nerve stalling and wandering, but restored C1 projection.** First arrow at 4 seconds: rescued C1 projection; second arrow at 28 seconds: abducens misfasciculation with the mandibular/cervical branch of the facial nerve.

**Movie 10: EphA4$^{fl/fl}$:Isla$^{Cre/+}$ E11.5 whole mount embryo exhibits abducens nerve wandering.** Arrow at 33 seconds: abducens misfasciculation with the mandibular/cervical branch of the facial nerve; note limited abducens projection to orbit. Trochlear and C1 nerves appear normal.

**Movie 11: EphA4$^{fl/fl}$:Twist2$^{Cre/+}$ E11.5 whole mount embryo exhibits abducens nerve stalling.** Arrow at 13 seconds: abducens stalling and turning back toward hindbrain; note the abducens nerve does not reach the orbit.