# Mapping of the binding domains of the axon guidance receptor Unc5B to Netrin-1 and FLRT3

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**Abbreviations**

VZ: Ventricular zone

FLRTs: fibronectin and leucine-rich transmembrane proteins

ECD: ectodomain

DCC: Deleted in Colorectal Cancer

EGFP: Enhanced green fluorescent protein

PEI: Polyethylenimine

CVS: coverslips

PFA: paraformaldehyde

DAPI: 4,6-diamidino-2-phenylindole

AP: alkaline phosphatase

TBS: Tris-buffered saline

IRES: Internal ribosomal entry site

HEPES: *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*

BSA: bovine serum albumin

## Introduction

Some landmarks of neural development include the birth and differentiation of neurons from stem cell precursors, the migration of immature neurons from their birthplaces in the embryo to their target areas, outgrowth of axons and dendrites from neurons, guidance of the motile growth cone through the embryo towards postsynaptic partners and the generation of synapses between these axons and their postsynaptic partners.

Neuronal migration is a fundamental process that determinates the final location of neurons in the nervous system, establishing the basis for the subsequent wiring of neural circuitry. From cell polarization to target identification, neuronal migration integrates multiple cellular and molecular events that enable neuronal precursors to move across the brain to reach their final destination. Instructed by extracellular cues, the activation of guidance receptors and their downstream signaling pathways enable newborn neurons to migrate through the developing nervous system until they reach their destination. The migratory cycle of neurons involves leading process dynamics, through which directional migration is achieved, and somal translocation, which involves the movement of perinuclear material and organelles as well as the nucleus.

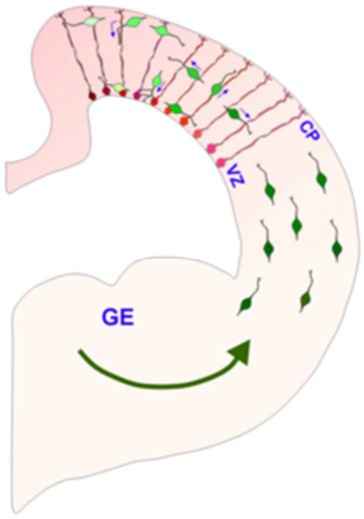


**Figure 1:** Tangential migration of the interneurons from ganglionic eminence.

Different types of neurons adjust and modify this basic migratory cycle depending on the specific requirements of their migratory pathway, which may also change through time. A good example of the variety of neuron migration is the cerebral cortex, which is the structure of the mammalian brain that has expanded the most in primates and is the responsible of our intelligence and cognitive capabilities. The total amount of neurons in the cortex can be divided into two groups: excitatory neurons and interneurons. On the one hand, the 80% of the neurons are excitatory glutamatergic also known as pyramidal neurons. They get born in the internal part of the adjacent ventricular zone (VZ) and display a radial migration to their final destination. The remaining 20% of neurons are gabaergic interneurons. In this case neurons get born in the VZ of the ventral telencephalon and therefore display a tangential migration towards the cortex. The interneurons control the activity and function of the excitatory neurons. Recent studies have established that some psychiatric pathologies

like schizophrenia and autism are bound to a delay on the migration of these interneurons inducing a non-controlled activity of the excitatory neurons. The molecular mechanisms controlling neuron migration are still poorly understood. Recently, the transmembrane proteins FLRT (FLRT1-3, fibronectin and leucine-rich transmembrane proteins) have been shown to regulate the migration of a subset of excitatory neurons in the mouse cortex. This effect is mediated by shedding of the extracellular domain (ECD) of FLRTs that binds to Unc5 receptors inducing repulsion (Yamagishi *et al*, 2011). Specifically, the Unc5B receptor interacts with FLRT-3 proteins which were widely expressed in the developing embryo, particularly in the central nervous system and somites (Robinson *et al*, 2004). Moreover, Unc5s are well characterized receptors for Netrins, the most well documented axon guidance cues that has been shown to be crucial for many axon guidance decisions in vivo. The axon guidance function of Netrin-1 is dependent on which receptor is involved and the intracellular signal transduction pathways activated by each receptor. (Bradford *et al*, 2009). We know that Netrin-1 has a dual function: when it interacts with DCC positive neurons, Netrin-1 promotes an attractive guidance. On the other hand, the interaction between the Unc5B positive neurons and Netrin-1 promotes a repulsive guidance. In addition, this Netin-Unc5B interaction is involved in the signaling of other processes such as angiogenesis or cell survival (Bradford *et al*, 2009). Neurons and growth cones are usually surrounded by multiple guidance cues so it is a big challenge in developmental neuroscience to understand how the neuron is able to integrate such information. In this context the present work aims to understand how a single receptor, Unc5B, interacts with FLRT3, Netrin-1 or both together. These results will be important to interpret the function of these molecules in vivo. In addition, the work also includes a short study based on the hypothesis FLRT3 and Netrin-1 may interact. With this interaction FLRT3 could act as a modulator of the function of Netrin-1.

To develop the experiments we used the extracellular part of the Unc5B receptor which contains some deletions in different domains to study in depth the interaction with both Netrin-1 and FLRT3. Previous studies established that the deletion of the TSP1 domain prevents the interaction between FLRT2 and the Unc5D receptor (Yamagishi *et al*, 2011). We also checked if with FLRT3-Unc5B the result was the same. Also we wanted to know if there was a direct interaction between FLRT3 and netrin-1. If this interaction exists, FLRT3 could modulate directly the activity and function of netrin-1.

With all this components we started to perform different analysis to evaluate if there were significant differences in the interactions between Netrin-1 and FLRT3 and the different types of Unc5B receptor. These assays can allow us to understand better the global process of neuronal migration and also, to study in depth the interactions between the different components implicated in the process.

## Materials and Methods

### *Plasmids transformation using chemicocompetent bacteria cells*

First of all the pieces of paper in which the external laboratory sent us the plasmids were put in different eppendorfs with 100µL of TE buffer to solubilize the DNA, while protecting it from degradation. After an hour of incubation at 37ºC, 2µL of each DNA were mixed with the chemicocompetent bacteria cells and let in ice during 30 minutes. After this time the eppendorfs were put in a bath at 42ºC during 45 seconds. Then the eppendorfs were put 5 minutes in ice and finally 500µL of LB media were added to each eppendorf. After an hour of incubation at 37ºC, the content of each eppendorf was plated in ampicillin p100 plates.

### *Transfection of HEK293T cells with FLRT3$^{ECD}$-AP, Netrin-1-AP and EGFP as a control*

The transfection was made using FuGene (Promega) to increase the efficiency of the transfection. In three different tubes were put: 200µL of OptiMEM (Invitrogen)+15µL of the DNA in the case of FLRT3$^{ECD}$-AP, 200µL of OptiMEM+60µL of the DNA in the case of Netrin-1-AP and finally 30µL of OptiMEM+10µL of DNA in the case of EGFP (Enhanced green fluorescent protein used as transfection efficiency control). In another tube were added 150µL of FuGene+3750µL of OptiMEM. The second tub was let 5 minutes at room temperature. After this time, 1,8mL of the second tub were added in both FLRT3$^{ECD}$-AP and Netrin-1-AP tubes. In the EGFP tube were added 200µL. After 20 minutes at room temperature, the content of each tube was shared out in 2 p100 plates that contained the HEK293T cells except the EGFP that was put in only one p35 plate. After a day of incubation at 37º the media was changed. 10mL of a media that contains OptiMEM, serum, P/S, glutamine and $Cacl_2$ was added. Passed 5 days of incubation at 37º the media that contained the proteins was collected.

### *Coomassie staining*

The medias that contain the FLRT3$^{ECD}$-AP and Netrin-1-AP proteins were homogenized in standard PAGE gel loading buffer and denatured at 95ºC for 5 minutes. Protein extracts were separated by electrophoresis on precast 8% polyacrylamide gel. Then, the proteins were fixed overnight using the fixative solution. After that, the gel was washed three times during 30 min with water and stained using the staining solution for 20 min with gentle agitation. After that time, the gel was washed with water.

### *Transfection with the different plasmids coding for the Unc5B deletion mutants*

In 6 eppendorfs were added 300µL of OptiMEM+a volume of each DNA to have a final concentration of 15,8µg/µL. In another tube, 1,8mL of OptiMEM were added+90µL of PEI. Then, 300µL of the tube were added in each eppendorf. After 10 minutes at room temperature, the 6-well cell culture plate was washed with OptiMEM and the content of each eppendorf was added in each well+1mL of OptiMEM. The plate was incubated at 37º and an hour later, the OptiMEM was changed for 2mL of neurobasal media.

### *Immunofluorescence*

A day after the transfection of HEK293T cells with the indicated constructs using PEI (Sigma), took place the cell fixation. First, 4%PFA+freshly added 4% sucrose was added in each well and was incubated 30 min on ice. Then we did a wash with PBS during 10 minutes, another with PBS+0,1% Triton X-100 to permeabilize the cells and a final wash with PBS+$NH_4Cl$. Finally we added PBS+$NaN_3$ and we left the plate at 4º. Two days after the cell fixation, the CVS were transferred to a clean parafilm and 200µL of blocking solution were added in each CVS. The blocking lasted 30 minutes at room temperature. After this time, the blocking solution was aspirated and 200µL of the solution that contained the primary antibody+PBS was added. After an hour of incubation in the dark, the solution was aspirated. Each CVS was washed 3 times with PBS during 5 minutes. Then 200µL of the solution that contained the secondary antibody+PBS was added. An hour of incubation later, the secondary antibody was aspirated. Finally, each CVS was washed 10 minutes with PBS, 10 minutes with PBS+DAPI and another 10 minutes with PBS. Then, after a wash using water, each CVS was placed over the slide with a drop of mounting media with antifade reagent.

***Cell lysis***

A day after the transfection of HEK293T cells with the indicated constructs using PEI, took place the cell lysis. First of all each well was washed two times with ice cold PBS. Then 300µL of Lysis buffer 1X+protease inhibitor was added over each well. After 20 minutes in ice, the lysate of each well was recollected in 1,5mL tubes. The tubes were put 20 minutes at 4ºC in the wheel and then centrifuged at 13000rpm during 10 minutes at 4º to remove cell debris. Then the cleared supernatant was recollected in another tube: 40µL of the supernatant were mixed with 10µL of loading buffer and stored at 4ºC for protein lysates control; the remaining volume was used in the lectin pull-down.

***Pull-down***

First of all we had to prepare the lectin or nickel-agarose beads (Invitrogen). 150µL of the suspension of the beads was spin-dried at 3000rpm during 3 minutes at 4ºC. Then the supernatant that contained ethanol was eliminated and the beads were washed with 1mL of lysis buffer an spin-dried to eliminate the supernatant. We repeated the wash 3 times. After the washes we left a volume of 150µL which was shared out in the different eppendorfs that contained the lysate. After 4 hours in the wheel at 4ºC the unspecific bindings were eliminated using lysis buffer (the same three washes than at the beginning). Finished with the washes, 20µL of each eppendorf were mixed with 5µL of loading buffer.

***Western blot analysis of the transfected HEK293Tcells with the different plasmids coding for the Unc5B deletion mutants***

Both the supernatant of the cell lyses and the pull-down were homogenized in standard PAGE gel loading buffer and denatured at 95ºC for 5 minutes. Protein extracts were separated by electrophoresis on precast 8% polyacrylamide gels, transferred to polyvinylidene difluoride membrane, and blocked at room temperature for at least 1 hour in 5% died skimmed milk in TBS. Blots were probed (4ºC, overnight) with goat Unc5B primary antibody (1/1000) and detected by anti-goat secondary antibody (1/5000).

### *AP-binding assay*

For the binding assays, HEK293T cells were transfected in collagen-coated p24 and p48 well plates with the indicated constructs. After 48 hours, the cells were incubated with the conditioned AP media that contains the recombinant proteins Netrin-1 and FLRT3 for 90 minutes at room temperature and washed with TBS (20mM Tris pH 8.0 + 120mM NaCl) 2 times. Cells were washed with the AP staining buffer (100mM Tris pH 9.5 + 100mM NaCl + 5mM $MgCl_2$) 1 time and then the cells of the p24 well plate were incubated for 1 hour in AP buffer containing naphtol FAST Red (Sigma) and then fixed with 4% PFA for imaging. Alternatively, the cells of the p48 well plate were incubated with *p*-nitrophenylphosphate and analyzed by absorbance at 405nm after 90 minutes of reaction. Background activity in non-transfected cells was subtracted.

### *Western blot analysis of the pull-down using FLRT3$^{ECD}$ and Netrin-1*

The supernatant of the pull-down was homogenized in standard PAGE gel loading buffer and denatured at 95ºC for 5 minutes. Protein extracts were separated by electrophoresis on precast 8% polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and blocked at room temperature for at least 1 hour in 5% died skimmed milk in TBS. The blot was analyzed directly using an anti-rabbit secondary antibody (1/5000) to detect the Fc domain. After the development, the membrane was washed during 15 minutes using TBS + sodium azide to eliminate the secondary antibody and 2 times with TBS during 5 minutes each time. The blot was analyzed again using a rabbit anti-Histidine primary antibody (1/5000) and detected by an anti-rabbit secondary antibody (1/5000).

The blot was then stripped and re-probed with rat anti-FLRT-3 primary antibody (1/5000) and detected with anti-rat secondary antibody (1/5000). After a wash, the blot was analyzed using goat anti-Unc5B primary antibody (1/1000) and detected with anti-goat secondary antibody (1/5000).

## Results

### *Results related to the confirmation of the structure of the plasmids*

To develop the experiments we used deletion mutants of the extracellular part of the Unc5B receptor. We worked with four plasmids which were constructed by an external laboratory. These plasmids were the $\Delta TSP1_{FULL}$ that encodes for an Unc5B receptor that contains a complete deletion of the

TSP1 domain (Figure 2B; Eichmann, A *et al*, 2005), the $\Delta TSP1_{MID}$ that encodes for an Unc5B receptor that contains a partial deletion in the TSP1 domain (Figure 2C), the ΔIg2 that encodes for and Unc5B receptor that contains a deletion of the Ig2 domain (Figure 2D) and finally, the full length that contains the complete receptor Unc5B and we could use it as a positive control (Figure 2A). Also, these plasmids contained an IRES (Internal Ribosome Entry Site) that allowed the simultaneous expression of EGFP together with Unc5B. In addition, we know that the Unc5B receptor contains a myc-epytope at C-terminus.

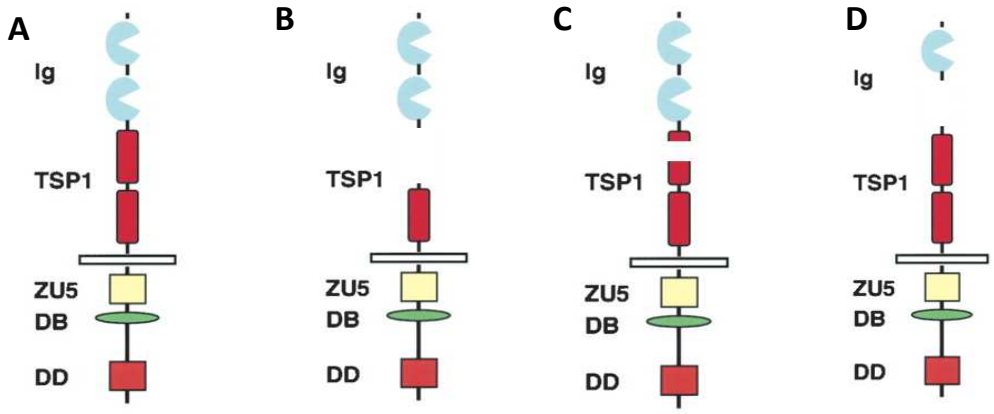


**Figure 2:** Schematic structures of the different mutants of the Unc5B receptor. In our work we only used the external part of the receptor, which includes both the Ig and the TSP1 domains. (**A**) Structure corresponding to the full length receptor. (**B**) Structure corresponding to the $\Delta TSP1_{FULL}$ mutant. (**C**) Structure corresponding to the $\Delta TSP1_{MID}$ mutant. (**D**) Structure corresponding to the ΔIg2 mutant.

Previous studies from the hosting group established that the deletion of the TSP1 domain of the Unc5D receptor impairs the binding to $FLRT2^{ECD}$ (Yamagishi *et al*, 2011). Since Unc5B is the preferred receptor for $FLRT3^{ECD}$ in the present work we checked if the interaction between the Unc5B mutants $\Delta TSP1_{FULL}$ or $\Delta TSP1_{MID}$ and $FLRT3^{ECD}$ was affected.

First of all we transform all the plasmids using chemicocompetent bacteria cells in order to amplify the constructs and then, we extracted the plasmid DNA. We had to do several tests because, at the beginning, the concentration of DNA was extremely low. Finally, we realized that the problem was the LB media that our laboratory was using and we decided to prepare another LB media using new reagents. The new LB media gave much better yield than our LB so we could obtain a higher concentration of DNA.

As the plasmids were constructed by an external laboratory, we checked the DNA by a digestion using restriction enzymes to be sure that the structures were correct before starting the experiments. Analyzing the initial information about the plasmids structure we decided to use two restriction enzymes which were HindIII and XhoI.

The pattern of the bands corresponding to the HindIII digestion didn't coincided with the fragments that we expected. For this reason we carried out several digestions using another two restriction enzymes (BamHI and EcoRI) and comparing enzymes of two different providers. Then analyzing all the results we could observe that the only enzyme with which we didn't obtain the result that we expected was HindIII. Searching information about the structure and construction of the plasmid (pCAGIG) we realized that it contained fragments of other plasmids. A possible explanation for our problem is that in the construction of the plasmid some sites in which HindIII cut were eliminated (Figure 4). Comparing the structure corresponding to the initial information about the plasmid with the results that we obtained after the digestion using HindIII, we established that probably both the 6546 and 6045 targets were eliminated during the plasmids construction.

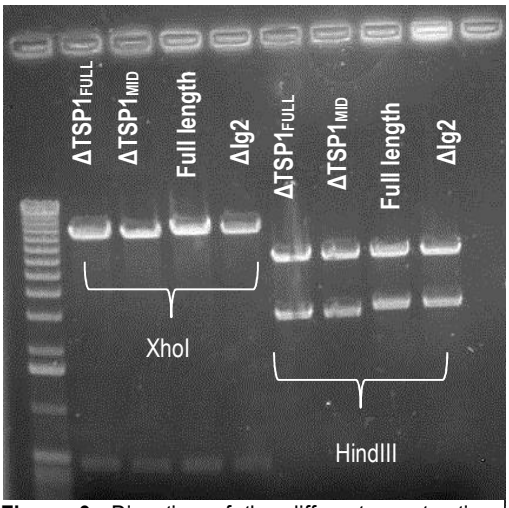


**Figure 3:** Digestion of the different constructions using the restriction enzymes XhoI and HindIII.

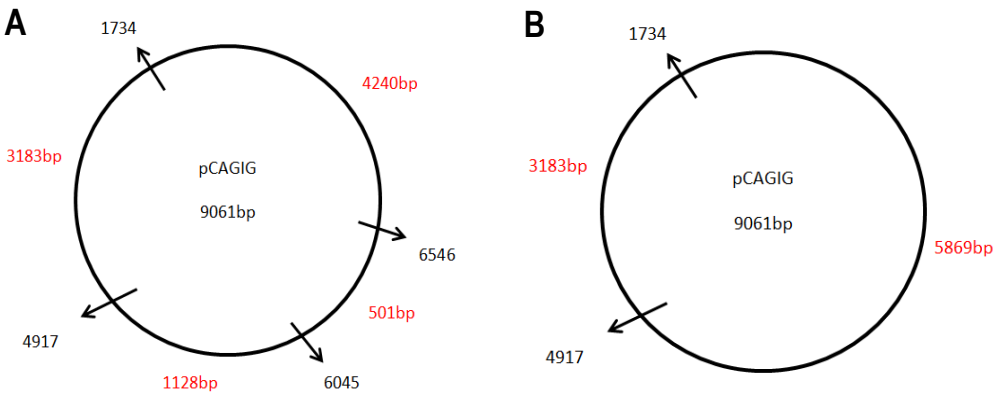


**Figure 4:** (**A**).Structure corresponding to the initial information about the plasmid. The numbers in black indicates the sites in which HindIII was expected to cut while the size of each fragment is indicated in red. (**B**) Possible real structure of the sites in which Hind III cut predicted by our analysis. The numbers in black indicates the sites in which HindIII really cut while the size of each fragment is indicated in red

***Results related to the correct production of Netrin-1-AP and FLRT3$^{ECD}$-AP***

In order to produce Netrin-1-AP and FLRT3$^{ECD}$-AP to be used in the binding assays we transfected HEK293T cells with the corresponding DNA. We performed the transfection using FuGene because it increases the efficiency of the transfection and we needed to produce high concentration of each protein. After the incubation we collected the media in four tubes. We added HEPES buffer in each tube to maintain the pH of the media to ensure that the proteins were stored in correct conditions. We stored the media at 4ºC until they were to the binding assay. Before the storage, an aliquot of each protein was analyzed to evaluate the presence and concentration of the recombinant proteins using a Coomassie staining. For that we loaded in parallel known amounts of BSA (Bovine serum albumin) as a standard. The molecular weight of the proteins that we studied were for FLRT3$^{ECD}$-AP 125kDa (FLRT3$^{ECD}$ had a molecular weight of 90 kDa and the AP showed a molecular weight of 35kDa, more or less), for Netrin-1-AP 115-120KDa (Netrin-1 had a molecular weight among 80 and 85kDa) and the molecular weight corresponding to the BSA standard was 65kDa. Comparing the bands corresponding to each protein (Netrin-1-AP and FLRT$^{ECD}$-AP) with the bands belonged to the BSA standard we could determinate more or less their concentration. In the case of FLRT3$^{ECD}$-AP, the band showed an intensity between 0,5µg and 1µg (more or less, 0,75µg). As the total volume was 35µL, we could establish a concentration of 0,021µg/µL (Figure 6). On the other hand, the band corresponding to Netrin-1-AP showed half intensity than the band corresponding to FLRT$^{ECD}$-AP. Therefore, we could establish a concentration of 0,0105µg/µL. (Figure 5). We could also observe that the molecular weights that we obtained for the BSA standards weren't correct. We thought that it could be a problem during the samples preparation.

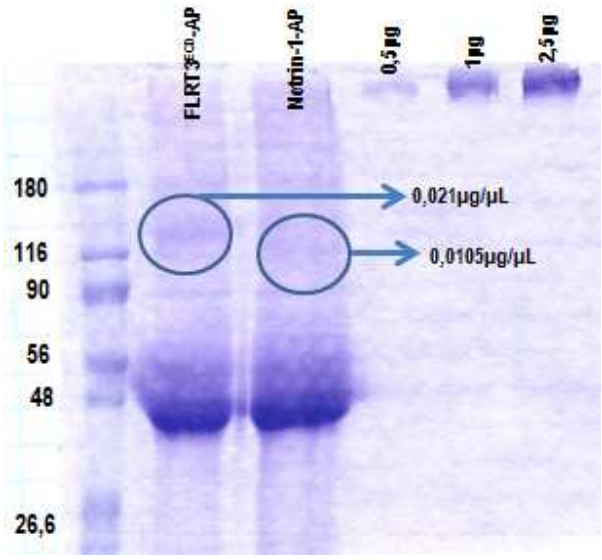


**Figure 5:** Coomassie staining. HEK293T cells transfected with FLRT3$^{ECD}$-AP and Netrin-1-AP. The standards of BSA (0.5, 1 and 2.5µg/µL) allowed the confirmation of the concentration of each protein.

***Results related to the correct expression of the Unc5B receptor***

To evaluate the expression of the Unc5B receptor in the membrane of the cells we carried out an immunofluorescence assay. We transfected the cells with the different constructions ($\Delta TSP1_{FULL}$, $\Delta TSP1_{MID}$, ΔIg2 and full length (Figure 2)) and also we used EGFP as a control. In this case we used a 6-well cell culture plate coated with collagen to attach the cells to the plate and to prevent their detachment during the washing steps of the immunofluorescence. The transfection was done with PEI because we only wanted to confirm the presence of the receptor so we didn't need to improve the efficiency of the transfection by using FuGene. After the incubation we started with the cell fixation to prepare the cells for the immunofluorescence. As we said before, the plasmids contained a myc-epytope so we used two primary antibodies: an anti-EGFP primary antibody (from rabbit) and an anti-myc primary antibody (from mouse) and the corresponding secondary antibodies. We also used DAPI to detect the nucleus of the cells.

Analyzing the results corresponding to the control (EGFP-only transfected cells; Figures 6A, 6B and 6C) we could observe the expression of EGFP and, as we expected, we didn't detect anything with the myc-epitope. Analyzing the different Unc5B mutants (Figures 6H, 6K and 6N) the synthesis of the EGFP protein by the IRES allowed us the confirmation of the correct expression of the receptor. However, in the images corresponding to the detection by the anti-myc primary antibody we couldn't observe the presence of the Unc5B in the membrane (Figures 6I, 6L and 6O). To solve the problem we decided to do another immunofluorescence assay using, in this case, EGFP and Unc5B (a plasmid from our laboratory, without an IRES) as controls. Again in the immunofluorescence controls (EGFP-only transfected cells; Figures 7A, 7B and 7C) we could observe the florescence belonging to the EGFP protein in the EGFP control but nothing in the image corresponding to the detection using the anti-Unc5B primary antibody, as we expected. The Unc5B receptor (Figures 7G, 7H and 7I) used as a control (without the IRES) allowed us the confirmation of the presence of the receptor in the membrane and the pictures corresponding to the full length receptor (Figures 7D, 7E and 7F) allowed us the comparison of the expression of the complete receptor with the mutants. The reason for why we only used the $\Delta TSP1_{FULL}$ mutants (Figures 7J, 7K and 7L) and the ΔIg2 mutants (Figures 7M, 7N and 7O) to repeat the experiment was because we received new information from the laboratory in where the design of the plasmids took place. In this information they said that there were some variations in the structures. These variations were that the ΔIg2 mutant receptor had a deletion as

well in the transmembrane domain. Most probably, this deletion prevents the receptor to be located at the membrane and therefore is not functional. Also, the plasmids that contained the mutations $\Delta TSP1_{FULL}$ and $\Delta TSP1_{MID}$ were the same; both of them presented a full deletion of the TSP1 domain. Therefore, in the following experiments we only worked with two functional plasmids: the full length as a positive control and the $\Delta TSP1_{FULL}$. However, we also used the ΔIg2 mutant to make some tests. In this case, we could observe (Figure 7O) that the ΔIg2 mutant was present in the membrane of the cell, contrary to what we expected from the information that we received and the fluorescence intensity was similar to the full length receptor (Figure 7F).

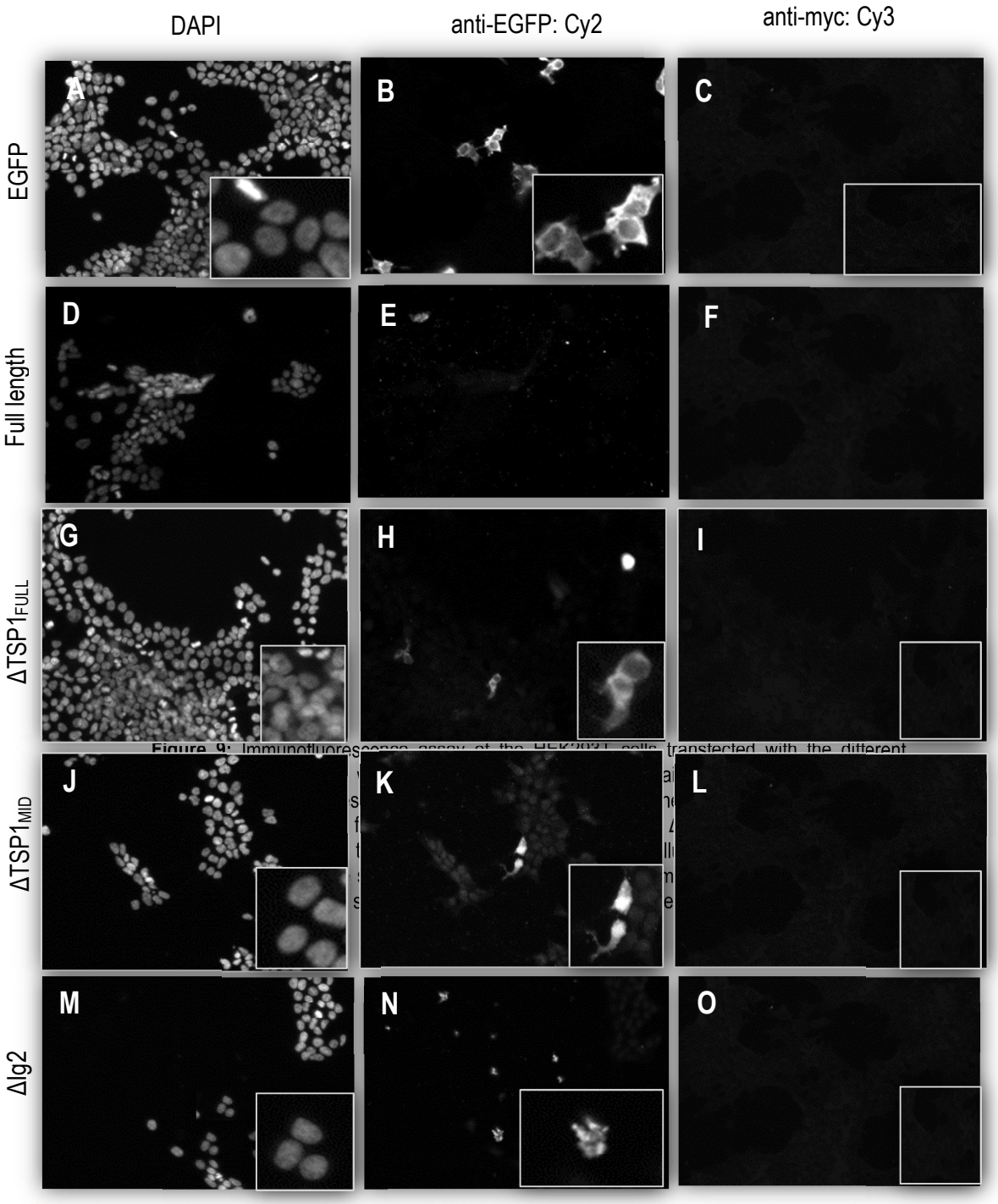


**Figure 6:** Immunofluorescence assay from the HEK293T cells transfected with the different constructs indicated on the left. Samples were analyzed using antibodies against EGFP (middle column of panels) and the myc-tag (right column of panels). The corresponding DAPI staining is shown in the column of panels on the left. The presence of EGFP (which was used as a control) was confirmed with the anti-EGFP antibody.. Analyzing the images corresponding to the other plasmids, we could detect (with less intensity) the presence of EGFP which was synthetized by the IRES and that

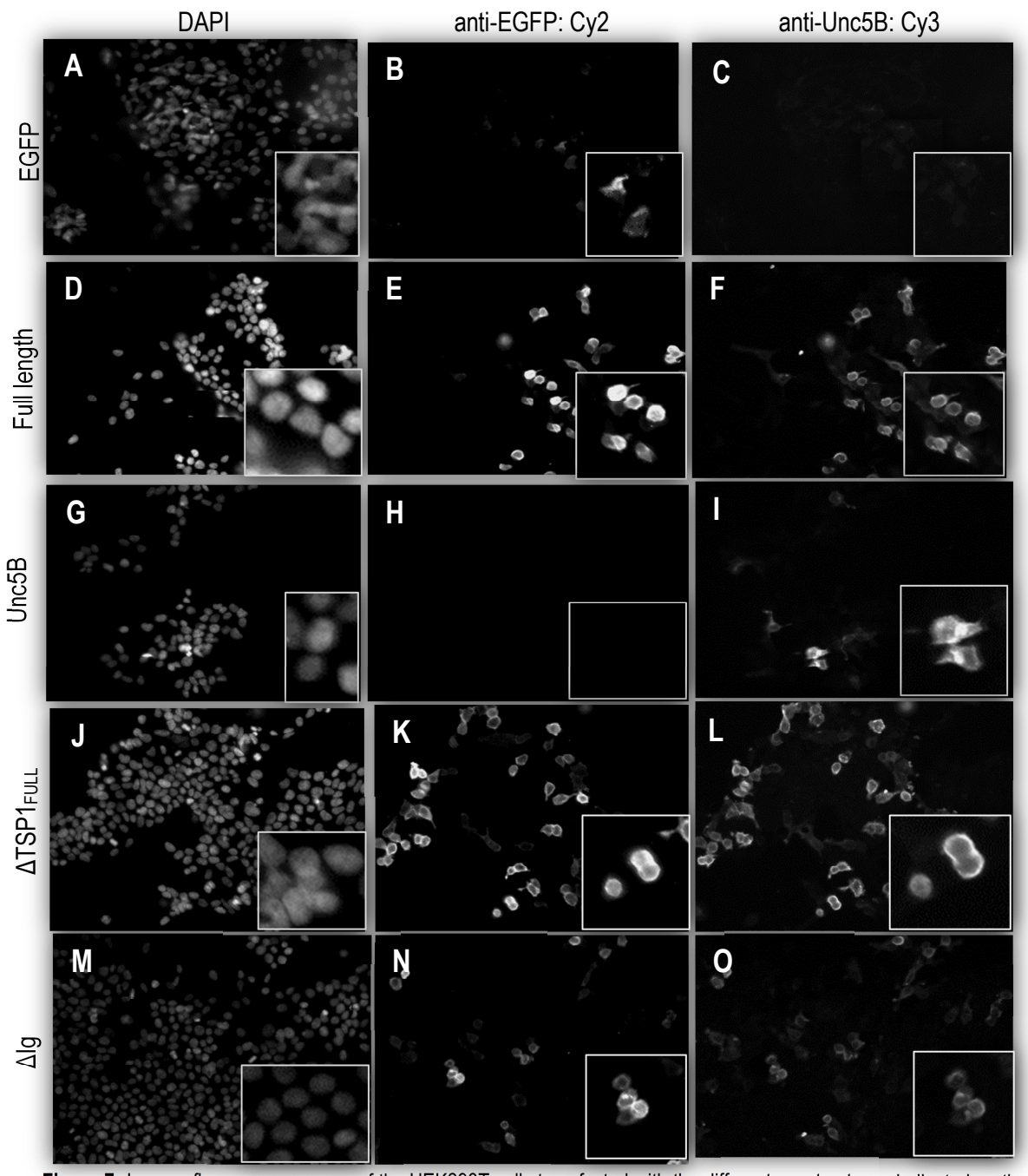


**Figure 7:** Immunofluorescence assay of the HEK293T cells transfected with the different constructs, as indicated on the left. Samples were analyzed using antibodies against EGFP (middle column of panels) and the Unc5B receptor (right column of panels). DAPI staining is shown on the left column of panels. The presence of EGFP (that we used as a control) was confirmed with the anti-EGFP antibody. The Unc5B from our laboratory allowed the confirmation of the correct function of the anti-Unc5B antibody. Analyzing the images corresponding to the other plasmids, we could detect (with less intensity) the presence of EGFP which was synthetized by the IRES and that confirms the fact that the transfection took place and also we

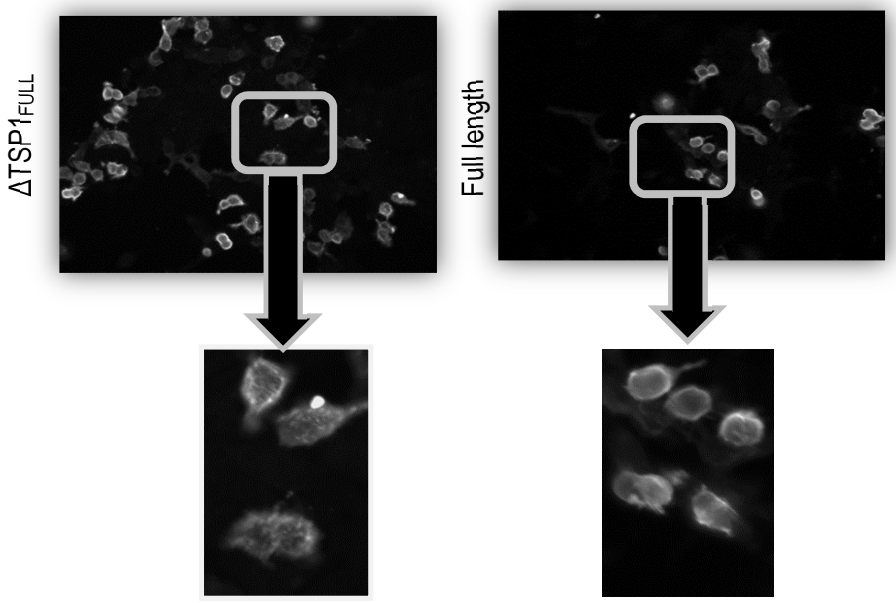


**Figure 8:** Immunofluorescence assay of the HEK293T cells transfected with the indicated constructs. Samples were analyzed using antibodies against the Unc5B receptor. In these images we could compare the presence of the Unc5B receptor in different parts of the cell. In the first image which corresponds to the $\Delta TSP1_{FULL}$ mutant, we could detect the presence of the Unc5B receptor in some intracellular compartments in which probably took places the synthesis. On the other hand, in the image corresponding to the full length receptor we could see the receptor in the membrane of the cells.

Studying in detail the results of the second immunofluorescence, more specifically, the pictures that belongs to the detection using the anti-Unc5B primary antibody, we could observe the presence of the full-length receptor in the membrane of the cells (Figure 8, Full length, right panel) to confirm its correct functionality. However, we observed some intracellular spots when we used the $\Delta TSP1_{FULL}$ that probably correspond to the compartments in which takes place the synthesis of the receptor (Figure 8, left panel).

To test the differences between the mutants and to compare the results of the immunofluorescence with another assay, we did a cell lysis and a lectin pull-down that allows precipitating only glycosylated proteins, among them, the receptor that have been properly matured and located on the cell surface. In this case we used two 6-well cell culture plates coated with collagen and we did the transfection using PEI because we didn't need a high efficiency, we only wanted to check the receptor. We transfected the cells with EGFP and Unc5B from our laboratory as controls, the two functional plasmids ($\Delta TSP1_{FULL}$ and Full length) and also we used the Δlg2 to test it. Then we began with the cell lysis. We divided the final volume of the total lysate in two eppendorfs and we used one for the lectin-pull down. With the lectin pull-down we could precipitate the receptor because lectins

interact with high affinity with glycoproteins. We analyzed the results from both the cell lysis and the lectin pull-down by a Western blot analysis using an anti-Unc5B primary antibody to detect the receptor.

The predicted molecular weight for the receptor is between 85-90KDa, so analyzing our results (Figure 9 and 10) we could observe a band located more or less in that position. In the case of the cell lyses (Figure 9), the bands presented a low intensity compared with the lectin pull-down (Figure 10) because in the second case we enriched the receptor fraction to separate it of the other cellular components. Comparing the bands corresponding to the full length receptor with the bands corresponding to the $\Delta TSP1_{FULL}$ mutant (Figure 10) we could observe a faster mobility of the band in the second case. This result allowed us the confirmation of the presence of the deletion. On the other hand, as we explained previously, the ΔIg2 mutant receptor had a deletion as well in the transmembrane domain. Most probably, this deletion prevents the receptor to be located at the membrane and therefore is not functional. However, in our results (Figure 10) we could see that the band corresponding to this deletion had similar size as the one corresponding to the full-length receptor. Due to the variety of the results compared with the information we decided that we didn't consider this mutant for performing the binding assays.

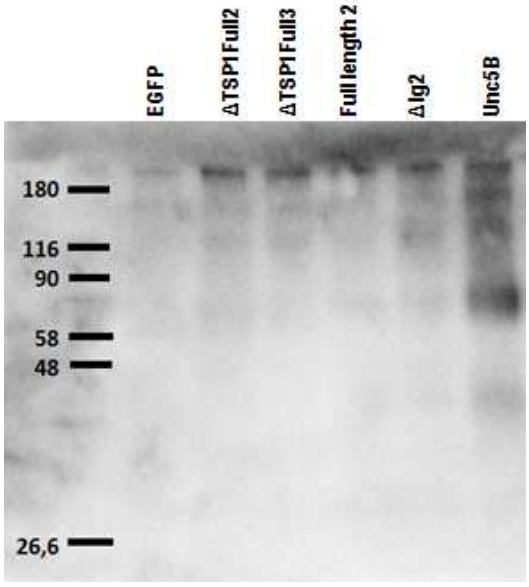


**Figure 9:** Western Blot analysis of the total lysates of HEK293T cells transfected with the different plasmids. Samples were analyzed using an antibody against the Unc5B receptor. We could observe significant differences between the intensity of the bands comparing the full length receptor with the mutants.

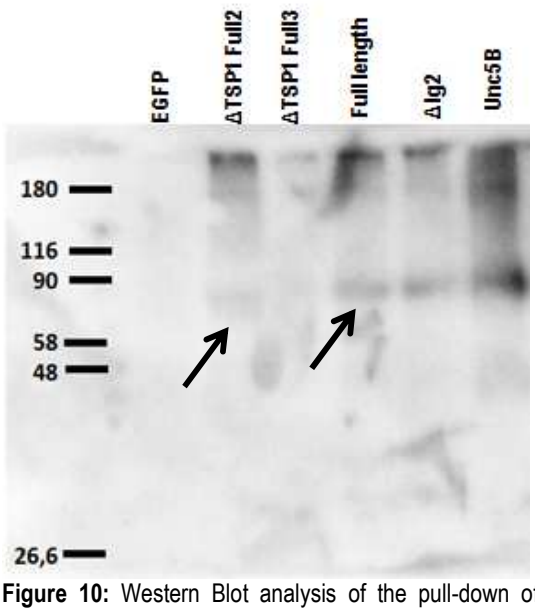


**Figure 10:** Western Blot analysis of the pull-down of HEK293T cells transfected with the different plasmids. Samples were analyzed using an antibody against the Unc5B receptor. We could observe significant differences between the intensity of the bands comparing the full length receptor with the mutants,

**Results related to the binding assays**

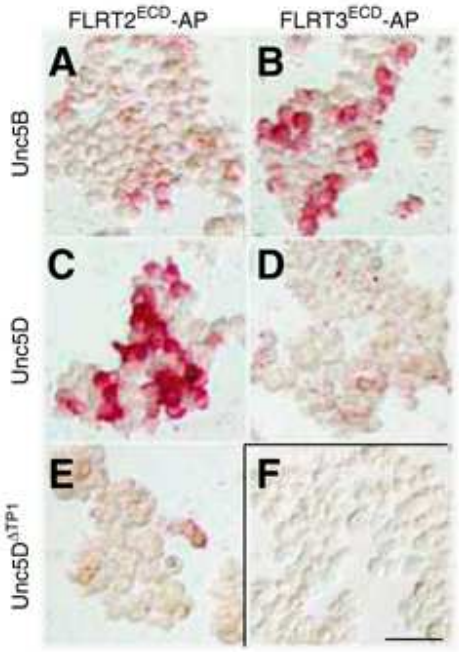


**Figure 11:** Soluble FLRT ECDs bind to Unc5 receptors. FLRT2 binds best to Unc5D and to a lesser extent to Unc5B, while FLRT3 shows strong preference to Unc5B.

After we completed the different analysis related to the viability and functionality of the different receptors and the production of both Netrin-1-AP and FLRT3$^{ECD}$-AP, we started with the binding assays. Previous studies established that the deletion of the ΔTSP1 domain of the Unc5D receptor impaires the interaction with the FLRT2$^{ECD}$ (Figure 11, Yamagishi *et al*, 2011). Also, in this study we could see higher affinity between Unc5D and FLRT2$^{ECD}$-AP compared with FLRT3$^{ECD}$-AP. On the other hand, the interaction between Unc5B and FLRT3$^{ECD}$-AP was higher than with FLRT2$^{ECD}$-AP (Figure 11). In our case, the study is based on a similar analysis and tries to analyze the domains that are important for the interaction between Unc5B and FLRT3$^{ECD}$-AP. Our assay consisted on a quantitative and qualitative analyses using different substrates for the alkaline phosphatase (AP). To carry out the qualitative analyses we used a p24 well plate and for the quantitative analyses we used a p48 well plate (both coated with collagen). In this case we used FuGene because we needed a high efficiency in the transfection to ensure the correct development of the assay. We transfected the well plates with EGFP as a control, ΔTSP1$_{FULL}$ and the full length receptor (Figure 2). The remaining wells were also used as a control (non-transfected condition). After one day of transfection we removed the media and incubated the cells with the conditioned media containing the AP-recombinant protein (FLRT3$^{ECD}$-AP or Netrin-1-AP). Cells were then washed and the corresponding substrate added to reveal AP activity (p-nitrophenylphosphate for the quantitative assay or naphtol FAST red for the qualitative assay). In the p24 well plate with naphtol FAST red the cells in which takes place the binding between the receptor and netrin-1-AP or FLRT3$^{ECD}$-AP presented a purple precipitate. On the other hand in the p48 well plate with p-nitrophenylphosphate the solution becomes yellow in those wells the binding occurs. The yellow color is proportional to the binding between the recombinant protein and the receptor expressing cells and can be measured by absorbance at 405nm.

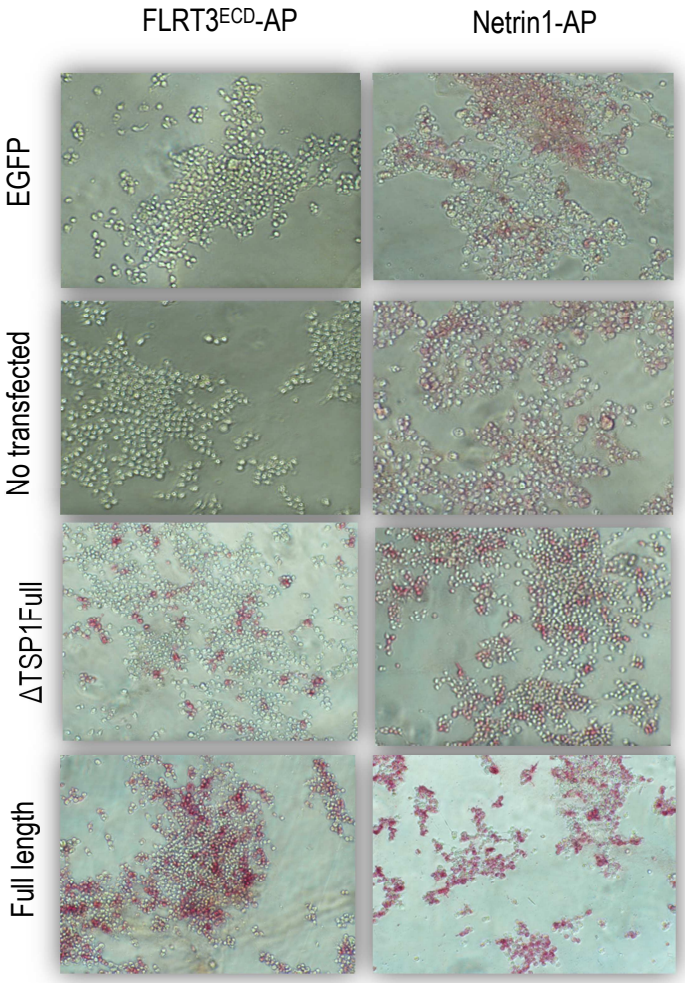


**Figure 12:** Quantitative analyses of the binding assay between the HEK293T cells transfected with the indicated plasmids and the proteins Netrin-1-AP and FLRT3$^{ECD}$-AP.

In the first experiment (Figure 12) we could observe that there was unspecific binding in the case of Netrin-1-AP in both controls (EGFP and the non-transfected cells). Moreover we could see that the interaction between FLRT$^{ECD}$-AP and the receptor was higher than with Netrin-1-AP, both in the case of the mutant and the complete receptor. This difference could be observed more clearly in the graphs (Figure 13). We did two repetitions with each plasmid to be sure that the results were similar and also to contrast it. In all the graphics the background was removed. Also, in the different graphics we could detect that there was a significant interaction between both the mutant and the complete Unc5B receptor and FLRT3$^{ECD}$-AP but not in the case of Netrin-1-AP because we obtained absorbance values around 0 when we substracted the background. Furthermore, analyzing only the case of FLRT3$^{ECD}$-AP we could see that the absorbance values were higher with the complete receptor, showing that there were more interactions than with the mutant.

We repeated the same analyses to compare the results. In this case we could observe that the unspecific binding between Netrin-1-AP and both controls was maintained (Figure 15). However, analyzing the graphics we could establish that, in this case, the absorbance values of the interaction between the ΔTSP1 mutant and FLRT3$^{ECD}$-AP were higher than with the complete receptor Unc5B (Figure 14), contrary to the result obtained in the first experiment.

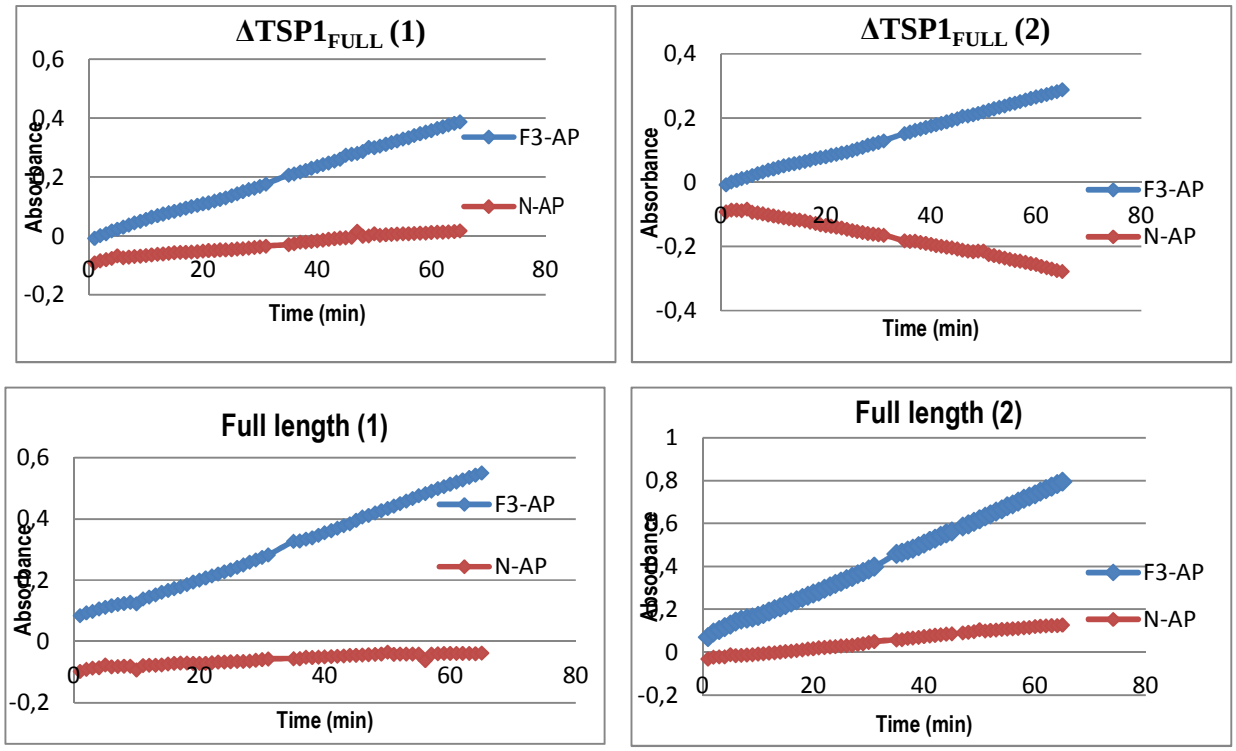


**Figure 13:** Graphics related to the absorbance measurements of the interaction between both the complete Unc5B receptor and the ΔTSP1$_{FULL}$ mutant and both Netrin-1-AP and FLRT3$^{ECD}$-AP. The background was removed in all the cases.

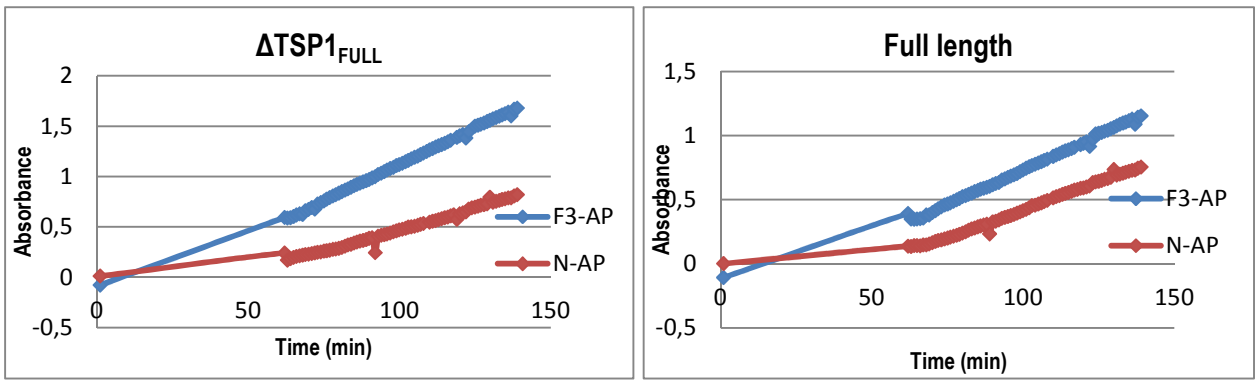


**Figure 14:** Graphics related to the absorbance measurements of the interaction between both the complete Unc5B receptor and the ΔTSP1$_{FULL}$ mutant and both Netrin-1-AP and FLRT3$^{ECD}$-AP. The background was removed in all the cases.

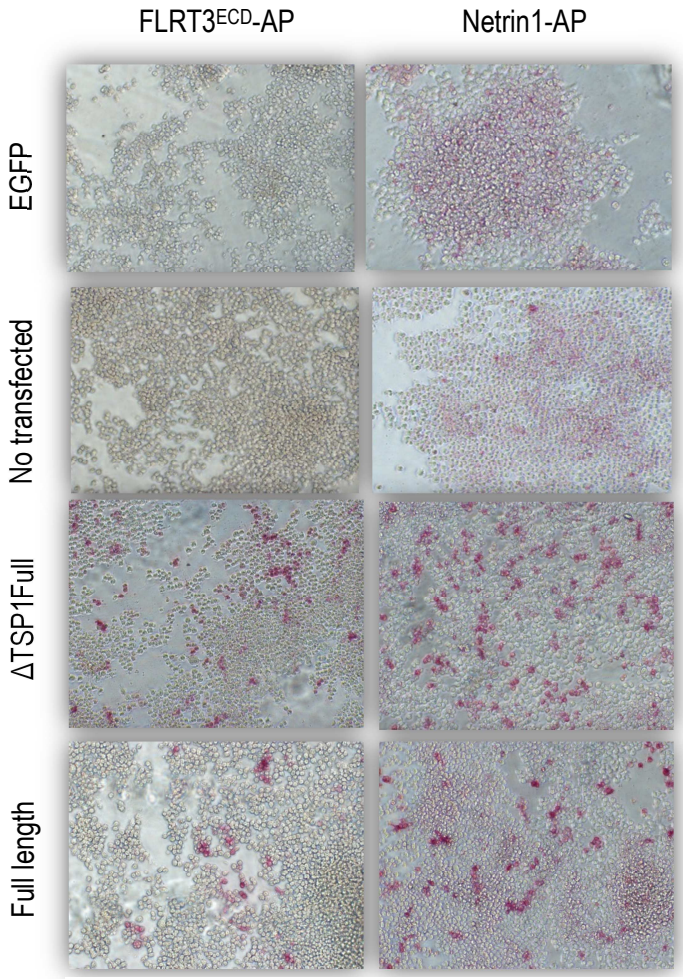


**Figure 15:** Quantitative analyses of the binding assay between the HEK293T cells transfected with the different plasmids and the proteins netrin-1-AP and FLRT3$^{ECD}$-AP.

**Results related to the binding assay between FLRT3$^{ECD}$ and Netrin-1**

In the experiments above we evaluated the competition between FLRT3$^{ECD}$ and Netrin-1 for the Unc5B receptor but another theory could be the possibility of an interaction between FLRT3$^{ECD}$ and Netrin-1 as a ligand competition or an interaction of Netrin-1 with FLRT3$^{ECD}$ when this is in the membrane of the cell. If these interactions exist, FLRT3 could act as a direct modulator of the activity of Netrin-1. To check this hypothesis we did an in vitro binding assay using different combinations of the recombinant proteins FLRT3$^{ECD}$-Fc, Netrin-1 with a His-tag, Unc5BFc and we also used the protein slit with a His-tag. Slit is another important protein involved in the migratory process. We did 5 different mix of the proteins above which were: FLRT3$^{ECD}$-Fc/Netin-1, FLRT3 as a control, FLRT3$^{ECD}$-Fc/Slit, Unc5BFc/Netrin-1 as a control and Unc5BFc as a control. We evaluated the binding using nickel beads to do the pull-down and then, we observed the results by Western Blot analyses. We used the Unc5B receptor as a control because we know that Netrin-1 binds to it. We also use different antibodies to confirm the results. We could detect the band corresponding to the FLRT3$^{ECD}$-Fc at 116KDa and the band corresponding to the Unc5B-Fc at 90KDa (Figure 16). Analyzing the controls which in this case were the Unc5B-Fc and the Unc5BFc/Netrin-1, we could see that Netrin-1 bound to the receptor because the intensity of the band related to the mix was higher than the band related to the Unc5B-Fc within Netrin-1. However, in the case of FLRT3$^{ECD}$-Fc, we couldn't detect significant differences between the controls and the samples. Also, we detected a band at 58KDa (more or less) which could belong to the Fc domain (Figure 16). As we didn't detect the bands corresponding to Netrin-1 and Slit, which should

be detected at 85 and 110 kDa respectively, we repeated the Western Blot analysis using, in this case, an anti-Histidine primary antibody, but we couldn't detect anything (Figure 17).

To compare the results of the first Western blot (Figure 16) with another experiment we did an stripping of the membrane and we used two different antibodies to do the detection (Figure 18), but the results were similar as in the other case, we couldn't detect significant differences between the different bands corresponding to FLRT3$^{ECD}$.

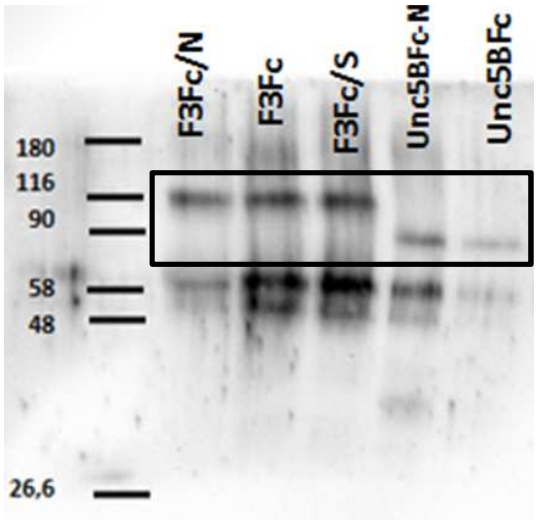


**Figure 16:** Western blot analyses of pull-down (glycoprotein enriched) from the binding assay between the different proteins. Samples were analyzed using an anti-Fc antibody.

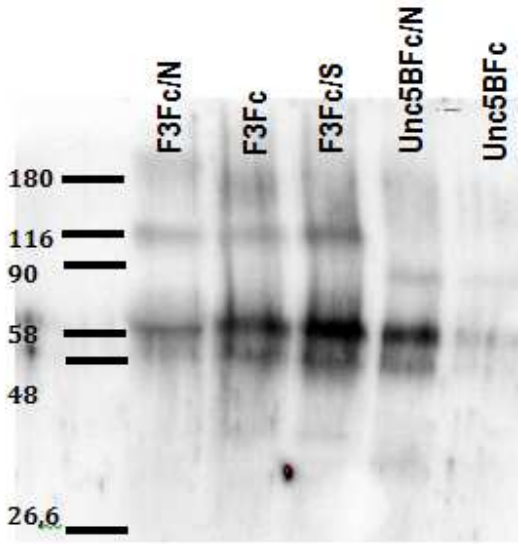


**Figure 17:** Western blot analyses of pull-down (glycoprotein enriched) from the binding assay between the different proteins. Samples were analyzed using an anti-Histidine primary antibody.

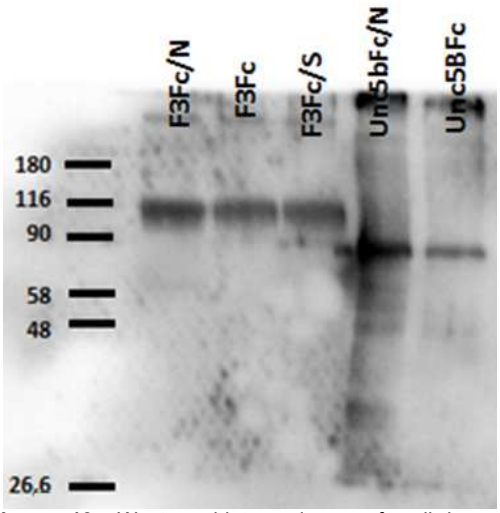


**Figure 18:** Western blot analyses of pull-down (glycoprotein enriched) from the binding assay between the different proteins. Samples were analyzed using an anti-FLRT3 and an anti-Unc5B primary antibodies.

## Discussion

During the first part of the assays, we analyzed the viability and functionality of all the receptors. In the first immunofluorescence (Figure 6F, 6I, 6L and 6O) we couldn't detect the receptor using an anti-myc primary antibody because, when we compared the sequence that encodes for the Unc5B to the sequence that encodes for the myc-tag, we realized that indeed, the Unc5B receptor didn't contain a myc-tag. As we explained before, the problem was solved using an anti-Unc5B primary antibody with which we could detect both the full length receptor and the different mutants. In this case the surprising result was the fact that we detected also the ΔIg2 mutant because this mutant had another deletion in the transmembrane domain and probably that we expected it will not allow the proper location of the receptor in the membrane and for that reason, at the beginning we thought that this mutant wasn't functional.

We used the cell-lysis and lectin pull-down to confirm both the presence of mutations in the different receptors and the results related to the immunofluorescence. The results related to the lectin pull-down (Figure 10) allowed us the confirmation of the presence of the mutation in the $\Delta TSP1_{FULL}$ mutant but not in the ΔIg2 mutant. This result suggested that perhaps the receptor didn't present the mutation and because of the contradictory results that we obtained compared with the initial information that we had about the structure of the different plasmids, we decided not to use it in the following binding assays.

The binding assays (Figure 12 and 15) allowed us the study and comparison of the different interactions between the complete and the mutant receptor and both Netrin-1-AP and $FLRT3^{ECD}$-AP. Previous studies established that the deletion of the TSP1 domain prevents the interaction between $FLRT2^{ECD}$-AP and the Unc5D receptor (Yamagishi *et al*, 2011) and, at the beginning, we thought that between $FLRT3^{ECD}$-AP and Unc5B the result could be the same because both were related proteins of the same family. Contrary to what we expected, the deletion of the TSP1 domain allowed the interaction between the receptor Unc5B and both Netrin-1-AP and $FLRT3^{ECD}$-AP. A possible explanation for this result could be that perhaps the domain that we needed to delete in the case of Unc5B to avoid its interaction with both proteins was different from the domain needed in the case of Unc5D to avoid its interaction with $FLRT2^{ECD}$-AP. To test this hypothesis would be necessary to make further experiments using different Unc5B mutants and comparing different results. Also, in this

analysis we obtained other contradictory results. In the first binding assay (Figure 13), the interactions between FLRT3$^{ECD}$-AP and the complete receptor were higher than with the mutant but, in the second assay (Figure 14) the results were completely opposite. Furthermore, we detected unspecific binding in the case of Netrin-1 both in the case of EGFP control and the non-transfected cells. These results mean that Netrin-1 interacts with other components presents in the HEK293T cells. Perhaps the cells expressed other Unc5 or DCC receptors that could also interact with Netrin-1.

In any case, these experiments suggest an intriguing difference in the way how FLRTs interact with Unc5 receptors: in the case of FLRT3, the TSP1 domain is not essential for the binding to Unc5B while to be performed in order to map better the binding domains between FLRTs and Unc5s and the biological consequences of these differences.

Finally, the hypothesis of a possible interaction between FLRT3$^{ECD}$ and Netrin-1 couldn't be confirmed because, with an accurate analysis of the results that we obtained (Figures 16, 17 and 18), we couldn't detect significant differences between the bands corresponding to the control and the bands corresponding to the mix proteins. This lack of variation in the intensity of the bands carried out to conclude that the interaction didn't occur.

**Conclusion**

In this work we studied the interaction between different mutants of the Unc5B receptor and both Netrin-1 and FLRT3$^{ECD}$ to understand better the neuronal migration and axon guidance processes. During the first part of the study, the experiments allowed us the confirmation of the functionality and the presence of the mutations in the different constructs. This experiments also allowed us the detection of contradictory results in the case of ΔIg2, comparing it with the initial information about this mutant.

Analyzing all the results we could conclude that unlike FLRT2, FLRT3 interacts with the ΔTSP1$_{FULL}$ mutant. We could also observe the same pattern in the case of Netrin-1 and this observation confirms previous results suggesting that Netrin-1 binds to the Ig domains of Unc5 receptors (Kruger *et al*, 2004). In addition we could detect unspecific binding in the case of Netrin-1 with both EGFP control and the non-transfected cells which means that Netrin-1 interacts with other components of the cells.

The results related to a possible interaction between FLRT3$^{ECD}$ and Netrin-1 as a ligand competition didn't confirm our hypothesis. We couldn't detect significant differences between the controls FLRT3Fc and the FLRT3Fc plus Netrin-1. However, more assays are needed to contrast the results and to be sure that the interaction doesn't exist.

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