

University of Nevada, Reno

# **Development of the ocular motor system in mouse embryos**

A thesis submitted in partial fulfillment of the  
requirements for the degree of Master of Science in  
Cell and Molecular Biology

By

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# **THE GRADUATE SCHOOL**

We recommend that the thesis  
prepared under our supervision by

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**Development of the Ocular Motor System in Mouse Embryos**

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## **Abstract**

The ocular motor system enables organisms to move their eyes to obtain precise visual feedback. In humans, six extraocular muscles form an oppositional pulley system to mediate slow, smooth tracking events as well as fast saccades. Their movement is dependent on three cranial nerves: the oculomotor, trochlear, and abducens. This study focuses on the oculomotor nerve (OMN), which innervates four of the extraocular muscles.

In early development, cranial nerves send pioneering axons into the peripheral tissue surrounding the neural tube. Axons must then respond to exogenous guidance cues to find their muscle targets. Precise axon projections are required, and miswiring or other abnormal development in the ocular system leads to strabismus, amblyopia, and impaired vision. Strabismus alone affects up to 2% of the population, and studies have shown that it greatly affects self-image, employment, and public perception. Studying the development of the ocular motor system can help identify the underlying cause of these defects.

Previous studies have explored oculomotor guidance in chick and zebrafish embryos; however, a time course of mammalian development has not been established. This study establishes a time course of the critical stages of oculomotor nerve outgrowth to the periocular area in mice at embryonic day (e) 9.5 – e14.5. In previous studies, targeting was thought to occur when the nerve selected between the 6 differentiated populations of the extraocular muscles. Via immunolabeling and tissue-specific genetic markers, we find that the oculomotor nerve grows out to and spreads out within a mass of muscle precursor cells expressing the myogenic determinants *Pitx2* and *Myf5* near the eye. Contact between nerves and muscle precursors occurs days before mature muscle fibers have formed. The nerve remains in a plexus with these muscle precursor cells past the point of primary myogenesis, then infiltrates the

mature fibers at e14.5. To examine whether EOM primordia were necessary for nIII guidance, we ablated muscle precursor cells by driving diphtheria toxin fragment A (DTA) with the early muscle differentiation factor *Myf5*. We found that the oculomotor nerve displayed ectopic branches, some of which projected beyond the eye, or a lack of plexus formation altogether.

Our research integrates the parallel fields of oculomotor guidance and extraocular muscle development to form a more complete understanding of the choate ocular motor system in mammals. Our results also identify extraocular muscle precursor cells as an intermediate target for the OMN, thus providing evidence for an additional pathfinding step in this poorly understood process.

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## Introduction

Eye movement is critical for a number of organismal functions, and the ocular motor system has evolved separately in several phyla as a unique amalgam of structures. The ability to smoothly track movement (pursuit movements), or snap to a point of interest (saccades) depends on precise motoneuron signaling to the extraocular muscles. This is a complex system, and errors are prevalent: eye movement disorders such as strabismus (commonly known as a “lazy eye”) affect up to 2% of the population (Baker 2002; data from the CDC). These conditions can be caused by congenital defects in ocular motor development, a tightly regulated process in which three cranial nerves target and innervate a group of six craniofacial muscles.

The formation of the ocular motor system is emblematic of craniofacial neuromuscular development: myoblasts and axons navigate through a shifting milieu of differentiating cells, and must respond very accurately to temporal cues in order to make precise connections to one another. Their maturation is interdependent (Hyatt et al. 2003; Eftimie et al 1991). Although muscular positioning occurs properly in the absence of innervation, lack of proper nerve input can lead to muscle fibrosis (Porter 1997). On the other hand, nerves are dependent on muscles for both positional information (unpublished data) and trophic signals (Steljes et al. 1999; Rind and von Bartheld 2002; Chen et al 2003). Therefore proper co-development of both systems is essential to an animal’s fitness, and establishing a baseline normal mammalian phenotype is useful for both clinical and molecular applications (Baker 2006). While studies have explored both chick and zebrafish embryos (Chilton and Guthrie 2004; Clark et al. 2013), mammalian research lags behind.

In the mouse, seven extraocular muscles are innervated by three cranial nerves: the oculomotor, trochlear, and abducens. The oculomotor nerve (OMN) is responsible for five of

these: the medial, ventral, and dorsal rectus; the ventral oblique, as well as the levator palpebrae (controls the eyelid). The trochlear nerve innervates the dorsal oblique, and the abducens innervates the medial rectus and retractor bulbi, an accessory EOM that has developed to protect the cornea (Porter 2006).

OMN cell bodies are born in the ventral midbrain, and axons extend laterally through the developing paraxial mesoderm and prechordal plate (Noden and Francis-West 2006). The path of the OMN is partitioned into conserved steps (Fig 8). It first exits the neural tube and travels ventrally until a point midway between the cephalic flexure and trigeminal nerve, where its path shifts medially. When it reaches the periocular region, it undergoes a stall behind the eye that is conserved across species (Clark et al. 2013). In the current paradigm, the nerve remains in a plexus behind the eye for several days before forming branches to adult extraocular muscles in their final positions (Chilton and Guthrie 2004; Clark et al. 2013). In this study, we find that the oculomotor plexus is colocalized with Pitx2-expressing muscle precursor cells well before differentiated myofibers have formed. The plexus remains in contact with these cells as they mature into myotubes and migrate to their eventual orientation around the eye.

Although a changing complement of guidance cues and receptors must be necessary to regulate the transitions of the OMN, only two ligand-receptor pathways have been identified *in vivo*, and none in mammals (Lance-Jones et al. 2012). Stromal cell-derived factor-1 (SDF-1) is an attractive cue localized in the mesenchyme surrounding the midbrain in chick embryos, and attracts axons from the oculomotor and trochlear nerves from the neural tube and into the surrounding cephalic tissue. SDF-1 is a ligand for CXCR4 receptors, and knockout mice for both molecules show a greatly reduced number of oculomotor and trochlear axons in the periphery (Lerner et al. 2010). No specific molecular cues have been identified for the second

transition point; however, a genetic screen for cranial nerve mutations preferentially affects this piece of the nerve in mice, suggesting that its migration is regulated by separate signals (Mar et al. 2005). Finally, Sema3A acts via the PlexinA receptor and alpha2-chimaerin signaling to mediate the transition from nerve plexus to organized and targeted branches that contact mature myofibers in zebrafish and chick embryos (Lerner et al. 2010; Ferrario et al. 2012).

In this study, we describe the time course of oculomotor nerve development in mouse embryos, from the initial outgrowth of the nerve on E9.5 through neuromuscular junction formation by E14.5. We find evidence that the population of extraocular myoblasts are responsible for initial plexus formation, and ablation of these cells causes ectopic branching and target deformations such that significant portions of the nerve don't reach the eye.

Extraocular muscle (EOM) development is spatially and temporally related to the oculomotor nerve, and a concurrent understanding of both systems must be developed to resolve the ocular motor system as a whole. In recent years, progress in fate-mapping techniques and molecular markers has enabled researchers to distinguish craniofacial muscles as an evolutionarily separate group of skeletal muscle (Sambasivan et al. 2011). Unlike the highly organized somites that give rise to trunk muscles, craniofacial muscles originate in poorly segmented somitomers of the head (Bothe and Dietrich 2006; reviewed Noden and Francis-West 2006), adjacent to the developing neural tube and contiguous with the medial splenic mesoderm (Tzahor 2009). Craniofacial muscles express a unique complement of myogenic regulatory factors (Hacker and Guthrie 1998; Mootoosamy and Dietrich 2002; Porter 2002; Porter and Kaminski 2006; Sambasivan et al. 2009), and share a common lineage with the cardiac muscle precursor cells that go on to become the second heart field (Tzahor 2009; Lescroart 2010). These populations have significant clinical impact because they govern

functions such as swallowing, tongue movements, and eye contact; in addition, they are differentially affected in myopathies such as myasthenia gravis and muscular dystrophy (Adrade 2000; Porter 2002) which can help researchers understand the molecular basis of trunk muscle fibrosis.

Within this subset of skeletal muscles, the EOM are further distinguished as their own allotype of muscle (Fischer et al. 2005), and express their own defining regulatory cascade (Sambasivan et al. 2009). Like cardiomyoblasts, differentiation of EOM precursors is dependent on the bicoid-like homeobox transcription factor Pitx2 (Diehl et al. 2006; Zhou et al. 2011) and retains this dependency postnatally (Hebert et al. 2013). EOM are derived from paraxial mesoderm and prechordal plate, directly adjacent to the midbrain and metencephalon, and migrate dorsolaterally to the periocular region. This proximity to the neural tube lends itself to comigration: in chick embryos, the developing abducens nerve exits the midbrain and contacts a mass of precursor cells, after which both migrate to the eye together (Carpenter and Walton 1904; Wahl et al. 1994). However, the potential for nerves to comigrate with their muscle precursors remains poorly understood, as well as any potential role for nerve or muscle precursors to regulate each other's development.

Fate determination of EOM primordia seems to be intrinsic, as chick embryos display distinct condensations for each muscle, each of which matures at its own rate (Noden et al. 1999). The extraocular muscle phenotype is complicated: each muscle has a global and orbital layer with different insertion points on the globe, and each muscle expresses a different array of fiber types including slow twitch, fast twitch, cardiac, embryonic, neonatal, and EOM-specific (Porter 2002, Zhou et al. 2010). However, long-term survival and proper fiber-type specification is dependent on proper innervation (Porter 1993, Porter 2006b). Additionally, congenital fibrosis

of the extraocular muscles—human pathologies once thought to solely originate from defects in muscle development—have now been proven to arise from atypical motoneuron development (Oystreck et al. 2011, Engle et al. 1997). These factors show that ocular motility is an interdependent process that cannot be fully resolved without considering both nerve and muscle.

This study finds that EOM precursor cells modulate an important step of OMN guidance, which suggests an additional intermediate target in its pathfinding.

## Methods

### *Mouse embryos*

Animal experiments were approved by the UNR IACUC and followed NIH guidelines. CD1 wildtype embryos were used. *Myf5-Cre;DTA* embryos were a gift from Tom Gould (University of Nevada School of Medicine). Embryos were collected at noon of day 9.5-14.5, and the 14.0 was collected at midnight. E0.5 was designated as the day of the vaginal plug. E13.5 and E14.5 embryos were fixed via cardiac perfusion, and all embryos were stored in 4% paraformaldehyde until dissection (at least one night).

### *Immunohistochemistry*

CD1 embryos ranging from stage e9.5 – e12.5 were dissected in cold .1M phosphate buffer, and fixed with 4% paraformaldehyde before embedded. Embryos were then embedded and cryostat sectioned as previously described (Farmer, 2008). 20 $\mu$ m cryostat sections were rinsed in warm 0.1M phosphate buffer, then blocked with 1% triton, 1% normal goat serum, and

1X sodium azide 3x quick rinse and 3x 5min. Primary antibody was diluted in Jessell and applied overnight at room temperature. Pitx2 antibody was from Capra, Sweden and diluted 1:2000;  $\beta$ III-tubulin was from (Covance/Millipore) and diluted 1:1000.

Slides were rinsed 3x quickly, then 3x 5min in Jessell the following day, then secondary antibody was applied at room temperature for four hours. Secondary antibodies were diluted in Jessell: Cy3 (anti-rabbit, Abcam) 1:200 and Alexa 488 (anti-mouse, Abcam) 1:200. Finally, slides were rinsed 3x quick and 3x 5min before imaging.

### *Axon tracing*

To anterogradely label the OM plexus, e12.5 embryos were dissected to reveal the nerve. A small crystal of lipophilic dye DiI was crushed and applied onto the OMN midway between the eye and the midbrain. Embryos were then left two nights in 4% PFA at 37°C. To visualize the nerve plexus, the embryo was bisected and the brain was lifted out, leaving a thin curve of tissue caudal to the eye. Embryos were equilibrated in graded glycerol solutions diluted in 4% PFA: 50% 30min, 75% 1 hr, 90% 1 hr, 100% 1.5 hr. The medial side of the eye was then imaged with confocal microscopy.

## **Results**

Mice have four extraocular muscles arranged at the cardinal positions of the eye and two oblique muscles that work in vestibuloocular movement. Their arrangement closely mirrors human EOM with one exception: mice have an accessory muscle called the retractor bulbi,

which retracts the globe and protects the cornea. The OMN innervates four muscles: the ventral oblique; and the ventral, medial, and dorsal recti. The trochlear nerve innervates the ventral oblique, and the abducens nerve innervates the remaining two muscles, the lateral rectus and retractor bulbi.

To visualize the formation of the ocular motor system, we used Pitx2 and  $\beta$ III-tubulin immunostaining on 20 $\mu$ M sections. Pitx2 encodes an early-activated homeobox transcription factor that is necessary for cardio- and craniomyoblast survival and differentiation (Evans and Gage 2005; Kitamura et al. 1999).  $\beta$ III-tubulin is a microtubule protein isoform found in axons, and is a general marker for all neurons.

No molecular markers were used to determine fiber formation in muscle precursor cells, but differentiation causes dramatic changes in cell morphology that are easily detectable by basic histology. Here, myoblasts are defined as round cells without polarity. Myotubes are immature, short, polarized cells. Myofibers are long cells that have reached tendinous attachment points at either end.

### **The oculomotor nerve contacts extraocular muscle precursor cells at e10.5**

The OMN exits the midbrain at e9.5 (Easter et al. 1993, corroborated by Fig 1B). By e10.5, it has arrived at the ventral periocular area. EOM primordia also coalesce in the infraorbital area at e10.5 (Cheng et al. 1992, Porter 2006a).

The tight fascicle of the OMN was easily identified exiting the midbrain in an e10.5 mouse embryo (Fig 1C). By tracing the nerve from its exit point through the subsequent 20 $\mu$ M sections, a 3D reconstruction (Fig 1A) was configured. The Pitx2-positive column of muscle precursor cells coursed from the metencephalon to an area ventromedial from the eye. No axons

were seen in the superior portion of the muscle precursor cells (MPCs) (Fig 1D-E); sparse axons were observed in one lateral plane in the center of the mass (Fig 1D). In Fig 1G, the inferior portion of the MPCs was located near the first branchial arch without innervation.

Sagittal sections enabled easy visualization of muscle positioning and morphology, which was sometimes difficult to infer from the transverse section plane. A sagittal section plane identified a solitary mass of precursor cells ventral to the eye (Fig 1 I-J), as well as a population of Pitx2-positive cells surrounding the optic cup. These cells exhibited a more diffuse Pitx2 expression pattern than the cells distal to eye. The eye is partially surrounded by a tendinous sheath known as Tenon's fascia, which is known to be conserved in humans and monkeys (Sevel 1988). Pitx2 colocalizes in previous studies with Six2, a transcription factor found in tendon precursors (Holmberg et al. 2008). Thus, these cells were interpreted as the anlagen of Tenon's fascia, which forms a fibrous layer around the globe and provides specific attachment points for the EOM. Development of Tenon's fascia is integral to the pulley system that underlies the implementation of Listing's law, which predicts torsion in gaze dynamics (Miller 2007). DAPI staining would determine if its Pitx2 expression pattern was cytoplasmic compared to the more discrete and presumably nuclear expression seen in EOM precursors.

The oculomotor nerve is the first nerve to make contact with the mass of MPCs ventral to the eye, which it does at e10.5. No overt segregation of the MPC mass is visible, and the nerve exists as several diffuse fibers within the mass with no major branches.

### **MPCs move dorsally as nIII fibers increase**

By e11.5, increased numbers of muscle and nerve cells have formed a distinct column on the ventromedial aspect of the globe (Fig 2A). To label the muscle and nerve cells, embryos

were immunostained with Pitx2 and  $\beta$ -tubulin (Fig 2C-F). A Myf5-Cre;Rosa26-tdTomato mutant mouse showed a labeling pattern very similar to the Pitx2 pattern (Fig 2G-J). Myf5 is the earliest transcription factor for EOM, and the Myf5-Cre;Rosa26-tdTomato mice create a fate map for all cells with Myf5 expression (Gensch et al. 2008). The labeling pattern corroborates the identity of the MPC mass.

The number of fibers in both the fascicle and plexus increased on E11.5 (Fig D,H), and branches were visibly intermingled with MPCs (Fig2 E-F;I-J). Myoblasts similarly proliferated, and tenuous boundaries could be ascribed to the mass. Nerve fibers contacted different aspects of the MPCs, and a majority of muscle cells were not in contact with axons. The oculomotor plexus was constricted laterally and spanned 40 $\mu$ M on the vertical axis. MPCs did not reach the dorsal side of the eye, but spanned at least 100 $\mu$ M vertically.

The trochlear nerve contacted the dorsomedial portion of precursors (Fig2 C,G), while the abducens nerve shared a similar plane of entry to the oculomotor nerve, but did not penetrate as far into the muscle mass(Fig 2 F,J).

All cranial nerves that innervate the extraocular muscles are present in the mass of muscle precursor cells at e11.5 in the developing mouse embryo. Although the mass is contiguous, each nerve enters and contacts a different portion. This suggests that muscle precursor cells are fate-locked before myofiber differentiation, and cranial nerves distinguish between distinct cell populations within the MPC mass.

### **The oculomotor nerve is static as myogenesis begins**

Individual, polarized myotubes were observed to migrate towards the globe by e12.5. Four of seven muscles were visible (Fig 3D): the lateral rectus (nVI), dorsal oblique (nIV),

ventral rectus and ventral oblique (nIII). Tenon's fascia showed four distinct swellings for muscle attachment (Fig 3C). Tendon attachment sites are a known source of guidance cues for developing muscles (Schnorrer and Dickson 2004).

Differences in maturation between littermates enabled a detailed analysis of this critical stage. The embryo depicted in Fig 3E was slightly more mature than the embryo in Fig 3F. The younger embryo (Fig 3F) displayed a sprawling plexus similar to stage e11.5. The dorsal rectus had not yet begun myogenesis, and a large group of undifferentiated myoblasts surrounded the optic stalk. In the more mature embryo (Fig 3E), the nerve plexus compacted and no longer infiltrating the diminishing population of myoblasts. A distinct condensation of myotubes formed the developing dorsal rectus, confirming that it is the fifth muscle to develop.

Towards the embryo midline and deep to the eye (G-I), more muscle precursor cells were colocalized with the bulk of the oculomotor nerve, and myotubes branched off the mass in two distinct masses. One mass gave rise to the lateral rectus (nIV), and the other separated into the ventral rectus, ventral oblique, dorsal rectus, and ventral rectus (nIII), indicating an organizational segregation.

Transverse sections (Fig 4A) enabled identification of the abducens (Fig 4D) and trochlear nerves (Fig 4B). In comparison to E11.5, the oculomotor nerve (Fig 4C-F) was relatively unchanged: the plexus remained constrained in the vertical axis, and nerve fibers infiltrated differentiating myotubes. The overall morphology of the MPCs was most apparent in this plane. Undifferentiated cells lay deep to the globe, while myotubes were adjacent in a more superficial position. The beginning of the tendinous annulus that serves as an attachment point for six of the seven EOM (Porter 2006) was seen in Fig 4F.

Interestingly, in contrast to the initial close contact on E10.5 between nerve and muscle precursors, the oculomotor nerve and associated cranial nerves do not follow their muscle precursor targets as they initially shift to their positions around the eye. As the embryo matures, the OMN plexus becomes more compact and dispartate from MPCs.

### **OMN plexus persists during and after primary myogenesis**

Transverse (Fig 5A-B) sections of an e13.5 embryo contained all muscles, and no round myoblasts remained. Interestingly, the OMN (Fig 5C-G) and abducens nerve (Fig 5H) were still coalesced and distinctly separate from the myofibers. Nerve fibers were no longer interwoven with myogenic precursor cells, suggesting that signaling between the two populations had changed. Nerve fibers that could be seen in contact with the inferior oblique (Fig 5A) came from the trigeminal ganglion and did not form a plexus in the EOM.

Medial and lateral recti connected to Tenon's fascia, which displayed large attachment points marked by increased cell numbers (Fig 5C,E,F,H). Tenon's fascia also formed a cone of cells along the equatorial axis of the eye, between the recti (Fig 5E,H). Innervation in these cells may correspond to smooth muscle fibers interspersed in the tendon, which form a smooth muscle pulley in the adult animal that mediates tension during eye movements (Demer et al. 1997).

After muscles reach their final positions around the eye, the OMN remains relatively stationary. It is no longer spread out in a mass of MPCs, but rather clustered along the side of maturing myofibers.

### **Nerves infiltrate EOM in conserved neuromuscular compartments .**

The e14.5 embryo has well-developed embryonic muscle fiber types (Porter 1995). To see the positions of muscles at this stage, I undertook gross analysis of sagittal sections. I found that all muscles were in their mature positions around the globe (Fig 6C-D). Muscles were spread out as they reached their attachment points in Tenon's fascia (Fig 6C), but grew more compacted as they reached their common origin at the tendinous annulus of Zinn adjacent to the brain (Fig 6D). The retractor bulbi partially encircled the optic nerve.

Although sagittal sections were more convenient to determine muscle development and positioning, the subtleties of innervation were more readily apparent in transverse sections (Fig 6 E-L). The infiltration of muscle fibers by cranial nerves was striking in contrast to e13.5; each muscle showed nerve fibers throughout its midbelly and deep towards the tendinous attachment point (Fig 6 E-F). The OMN was observed branching into the ventral rectus, ventral oblique (Fig 6D; I-K), and medial rectus (Fig 6 G-H). The abducens nerve displayed a very similar branching in the lateral rectus as the OMN in the medial rectus (Fig 6E).

The pattern of infiltration was consistent across embryos, indicating the presence of conserved neuromuscular compartments (Schieber et al. 2001). These compartments add another layer of complexity to axon targeting; one branch of a nerve will target a specific subset of myofibers, enabling partial contraction in skeletal muscles through conserved compartments (Donahue and English 1987). This pattern has been meticulously documented for the abducens nerve in the lateral rectus (Peng et al. 2010), and is typified in my observations by the oculomotor nerve. In every embryo examined, nIII infiltrated the medial rectus approximately three muscle fibers away from the retractor bulbi (Fig 6G-H); it inserted in the dorsal oblique close to the globe and sent out characteristic axons (Fig 6I-K).

Somewhat anomalously, both the medial and lateral recti appeared to have a small dorsal projection (Fig 6E-F). This smaller mass seemed to have a separate attachment point on Tenon's fascia; however, it was difficult to resolve the three dimensional nature of these muscles in 20 $\mu$ M sections. Two alternative interpretations may explain these smaller muscle masses. First, adult EOM possess two different layers—global and orbital—with distinct fiber types and attachment points. This process was thought to take place postnatally, but has not been closely examined (Porter 2006). The smaller muscles are likely not accessory muscles. Although some mammals such as monkeys and cats have an accessory later rectus that arises from a chimaera of two earlier myotomes (Narasimhan et al. 2007), adult mice display no evidence of this additional muscle, indicating that it does not persist through development. The dorsal muscle projections were taken as protrusions of the larger muscle mass for the purpose of this study.

Overall, the transition from a separate nIII plexus in E13.5 embryos to muscle midbelle innervation in E14.5 shows that the innervation of the OMN takes place quickly, and in a conserved fashion. Nerves target their intended neuromuscular compartments and spread characteristic branches out through the myofibers.

### **MPCs provide an intermediate cue that governs OMN plexus formation**

Because the OMN initially targeted the MPC mass, we tested whether or not the MPC mass was required for oculomotor nerve guidance. Our strategy was to specifically ablate muscle precursors using a Myf5-Cre;DTA transgene, in which early muscle precursors express Cre recombinase. The Cre then activated the expression of a diphtheria toxin (DTA) transgene by deleting a lox-GFP-STOP-lox cassette to drive DTA expression by the ubiquitous Rosa 26 promotor. Myf5 is one of the earliest transcription factors to be expressed in EOM, and is

present before MyoD or subsequent differentiation to myotubes. Therefore, Myf5 acts as a promoter to drive diphtheria toxin in muscle precursor cells. To specifically label oculomotor neurons, I used DiI—a lipophilic dye that can be crushed onto an axon and travel retro- and anterogradely. For this preliminary study, three E12.5 littermates were analyzed by DiI labeling of the oculomotor nerve: one control lacking the Myf5-Cre driver, and two embryos positive for the Myf5-Cre;DTA transgenes. Genotypes were confirmed via PCR.

In the WT littermate control, the labeled nerve fibers grew out to form a large plexus located on the ventromedial side of the eye. This plexus was identical to what was observed in CD1 mice, indicating that animals without the Myf5 driver were phenotypically normal (Fig 6A). A small branch before the eye may contact the ciliary ganglion. Research on this event in mice is scant and incidental: a study of Wnt-1 signaling incidentally hypothesized this contact happened at e14.5, but wasn't conclusive (Fritsch et al. 1995). One longer branch extended to the ventromedial aspect of the globe, where previous Pitx2 immunolabeling indicated a large muscle precursor mass would be beginning to differentiate into several oculomotor targets (Fig 3).

In the first Myf5-Cre;DTA mutant, the oculomotor nerve prematurely formed two large branches on the lateral side of the eye (Fig 6B). One branch traveled to the dorsal aspect of the eye and ended in a broad stub, while the other split again. One of these branches missed the eye entirely, while the other formed what could be a small plexus behind the globe. The other side of the embryo (not pictured) showed an independent pattern: while the nerve reached the periorbital area, it was small and ended in a stunted fascicle.

The second Myf5-Cre;DTA mutant also displayed no plexus. (Fig 6C). The fascicle was correctly oriented and positioned, but split aberrantly, then reformed and failed to branch

entirely. Again, no bilateral symmetry was observed, and the other side showed ectopic branches behind the eye.

In summary, muscle ablation experiments reveal major guidance defects in OMN plexus formation, indicating that extraocular muscle precursor cells are a source of directional cues for the OMN. These errors are present as early as E12.5, which means that guidance signaling occurs prior to the formation of mature myofibers.

## **Discussion**

The key finding of this study is that the oculomotor nerve contacts a population of Pitx2-expressing muscle precursor cells in the periocular area, and remains in close contact with those cells during their concurrent development into the ocular motor system. The oculomotor plexus stalling point has been observed in several model systems, including chick and zebrafish embryos (Chilton and Guthrie 2004; Clark et al. 2013; Ferrario et al. 2012). While this evolutionary conservation could be coincidental, the otherwise tightly regulated and highly specialized development of the ocular motor system indicates that critical functionality may underlie this process (Block 1994). The close contact between the oculomotor nerve and the muscle precursor mass suggests critical signaling events in oculomotor system development.

### **Oculomotor axons contact myogenic precursor cells prior to primary myogenesis**

Using immunohistochemistry and tissue-specific promoters, we visualized oculomotor nerve migration to the eye, creating a time course of EOM innervation in mammals.

Surprisingly, we found that the oculomotor nerve remained stalled for several days behind the eye. Although little is known about motoneuron targeting in the periphery chick lumbosacral

spinal cord motoneurons also stall before they infiltrate their intended targets in the hindlimb (Lance-Jones and Landmesser 1981b; Wang and Scott 2000). Using an antibody for the early myogenic regulatory factor Pitx2, we found a large mass of undifferentiated cells colocalized with the oculomotor nerve. The mass was traced back to the area of the first branchial arch (Fig 1G), with a large portion migrating to the dorsal periocular region. Although innervation was sparse, axons from the OMN nucleus were interspersed throughout the muscle anlagen ventral to the eye.

Axons traveled preferentially target muscle precursor cells before retracing their steps back to the optic cup in the following few days of development. This movement suggests a type of axon guidance known as “stretch growth” or “towing” (Franze 2013; Gilmour et al. 2004). In this model, axons use cell-cell adhesion to connect with their target tissue, which then actively pulls them to their final positions.

Previous studies (Chilton and Guthrie 2004; Clark et al. 2013) used markers for adult myosin isotypes when mapping OMN development in chick, which would not detect the interaction of the oculomotor nerve with the muscle precursor mass. Our evidence shows that the oculomotor nerve makes contact with the dorsal portion of extraocular muscle precursors, then travels with those cells to the eye. Staining for acetylcholine receptors would determine whether or not active signaling occurs during this time period.

Innervation has long been known to influence myogenic differentiation factors and survival in skeletal muscle (Wang and Washabaugh 2003; Yablonka-Reuveni et al. 1999; DiMario and Stockdale 1997; Eftimie et al. 1993; Fredette and Landmesser 1991; Dennis et al. 1981), although its role as a primary regulator of myogenesis is contested (Fredette and Landmesser 1991; Vogel and Landmesser 1987). Regardless of whether nerve input is the sole

source of trophic and fiber-type cues during the early stages of development, it is unquestionably important. In the ocular motor system, ablation of nIII via *Phox2A* mutation or *Wnt1* mutation causes muscle cell death (Hasan et al. 2010; Bosley et al. 2006; Porter 1997) or aberrant fiber morphology when muscles are cocultured with improper nerve input from the spinal cord (Porter 1993). However, initial muscle positioning and primary myogenesis in EOM is unaffected by nerve ablation in *Wnt-1* mutant mice that lack a midbrain, indicating that muscle migration is not nerve-dependent (Porter 1997). Examining the nature of interaction between muscle and nerve during this early stage in development could provide clues about myogenic differentiation pathways, as well as further the field of craniofacial myogenesis.

#### **Nerve and muscle separate during muscle differentiation.**

Interestingly, our study finds that the oculomotor plexus persisted as muscle precursors differentiated into myofibers and found their adult positions around the eye. While the nerve plexus at e11.5 was a sprawling entity with growth cones extending the width of the MPC mass, it became increasingly compacted and separate as muscle cells matured. By e13.5, the nerve plexus was completely isolated from new myofibers and no infiltration was observed whatsoever. At e14.5 a striking change occurred, and axons infiltrated muscle fibers extensively.

This modified behavior indicates that signaling between muscle and nerve is altered during or directly following primary myogenesis. Axons change their protein expression in response to target-derived signals (Tessier-Lavigne et al. 1988, Brittis et al. 2002). In oculomotor neurons, the transition from plexus to arborization is known to involve the RACgap protein  $\alpha 2$ -chimaerin (Clark et al. 2013), which regulates—along with other cytoskeletal components—tubulin dynamics in axonal growth cones (Yang and Kazanietz 2007).

Overexpression of  $\alpha 2$ -chimaerin leads to extended stalling of the OMN in zebrafish, while knockdown assays lead to ectopic branching (Clark et al. 2013; Miyake et al. 2008), indicating that this protein is expressed in oculomotor axons during their stall.

Acting downstream of the PlexinA receptor,  $\alpha 2$ -chimaerin integrates chemoattractive and chemorepellent signals during OMN migration (Ferrario et al. 2012). When cultured with HGF or CXCL12, OMN axons show increased outgrowth, which is inhibited by  $\alpha 2$ -chn knockdown (Lerner et al. 2010). *In situ* hybridization reveals Sema3A expression outlining each newly formed muscle just prior to innervation in chick embryos. This mechanism is thought to be at least partially contact-mediated, making  $\alpha 2$ -chimaerin signaling a likely candidate for the anomalous OMN plexus during primary myogenesis.

Mapping a time course of expression for these ligands, or generating a temporally controlled expression pulse, could determine whether or not muscles begin to express Sema3A as they form myotubes. The molecular pathways underlying OMN guidance are poorly defined, and this information could also resolve the chronological expression of PlexinA1 and  $\alpha 2$ -chimaerin ligands, providing insight into the complex signal integration that occurs during this transitional stage.

Finally, the separation of muscles and nerves suggests a complicated selection process. Primary nerve contact occurs in a conserved location of the MPC mass, with distinctive areas for the abducens, trochlear, and oculomotor nerve. Like many other propagating cell populations, it is possible that myogenic precursors possess their fate determination before they differentiate, and the localized contact of the cranial nerves suggests that this type of prepatterning is present. However, differentiating myotubes move away from the nerve plexus, which means that axons must once again find their targets amongst the seven EOM. This could be caused by repulsion,

loss of a chemoattractant, or cell-cell signals that disable axons from leaving the plexus.

Identifying molecular markers for each muscle would enable researchers to map the precursor mass, as well as ablate muscles specifically to determine differential guidance defects.

### **Muscle anlagen are a source of guidance cues for the OMN.**

To test whether muscle precursor cells were involved in oculomotor nerve guidance Myf5-Cre;DTA mice were generated. In mice with ablated muscle cells at e12.5, the OMN shows premature branching, ectopic branching behind the eye, and collapsed growth cones. In Fig 6B, one branch bypasses the eye entirely. Embryos show no bilateral symmetry, suggesting that axons are responding in a random manner, rather than to any dominant positioning signals that remain in the absence of the muscle precursors. Because Myf5 is an early myogenic determinant and no mature myofibers are present at E12.5, this phenotype suggests that the extraocular muscle precursor cells provide an important guidance cue that is responsible for the OMN plexus.

In previous time courses mapped out in chick and zebrafish, it was hypothesized that there was no signaling between the oculomotor nerve and extraocular muscles until the OMN chooses between myofibers located at their mature positions around the globe (Chiton and Guthrie 2004; Clark et al. 2013). Our results suggest that functional signaling occurs before primary myogenesis, and explains why the oculomotor nerve maintains a plexus behind the eye during multiple stages of development.

Myf5-Cre;DTA mice have no early myogenic cells, but eventually develop a rescued skeletal muscle phenotype by E14.5 (Gensch et al. 2008). This is due to the functional

redundancy of MyoD and Myf5, two myogenic regulatory proteins that are frequently expressed in all muscle precursor cells, and do not require sequential activation in order to develop a mature muscle phenotype (Haldar et al. 2008). Cre-driven GFP expression in E12.5 mice shows that myoblasts are nonexistent at this stage, and MyoD compensation does not occur until later in development.

It is interesting to note that the OMN unerringly reaches the periocular region even when defasciculated, disrupted, or in the absence of muscle signaling (Caton et al. 2000; Mar et al. 2005; our data). This indicates that periocular migration is independent of the muscle precursor mass. The migration of the OMN to the eye is robust and conserved not only when muscle precursor cells are ablated, but also in a genetic screen for cranial nerve mutations (Mar et al. 2005). The OMN shows various defects such as zigzagging and defasciculation in the periphery, but axons still manage to find the eye, indicating that this process is tightly regulated by an early and indispensable gene, or has multiple compensatory pathways to rescue its function. Some spinal cord motoneuron populations have difficulty migrating into the periphery in the absence of skeletal muscle, and motor pools undergo sequential ablation when muscles are removed (Lewis and Chevallier 1981; Kablar and Rudnicki 1999).

Determining the fate of the misdirected OMN in later stages might prove interesting, particularly in light of MyoD rescue. The OMN is regenerative, and displays some level of target specificity after being severed (Bender and Fulton 1938; Sperry and Arora 1965; Metz and Scott 1970; Scott 1977). Whether or not it could be redirected after muscle ablation at an earlier stage could provide some insight into innervation specification.

Neuromuscular targeting is a complex question, made additionally complicated in the oculomotor nerve by a number of intermediary steps and multiple targets. It is largely assumed

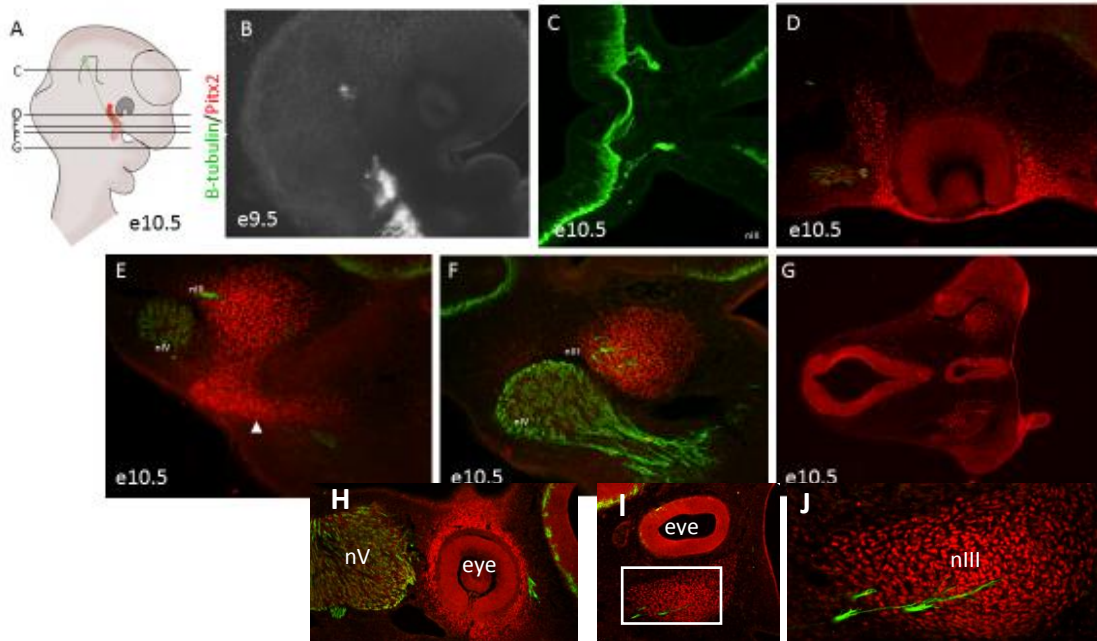
that some level of competitive innervation exists, meaning that over-innervation occurs at an early stage in development, and then synapses from foreign nerves are trimmed back during a period of neuronal death (Landmesser 1980; Lance-Jones and Landmesser 1981a). Even neurons from the same motor pool will be eliminated: each mature myofiber has just one nerve input (Hamburger and Oppenheim 1990; Sun et al. 2003). The current research supports a hypothesis in which axons are thought to show target specificity, but have secondary responses to other muscles due to the commonality of their receptors and adhesion molecules. In the absence of their preferred cue, they will often respond to any muscular target.

Given the segregation of the individual extraocular muscle primordia in chick embryos, their differential expression of myogenic regulatory elements, and their diverse evolutionary origins, it seems likely that these cells are fate-locked before primary myogenesis begins (Noden and Francis-West 2006; Sambasivan et al. 2009; Rios and Marcelle 2009). Therefore, the incoming OMN must distinguish between a large mass of adjacent cells, in close proximity with two other nerves, in order to find its targets. This is a miracle of guidance, and a unique environment for researchers to manipulate. Finding molecular markers for each EOM would enable us to study this system in great detail. Perhaps ablating the muscles that give rise to OMN targets would result in the same phenotype as *Myf5* ablation, or the remaining precursors of the lateral rectus and superior oblique would rescue the phenotype. Compensatory innervation in this system would enable a better understanding of certain types of amblyopia, as well as increased evidence for the competition theory.

## **Acknowledgements**

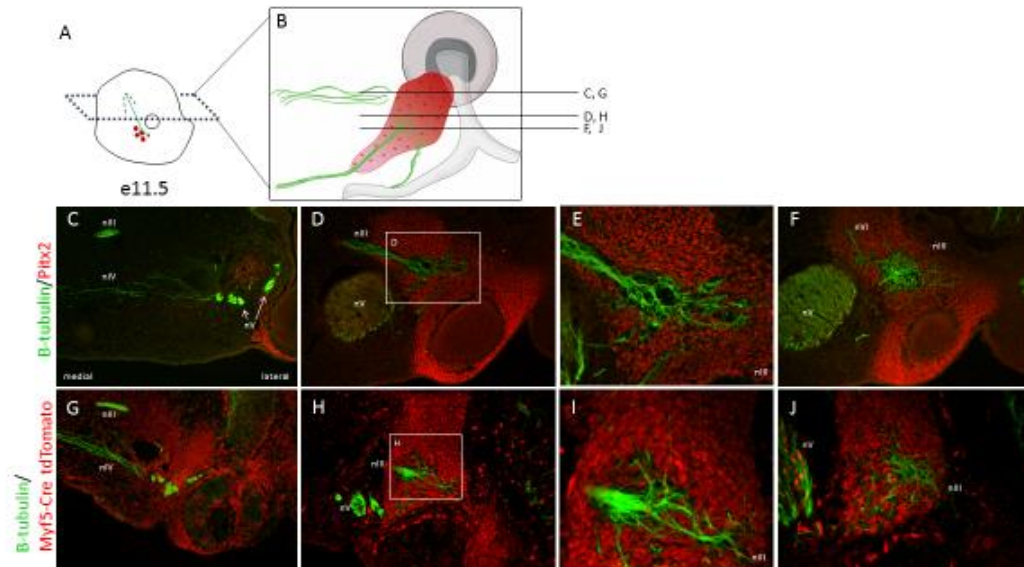
Funding was provided to Grant Mastick by NIH grants R21NS077169, with core facility support by NIH COBREs 1 P20 RR024210 and 1 P20 GM103650, and the Nevada INBRE 8 P20 GM103440-11.

## Figures



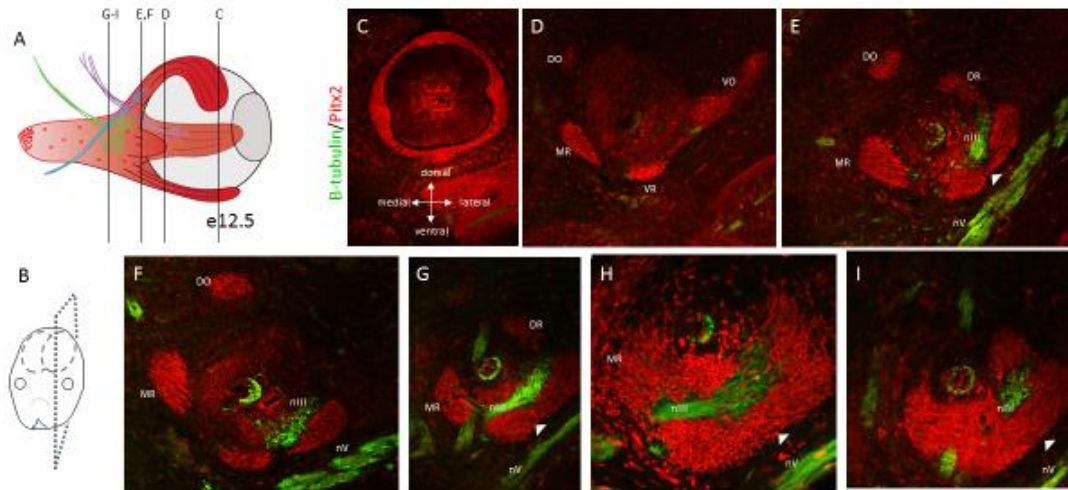
**Figure 1. The oculomotor nerve is colocalized with a mass of undifferentiated muscle precursor cells at e10.5.**

(A) Schematic of transverse section plane. (B) Whole-mount image of e9.5 embryo labeled with GFP. (C-J) Transverse sections through an e10.5 embryo labeled with Pitx2 (red) and  $\beta$ -tubulin (green). (C) Exit point of the OMN in the midbrain. The tight fascicle is easily traced to the periocular area. (D) No innervation is present directly behind the optic cup. A single lateral column of muscle precursor cells is contiguous with the larger ventral mass. (E) The oculomotor nerve enters the lateral aspect of the muscle precursor mass. Arrowhead indicates Pitx-2 labeled cells with cytoplasmic labeling. (F) nIII fibers are fewer than what is seen at later stages. (G) Pitx2-labeled cells extend down to the first branchial arch. (H) Although the precursor cells for Tenon's fascia are present, no attachment points are visible. (I) The precursor mass is entirely ventral to the eye. (J) The precursor mass is entirely ventral to the eye.



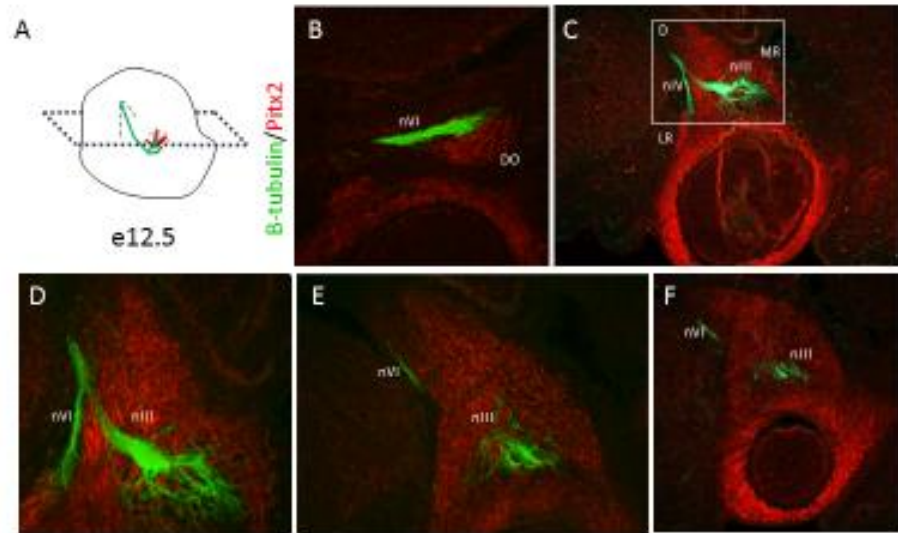
**Figure 2. The oculomotor plexus grows in size, colocalized with a column of muscle precursor cells at e11.5.**

Pitx2 antibody labeling identifies the same set of cells as a Myf5-Cre driver with a tdTomato reporter (A) Schematic of transverse section plane. (B) 3-dimensional schematic of e11.5 embryo. (C, G) The trochlear nerve contacts the dorsal aspect of the MPC mass. This nerve is not fasciculated. (D, H) The oculomotor nerve enters the MPC mass as a tight fascicle and spreads out in a deep column of muscle precursor cells. (E, I) A 40x image of the OMN plexus. (F, J) The abducens nerve innervates the deepest portion of the MPC mass, indicating that some inherent segregation of these cells may exist.



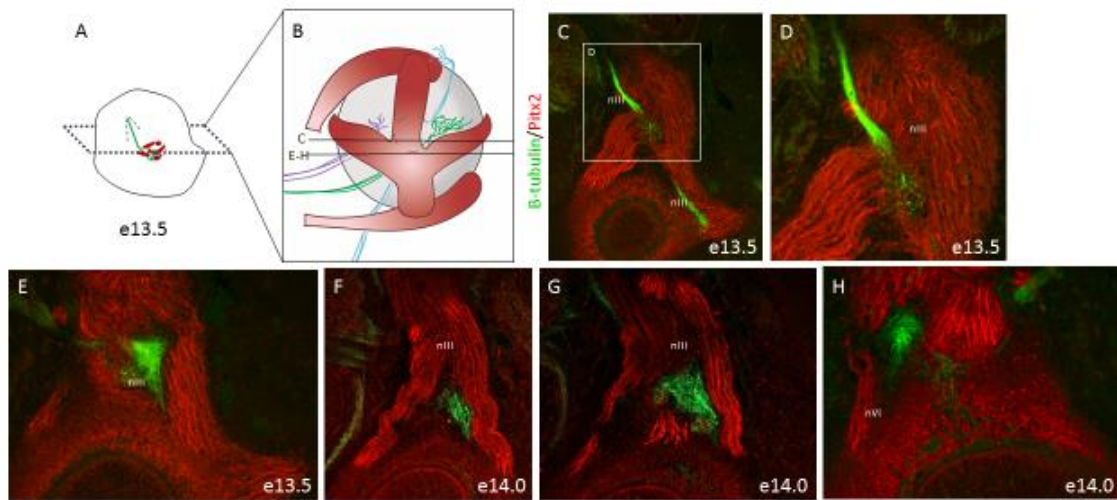
### Figure 3. Organized myofibers begin to appear at embryonic day 12.5

(A) 3-dimensional schematic of the e12.5 embryo. (B) Schematic of sagittal section plane. (C) Tenon's fascia develops four distinct swellings, causing invagination of the optic cup. These will later become attachment points for the extraocular muscles. (D) Four of eight EOM have formed myofibers and are visible as individual condensations on the superficial aspect of the muscle mass. Nerves are absent. (E+F) Images from two littermates (E) nIII is compacted and not spread out in the myofibers. A small, poorly delineated mass forms the beginnings of the dorsal rectus. (F) nIII sends tendrils into undifferentiated myoblasts. Anlagen has not yet condensed to form the dorsal rectus in this particular embryo, indicating that it is the fifth muscle to mature. (G) Deeper in the muscle mass, muscles merge and undifferentiated myoblasts are present. (H - I) Differentiated myofibers from the medial rectus are contiguous with the greater muscle precursor mass.



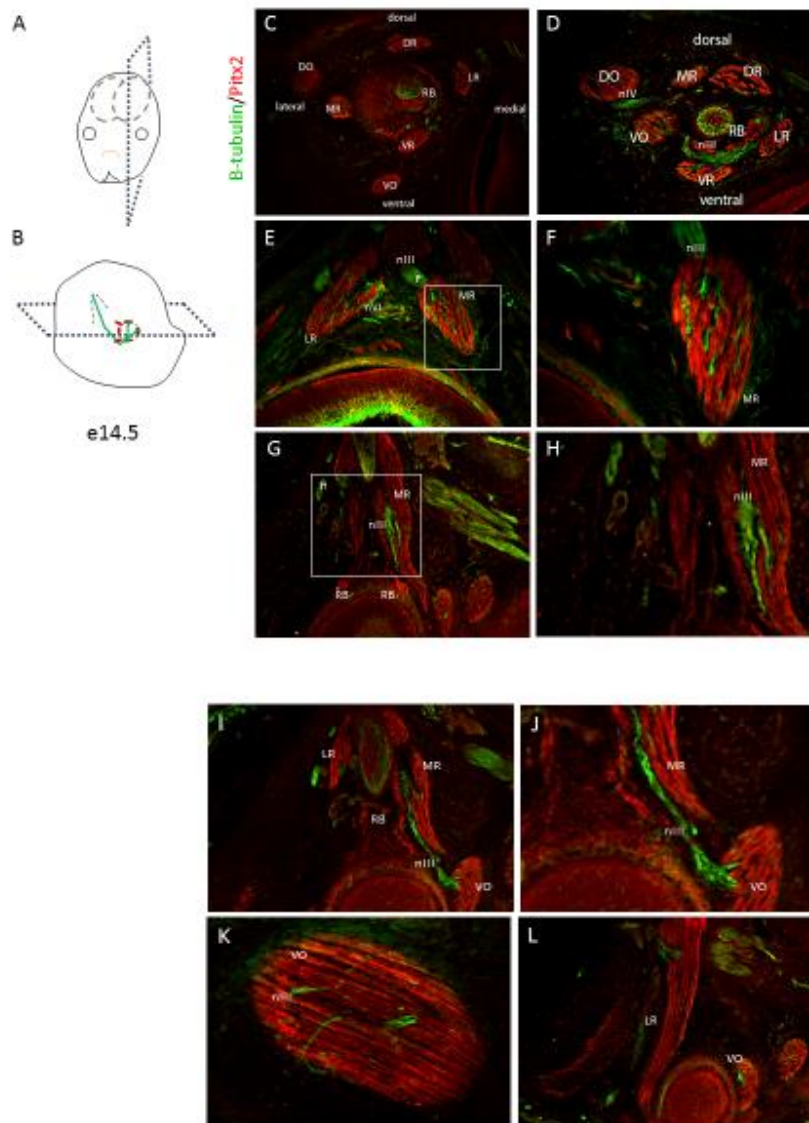
**Figure 4. Undifferentiated MPCs remain deep behind the eye, while fibers begin to form in the periocular area.**

(A) Schematic of transverse section plane. (B) The dorsal oblique has differentiated and migrated towards the superior aspect of the eye. (C-D) The oculomotor nerve and trochlear nerve share the same entry point, but can be distinguished in other sections as separate fascicles. The oculomotor nerve remains in a stationary plexus. The medial rectus has formed fibers and made contact with Tenon's fascia. (D) Higher resolution shows that the muscle mass is segregated into deeper, undifferentiated cells and migrating myofibers. (E-F) Ventral to the eye, muscle segregation lessens. Whereas the muscle mass accommodated to fit the OMN in previous stages, here the OMN is shaped by the muscle mass.



**Figure 5. OMN plexus persists after muscles have initiated primary myogenesis.**

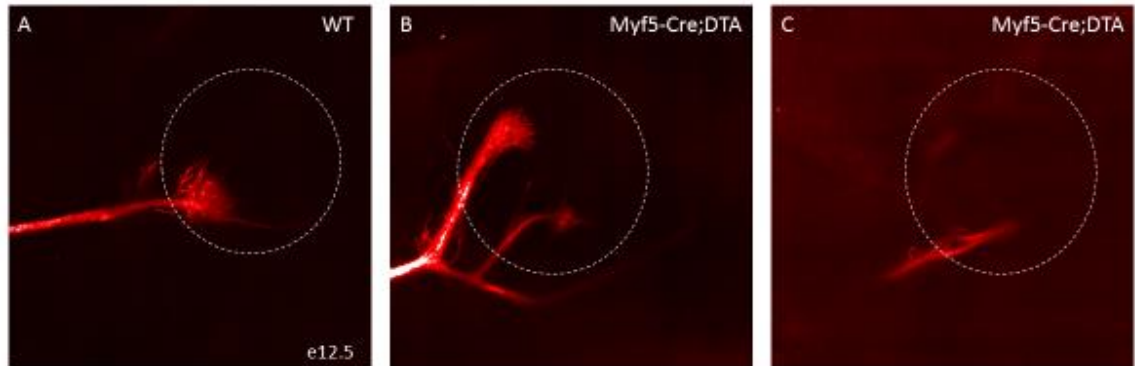
(A) Schematic of transverse plane. (B) 3-dimensional schematic of e13.5 embryo. (D) The entry point of the OMN. (E) Nerve and muscle fibers are distinctly separate. (F-G) At e14.0, nerve still forms tight plexus adjacent to—but not infiltrating—maturing myofibers. (H) The abducens nerve is adjacent to the lateral rectus, but displays the same lack of infiltration as the OMN.



**Figure 6. Nerve infiltration of EOM occurs at e14.5.**

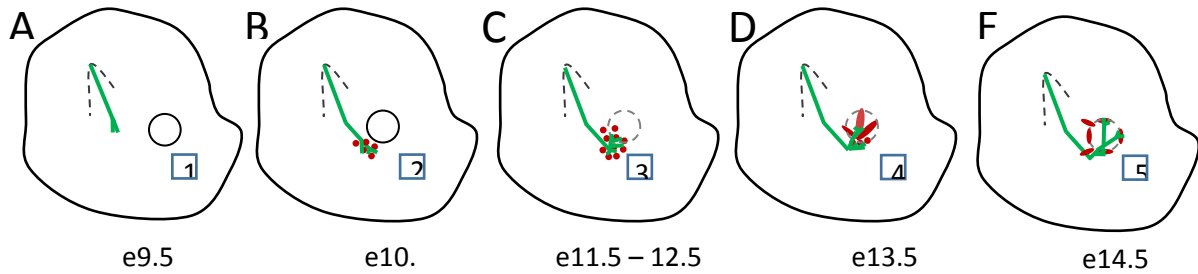
(A-B) Schematic of the sagittal and transverse plane, respectively. (C) Superficial section near the optic cup. Primary myogenesis is complete and all muscles have reached their attachment points in Tenon's fascia (seen here as diffuse red staining directly behind the globe). (D) Deeper in the tissue, innervation is observed in all muscles. The plexus has dispersed into directed branches, forming the inferior arm of the OMN. (E-F) Consistent with some anatomical studies, the lateral and medial recti have two attachment points. Observed here is the superior,

smaller arm of both muscles. (F) OMN axons are interspersed with myofibers. Pattern is generally consistent between embryos. (G-H) The larger, inferior arm of the medial rectus is infiltrated by the OMN in a consistent manner. The retractor bulbi can be visualized as two diminutive arms. (I-J) An extension of the inferior branch of the OMN innervates the ventral oblique. (K) Axons spread throughout the muscle fibers. (L) Innervation of the lateral rectus by the abducens nerve.



**Figure 7. Ablation of myogenic precursor cells causes gross guidance defects in OMN plexus formation.**

A myf5-Cre driver drives DTA expression in myogenic precursors. (A) Control embryo shows normal plexus formation at the ventrolateral aspect of the globe. (B-C) Gross guidance defects are observed in mice with myf-5 driven muscle ablation. (B) Aberrant branches form in the periocular region, while some miss the eye entirely. (C) A plexus fails to form entirely. Neither embryo shows bilateral symmetry.



**Figure 8. The oculomotor nerve displays five distinct transition points on its way from the midbrain to the developing eye.**

Halfway between the eye and the midbrain (A) the nerve makes a slight medial shift and becomes more susceptible to congenital deformation (B). Nerve and muscle fibers intermingle for several days (C), but separate as myogenesis nears completion (D). Finally, nerves infiltrate differentiated muscle fibers at e14.5 (E).

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