

University of Nevada, Reno

**Multiple roles for Robo1/2 repellent receptors in
guiding the facial nucleus and nerve**

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by

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Abstract

The facial nerve is necessary for our ability to eat, speak, and make facial gestures. To perform these vital functions, the nerve must accurately navigate from the hindbrain to the correct muscles during embryonic development. In the development of the facial nerve, both the cell bodies and the axons undergo specific migration patterns to assemble central neuron patterns and peripheral nerve projections to target muscles. Our goal in this study was to define the molecular signals by which the facial cell bodies and their axons correctly make this long journey. By analyzing facial neuron migration patterns in mouse embryos carrying mutations in the chemorepulsive receptors *Robo1* and *Robo2*, we found that these proteins are necessary for guiding the cell bodies and axons of the branchiomotor aspect of the facial nerve. In mutants, most axons do not make it to their motor exit point, and instead project into the floorplate. Mutant axons that exit the hindbrain appear misguided, with some branches becoming disorganized. In addition, a subset of mutant facial cell bodies fail to undergo the correct migratory patterns and instead appear to be misguided in various locations throughout the hindbrain. Even more, a subdivision of mutant cell bodies extend multiple long neurites far away from the normal target of facial axons. Overall, we found that *Robo1/2* are important for guiding multiple aspects of the complex cell migration of the facial nucleus as well as regulating the position and number of neurites that reach the periphery.

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1.1 Introduction

In the developing nervous system, neural progenitors must accurately navigate the complex environment of the embryo to arrive at their final target. These precursors differentiate in specific locations and send out projections that are directed by an amalgam of guidance cues (Tessier-Lavigne et al., 1988). In the hindbrain, various cell populations emerge from a series of subdivided structures deemed rhombomeres. These boundaries specify the differentiation of many of the cranial nerve populations, including the facial nerve (Lumsden and Keynes, 1989). From their birthplace in rhombomere 4 (r4), both the cell bodies and axons of the facial nerve simultaneously navigate long distances to their respective targets. A subpopulation of this nucleus, the facial branchiomotor nucleus (FBMN), undergoes a complex series of migrations while simultaneously sending out projections to innervate the facial muscles derived from the second branchial arch (Auclair et al., 1996; Fritsch, 1998; Fritsch and Nichols, 1993). Several mechanisms underlying this complex migratory pattern have been suggested. however, it remains unclear how the cell bodies and axons projecting from them correctly assemble in the CNS and second branchial arch,.

The intricate migratory pattern of the facial nucleus and nerve suggests the integration of multiple guidance pathways to guide this population. In mouse embryos, cell bodies of the branchiomotor (BM) aspect of the facial nucleus first differentiate in r4 via *Hoxb1*, *Islet-1*, *Phox2b*, and hedgehog activity (Chandrasekhar et al., 1998; Goddard et al., 1996; Pattyn et al., 2000; Studer et al., 1996; Varela-Echavarría et al., 1996;

Zhuang et al., 2013). Once established in the ventral r4 on embryonic day 9.5 (E9.5), cell bodies of the facial nucleus start sending their axons out towards the developing second branchial arch.

Shortly after their differentiation (E10.0), facial axons have already reached their motor exit point in dorsal r4 (Niederländer and Lumsden, 1996). These initial projections by motor neurons are thought to occur through an unknown attractive cue secreted by the exit point, as well as repulsive cues restricting where axons can migrate (Bravo-Ambrosio and Kaprielian, 2011; Bravo-Ambrosio et al., 2012; Guthrie and Lumsden, 1992; Xiao et al., 2003). On E10.5-E12.5, the chemorepulsive receptor Neuropilin-1 (Nrp1) and its ligands Sema-3A and VEGF164 guide and organize the facial axons and cell bodies, respectively (Kawakami et al., 1996; Kitsukawa et al., 1997; Schwarz et al., 2004a; Varela-Echavarría et al., 1997). Similarly Netrin-Unc5a signaling repels facial axons away from the wrong targets whereas a non-characterized cue(s) secreted by the branchial arches directly attracts the facial nerve (Caton et al., 2000; Murray et al., 2010a). By E13.5, the facial nerve diverges into five unique branches found in the adult animal. The nerve branches form through uncharacterized guidance mechanisms, exemplifying the need to further investigate the guidance of facial axons.

While the facial axons are being guided in the periphery, their corresponding cell bodies are also undergoing complex migrations in the hindbrain. On E10.5, cell bodies shift from their ventral position in r4 to a more caudal location in r6. This translocation occurs via tangential migration, by which cell bodies translocate behind small protrusions that direct the cell bodies migration. Members of the Polar Cell Polarity (PCP) pathway

(Prickle1, Prickle1b, Vangl2, Protein tyrosine kinase 7, Celsr1-1a-2-3, Frizzled3a, Frizzled7, Tbx20, Wnt5a, Scribble) are required to mediate this first step of migration in mouse and in zebrafish (Carreira-Barbosa et al., 2003; Glasco et al., 2012; Mapp et al., 2010; Qu et al., 2010; Song et al., 2006; Vivancos et al., 2009; Yang et al., 2014). Mutating these genes prevents or limits facial cell bodies from successfully translocating to r6, implying these are necessary to promote caudal migration. Interestingly, the PCP proteins appear to act independently of Dvl1/2, suggesting a unique signaling cascade that utilizes different signaling molecules than most known Wnt pathways (Glasco et al., 2012). In most Wnt signaling pathways Dvl is necessary to activate Rho GTPase effectors (Rosso et al., 2005; Strutt et al., 1997), which are necessary for neuronal migration (Kit Wong et al., 2001; Spiering and Hodgson). This indicates other effectors, perhaps activated by Robo-Slit signaling, are utilized in the facial nucleus to activate these effectors.

The subsequent ventral to dorsal and ventricular to pial migrations are not as well defined mechanistically, but initiate on E12.5 and finish at E14.5. One factor identified for the dorsolateral migration is the transcription factor Ebf1, as knockouts exhibit premature dorsolateral migration of the FBMN (Garel et al., 2000). The Dachshous-Fat PCP pathway is necessary for dorsolateral migration, as Fat4/Dchs1 mutants fail to initiate this dorsolateral movement (Zakaria et al., 2014). As the dorsal translocation is occurring, the cells also migrate radially from the ventricular surface to the pial surface. In Reeler mutant mice, the facial nuclei become disorganized and do not reach the pial surface, suggesting this migration is radial glial cell dependent (Goffinet, 1984; Rossel et

al., 2005). Similarly, Cdk5, Dab1, and p35 are necessary for facial cell bodies to migrate to the pial surface (Ohshima et al., 2002). Although much is known about the migration of these neurons, our current knowledge paints an incomplete picture of the external signaling pathways involved. In addition, the extent to which cell body and axonal migration are separable or coupled events is unclear.

Slit-Robo repellents are highly conserved guidance molecules necessary for guiding many populations of cells during development (Brose et al., 1999; Kidd et al., 1998; Zallen et al., 1998). This widely used signaling pathway guides a multitude of neuronal populations (Dickson and Gilestro, 2006; Mastick et al., 2010; Ypsilanti et al., 2010), but has been implicated in only a few cases to guide cranial nerves (Cho et al., 2007; Fouquet et al., 2007; Li et al., 1999; Prince et al., 2009). The FBMN express Robo1/2 during development (E12.0 rat), while their ligands Slit1/2 are expressed by the adjacent floorplate and rhombic lip (Hammond et al., 2005).

Using a variety of approaches, including explant assays exposing rat hindbrains to Slit1/2, dye tracing of E11.5 mouse FBMN in both Robo1 and Robo2 single mutants, dye tracing of E11.5 Slit1/2 double mutants, and *in ovo* electroporation of dominant negative Robo1/2, Hammond et al. discovered that Slit-Robo signaling guides facial axons (Hammond et al., 2005). Through these varied techniques they found Slit1/2-Robo1/2 signaling organizes and guides facial axons toward the dorsal motor exit point at r4, while also repelling axons out of the Slit-producing floorplate. They also discovered that Robo2 is of particular importance for restricting facial and commissural axons to r4, preventing them from coursing rostrally or caudally relative to r4. How Robo-Slit

signaling influences the facial nerve and nucleus at earlier or later stages, as well as how this pathway complements the function of known pathways, remains ambiguous.

We set out to more closely examine the role of Robo-Slit signaling during the development of the facial nerve and nucleus by 1) using multiple time points during development, and by 2) analyzing FBMN defects in Robo1/2 double mutant mice. We found Slit-Robo signaling guides and organizes facial axons throughout their development. Unexpectedly, we also discovered defects in the positioning of FBMN cell bodies in Robo1/2 mutant mice. Our data suggests a unique guidance mechanism by which a population of neuron cell bodies and their axons use Robo signaling to control a range of different aspects of their migrations.

1.2 Results

Robo1 and Robo2 guide initial facial axon outgrowth towards the exit point

Our goal was to compare the migration of the facial nucleus and nerve throughout development between wildtype and Robo1 and Robo2 double mutant embryos.

Heterozygotes were examined, but were not significantly different than wild type, and thus have been excluded from further discussion. To map the development of the facial nerve projections and the migrations of the facial branchiomotor neurons, we used transgenic *Isl^{MN}:GFP-F* embryos were examined during various stages of development. This transgenic line expresses EGFP specifically in motor neurons under the control of the *Isl1* enhancer, *crest1* (Lewcock et al., 2007). On E10.0, facial cell bodies

differentiated adjacent to the floorplate in r4 (Figure 1A). These cells had already sent pioneer axons to their ipsilateral dorsal motor exit point in r4 (Figure 1B). Some of the initial projections to the exit point had already formed thick bundles, however there were still individual axons not yet fasciculated. On E10.0, facial cell bodies are stationary along the ventral aspect of r4, and have not yet begun migrating caudally.

To test the function of Slit-Robo1/2 signaling in the facial nucleus, we studied Robo1/2 double mutant mouse embryos. In *Robo1^{-/-};Robo2^{-/-}* E10.0 embryos, early facial axon and cell body guidance defects were already evident. The majority of pioneer facial axons projected into floor plate in mutant animals (Figure 1C,K). Bundles of axons coursed through the floorplate both rostrally and caudally relative to r4. On E10.0 these misguided axons did not appear to stall, but continue migrating. The number of axons reaching the dorsal motor exit point in r4 was greatly reduced (Figure 1D,K). Interestingly the distance between the nuclei was also reduced in *Robo1^{-/-};Robo2^{-/-}* embryos, suggesting that facial neurons shifted toward the ventral midline and thus that cell body position may be regulated by Robo signaling (Kim et al., 2014).

On E10.5 in wild type, facial cell bodies started migrating tangentially from r4 to r6. This rostral to caudal migration was initiated by a few pioneer cell bodies that were located in r5 at this stage in development (Figure 1E). Additional differentiated facial cell bodies appeared in the nucleus in r4. Axons that coursed to the exit point in r4 appeared more tightly fasciculated on this stage. On E10.5 the initial outgrowth of the inner ear efferent (IEE) commissural axons crossed the floorplate at r4 (Figure 1F).

In *Robo1^{-/-};Robo2^{-/-}* E10.5 embryos facial axons accumulated additional guidance defects. Some facial cell bodies initiated rostral-caudal migration in mutant animals (Figure 1G), though *IslMN:GFP* was expressed in all developing somatic motor neuron populations at this stage making the facial nucleus difficult to discern among the massive amounts of other motor neurons present at this stage. Axons did not appear to self-correct their guidance errors from E10.0. The width of fascicles in the floorplate increased due to newly born axons that used pioneer axons already in the floorplate as a scaffold to migrate onto (Figure 1H). Some new axons also migrated onto bundles that already correctly exited the hindbrain into the periphery, but did not forge a new pathway to the motor exit point. Interestingly small neurites that projected off the cell bodies in r4 were present on this stage, which I did not see in wild type controls, suggesting stalling of the growth cone also occurs in the absence of *Robo1/2*.

Robo1^{-/-};Robo2^{-/-} facial cell bodies make several migration errors during tangential migration

To observe the tangential migration in wildtype and mutant animals we again utilized the *Isl^{MN}:GFP* transgene for E11.5, and then used lipophilic dye tracing to follow their migration trajectories in E12.5 embryos. In wild type, facial cell bodies started migrating caudally towards r6 on E11.5 (Figure 2A). Cell bodies were present in r4 on this stage, however the majority of cell bodies were migrating tangentially (Figure 2B, O). Some leading cell bodies had arrived in ventral r6 by this stage, though in this early stage of rostral-caudal migration, many cell bodies were still translocating through r5. In the process of moving, cell bodies laid down trailing axon tracts indicative of their

migratory route. This created a mixture of cell bodies and axon tracts in ventral r4-r6 at this stage. In *Robo1^{+/+};Robo2^{+/+}* wild type animals the separate IEE commissural axons had already traversed across the floorplate on this stage (Figure 2C). There were few longitudinal axons, attributed to the stochastic nature of axon guidance, visible in the embryonic floorplate.

In *Robo1^{-/-};Robo2^{-/-}* E11.5 facial nuclei, migratory defects of the cell bodies were apparent. Ectopic cell bodies were seen along the axon bundles projecting towards the exit point (Figure 2D). The nucleus also appeared larger in r4 in comparison to wildtype, suggesting cell bodies stalled in addition to be mispositioned. In agreement with cell bodies not initiating migration in r4, there were significantly less cell bodies in mutant embryos that had migrated caudally to r5 compared to wild type (Figure 2E,O). Despite there being fewer cell bodies, those that started migrating along the rostral–caudal axis appeared to successfully do so and migrated to r6. Surprisingly, cell bodies also had migrated into the floorplate in addition to the thickened longitudinal fascicles already there (Figure 2F). *Slit1/2* is expressed from the floorplate as well as the regions flanking the exit points during earlier (E10.0) and stages of development and also during tangential migration (Hammond et al., 2005; Holmes et al., 1998; Yuan et al., 1999). The presence of cell bodies in the floorplate and in other unusual positions implied *Robo1/2*-*Slit* signaling is required guide facial cell bodies during migration. *Slit1/2* repulsion may have created a boundary for cell bodies and axons during tangential migration, thus corralling them to the correct route.

On E12.5, to clearly visualize facial neurons in the increasingly complicated

hindbrain, various dye tracing experiments were performed in both wildtype and mutant embryos, though only mutant labels are shown for clarity. On E12.5 the rostral-caudal migration is mostly completed in wild type embryos. Exit point fascicles merged into large bundles on this stage (Figure 2G). In *Robo1^{+/+};Robo2^{+/+}* embryos, most cell bodies were seen in r6 (Figure 2H). Though tangential migration is documented to be complete on E12.5, some cell bodies were still in r5. The presence of cell bodies en route to r6 was confirmed in both Islet-GFP embryos and dye tracing experiments.

To visualize the morphology and number of cell bodies that had successfully projected axons out to their exit point, different colored dyes were placed on either side of the peripheral facial nerve (Figure 2I,J). To trace where the longitudinal fascicles in the floorplate originated from different colored dyes were placed in the floorplate more caudal and rostral to r4 (Figure 2K,L). The migratory path of the cell bodies toward r6 appeared disorganized as evidenced by the axon tracts that followed the cell bodies spanning r5-r6 (Figure 2I). Atypically located cell bodies were in E12.5 similar to E11.5 (Figure I,J). Retrograde dye labeling experiments were able to elucidated axon guidance errors that were not as evident in IslMN;GFP embryos. FBMN cell bodies contralateral to the dye label site were labeled along with ipsilateral cells (Figure 2I), suggesting these axons initially projected across the floorplate to the incorrect motor exit point or cell bodies migrated contralaterally. Other abnormal axon behaviors such as looping around in the floorplate were also clearly seen. Interestingly, a small set of cell bodies possessed multiple long neurites (Figure 2J,L). Multipolar cell bodies labeled in this experiment possess an axon that successfully made it out to the exit point, as

evidenced by their being labeled by dye placed in the periphery, and also another neurite that extend long distances into the floorplate. Facial cell bodies normally extend small neurites to guide tangential migration, however in wildtype animals the neurites guiding this process never extended as long as in *Robo1^{-/-};Robo2^{-/-}* animals.

To confirm that longitudinal tracts in the floorplate had indeed come from the facial nucleus, floorplate dye labels more rostral (r1) and caudal (r8) relative to r4 were performed (Figure 2K,L). Back-labeled axons were traced back to cell bodies stalled in r4 (Figure 2K), or cell bodies in r6 (data not shown) The majority of cell bodies in r4 possessed short growth cones. Despite possessing long neurites that coursed longitudinally through the floorplate, some cell bodies back-labeled to r4 also possessed filaments that extended towards the r4 motor exit point, once again supporting the idea that a proportion of these cell bodies extended unusually long neurites (Figure 2L).

Facial Branchiomotor cell bodies extend multiple neurites in the absence of Robo1/2

On E13.5 facial cell bodies and corresponding axons were generally completing their migration through the hindbrain. To specifically back label and visualize the facial nucleus lipophilic dye was placed on the peripheral facial nerve to specifically back label and visualize the facial nucleus (Figure 3A-H). Cell bodies were no longer migrating along the tracts of facial axons laid down between r4-r6 (Figure 3A). Axon bundles exiting in r4 had tightly fasciculated on this stage. Immediately after E12.5 the facial nuclei started migrating away from the ventral location to a new dorsal-lateral position,

still in r6. Concurrently cell bodies also migrated radially away from the ventricular surface to the pial surface of r6 (Figure 3B). In wildtype embryos the only axons in the floorplate or contralateral side were those of the inner ear efferent (IEE) population, which is another r4 derived group that is separate from nVII that migrate contralaterally across r4 instead of caudally (Figure 3E,F).

In *Robo1^{-/-};Robo2^{-/-}* embryos, a subpopulation of the FBMN embryos migrated successfully to the correct location, however many mutant cell bodies were displaced. Tracks of axons spanning r4 to r6 were disorganized, much like what occurred on E12.5 (Figure 3C). Axon tracts that successfully arrived at the exit point appeared to merge into one or two bundles that exited the hindbrain. Radial migration appeared largely unaffected in mutant neurons, as some cell bodies reached the pial surface of r6 (Figure 3D). Similarly, cell bodies correctly located on the pial surface were also in the correct dorsal-lateral position. Stalling cell bodies were still present in r4 and r5 in mutant embryos. Additionally ectopic cell bodies migrated along the axon bundles reaching the r4 motor exit point. Axon guidance defects within the hindbrain remain prevalent at this stage as well. *Robo1^{-/-};Robo2^{-/-}* embryos had extensive neurons and processes growing into the floor plate, including longitudinal tracts, looping axons, and cell bodies in the floorplate (Figure 3G,H). Facial cell bodies on the contralateral side of the eye site extended axons to the wrong contralateral exit point (Figure 3G). Cell bodies located in other rhombomeres also incorrectly sent axons to the r4 exit point and possessed multiple long neurites (Figure 3H).

Robo1/2 guides peripheral facial nerve migration

We next wanted to observe the development of the peripheral facial nerve to see if the removal of *Robo1* and *Robo2* affected its guidance. To observe the dynamics of the nerve we examined wild type and mutant embryos on E10.0, E10.5, and E11.5. The same transgenic IslMN:GFP-F embryos were used as mentioned previously. On E10.0 few pioneer axons were already in the second branchial arch in wildtype animals (Figure 4A). Axons weren't fasciculated yet as single axons were easily seen. In *Robo1^{-/-};Robo2^{-/-}* facial nerve outgrowth was disrupted. Some mutants did not possess a peripheral facial nerve (3/7), which was not observed in wild type embryos (3/3). This suggests facial axons are not correctly guided by Slits in the floorplate or dorsal aspect of the hindbrain. Interestingly, of those mutants that did possess facial nerves, many possessed an ectopic cell body along the peripheral facial nerve (Figure 1B). This occurred significantly more (87.5%) in mutant animals compared to wildtype (12.5%) (Figure 1C).

Facial nerves on E10.5 already look dramatically different than half a day earlier. The nerve had increased in size, owing to more axons that migrating unto the pioneer facial axons. Facial had also grown out further into the second branchial arch at this stage, and are fasciculated into one large main bundle (Figure 1D). On the most anterior aspect of the nerve there were some axons that were not apart of the large bundle, however. In *Robo1^{-/-};Robo2^{-/-}* facial nerves the morphology appeared different than in wildtype. The nerve in mutant animals was significantly more defasciculated compared to *Robo1^{+/+};Robo2^{+/+}* animals (Figure 2E,F). *Slit2* is expressed in the posterior aspect of the second branchial arch at this stage (Yuan et al., 1999), which may imply *Robo1/2-Slit2* are acting to fasciculate the nerve.

Lastly, on E11.5 the facial nerve started forming unique branches that will make up the five unique branches of the facial nerve in the adult mouse. The nerve was split into two main bundles on the most distal end (Figure 4G). A small axon bundle branched anteriorly from the middle of the nerve on this stage. Again, due to more axons migrating unto the nerve already there, on E11.5 nerve appeared more dense compared to E10.5. In mutant animals the bundles were not as thick compared to wild type, suggesting more axons were not able to self-correct from E10.5. The most distal anterior branch appeared to be greatly reduced in *Robo1^{-/-}; Robo2^{-/-}* (Figure 4G). A posterior nerve branch not present in *Robo1^{+/+}; Robo2^{+/+}* branched out from the middle of the nerve in several mutant embryos, suggesting Robo1/2 may partially guide the nerve. The overall size of the mutant nerve was significantly lower than in wild type (Figure 4I).

1.3 Discussion

In our study we found that Robo1/2 has a multifunctional role in regulating both the facial nucleus migration and the periphery nerve. How Robo integrates and interacts with other known facial guidance factors is an interesting question. First, the caudal migration of the nucleus is disrupted in Robo1/2 mutants. This suggests there is a joint effort between the known PCP proteins and Robo-Slit signaling to guide facial cell bodies caudally. In misguided Robo1/2 cell bodies dorsolateral migration doesn't occur, though this may be due to earlier defects in migration. Next, Radial migration isn't affected in Robo1/2 mutants as cell bodies are seen on the pial surface. Interestingly, we observed multiple long neurites projecting off facial cell bodies during its migration,

suggesting Robo is important for regulating neurite formation or length. Lastly, Slit-Robo1/2 signaling appears to guide facial axons out of the neural tube and may influence nerve morphology in the periphery.

Numerous motor neuron populations express Robo1 and Robo2 and are responsive to Slit2 (Bravo-Ambrosia and Kaprielian, 2011; Brose et al., 1999a; Holmes et al., 1998; Jaworski et al., 2010), including the branchiomotor aspect of the facial nerve and nucleus (Hammond et al., 2005; Murray et al., 2010). All three Slits (Slit1, Slit2, Slit3) are highly expressed in the floorplate, spanning the length of the midbrain through the spinal cord (Holmes et al., 1998; Yuan et al., 1999). Slit2 is expressed during the entirety of migration of the facial nucleus, from E8.5 before facial cell bodies are even differentiated through E14.5 when the nucleus has settled to its final location (Geisen et al., 2008). Some motor neuron populations secrete Slits, thus regulating themselves in an autocrine fashion (Jaworski and Tessier-Lavigne, 2012). Interestingly, at later stages the facial nucleus itself expresses Slit (Geisen et al., 2008), suggesting the facial nucleus may regulate its guidance or organization at later stages of development.

Caudal migration requires multiple signaling cascades, including Robo1/2

In our study, we found that Robo-Slit signaling is important for caudal migration of the facial nucleus, suggesting that Robo1/2 signaling integrates with other previously known guidance pathways to guide this complex migration. This is in contrast to Hammond et al., who found Robo mutants only have facial axon defects (Hammond et al., 2005). This discrepancy is most likely attributable to using Robo1/2 double knockout mice and previous work being done in single Robo1 or Robo2 knockouts. Interestingly,

our results do agree with an experiments in which Hammond et al. found mispositioned cell bodies when electroporating dominant-negative Robo constructs into chick embryos (Hammond et al., 2005). We found that in Robo1/2 mutant mice, some facial cell bodies remain in ventral r4 and do not undergo tangential migration. A few mutant cell bodies migrate into the periphery along their axons, whereas others move into the floorplate. These abnormal migrations could indicate that Robo1/2 signaling either acts to instigate, allow, or modulate caudal migration. The PCP pathway is necessary for the initial migration of the facial nucleus. Because the migration of the facial nucleus is not dependent on Disheveled (Dvl) (Glasco et al., 2012), an essential component of most Wnt pathways (Gao and Chen, 2010), the PCP pathway guiding the facial nucleus must function in an atypical manner. Our results indicate Robo-Slit may integrate with the PCP pathway to mediate the caudal migration of the facial nucleus.

The migration defect in Robo1/2 mutants does not occur in all mutant facial cells nor are cell bodies mispositioned in a consistent way, making it difficult to ascertain how Robo1/2 is regulating cell body position. One mechanism by which Robo-Slit signaling could affect tangential migration is by guiding the cell bodies. During this tangential migration, neurites guide the cell body similar to what occurs in axon guidance. Robo may be localized to a discrete region of the leading edge to directly guide these neurites away from the floorplate and away from the Slit-expressing dorsal neural tube, thus limiting their growth to the anterior-posterior axis. Another strong possibility is Robos may instead help create an asymmetric localization of other receptors necessary for the correct growth orientation, as noted in other organisms (Tang and Wadsworth, 2014).

Robo was indeed found to be particularly important and necessary for directed migration of axons (Quinn et al., 2006; Tang and Wadsworth, 2014) .

One possible attractive receptor and ligand complex that Robo-Slit signaling or binding may modulate to orient cells caudally is Frizzled (Fz) and Wnt. Wnt5a attracts migrating facial neurons, however when this molecule is removed the nucleus is mostly unaffected (Vivancos et al., 2009). This result suggests that other guidance cues lead this caudal migration. The downstream signaling proteins ROCK, MLCK, and JNK activity are necessary to transduce the attractive response of Wnt5a (Vivancos et al., 2009). When inhibiting these effectors, the migration of the FBMN was more severe than in Wnt5a knockouts alone. This suggests that other axon guidance receptors, such as Robo, also redundantly use these effectors to guide the facial nucleus. Evidence that the repulsive effects of Slit and Netrin on facial axons requires MLCK, ROCK, and Myosin II (Murray et al., 2010) supports the idea that Robo may also utilize these same effectors to guide facial cell bodies. Because Dvl is not necessary for migration of the nucleus (Glasco et al., 2012), but is required downstream of Wnt signaling to activate GTPases that activate MLCK, ROCK, and MyosinII (Rosso et al., 2005; Strutt et al., 1997), perhaps this means Robo1/2-Slit are necessary to activate these GTPases in order for Fz-Wnt signaling to polarize the neuron outgrowth. This possibility is feasible due to Slit1/2 being expressed during the entirety of the facial nucleus migration in the floorplate adjacent to the tangentially growing cell bodies (Hammond et al., 2005; Holmes et al., 1998). It would be interesting to test the effect of knocking out both Robo1/2 and Wnt5a to see if these redundantly guide the facial nucleus.

Another signaling cascade Robo may modulate is the PCP protein Vangl2, which is necessary for the tangential migration of the facial nuclei in both mouse and zebrafish. Vangl2 and other PCP proteins Pk1, Ptk7, and Scrib bind and are thought to activate one another. This binding mediates their correct localization in the cell, and also potentiates the PCP pathway by inhibiting the canonical Wnt pathway (Carreira-Barbosa et al., 2003; Glasco et al., 2012; Hayes et al., 2013; Mapp et al., 2010, 2011; Shafer et al., 2011; Wada et al., 2005). The downstream mechanism by which Vangl2 affects axon guidance is not well known. Evidence from non-neuronal tissue suggests that Vangl2 requires RhoA (Phillips et al., 2005) and Rac1 (Lindqvist et al., 2010), which are GTPases also activated by Robo-Slit signaling (Fan et al., 2003; Guan et al., 2007; Wang et al., 2013). Robo and Vangl2 signaling may cooperate to regulate the proper localization and activation patterns of these shared GTPases.

Another potential mechanism Robo1/2 may be necessary for tangential migration is that it may attenuate cell adhesion at the start of migration, thus acting as a permissive signal. In *Robo1^{-/-}*; *Robo2^{-/-}* mutants we observed facial cell bodies that appear to be stuck in r4 through the time period of E11.5 to E13.5, thus suggesting the adhesion between the neuroepithelium and the facial nuclei are not alleviated. The scaffold protein Scrib is responsible for propagating caudal migration as well as restricting where the facial nucleus can migrate (Wada et al., 2006). Because knockouts of Scrib and Vangl2, exhibit wandering behavior and have been shown to act non-cell autonomously, it is theorized they may help establish a boundary to limit where the facial nucleus can migrate (Glasco et al., 2012; Jessen et al., 2002; Vivancos et al., 2009; Wada et al., 2005). Vangl2 may

also be recruited to bind to Celsr1 to mediate this non-cell autonomous boundary and polarity (Devenport and Fuchs, 2008).

Another way Robo regulates cell migration is attenuating the cadherin adhesion, thus breaking apart junctions between cells (Rhee et al., 2007). The atypical cadherins Celsr1 and Celsr2 are expressed in the facial nucleus itself and in the surrounding neuroepithelium. They restrict the migrating facial nuclei to r4-6, presumably through cell adhesion interactions (Qu et al., 2010; Wada et al., 2006). Celsr1 appears to be particularly important for creating this boundary, as knocking it out creates rostral migratory streams of facial nuclei. Perhaps Robo signals may help mediate breaking apart the adhesion caused by these cadherin-like proteins, explaining why facial nuclei appear to stall in r4 when Robos are knocked out. Note that Celsr1 contains several Laminin G (LamG) domains and an EGF domain on its extracellular surface (Wang et al., 2014), which are also present in Slits (Howitt et al., 2004). It would be interesting to investigate a possible role for Robo or Slit binding to and modulating Celsr1 adhesion. Celsr proteins and Fzd receptors also collaborate in forming boundaries for cell migration. Celsr2; Celsr3 mutants have a similar block in caudal migration as Fz3a knockouts (Qu et al., 2010). These proteins are thought to function non-cell autonomously to prevent the facial nucleus from migrating rostrally or towards the ventricular surface (Wada et al., 2006). Yet another enticing scenario is Robo may act to inhibit N-Cadherin mediated adhesion, as demonstrated in other systems (Rhee et al., 2007). Indeed, in zebrafish N-Cadherin is necessary for preventing facial cell bodies from entering the floorplate and is expressed in the surrounding neuroepithelium (Stockinger et al., 2011). Facial cell bodies stalling in

r4 in Robo mutants is not uniquely explained by interactions with cadherins to modulate cell adhesion. However due to the importance of these molecules in caudal migration of the nucleus, it is worth consideration.

Lastly, the facial nucleus migration errors may be secondary or indirect effects of other substrates that were misplaced in Robo1/2 mutants. In our study, we observed cell bodies misguided into the floorplate, which may be due to them following other misguided populations to the floorplate. In zebrafish, facial neurons undergoing tangential migration through r5 and r6 use the medial longitudinal fasciculus (MLF), an ipsilateral tract of axons connecting visual and auditory nuclei of the brain to the spinal cord, as a substrate to migrate on (Wanner and Prince, 2013). Without the MLF, non-pioneer facial cell bodies did not migrate successfully to r6. Though it has been suggested that the FBMN is located too dorsally in mouse to contact the MLF (Wanner et al., 2013), this has never been experimentally validated. Our previous work has shown without Robo-Slit signaling the MLF collapses into the floorplate and forms long fascicules coursing through the floorplate (Farmer et al., 2008; Kim et al., 2014). It would agree with our results if facial cell bodies adhered unto the MLF and thus migrated into the floorplate with it. Alternatively, it is possible that facial axons may make independent ventral errors, because of the lack of Slit-mediated floor plate repulsion, but turn longitudinally once they contact the ectopic MLF bundles within the midline.

Dorsolateral and radial migration act independently of Robo signaling

When facial cell bodies reach r6 on E12.5 they turn perpendicular towards the

dorsal aspect of r6, a process complete on E14.5. Without Robo1/2 signaling, facial neuron cell bodies become misplaced during the period when caudal migration should be occurring and remain displaced in later stages. Though mispositioned cell bodies do not appear to undergo dorsolateral migration on E12.5, it is not easy to determine if Robo is particularly important in regulating tangential migration as they are already strongly affected earlier in development. To ascertain whether Robo is needed in these later stages, an inducible or temporal specific knockout would be needed to see if the lack of tangential migration is due to earlier defects or a separate guidance mechanism. The mechanisms underlying dorsolateral migration of the facial nucleus are not as well defined as those guiding caudal migration. The transcription factor Ebf1 appears inhibit the transcription of r6-specific genes from being expressed at inappropriate times, thus contributing to correct dorsolateral migration (Garel et al., 2000). Another PCP pathway variant, the Fat-PCP pathway, is necessary for tangential migration. Without the protocadherins Dachshous and Fat, facial nuclei can undergo caudal migration but no longer initiate dorsolateral migration (Zakaria et al., 2014).

In Robo mutant mice, we observed the mutant cell bodies affected earlier in development do not migrate to the pial surface, though unaffected facial cell bodies do correctly migrating to the pial surface of r6. Similarly to the disruption of dorsolateral migration in Robo1/2 mutant cell bodies, it is difficult to know if the lack of ventricular to pial migration in cell bodies are due to earlier defects or are indeed important for radial migration. Pk1 is a conserved nuclear receptor necessary for caudal and radial migration of the facial nucleus (Carreira-Barbosa et al., 2003; Mapp et al., 2010, 2011; Yang et al.,

2014) . In addition to potentiating the migration from r4 to r6, Pk1b also appears important for paring down the number of exploratory neurites and limiting the boundaries of FBMN in zebrafish (Mapp et al., 2010). Furthermore, Pk1b localizes the transcriptional repressor REST to the nucleus during the rostral -caudal migratory phase. REST is thought to prevent genes necessary for radial migration such as Reelin, Dab1, and Cdk5 from being transcribed at early stages of development (Mapp et al., 2011). The final radial migratory step of the facial nucleus is in fact dependent on Reelin, Cdk5, Dab1, and p35 (Goffinet, 1984; Ohshima et al., 2007; Rossel et al., 2005), albeit removal of Cdk5 also affects caudal migration. Thus, coinciding with the end of tangential migration, Pk1b and REST translocate out of the nucleus and allow transcription of these radial migration-inducing genes. Though Robo doesn't appear necessary for dorsolateral and radial migrations, the initial correct caudal location of the cell bodies may influence the ability of mutant facial cells to undergo dorsolateral migration.

Robo1/2-Slit signaling regulates multipolar neurite length

In Robo mutant animals spanning E10.0-E13.5, we observed that some facial cell bodies possessed multiple long projections. Through our dye labeling experiments of the peripheral nerve, we observed facial cell bodies sending axons to their motor exit point while at the same time making long projections in the floorplate. Previously, these longitudinal tracts were observed in lower numbers in single Robo1 and Robo2 mutants (Hammond et al., 2005), though they were attributed to axons of the IEE which normally send commissural axons across r4. (Fritzsich and Nichols, 1993). Though some of the longitudinal axons in the floorplate could be from the IEE in our mutant animals, our

floorplate back labeling experiments on E12.5 showed that a lot of longitudinal fascicles in the floorplate traced back to the facial nucleus.

Radial migration and the regulation of multipolar migration are tightly intertwined and regulated by similar molecules, such as Reelin. Reelin activating its effector Dab1, results in neurons switching from multipolar migration to radial migration (Franco et al., 2011). Dab itself activates Rap1, which in turn activates N-cadherin (Jossin and Cooper, 2011). Interestingly, N-cadherin is necessary to orientate the neuron for radial migration, and without it the cell remained multipolar (Jossin and Cooper, 2011). Though N-Cadherin hasn't been shown to function in the facial nucleus in this way, it is expressed and utilized during FBMN development, as mentioned previously (Stockinger et al., 2011). Robo may act to modulate the activity N-cadherin in specific regions of the cell (Rhee et al., 2007), thus perhaps tweaking where neurites can form and where they're collapsed.

Another possible scenario is Robo may also regulate the length of neurites through activation of Cdk5. The kinase Cdk5 is well known to modulate and potentiate Reelin/Dab signaling (Ohshima et al., 2001), but is also well characterized to regulate neurite length (Ohshima et al., 1996, 2007b; Tang and Wang, 1996). Some *Robo1*^{-/-}; *Robo2*^{-/-} mutant facial nuclei successfully migrate to the pial surface of r6, indicating that Robo activation of Cdk5 may not be necessary for successful radial migration but for regulating neurite length and number. Robo-Slit signaling limits the number of neurites cell bodies extend (Quinn et al., 2006). Perhaps this may occur in part through Robo activating Abl and Cables (Bashaw et al., 2000; Rhee et al., 2007), the latter of which

activates Cdk5 (Zukerberg et al., 2000). Cdk5 may in turn regulate the number of neurites projecting off cell bodies. A more simple mechanism may just be that Robo-Slit signaling normally repels these multipolar neurites out of the floorplate much like it repels longitudinal and post crossing commissural populations (Brose et al., 1999; Farmer et al., 2008; Long et al., 2004). Without this repulsive signaling coming from the floorplate, neurites and the cell body that follows may be attracted to the floorplate by unidentified cues, and continue extending as seen in other populations (Farmer et al., 2008).

Facial nerve projections in the periphery are guided by Robo-Slit signals

Our study validates that Robo-Slit signaling guides branchiomotor axons out of the floorplate. In our study, *Robo1^{-/-}; Robo2^{-/-}* double mutants exhibited more severe defects than single Robo1 or Robo2 knockouts (Hammond et al., 2005). Our extreme phenotypes with the axons, and cell bodies alike, suggests Robo1 and Robo2 largely act redundantly to regulate this population. The previous group had found that some mutant axons migrated the floorplate and also crossed between the exit point fascicles in Robo mutants (Hammond et al., 2005). Agreeing with these results, our Robo1/2 double knockout animals we found most axons of the facial nucleus migrate into the floorplate. The in ovo electroporation of a dominant-negative Robo receptor experiments performed by Hammond et al. strongly agree with the phenotypes we saw in our *Robo1^{-/-}; Robo2^{-/-}* animals. They found axons that stalled, looped around, and failed to project away from the floorplate and to the exit point in their chicken experiments, which are all effects we saw in our mutant animals. In another study, the chemorepulsive effects of the Robo ligand Slit, and the Unc5a ligand Netrin, were previously both found to guide facial

axons and which was further demonstrated to be dependent on MLCK, ROCK, and Myosin II (Murray et al., 2010). Netrin was found to be particularly important in guiding facial neurons to their dorsal exit point, as facial axons in Netrin mutants turn towards the exit point too early or migrate into the floorplate (Murray et al., 2010). This is very similar, though less severe, than what we see in Robo1/2 double mutants suggesting there may be redundancies or interactions between these signals.

Slit-Robo signaling is important for motor exit point pathfinding. For instance Robo2, under the regulation of Nkx2.9, is necessary for spinal accessory motor neurons to correctly exit the neural tube (Bravo-Ambrosio et al., 2012). The neural crest-derived motor exit point is joined later in development by boundary cap cells that keep cell bodies from leaving the periphery where at the same time are thought to facilitate axon exit out of the neural tube by secreting an attractant (Niederländer and Lumsden, 1996). Boundary cap cells of the spinal cord produce various Semaphorins (Sema3B/3G/6A) that are thought to repel the Neuropilin-2 (Npn-2) and PlexinA1 expressing motor cell bodies (Bron et al., 2007; Mauti et al., 2007). Motor axons are not thought to express these chemorepulsive receptors and are therefore attracted to the motor exit point and leave the neural tube. In agreement is the finding that facial motor axons are not repelled from the motor exit point expressing Sema3A, but are instead repelled by Slit which appears to flank the exit point (Murray et al., 2010; Yuan et al., 1999). The expression pattern of Slit1/2 being adjacent to the motor exit points suggests Slits may be corralling axons to the correct exit point in the right rhombomere level.

Aside from guiding the facial nerve initially out of the CNS, Robo-Slit signaling may

also regulate the fasciculation and guidance of the nerve at later stages. On E10.5-E11.5 Robo mutants possess less dense facial nerves that are bundled less tightly than wild type. We also have tantalizing preliminary data suggesting Robo-Slit signaling is necessary to guide the facial nerve at older stages (data not shown). In Robo1/2 mutants we see branches of the facial nerve that are migrating in the wrong direction, suggesting they will not make contact with their corresponding muscles. On E12.5 and E13.5, Slit2 mRNA is clearly seen in the same regions of the leading edge of the facial nerve in whole in situ preparations of mouse embryos (Holmes et al., 1998). This exciting observation suggests Slit2 may be produced and secreted by the facial nerve itself, thus guiding or fasciculating itself in an autocrine manner. On E14.5 the facial nucleus has already been found to express Slit2 itself (Geisen et al., 2008), thus again suggesting Slit2 may be also used in the peripheral nerve. Indeed, other motor neuron populations have been found to regulate their own fasciculation through the expression of Slit2 (Jaworski and Tessier-Lavigne, 2012).

Few other guidance factors are known to mediate the organization of the facial nerve, although removing Npn causes the facial nerve to become defasciculated (Kitsukawa et al., 1997). This suggests spinal motor neuron guidance may be regulated by different factors than cranial nerves. Similarly, mutants for the Npn ligands Sema3A and Sema3C, did not affect cell body migration (Schwarz et al., 2004b). It is known that facial axons are attracted to explanted branchial arch tissue, however the cue responsible for this attraction remains undetermined (Caton et al., 2000). One potential attractive factor, HGF, was found to attract other cranial nerves, however its receptor MET is not

expressed in the facial nucleus. We currently have an incomplete list of the cues responsible for guiding facial axons. However, from our study, it appears that Slit-Robo signaling may contribute to the nerve guidance during multiple stages of development. Our work suggests Slit repulsion drives axons out of the floorplate and targets them to the exit point, while at later stages appears to fasciculate and guide the more mature facial nerve branches.

1.4 Materials and Methods

Mouse embryos

Animal protocols were approved by the University of Nevada, Reno's Institutional Animal Care and Use Committee (IACUC) and in accord to the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Embryonic mice were collected at embryonic day 10.0 (E10.0), E10.5, E11.5, E12.5, and E13.5. Noon of the day a plug was discovered was counted as E0.5. If between E10.0-E12.5 embryos were obtained through uterine dissection, put in fresh (made the same day) 4% paraformaldehyde (PFA) and fixed for a minimum of 24 hours. If embryos were E13.5 cardiac perfusion was performed. We bought CD-1 mice from Charles River Laboratory (Wilmington, MA). Limb buds were removed from freshly collected embryos to genotype Robo mutant embryos as previously described (Grieshammer et al., 2004; Plump et al., 2002). Robo1/2 founder mice were a generous gift of Marc Tessier-Lavigne, of Rockefeller University. The Isl1^{MN}-EGFP strain was a gift of Samuel Pfaff (Lewcock et al., 2007), of the Salk Institute. To create stable heterozygous lines, Isl1-GFP mice

were crossed with *Robo1/2* mutant mice as well as CD1 mice.

NeuroVue facial nerve tracing

The lipophilic fluorescent axon tracers NeuroVue™ Orange and NeuroVue™ Jade were used to label the facial nucleus and trace facial axon trajectories in the peripheral branchial arches (Fritzscht et al., 2005). To trace the facial nerve a small piece of dye was cut into a triangle shape, and inserted immediately anterior to the otic vesicle on E10.0 and E10.5. Jade dye was placed above the left vesicle whereas orange was placed above the right vesicle. Similarly on E11.5 dye was anterior to the otocyst, and lastly on E12.5 and E13.5 dye was placed in the most anterior region within the developing ear. For peripheral nerve tracing experiments *Robo*^{+/+}; *Robo*^{+/+} and *Robo*^{-/-}; *Robo*^{-/-}, dye was placed in same location to assure consistency. For floorplate fascicle tracing experiments a smaller piece of dye than used in peripheral tracing experiments was placed directly in the floorplate several rhombomeres caudally or rostral relative to r4. After dye was placed embryos were incubated for 24hrs (E10.0, E12.5), or 48 hrs (E13.5) at 37° C to allow the dye to diffuse to the distal nerve and nucleus.

Imaging and preparations of tissue

Embryos were assessed for successful fluorescence labels under a Leica upright fluorescence microscope. Neural tubes were dissected out while preserving the peripheral branchial arch. Hindbrains were prepared in an “open-book” conformation by cutting the dorsal most aspect of the neural tube along the dorsal midline. Neural tubes were incubated in 80% Glycerol in 4% PFA for several hours before imaging. Heads of

embryos were bisected to image the peripheral facial nerve (E10.0-E11.5). Older embryos (E12.5, E13.5) were put in melted 2% agar to vibratome section. Embryos were sectioned into 200 μ m thick slices and mounted on slide with agar attached. Once mounted, embryos were imaged using the Leica TCS-SP8 confocal microscope within 2 days of the end of dye diffusion to limited the amount of unwanted additional diffusion of the dye.

Analysis and quantification

Images were analyzed using the NIH's ImageJ software. Neural tube analysis: E10.0-E10.5 neural tubes were analyzed by counting the number of dorsal projections coming immediately off the facial nucleus. All sizes of fascicles or axons were counted as equal. For E11.5 analysis migrating cells were counted as the area facial cell bodies in r5 and r6, while stationary cell bodies were counted as the area of the facial nucleus in r4. For E13.5 cell bodies were counted as multipolar if axons were labeled in the periphery and neurites also present in the floorplate.

Peripheral nerve analysis: In E10.0 embryos the number of ectopic cell bodies located in the second branchial arch were counted. These were defined as cell bodies immediately attached to the facial nerve and outside of the neural tube. In E10.5 embryos the relative defasciculated was measured by comparing the average length of space in between bundles of facial nerve to the average nerve length. In E11.5 the area of the facial nerve was determined by setting an intensity threshold of the image to eliminate background noise. "Wand (tracing) tool" was then used to determine the area of the facial

nerve. The first and second branchial arches were removed consistently where the neural tube and branchial arch meet, and the entire peripheral facial nerve was imaged for all cases. The area included the entire facial nerve present in the second branchial arch.

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1.6 Figure Legends

Fig 1. Robo1 and Robo2 are required to guide pioneer facial axons toward their r4 dorsal exit point. Transgenic mice, $Isl^{MN}:GFP$, expressing EGFP under the control of somatic motor neuron specific enhancer, $Isl1$, were utilized to visualize the facial nucleus and nerve. E10.0-E10.5 open-book $Isl1$ -EGFP hindbrains comparing wild type (**A,B,E,F**) and (**C,D,G,H**) embryos. (**A-D**) E10.0 mouse hindbrains arranged rostral, top; floorplate (FP), center. (**A, B**) In wild type embryos, facial axons coursed from the facial branchiomotor nucleus (FBMN) towards the dorsal motor exit point (denoted as exit) in r4; a higher magnification view is shown in panel B. (**C**) In $Robo1^{-/-};Robo2^{-/-}$ embryos, motor axons were misguided into the floor plate. Likewise, the nucleus was shifted closer to the floor plate. (**D**) A higher magnification view in $Robo1^{-/-};Robo2^{-/-}$ mice to show that few axons projected dorsally from the FBMN to their exit point. (**E-H**) By E10.5, facial axons did not self correct after initial projection errors. (**E,F**) On E10.5 in wildtype, FBMN cell bodies migrated caudally (arrow). The commissural axons of the inner ear efferents (IEE) nerve (arrowhead) also crossed the midline. (**G**) E10.5, facial axons did not project towards the exit point and instead projected in bundles rostrally and caudally in the floorplate. (**H**) The nucleus was wider and closer to the floor plate relative to control. (**I, J**) Schematics of E10.0-10.5 FBMN nerve projections. In wild type many axons project to the exit point in rhombomere 4 for wild type (**I**). In $Robo1/2$ mutants, axons collapsed into the floor plate and nuclei shifted towards the floor plate (**J**). The tangential caudal migration of cell bodies started on E10.5, and is characterized by a

subpopulation of FBMN pioneering this route. **(K)** Graph quantifying the number of facial axon bundles projecting to the r4 exit point in E10.0 and E10.5 wild type (black) compared to *Robo1^{-/-};Robo2^{-/-}* mutants (diagonal). Scale 50 μ m. Error bars show S.E.M.; significance was measured using students t-test, * $p < 0.05$; ** $p < 0.01$. n=3, E10.0 *Robo1^{+/+};Robo2^{+/+}*. n=2, E10.0 *Robo1^{-/-};Robo2^{-/-}*. n=3, E10.5 *Robo1^{+/+};Robo2^{+/+}* E10.5. n=5, *Robo1^{-/-};Robo2^{-/-}*.

Fig 2. Robo1 and Robo2 regulate facial branchiomotor cell body position. Wild type **(A-C,G,H)** and *Robo1^{-/-};Robo2^{-/-}* **(D-F,H-L)** E11.5 and E12.5 open-book hindbrains expressing Isl^{MN}:GFP or labeled with NeuroVue (Orange), respectively. **(A-I)** Mouse hindbrains arranged as in Fig 1. **(A)** On E11.5, facial axons are added to fascicles already projecting to the motor exit point in wild type embryos. **(B)** FBMN cell bodies migrate tangentially from ventral r4 towards r6. The majority of cell bodies are in r5, however some cell bodies had already made it to r6 by E11.5. **(C)** Commissural axons of the IEE were the only axons seen crossing the floorplate in wild type embryos. **(D)** In mutant E11.5 animals, FBMN cell bodies are ectopically positioned more dorsally in r4. In *Robo1^{-/-};Robo2^{-/-}* embryos, axons are added to the existing pioneer axons projecting to the exit point or floorplate. The fascicles coursing through the floorplate had increased in size relative to E10.5 embryos, suggesting that some newly projecting axons do not find the r4 exit point and instead follow axons into the floorplate. **(E)** There are significantly less cell bodies migrating caudally in mutant embryos, with the majority still residing in r4 (quantified in O). **(F)** On E11.5, cell bodies are ectopically located in the floor plate. **(G)** By E12.5, facial axons projecting to the exit point are tightly fasciculated at r4, and have

laid down axon tracts tracing the cell bodies migration to r6. **(H)** Cell bodies have largely completed their journal to r6. **(I,J)** To visualize facial axons, or aberrant axons that also exited r4 motor exit point, lipophilic dye was applied in the periphery (as described in methods). On E12.5 mutant embryos facial cell bodies appear mispositioned (arrow) and disorganized. **(I)** Axon tracks spanning ventral r4 to r6 laid down from the migrating cell bodies appear disorganized. Additionally, cell bodies from the contralateral side projected axons to the opposite exit point. **(J)** A magnified view of **I** demonstrating a cell body (asterisk) in r3 that had either sent an axon to the wrong exit point in r4, or had itself become misguided rostrally in r3. Ectopic facial cell bodies (arrows) in r4 were visible using lipophilic back labeling **(K,L)** *Robo1^{-/-};Robo2^{-/-}* E12.5 hindbrains were labeled with lipophilic dye in the floorplate of r8 to trace fascicles coursing caudally in the floorplate. **(K)** Back-labeling neurite bundles in the floor plate revealed these originate in the facial nucleus at r4. **(L)** *Robo1^{-/-};Robo2^{-/-}* cell bodies maintain long neurites in the floor plate. These same cell bodies also possess axon-like filaments headings towards the motor exit point, or stalling closely to the soma. **(M,N)** Schematics of the rostral-caudal migration of the FBMN cell bodies, as well as the nerve morphology, that occurs from E11.5 to E12.5 in both wild type **(M)** and *Robo1^{-/-};Robo2^{-/-}* **(N)**. **(M)** In wild type embryos the majority of cell bodies have migrated to r5 and r6. Concurrently, newly differentiated cell bodies send out axonal projections that adhere and migrate unto the pioneer tracts that had went through the motor exit point. The IEE axons are still the only axons in the floorplate. **(N)** In *Robo1^{-/-};Robo2^{-/-}* mutant facial cell bodies stall in r4 and do not have as many migrating cells as wild type. A subpopulation of cell bodies that remain in r4 are mispositioned dorsally, some of which possess multiple neurites. Newly projecting axons

thicken the already misguided fascicles that continue to migrate rostrally and caudally within the floorplate. **(O)** Bar graph quantifying the proportion of migrating cell bodies (present in r5 and r6) over the number of stationary cell bodies (present in r4) in E11.5 embryos (area of cell bodies in r5 and r6 and divided by the area of cell bodies in r4). It was found using a student's t-test that there are significantly ($p=0.0104$) more cell bodies migrating caudally in wild type (black) compared to mutant (diagonal) FBMN. Scale bar 50 μm . Error bars show S.E.M. * $p<0.05$ $n=5$ wild type, $n=3$ *Robo1^{-/-};Robo2^{-/-}* .

Fig 3. Subpopulations of facial cell bodies extend multiple long neurites in the absence of Robo1/2. E13.5 open-book *Robo^{+/+};Robo2^{+/+}* (A,B,E,F) and *Robo1^{-/-};Robo2^{-/-}* (C,D,G,H) facial nuclei back labeled with NeuroVue Orange dye. **(A-H)** E13.5 mouse hindbrains arranged rostral, up; floorplate (FP), center. Embryos imaged ventricular side up **(A,C,E-H)** or pial side up **(B,D)**. **(A-D)** Demonstrate the final dorso-lateral and radial migration patterns in both wildtype and mutant embryos. **(A,B)** *Robo^{+/+};Robo2^{+/+}* facial axons exiting in r4 **(A)**, while the cell bodies **(B)** have radially migrated to the dorsolateral pial surface of r6. **(C)** Axons in *Robo1^{-/-};Robo2^{-/-}* animals are reduced in number and appear disorganized in the rostral caudal tract. **(D)** Cell bodies that correctly migrate rostral-caudally in *Robo1/2* double mutants also successfully migrate to the pial surface of r6. **(E-H)** Illustrates the axon migration patterns of wildtype and mutant embryos in a late stage. **(E,F)** *Robo^{+/+};Robo2^{+/+}* facial nerves have followed their axons to the lateral side of r6. The contralateral projections of the IEE are the only axons seen in or across the floorplate. **(G)** E13.5 *Robo1^{-/-};Robo2^{-/-}* facial axons migrate long distances incorrectly throughout the hindbrain. Cell bodies of the contralateral side incorrectly sent

out axons (arrow) to the exit point of the opposite side. Cell bodies extended axons to exit point and also a separate neurite into the floorplate. Cell bodies with neurites in the floorplate as well with projections in the floorplate were observed in 3/4 *Robo1/2* mutant embryos and 0/6 in wild type. **(H)** A magnified view of a cell body in the floor plate that possess two long neurites. **(I, J)** Schematics of E13.5 FBMN nerve projections demonstrating the dorsolateral and radial migrations of the cell bodies and axons that followed. **(I)** In wildtype, axons follow the migration path of the cell bodies to the pial surface of r6 while bundles continue to fasciculate on their way to the r4 exit point. Cell bodies are arranged lengthwise across the entire pial surface of r6. **(J)** In *Robo1^{-/-};Robo2^{-/-}* animals, cell bodies send axons out to the contralateral exit point and are sometimes ectopically located in the floorplate. A subpopulation of cell bodies also extend long and multiple neurites.

Figure 4. *Robo1/2* signaling regulates peripheral facial nerve dynamics. To visualize the peripheral nerve during its outgrowth *Isl1MN;GFP* embryos were used. Embryos arranged rostral, left; dorsal, right; anterior top, posterior bottom. **(A,B)** E10.0 whole embryos comparing the pioneer peripheral facial axons of wild type **(A)** and *Robo1^{-/-};Robo2^{-/-}* animals **(B)**. Pioneer facial axons were guided to the second branchial arch by E10.0. Few axons are present and are not fasciculated, however they all were migrating within a short distance of each other. **(B)** Without *Robo1/2* there were fewer axons present, probably because the majority of axons had not exited the hindbrain. In some cases (3/7) no facial cell bodies were seen in the branchial arch at all, unique from wild type animals which all displayed peripheral axons (3/3). Among some of the mutants that

possessed a peripheral nerve, an ectopic cell body (arrowhead) was located along the facial axons. **(C)** Significantly more Robo mutants (4/7) possess an ectopic cell body located along the peripheral facial nerve, compared to wild type (1/8). **(D,E)** E10.5 embryos possess larger more fasciculated facial nerves. **(D)** At this stage the facial nerve grew out further and also was thicker as a result of additional axons growing along the pioneers. **(E)** Mutant facial axons were defasciculated and less dense than in wild type. **(F)** *Robo1^{-/-};Robo2^{-/-}* animals were found to have less fasciculated facial nerves relative to the total width of the nerve, compared to *Robo1^{+/+};Robo2^{+/+}*. **(G,H)** By E11.5 the facial nerve had branched into distinct bundles that will give the branches found in adult animals. **(H)** On E11.5 there were less axons comprising the facial nerve in mutant animals. **(I)** The area of peripheral facial axons in mutant animals was significantly less than wild type. **(J-K)** Diagrams of peripheral facial nerve outgrowth of wildtype **(J)**, and mutant **(K)** animals spanning E10.0-E11.5. The facial nerve has reached the second branchial arch during these stages and was continuously growing. **(K)** Less facial axons reach the periphery in Robo1/2 mutants and are more defasciculated when they do. E10.0 *Robo^{+/+};Robo2^{+/+}*, n=8; *Robo1^{-/-};Robo2^{-/-}*, n=7; error bars are Standard Error of the Proportion (S.E.P) . E10.5 *Robo^{+/+};Robo2^{+/+}*, n=6 *Robo1^{-/-};Robo2^{-/-}*, n=4; error bars are S.E.M. E11.5 *Robo^{+/+};Robo2^{+/+}*, n=5 ; *Robo1^{-/-};Robo2^{-/-}* n=3; error bars are S.E.M. Significance calculated by t-test. *p<0.05 , **p<0.01 Scale bar is 100µm.







