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The Robo3 receptor, a key player in the development, evolution and function of commissural systems

Running title : Function and dysfunction of Robo3

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ABSTRACT

Roundabout receptors are known to mediate Slit-dependent repulsive signaling. However in vertebrates, mounting evidence suggest that Robo3 is an unconventional Robo receptor regarding both its expression and function. From its initial description, Robo3 receptor has been tightly associated with the development of specific axons, called commissural, that connect both sides of the nervous system. Many studies using transgenic mouse models showed that Robo3 expression is mandatory for commissural axon guidance to the floor plate. Moreover, mutations in human ROBO3 are responsible for a rare neurological disease in which patients also display midline crossing defects. Robo3 was initially thought to counteract Slit/Robo repulsion. However, recent studies support an alternative model where Robo3 potentiates midline attraction. These studies support a complex, central and multifaceted role of Robo3 in controlling the development of commissural circuits. Furthermore, the analysis of Robo3 evolution in vertebrates points out the specificity of this receptor in the mammalian lineage, suggesting mechanistic and functional divergence of Robo3 in mammals compared to a more traditional function in other vertebrates. Here, we review the current knowledge about Robo3 function, from the regulation of its expression to signaling. We also present evidence for a high variability of Robo3 splice variants in vertebrates.

Keywords: Axon guidance, Robo, Slit, receptor, commissure, HGPPS

INTRODUCTION

The central nervous system of all species with bilateral symmetry (the so-called *bilateria*) contains two main types of projection neurons: some connected to neurons located on the same side than their cell body, others connected to the opposite side. The latter are called commissural neurons and they allow coordination and integration of information coming from both sides of the body, which is essential for multiple functions such as binocular vision, sound localization or integrated sensory-motor responses. Abnormal axon midline crossing during development can cause a whole range of neurological disorders. This is for instance the case of congenital mirror movements (CMMs) in which patients exhibit bilateral and symmetric movements when intending to perform an unilateral motor task (Meneret et al., 2015). In some patients, the motor cortex was found to be connected to motor neurons on both sides of the spinal cord, instead of the contralateral side only, although abnormal midline crossing at the level of the spinal cord and corpus callosum might also be involved. Abnormal connections of the motor cortex to the spinal cord motor neurons were also observed in patients having horizontal gaze palsy with progressive scoliosis disorder (HGPPS) (Jen et al., 2004). In these patients, a reduction of commissural projections in the hindbrain is associated with an absence of conjugate horizontal eye movements. In albino patients, the severe reduction of the contingent of visual axons projecting to the ipsilateral side of the brain (which is normally around 50% in humans) leads to a severe impairment of binocular vision (Neveu and Jeffery, 2007). Partial or complete corpus callosum agenesis (CCA) are some of the most common brain malformations in children (Paul, 2011) and can now be diagnosed as early as 20 weeks of gestation using magnetic resonance imaging (MRI) and *in utero* diffusion tensor imaging (Huang and Vasung, 2014). The genetic basis of CCA is still largely unknown and the deleterious neurological outcome is variable. Therefore, getting a better understanding of the mechanisms that control midline crossing by commissural axons should help understanding the etiology of these complex diseases.

Basic neuroscientists as well have been fighting for decades to solve the midline crossing enigma and to characterize the cellular and molecular mechanisms involved (Tessier-Lavigne and Goodman, 1996; Chédotal, 2011). In the last quarter-century, ENU-based mutagenesis screens in *C.elegans* (Hedgecock et al., 1990), *Drosophila* (Seeger et al., 1993) and zebrafish (Karlstrom et al., 1996), together with biochemical and genetic studies in other vertebrate species have led to rapid progress and the identification of key receptors and ligands which control midline decussation (Tessier-Lavigne and Goodman, 1996; Chédotal, 2011). In all bilaterian species, some of these axon guidance molecules attract commissural axons to the midline when others expel or repel them away from it.

The Roundabout receptor (Robo) was first identified in *Drosophila* as the product of a gene whose mutation leads to multiple crossing and recrossing of commissural axons that wander around the nerve cord midline (Seeger et al., 1993). This receptor mediates axonal repulsion upon binding to its ligand Slit, which plays an important role in the midline crossing process (Kidd et al., 1999). Two other Robo genes are presented in *Drosophila*, Robo2 and Robo3 and are also associated with Slit-mediated axon guidance (Rajagopalan et al., 2000). Robo homologues have since been identified in vertebrates where they also control midline crossing (Kidd et al., 1998). At least 4 *Robo* genes, *Robo1-Robo4* exist in all vertebrates, sharing no orthology with *Drosophila Robo1-Robo3* genes as they derive from two rounds of duplication in vertebrate phylum (Evans and Bashaw, 2012; Zelina et al., 2014) (Figure 1). Three of them, Robo1, Robo2 and Robo3 being expressed in the CNS of vertebrates (Lee et al., 2001; Marillat et al., 2002; Camurri et al., 2004; Sundaresan et al., 2004). Robo1 and Robo2 were identified by homology with *Drosophila Robo* (Kidd et al., 1998; Brose et al., 1999), but Robo3, also known as Rig1, was found by differential display as a gene up-regulated in retinoblastoma-deficient cells (Yuan et al., 1999). Like all Robo receptors, Robo3 is a transmembrane receptor with an extracellular domain composed of five immunoglobulin (Ig) domains and three fibronectin type III (FNIII) repeats followed by a cytoplasmic domain containing 3-4 conserved motifs (CC) (Figure 1). Mounting evidence shows that Robo3 is one of the master

regulators of midline crossing in vertebrates, with unique signaling properties, mechanisms of action and function. Here, we will summarize the current knowledge on Robo3 in the normal CNS and in neurological diseases.

ROBO3 FUNCTION: FROM MOUSE TO FISH

Robo3 expression in the vertebrate nervous system was first studied using *in situ* hybridization. In zebrafish, *Robo3* is mostly expressed in the nervous system, including spinal cord, hindbrain, cerebellum, tectum with a low expression in retinal ganglion cells (Challa et al., 2001; Lee et al., 2001). This is also the case in xenopus tadpoles (Hocking et al., 2010). On the contrary, Robo3 expression pattern in mouse embryos appears extremely restricted in time and space, with a strong expression level in the dorsal spinal cord, hindbrain and cerebellum (Camurri et al., 2004). Immunohistochemical studies using Robo3-specific antibodies have shown that in the mouse, Robo3 is selectively expressed on developing commissural axons in the spinal cord, hindbrain, midbrain and epithalamus (Marillat et al., 2004; Sabatier et al., 2004; Belle et al., 2014) (Figure 2A). It was also detected in tangentially migrating neurons including cortical interneurons during their migration from the ganglionic eminence (Nguyen-Ba-Charvet et al., 2004; Barber et al., 2009; Kaneko et al., 2010; Cariboni et al., 2012), but it is not expressed by forebrain commissural neurons such as those of the anterior commissure or corpus callosum. Strikingly, Robo3 expression is rapidly down-regulated after axons have crossed the ventral midline (the floor plate).

Understanding Robo3 receptor function was first addressed in the mouse through the generation of a *Robo3* knockout (KO) line, by homologous recombination (Sabatier et al., 2004). The phenotypic analysis of *Robo3* KO embryos revealed that they lack commissures in the posterior part of the CNS, including spinal cord, hindbrain and some midbrain commissures (Marillat et al., 2004; Sabatier et al., 2004; Tamada et al., 2008). This observation was surprising, as it could not be explained by a reduction of commissural axon midline repulsion as expected from a classic Roundabout receptor. Inspired by the drosophila *Commissureless/Robo* model (Keleman

et al., 2002), it was then proposed that Robo3 receptor would inhibit repulsive signaling of Robo1/2 receptors in commissural axons before they cross the midline, thereby facilitating axonal extension towards the floor plate (Sabatier et al., 2004) Figure 2B. Accordingly, *Robo1/2/3* triple mutants display partial rescue of commissural crossing in the spinal cord and some hindbrain nuclei (Sabatier et al., 2004; Di Meglio et al., 2008; Jaworski et al., 2010). However, other commissural axons, such as those of inferior olivary neurons (IO) still fail to cross in *Robo1/2/3* triple mutants (Di Meglio et al., 2008). The possible mechanism underlying Robo3 inhibition of Robo1/2 repulsion remains unclear. One hypothesis was that Robo3 would compete with Robo1/2 for Slit binding (therefore titrating it) but the mouse Robo3 receptor does not bind Slit proteins (Mambetisaeva et al., 2005; Zelina et al., 2014). *In vitro* data also suggest that Robo3 might trigger Robo1/2 degradation (Li et al., 2014), but this hypothesis would need to be further explored *in vivo*. Finally, Robo3 could interfere with Robo1/2 downstream signaling pathways. An impairment of midline attraction provides an alternative explanation for the lack of commissures in Robo3 KO mice. Direct evidence supporting this hypothesis comes from the analysis of pontine neurons (PN), a class of precerebellar neurons which migrate from the rhombic lip in the dorsal hindbrain towards the floor plate. In *Robo3* KO, pontine neurons are unable to reach the midline and their response to floor plate and netrin-1 attraction is abrogated (Zelina et al., 2014). It was shown that Netrin-1 does not directly bind to Robo3, but that it indirectly activates Robo3 through the deleted in colorectal carcinoma (DCC) receptor which can form a complex with Robo3 (Figure 2B).

Altogether, these models suggest that mammalian Robo3 promote midline attraction, directly or indirectly, which is surprising from an evolutionary point of view as it belongs to a family of repulsive axon guidance receptors (Ypsilanti et al., 2010). *Drosophila* Robo2 receptor was also associated with midline attraction (Evans and Bashaw, 2010), yet the mechanism involved is different from what is proposed for mammalian Robo3 (Evans et al., 2015). Interestingly, Robo3 displays specific features in mammalian lineage compared to other vertebrates including the inability to bind Slits and important modifications of its intracellular domain associated with the loss of CC1

domain (Figure 1C). This supports distinct Robo3 signaling mechanisms between mammals and other vertebrates. How Robo3 receptors function in non-mammalian species is still largely unknown but they were shown to bind Slits, suggesting that they could mediate repulsion or titrate Slits from Robo1/2. Interestingly, in zebrafish, a large scale genetic screen for genes controlling embryo motility (Granato et al., 1996) identified the *twitch twice* mutant (*twf*) which exhibits abnormal escape response. It was later found that *twf* mutants carry mutations in the *Robo3* gene (Burgess et al., 2009). In these fish, the motor response defect is probably associated with misguidance of hindbrain Mauthner axons that fail to cross the midline. Likewise, overexpression of Robo3 in these cells using a heat-shock transgenic line induces midline recrossing of Mauthner cells axon (Zelina et al., 2014). These data suggest that fish Robo3 might also enhance midline attraction. However, in zebrafish, Robo3 is also expressed by longitudinal and ipsilaterally projecting axons where it seems to repress Robo2-mediated midline repulsion (Schweitzer et al., 2013). Likewise, Robo2 and Robo3 influence the lateral positioning of post-optic commissure, in an opposite manner (Devine and Key, 2008). These results suggest that in zebrafish and other non-mammals, Robo3 could function as an anti-repulsive receptor. Interestingly, there is also evidence in zebrafish and xenopus, that Robo3 might control the development of RGC dendrites and apical process retraction (Hocking et al., 2010; Wong et al., 2012). These data suggest that in non-mammals, Robo3 function is more diverse than in mammals and varies between neurons.

The analysis of *Robo3* KO and *Robo3* conditional KO mice showed that the axons that become unable to cross the midline in absence of Robo3, still connect to their appropriate targets but on the ipsilateral side (Marillat et al., 2004; Comer et al., 2015). The physiological impact of this ipsilateral rewiring has been investigated in the cerebellum and auditory system. The inferior olive contains commissural neurons which project their axons (the climbing fibers) to Purkinje cells (PCs) in the contralateral cerebellum. In the adult, one PC is innervated by a single climbing fiber. However, during postnatal development, there is a transient phase of multiple innervation during which 3-

5 climbing fibers synapse on the same PC (Crepel et al., 1976). Activity-dependent synaptic elimination is completed by the end of the second postnatal week. Interestingly, Robo3-deficient olivary axons aberrantly project to the ipsilateral cerebellum and exhibit severe and permanent motor deficits. However, CF elimination still occurs and PC responses to CF stimulation are not affected (Badura et al., 2013). A different outcome of ipsilateral rewiring was described in the auditory system. Globular bushy cells (GBCs) in the ventral cochlear nucleus receive inputs from the inner ear and project onto neurons of the medial nucleus of the trapezoid body (MNTB) in the opposite side (Borst and Soria van Hoeve, 2012). The very large synapse of GBC axons on MNTB neurons is called calyx of Held and after a transient phase of multiple innervation during development, the adult MNTB neurons becomes mono-innervated. This circuit plays a major role in sound localization. In absence of Robo3, GBC axons project to the ipsilateral MNTB (Renier et al., 2010; Michalski et al., 2013). Patch-clamp recording showed that many MNTB neurons remain multi-innervated and synaptic transmission and release are significantly altered. This shows that in some cases, axons that are prevented from crossing the midline might stay in an immature stage (Michalski et al., 2013), suggesting that the lack of Robo3, which is not expressed during synaptogenesis, might have some long-term functional impact on neuronal circuits. Importantly, the lack of Robo3 has even more drastic consequences for the conventional *Robo3* KO mice as they all die a few hours after birth. It was found that lethality is most likely due to a decoupling of bilateral hindbrain premotor respiratory rhythm generators, which control the activity of phrenic nerve motor neurons innervating diaphragm muscles. In *Robo3* KO, left-right rhythms are asynchronous and asymmetric diaphragm contractions severely perturb breathing (Bouvier et al., 2010).

ROBO3 MUTATIONS IN HUMAN CAUSE THE HGPPS SYNDROME

Shortly after Robo3 was characterized in the mouse, a genetic study of human patients suffering from a rare neurological disorder name HGPPS (Jen et al., 2004), identified ROBO3 as the causal gene. HGPPS is characterized

by an absence of conjugate horizontal eye movements associated to a severe progressive scoliosis developing in childhood and adolescence.

Frequent consanguinity in patient families, and the absence of any symptoms in preceding generations suggested autosomal recessive inheritance. Genetic analysis first mapped the disorder locus on chromosome 11q23-25 (Jen et al., 2002). Further analysis identified multiple mutations in *ROBO3* gene, homozygous in most HGPPS patients and heterozygous in non-affected relatives (Jen et al., 2004). Subsequent genetic studies of HGPPS individual has increased the list of *ROBO3* mutations associated with HGPPS disorder, all listed in Table1 (Jen et al., 2004; Amoiridis et al., 2006; Chan et al., 2006; Sicotte et al., 2006; Haller et al., 2008; Khan et al., 2008; Abu-Amero et al., 2009; Amouri et al., 2009; Volk et al., 2011; Ng et al., 2011; Wang et al., 2013; Yamada et al., 2015; Fernández-Vega Cueto et al., 2016). Surprisingly, the mutations are highly diverse, and the positions of the mutated amino acids spread along the *ROBO3* protein (Figure 3B). Some mutations are predicted to induce a premature stop-codon, associated with expression of a truncated protein or mRNA degradation via nonsense mediated decay (NMD). In these cases, the absence of commissures in HGPPS patients could be easily explained by the absence of *ROBO3* protein, as observed in *Robo3* KO mice. However, most mutations are missense variants affecting multiple subdomains of the *ROBO3* receptor (Figure 3B). Some variants impact *ROBO3* targeting to the membrane or *ROBO3* conformation leading to receptor degradation (Wang et al., 2013), but the consequences of most missense mutations on *ROBO3* expression and function remain to be investigated. Additionally, numerous *ROBO3* variants have been detected in a large-scale sequencing project ((Auton et al., 2015); Ensembl 84), most being rare variants when population frequency data was available. These variants are equally distributed along the *ROBO3* sequence, with important accumulation of missense and frameshift mutations in the last exons coding for the C-terminal part of the receptor. This observation contrasts with HGPPS-associated variants, which are mostly located on the extracellular part of the protein. This difference suggests an important role of the extracellular domain in *ROBO3* function.

Although no detailed neuroanatomical study of the organization of the CNS of *ROBO3* patients was performed, they seem to exhibit commissural defects as *Robo3* mutant mice, with one important difference, which is an apparently normal postnatal viability (there is not yet any evidence linking *ROBO3* mutations to sudden death syndrome). The pontine nuclei is hypoplastic, which, as in *Robo3* KO mice, is most likely due to the aborted ventral migration and dorsal spreading of these neurons along the hindbrain surface (Zelina et al., 2014). Likewise, diffusion tensor imaging and physiological studies showed that the corticospinal tract connecting the motor cortex to motor neurons project ipsilaterally instead of contralaterally in HGPPS patients (Jen et al., 2004; Bosley et al., 2005; Amoiridis et al., 2006). This is also the case of ascending sensory inputs from the spinal cord to the brain (Haller et al., 2008). The lack of CST decussation is surprising as, in the mouse, *Robo3* is not expressed by projection neurons of the motor cortex (Barber et al., 2009). Although *Robo3* expression in the developing CST has not been tested, it is likely that CST crossing defects are secondary and probably linked to an abnormal development of the ventral midline. Unfortunately, *Robo3* KO mice die at birth precluding from analyzing CST projections, which enter the spinal cord postnatally. The origin of scoliosis onset in HGPPS patients is unknown. Neuromuscular disorder or imbalance in posture control pathways with vestibular system defects have been proposed to cause scoliosis (Ouellet and Odent, 2013), but no model is available to test this hypothesis. By contrast, horizontal gaze palsy is probably caused by commissural defects at the level of the hindbrain. A selective perturbation of horizontal eye movement in mice, mimicking what is found in HGPPS patients, was obtained using conditional *Robo3* KO. The absence of *Robo3*, prevents the development of the olivocerebellar commissure (Badura et al., 2013) and of the commissural connections between abducens interneurons and the principal oculomotor (PO) nucleus (Renier et al., 2010) and impairs horizontal compensatory eye movements (Figure 3C-D) , suggesting that these two circuits might be altered in humans.

In addition to HGPPS, there is evidence for *ROBO3* association with psychiatric or developmental disorders such as a correlation of certain

widespread intronic polymorphism with Autism (Anitha et al., 2008; Suda et al., 2011). Dys-regulation of *Robo3* has also been reported in models of peripheral nerve injury, hypothyroidism and rheumatoid arthritis (Denk et al., 2010; Alvarado et al., 2013; Shiraki et al., 2016). The functional relevance of these modifications is still unknown.

STRUCTURAL AND FUNCTIONAL DIVERSITY OF ROBO3 ISOFORMS

Like most receptors, multiple isoforms of Robo3 receptor have been described (Figure 4A). Two alternative first exons have been identified in zebrafish, mouse and human, leading to two N-terminal protein isoforms (Camurri et al., 2005; Challa et al., 2005). These N-terminal isoforms are referred to as Robo3A and Robo3B (Figure 4A). Although both isoforms display a signal peptide and are at the membrane, little is known about the expression pattern and biological function of these two variants. Additionally, an alternative intron retention has been identified in mouse embryonic dorsal spinal cord full-length cDNA collection (Chen et al., 2008). Alternative retention of intron 26 during Robo3 mRNA splicing inserts a premature stop codon shortly after the CC3 domain, resulting in a shorter version of Robo3 receptor (Figure 4B). The two C-terminal isoforms are called Robo3.1 and Robo3.2 (Figure 4A). The comparative analysis of their expression pattern in the spinal cord of mouse embryos using Robo3.1 and Robo3.2 specific antibodies showed that Robo3.1 is only expressed in pre-crossing commissural axons and immediately down regulated as they reach the midline, whereas Robo3.2 is transiently expressed in post-crossing axons, immediately after midline crossing (Chen et al., 2008; Colak et al., 2013). This differential expression was attributed to Robo3.2 mRNA translocation and local translation in post crossing commissural axons. Intron retention in Robo3.2 mRNA is associated with nonsense-mediated decay (NMD), leading to elimination of this transcript shortly after translation, resulting in transient expression of this isoform (Colak et al., 2013). However, no mechanism has been proposed yet to explain Robo3.2 mRNA protection from NMD prior floor plate induced local translation. Furthermore, functional studies showed that

only Robo3.1 is able to restore midline attraction in Robo3-KO rescue experiments (Chen et al., 2008). Robo3.2 has been proposed to participate to post-crossing axons midline repulsion (Chen et al., 2008; Colak et al., 2013), although functional evidences are still weak and no mechanism is proposed to explain this repulsive action considering that neither Robo3 isoform binds Slits (Zelina et al., 2014). An alternative possibility would be that intron 26 retention is a mechanism that inactivates Robo3.1 expression and therefore decrease midline attraction.

Here we focus on the phylogenic conservation of Robo3.1/Robo3.2 variants in vertebrates, which had not yet been addressed. As mentioned before, Robo3.2 has only been identified in mouse and rat embryonic cDNA banks (NCBI database, respectively AF060570.1 and NM_001108135.1), but never been described in human or any other species. However it is possible to forecast this variant from existing *Robo3* gene sequences. On the one hand, we collected sequences of Robo3.1 proteins in Ensembl and NCBI databases. On the other hand we generated predicted Robo3.2 protein sequences by doing artificial intron26 retention from vertebrates *Robo3* genes. Alignment of the C-terminal parts of Robo3.1 and Robo3.2 proteins are presented in Figure 4. We found that Robo3.1 is highly conserved in vertebrate species (60% identity between residues 1319 to 1386, see Figure 4C). In contrast, predicted Robo3.2 sequences are highly variable (17% identity between residues 1319 to 1351, Figure 4D). This difference is even present between closely related mammals (see rabbit, mouse and marmot sequences, Figure 4D), demonstrating poor conservation of this intron between mammals. Such variability does not seem to support a conserved functional role of Robo3.2 variants widely in vertebrate axon guidance. Moreover, intron 26 splicing sites appear to be modified in glires phylum (a group of mammals including mouse, rat and rabbit) leading to an elongation of 8 amino acids at the splicing site (Figure 4C). Both versions of intron 26 splicing can even be found in some glires species like in the rabbit (Ensembl84 database: ENSOCUT00000005842 and ENSOCUT00000002640). This observation suggests that intron 26 splicing is divergent in glires, and particularly in rodents, and therefore might be more

likely to give intron retention during splicing. Altogether, this suggests that Robo3.2 is most likely a rodent-specific isoform, belonging to lineage-specific alternative splicing category (Necsulea and Kaessmann, 2014).

ROBO3 GENE EXPRESSION IS TIGHTLY REGULATED

In addition to sequence and splice variation, Robo3 receptor expression is tightly regulated at different levels including transcriptional and translational regulation. As discussed before, Robo3 receptor is transiently expressed by commissural axons before they cross the midline, and rapidly down-regulated shortly after crossing (Marillat et al., 2004). This expression in a narrow developmental window and in restricted neuronal populations would support a basal transcriptional repression of this gene. Accordingly, Robo3 was first identified in mRNA extracts from Retinoblastoma protein (RB) knockout mouse embryos, where the transcriptional regulator RB protein is absent (Yuan et al., 1999). Luciferase assay with Robo3 promoter confirmed that RB induces a basal repression of Robo3 transcription (Yuan et al., 2002). Likewise, Robo3 up-regulation was detected in embryonic fibroblast cultures from mice deficient for the ubiquitous transcription factor Sp3 (Stielow et al., 2010). Sp3 is associated with gene silencing via heterochromatin formation and inhibition of RNAPol-II effective transcription (Valin and Gill, 2013). Loosening of Robo3 constitutive repression could be induced by the transcription factor Pax2, binding to RB and inhibiting its repression activity on *Robo3* (Yuan et al., 2002). However, Pax2 is not specific of commissural neurons (Wehr and Gruss, 1996) and no commissural phenotype was reported in *Pax2* knockout (Torres et al., 1995), suggesting other transcription factors are certainly involved in this release of inhibition.

The genetic program controlling Robo3 expression in commissural neurons is still unknown, although several transcription factors (TFs) that either repress or induce its expression in some neurons have been identified (Chédotal, 2014). In the mouse spinal cord, ipsilateral and contralateral projecting

interneurons are intermingled (Goulding, 2009), and *Robo3* expression in these neurons is directly correlated to contralateral projection. For example, dl1 interneurons expressing *Robo3* are commissural interneurons (called dl1c), whereas others stay ipsilateral (called dl1i) (Wilson et al., 2008). This duality is controlled by TFs differential expression regulating *Robo3* expression: LIM homeobox TFs *Lhx2* and *Lhx9* expression (Wilson et al., 2008), and upstream *Barhl2* expression (Ding et al., 2012) (Figure 5A). *Lhx2* can directly bind to *Robo3* intronic region (Intron1) and possibly induce its expression in a dose-dependent manner (Wilson et al., 2008). This potential activation depends on other factors controlling *Robo3* chromatin accessibility as *Lhx2* does not bind to the *Robo3* gene in thalamic neurons compare to spinal cord neurons (Marcos-Mondejar et al., 2012). However, this transcriptional cascade cannot be extended to all commissural systems as *Lhx2* is only expressed by a sub-population of commissural neurons in the spinal cord, and is equally expressed by contralateral and ipsilateral neurons in the midbrain (Wilson et al., 2008; Inamata and Shirasaki, 2014).

In the mouse midbrain and hindbrain, neurons derived from progenitors expressing Developing Brain homeobox protein 1 (*Dbx1*) express *Robo3* and send commissural projections (Bouvier et al., 2010; Inamata and Shirasaki, 2014). Overexpression and silencing experiments in midbrain showed that the *Dbx1* TF is necessary and sufficient to trigger *Robo3* expression in neurons and midline crossing (Inamata and Shirasaki, 2014). *Dbx1* regulation of *Robo3* expression acts through Even-skipped homeobox 2 (*Evx2*)(Figure 5A), but weather this is direct or not is still unclear. Interestingly, this correlates with studies showing that *Dbx1*-derived spinal cord V0 commissural neurons express *Evx1/2* TFs and that deletion of *Dbx1* prevent midline crossing (Moran-Rivard et al., 2001; Pierani et al., 2001). This suggests that *Dbx1* and *Evx* TFs might also control *Robo3* expression in some spinal cord interneurons population, representing an important *Robo3* gene regulatory pathway. Other important players controlling *Robo3* expression have been identified in the mouse, including *Hox* TFs. As it has been shown for other Robos (Geisen et al., 2008), *Hox* genes are necessary to induce *Robo3* expression. In *Hoxa2* deficient mice, *Robo3* expression in cochlear nucleus

neurons is altered and these neurons send aberrant projections to the ipsilateral MNTB (Di Bonito et al., 2013). However, the association of these TFs with Robo3 expression in non-mammalian vertebrates has not been investigated yet.

In parallel of these findings, diverse TFs were found to maintain ipsilateral targeting of axons by repressing Robo3 expression in various vertebrates (Marion et al., 2005; Escalante et al., 2013; Schweitzer et al., 2013). Both in chicken and mouse spinal cord, over-expression of zinc finger protein Zic2 induces strong reduction of Robo3 expression (Escalante et al., 2013). As a consequence, the down-regulation of Zic2 in dLL_B ipsilateral interneurons in the mouse spinal cord induces Robo3 up-regulation and contralateral projections. Correspondingly, Zic2 overexpression in chicken dl1 and dl2 contralateral interneurons induces ipsilateral projections (Escalante et al., 2013). In mammillary body neurons, the absence of single-minded proteins Sim1 and Sim2 TFs induces Robo3 up-regulation and axon midline attraction in the mouse (Marion et al., 2005). Likewise, in zebrafish, Robo3 receptor expression in hypothalamo-spinal axons is up-regulated in *Sim1a* mutants (Schweitzer et al., 2013). However, how Sim1/2 or Zic2 suppress Robo3 expression is still unknown.

Altogether, these different results present multiple regulatory networks of *Robo3*, based on combinatorial expression of enhancing and repressing TFs (Figure 5A). Further evidence supporting the heterogeneity of the molecular mechanisms regulating *Robo3* expression comes from the analysis of post-transcriptional regulation in pre-cerebellar nuclei (Kuwako et al., 2010). Inferior olive (IO), lateral reticular nucleus (LRN) and external cuneatus nucleus (ECN) precerebellar neurons express the RNA-binding protein Musashi 1 (Msi1), which binds to Robo3 mRNA and recruits it to heavy polysomes (Figure 5B). In Msi1-deficient animals, IO, LRN and ECN neurons do not cross midline due to insufficient Robo3 expression, but surprisingly other commissural projections in the pons and spinal cord remain unaffected (Kuwako et al., 2010). This suggests that different RNA-binding proteins might influence the post-transcriptional regulation of Robo3 in various types of commissural neurons. Of note, nothing is known about the mechanisms

responsible for the rapid silencing of Robo3 expression in post-crossing axons.

CONCLUSION AND PERSPECTIVES

Many questions about Robo3 receptor function and mechanism of action remain to be answered.

First, the identity of the Robo3 ligand(s) is still unknown in mammals as neither Netrin-1 nor Slits bind to it (Zelina et al., 2014). It was recently proposed that neural epidermal growth factor-like-like 2 (NELL2), a glycoprotein secreted by motor neurons, is a Robo3 ligand (Jaworski et al., 2015) mediating repulsion. However no clear axon guidance phenotype is associated with NELL2 deletion in mice and how Robo3 receptor would transduce repulsive signaling remains unclear. Furthermore, NELL2 is not expressed by the floor plate and can't mediate Robo3 attractive activity. Another molecule, Secreted Frizzled Related Protein (SFRP), has been shown to bind to ROBO3 in pancreatic cancer cells (Han et al., 2015), but the physiological relevance is unknown. As a member of the IgCAM protein family, Robo3 could interact with many different receptors, including itself. Potential Robo3 oligomerisation could affect Robo3 activity and downstream signaling, as proposed for other Robo receptors (Hohenester, 2008; Zakrys et al., 2014). Heterophylic interaction with Robo1 have been reported (Camurri et al., 2005). Last but not least, Robo3 interacts with DCC via its intracellular domain (Zelina et al., 2014),

What are the components of the intracellular signaling cascade downstream of Robo3? Robo3 intracellular domain can be phosphorylated by different proteins, including Src-family kinases and protein kinase C (Zelina et al., 2014; Samelson et al., 2015). And strong divergence of Robo3 intracellular domain, with the CC1 domain loss in mammals, suggests that Robo3 signaling pathways differ from other Robo receptors (Figure1C). Yet, there is no clear and direct association of Robo3 with any intracellular signaling cascade.

How Robo3 functions in non-mammalian species is also largely unknown. Unlike mammals, non-mammalian Robo3 receptors bind and respond to Slits (Zelina et al., 2014), and their intracellular domain is more similar to other Robo receptors (Figure 1C). In non-mammals, Robo3 expression does not seem to be restricted to caudal commissural neurons, and it was found in retinal ganglion cells, ipsilaterally projecting neurons or muscles (Challa et al., 2001; Hocking et al., 2010; Wong et al., 2012; Schweitzer et al., 2013). In Robo3 mutant zebrafish, commissural defects are variable and primarily affect Mauthner neurons (Burgess et al., 2009) and ipsilateral axons (Schweitzer et al., 2013). Last, injection of Robo3 morpholinos in *Xenopus* leads to axonal fasciculation defects (Devine and Key, 2008), supporting additional functions for this receptor outside of midline axon guidance.

In conclusion, a better understanding of Robo3 function, its regulation and downstream signaling pathways will shed new light on our knowledge of the molecular mechanisms controlling the development of commissural circuits and their evolution in vertebrates.

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Figures legends

Figure 1: Robo3 receptor structure and evolution. **A.** Robo3 receptor is composed of 5 immunoglobulin domains (Ig1-5), 3 fibronectin type III domains (FnIII1-3) and an intracellular domain containing 3 or 4 cytoplasmic conserved motifs (CC). **B.** Robo3 belongs to Robo family of receptors, which are present in all bilateria. There are 4 *Robo* genes in vertebrates, all deriving from a common ancestral *Robo* sequence. **C.** Vertebrate Robo receptors display conserved features including highly conserved Ig1 domain involved in Slit binding, and cytoplasmic domains involved in intracellular signaling. However, mammalian Robo3 receptors differ from other Robos on these two aspects with numerous mutations in the Ig1 domain, related to loss of Slit binding, and intracellular modifications including a CC0 domain loss (identified by a red star). Percentages indicate amino acid identity between Robo Ig1 domains.

Figure 2: Robo3 expression and function in commissural axons in the mammalian nervous system. **A.** Immunostaining against Robo3 (in green) and Neurofilament (NF, in magenta) in a mouse embryo spinal cord (E11). Robo3 expression is restricted to commissural projections before they cross the midline. **B.** Dual mechanisms of action proposed for Robo3 in commissural axon guidance. Commissural neurons are attracted to the midline, which is secreting multiple guidance molecules including Netrin-1 and Slit proteins. In the anti-repulsive model, Robo3 inhibits Slit/Robo1/2-mediated midline repulsion. In the pro-attractive model, Robo3 potentiates Netrin-1 attractive signaling in cooperation with the Netrin-1 receptor DCC.

Figure 3: Horizontal Gaze Palsy with Progressive Scoliosis (HGPPS) is associated with *ROBO3* mutations and commissural defects.

A. Signature symptoms of HGPPS patients: A severe progressive scoliosis developing in childhood, hypoplasia of basilar pons and absence of conjugate horizontal eye movements. Adapted with permission from Jen et al., 2004. **B.** *ROBO3* mutations associated with HGPPS and their position along the *ROBO3* protein. Missense mutations are presented by a red and yellow circle, Nonsense mutations by a red circle, Frameshift by a red star and splicing mutations by a brown hexagon. Complete list of *ROBO3* mutations and their amino acid equivalence is presented in Table 1. **C.** Comparison of horizontal gaze palsy in human HGPPS patients with vestibulo-ocular reflex defects in mice from two different conditional *Robo3* knockout mouse lines: Pf1aCre-*Robo3lox* and *Krox20Cre-Robo3lox*. **D.** Oculomotor network controlling horizontal eye movement involves many different commissural systems. Simultaneous activation of abducens nucleus and pre-optic nucleus (PO) results in conjugate contraction of LR (Lateral Rectus) from the right eye and MR (Medial Rectus) from the left eye, leading to conjugate lateral eye movement. Perturbation of this system induces horizontal eye movement defects similar to those observed in HGPPS patients. In *Krox20Cre-Robo3lox* mice, the median longitudinal fascicle (mlf) is disrupted; in *Ptf1Cre-Robo3lox* mice, inferior olivary neurons (IO) project to the ipsilateral cerebellum. Both defects are indicated by red stars. VN: Vestibular nucleus.

Figure 4: *Robo3* receptor isoforms and their conservation in vertebrates.

A. Schematic representation of mouse *Robo3* isoforms *Robo3A*, *Robo3B*, *Robo3.1* and *Robo3.2*. **B.** Alternative intron 26 retention during mouse *Robo3* splicing produces two different *Robo3* mRNAs. Premature stop codon (red star) in intron 26 produces a truncated version of *Robo3* receptor identified as *Robo3.2*. **C.** Alignment of vertebrate *ROBO3.1* sequences from NCBI and Ensembl databases (references are listed in E) using ClustalW. Only C-terminal sequences are displayed, starting amino acid 1312 of human *ROBO3A.1*. Amino acids coded by exons 26 and 28 are presented over a blue background and amino acids coded by exon 27 over a green background. Amino acid conservation was calculated for each position using

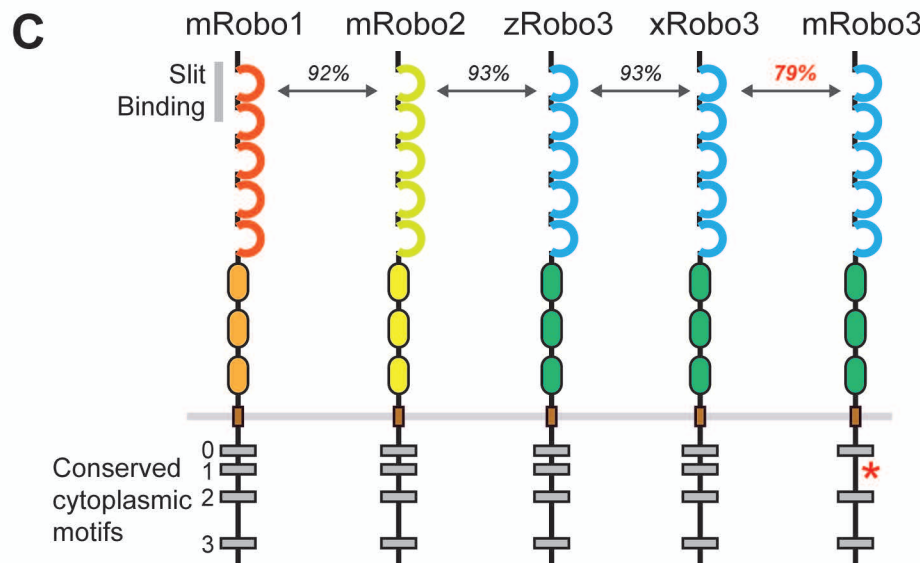
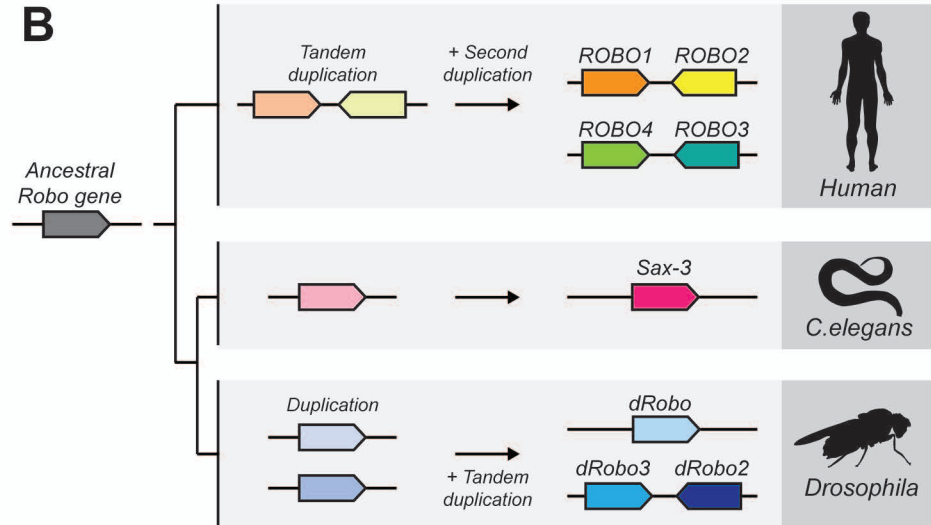
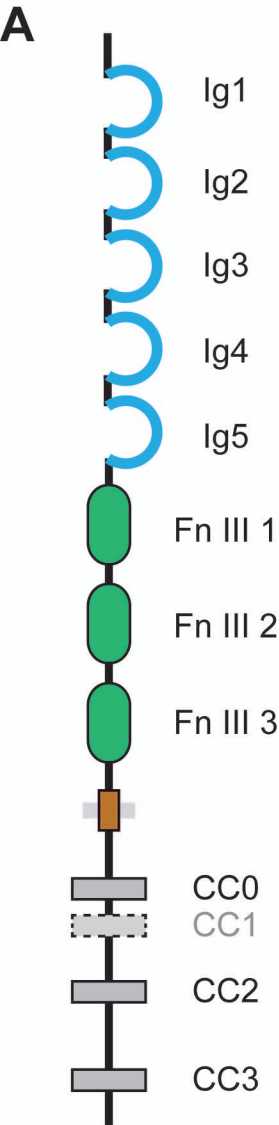
the alignment ; conservation index is represented in the graph below the alignment. **D.** ROBO3.2 sequences were produced from *ROBO3* genomic sequences (references are listed in E) by translating exons 26 and following intron 26 until stop codon was reached (except from mouse Robo3.2 protein sequence already available on NCBI). Vertebrate ROBO3.2 sequences were aligned using ClustalW. Only C-terminal sequences are displayed, starting amino acid 1312 of human ROBO3A.1. Amino acids coded by exon 26 are presented over a blue background and amino acids coded by intron 26 over a purple background. Red stars represent premature intron 26 stop codons. As for ROBO3.1, conservation index was calculated for each position using the alignment and represented in the graph below the alignment. **E.** References of sequences used for ROBO3 protein alignments. Genomic sequences references used for predicted ROBO3.2 translation are in italic.

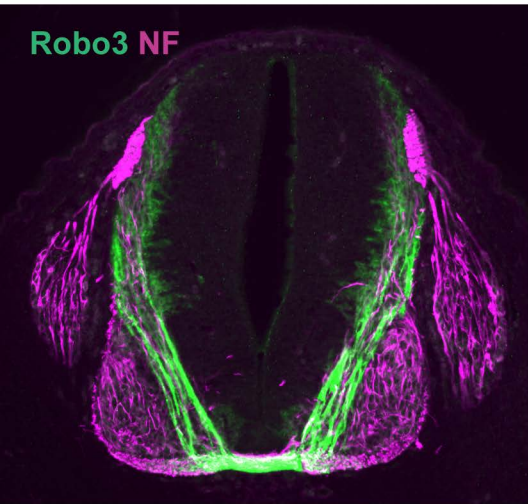
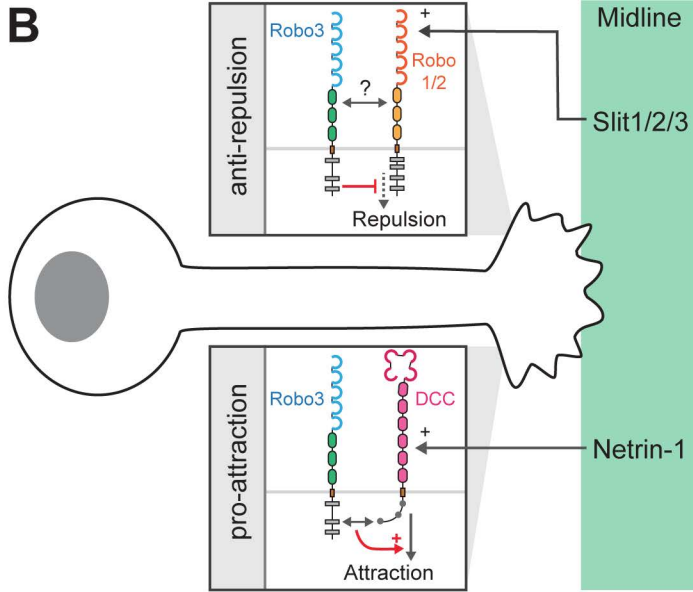
Figure 5: Regulation of *Robo3* expression in mammals. **A.** Transcriptional regulation of *Robo3* expression. Multiple pathways have been described to regulate *Robo3* expression, depending on commissural systems. Here are presented different transcriptional cascades leading either to *Robo3* expression or silencing, respectively represented in green and yellow. **B.** Post-transcriptional regulation of *Robo3* mRNA. In inferior olive neurons, Musashi protein binds to *Robo3* mRNA and recruits it to heavy polysomes for high-level translation. Floor plate-mediated Musashi down-regulation would contribute to *Robo3* expression decrease after midline crossing. In other commissural circuits, Musashi protein is absent and no protein have been associated yet with *Robo3* post-transcriptional regulation.

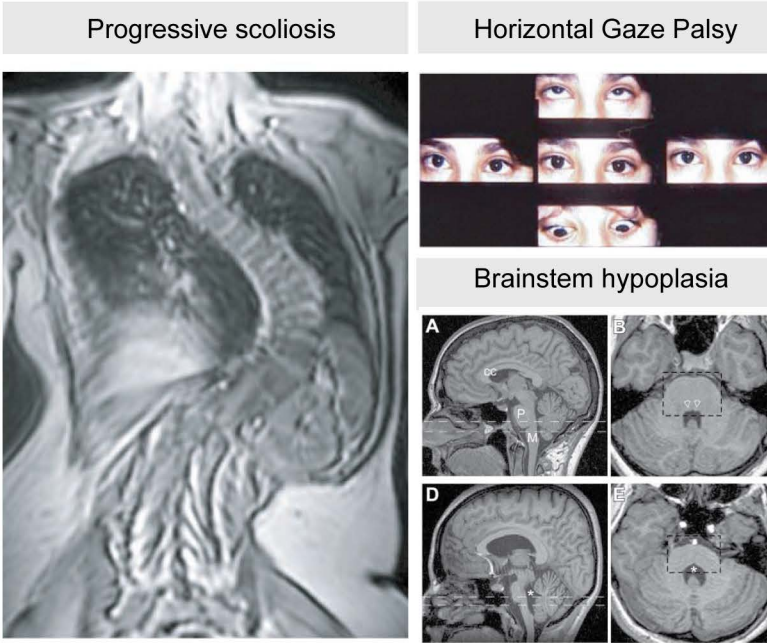
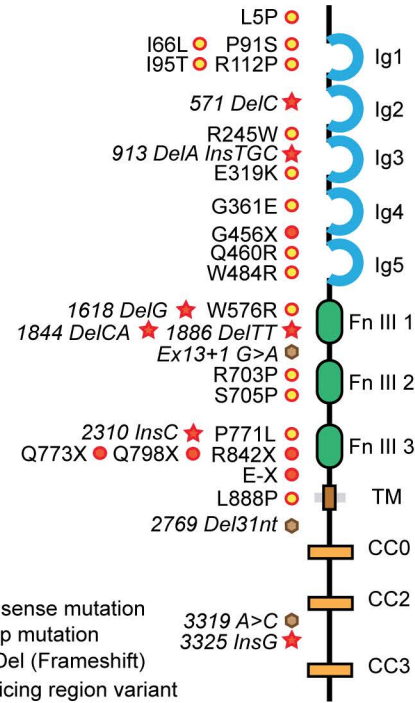
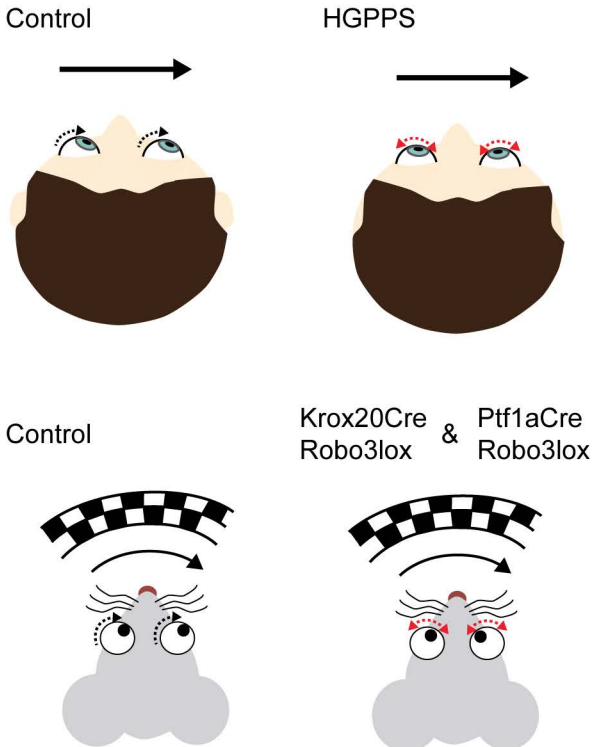
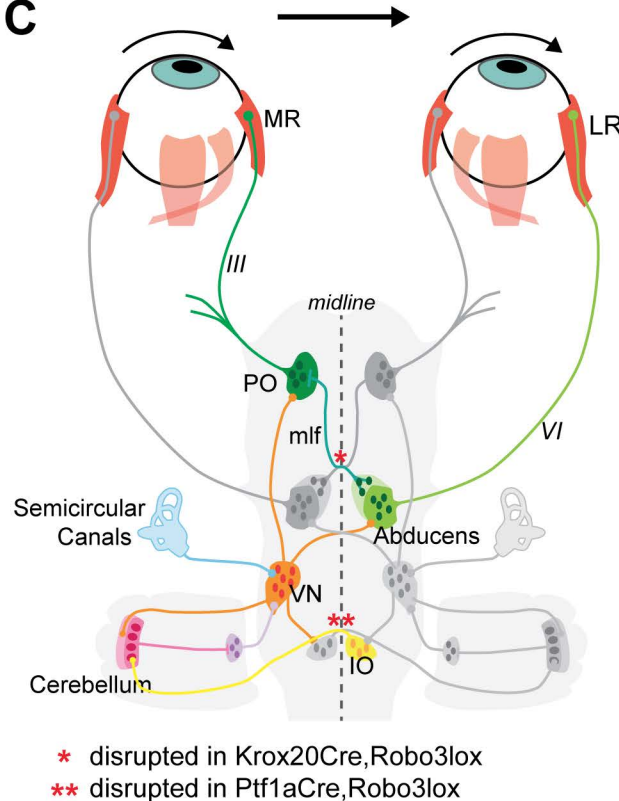
Table 1: HGPPS associated *ROBO3* mutations. Nonsense mutations and frameshift mutations leading to premature stop codon and potentially truncated proteins are indicated in orange.

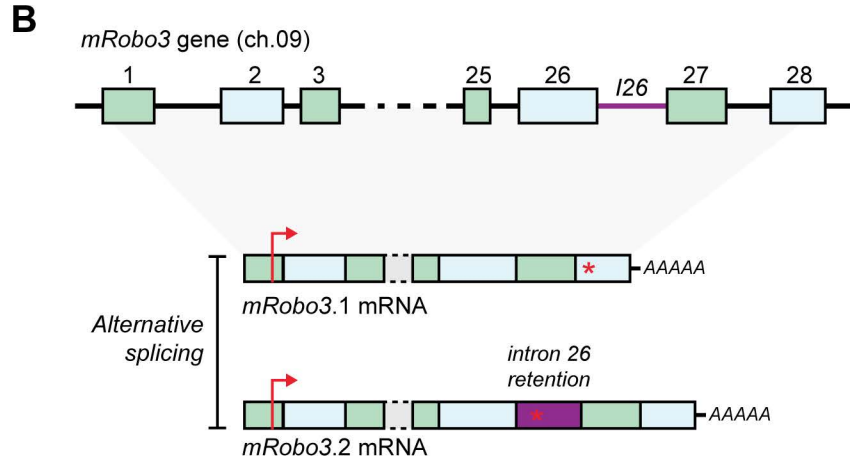
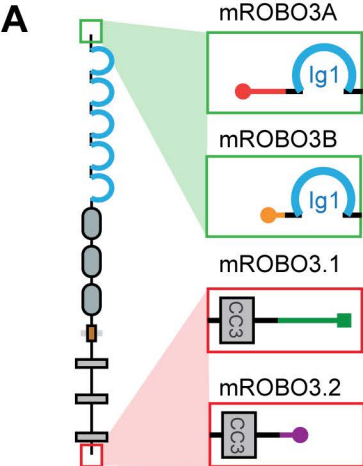
Nucleotide change	Amino acid change	Domain	Publication
14T>C	L5P	Nter	<i>Jen et al., 2004</i>
196A>C	I66L	Ig1	<i>Jen et al., 2004; Wang et al., 2013</i>
271C>T	P91S	Ig1	<i>Abu-Amero et al., 2009</i>
283C>T	I95T	Ig1	<i>Amouri et al., 2009</i>
335G>C	R112P	Ig1	<i>Abu-Amero et al., 2009</i>
571 DelC	Frameshift	Ig2	<i>Abu-Amero et al., 2009</i>
733C>T	R245W	Ig2-3	<i>Chan et al., 2006; Amouri et al., 2009</i>
913 DelAinsTGC	Frameshift	Ig3	<i>Volk et al., 2011</i>
955G>A	E319K	Ig3	<i>Jen et al., 2004; Amoiridis et al., 2006</i>
1082G>A	G361E	Ig4	<i>Jen et al., 2004</i>
1158G>C	Q386H	Ig4	<i>Fernández-Vega Cueto et al. 2016</i>
1366G>T	G456X	Ig4-5	<i>Jen et al., 2004</i>
1379A>G	Q460R	Ig5	<i>Abu-Amero et al., 2009</i>
1450T>C	W484R	Ig5	<i>Amouri et al., 2009</i>
1618 DelG	Frameshift	Fn III 1	<i>Amouri et al., 2009</i>
1726T>C	W576R	Fn III 1	<i>Abu-Amero et al., 2009</i>
1844-45 DelCA	Frameshift	Fn III 1	<i>Chan et al., 2006</i>
1886-87 DelTT	Frameshift	Fn III 1	<i>Chan et al., 2006</i>
Ex13 + 1 G>A	Splicing defect	Fn III 2	<i>Jen et al., 2004</i>
2108G>C	R703P	Fn III 2	<i>Jen et al., 2004; Haller et al., 2008</i>

2113T>C	S705P	Fn III 2	<i>Jen et al., 2004</i>
2310 InsC	Frameshift	Fn III 3	<i>Jen et al., 2004; Sicotte et al., 2006</i>
2312C>T	P771L	Fn III 3	<i>Khan et al., 2008</i>
2317C>T	Q773X	Fn III 3	<i>Chan et al., 2006</i>
239C>T	Q798X	Fn III 3	<i>Yamada et al., 2015</i>
G>T (Ex17)	E-X	TM	<i>Ng et al., 2011</i>
2663 T>C	L888P	TM	<i>Khan et al., 2014</i>
2769 Del31nt (11Ex17 + 20Int17)	Splicing defect + frameshift		<i>Volk et al., 2011</i>
3319A>C	Ex22 skip + frameshift	CC2-CC3	<i>Volk et al., 2011</i>
3325 InsG	frameshift	CC2-CC3	<i>Jen et al., 2004</i>



A**Robo3 NF****B**

A**B****D****C**



C

ROBO3.1 protein



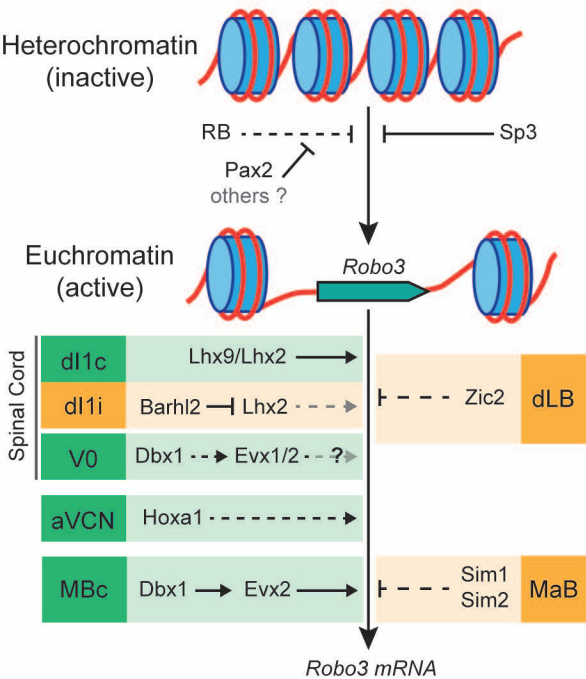
D

ROBO3.2 protein



E

	ROBO3.1	ROBO3.2
Human	ENSP00000380903	ENSG00000154134
Rabbit	ENSOCUP00000002281 ENSOCUP000000023052	ENSOCUE000000293660
Mouse	ENSMUST00000115038	AAD11628.1
Marmot	XP_015354478.1	NW_015351268.1
Horse	ENSECAP00000006275	ENSECAG000000013956
Cat	ENSFCAP00000006275	ENSFCAG00000006751
T. devil	ENSSHAP00000018216	ENSSHAE000000154162
Turtle	XP_008169327.1	NW_007281402.1
Gekko	XP_015284878.1	NW_015161948.1
Xenopus	NP_001164069.1	ENSXETG00000003938
Gar	ENSL0CP00000006335	ENSL0CG00000005170
Fugu	ENSTRUP00000008702	ENSTRUG00000003711
Zebrafish	ENSADAR00000027377	ENSADARG00000005645

A**B**