UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA
Facoltà di Scienze Matematiche, Fisiche e Naturali
Dipartimento di Biotecnologie e Bioscienze
Dottorato di Ricerca in Biotecnologie Industriali

Functional and structural characterization of Slitrk-2
(Bio-10)

Dottorando: Dr. Daniele COLOMBO

Coordinatore: Chiar.mo Prof. Marco VANONI
Tutor: Dr.ssa AnnaMaria COLANGELO
Co-Tutor: Dr.ssa Ileana ZUCCHI

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Ente Ospitante: Istituto di Tecnologie Biomediche - CNR -

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ABSTRACT

Background: Leucine-Rich Repeat (LRR) proteins play a key role in several systems, from immune response to neuritogenesis. Some years ago, our laboratory identified a gene located in the q27 region of the human X Chromosome that encodes for a LRR-enriched protein. Interestingly, this cytogenetic band contains the gene responsible for the Fragile X syndrome, the most common form of inherited intellectual disability, and other several tissue-specific genes, in particular several brain-specific genes. The function of the gene identified in our laboratory, located to on the q27.3 region and named Slitrk2, is to date largely unknown. The goal of this work was to characterize structurally and functionally Slitrk2.

Principal Findings: Slitrk2 is a member of the Slitrk family. Slitrk family has 6 members whose function is still unknown. In order to achieve a better understanding about Slitrk2 function, we carried out integrated analysis at the transcriptomic, proteomic and bioinformatic level. At a transcriptomic level, we found that Slitrk2 expression is mainly restricted to the Central Nervous System. Slitrk2 is also expressed, although at low level compared to brain, in mammary gland, pancreas, prostate, testis. This expression pattern suggest a role for Slitrk2 not only in brain, but in general in organogenesis and morphogenesis.

Slitrk2 expression during mouse embryogenesis starts at Embryonic Day 11, indicating that Slitrk2 acts in post-gastrulation mechanisms. By *in situ hybridization* in adult mouse brain, we found that Slitrk2 is expressed mainly in the Dentate Gyrus of the hippocampal formation.

The *in silico* analysis of the protein reveals that Slitrk2 is likely to be located at the plasma-membrane. Some Trans-Golgi retrivial signals were found in the Slitrk2 sequence. With a multilevel computer-assisted approach we identified two potential phosphorylation sites: Threonine 806 and Tyrosine 832. Tyrosine 832 is in an aminoacidic environment able to be recognised by the COP-I coatomer, indicating that a Golgi-Retrivial of Slitrk2 is possible. The three-dimensional structure of Slitrk2 obtained with Phyre and I-TASSER calculations showed that the predicted extracellular region of Slitrk2 folded similarly to the ectodomain of other key neuronal protein such as Lingo-1 and NogoReceptor.

With *in vitro* analyses, we found that Slitrk2 protein is mainly located intracellularly, being polarized to one side of the perinuclear boundary in PC12 cells.

Slitrk2 expression is regulated by Nerve Growth Factor during PC12 cells differentiation, being downregulated after NGF exposure. The forced expression of Slitrk2 during NGF differentiation of PC12 cells lead to a reduction of the neuritic network caused by an impairment in Intermediate Filaments assembly (Peripherin). During NGF deprivation of neurotrophin-differentiated PC12 cells Slitrk2 transcript is upregulated. The knocking-down of Slitrk2 with short-interfering RNA recovered the drop in cell viability caused by neurotrophin depletion in NGF-differentiated PC12 cells.

Outside the nervous system, Slitrk2 is expressed at low levels also in mammary gland. Also in mammary cell lines, both normal and tumoral (MCF10A and LA7/3F12, respectively), Slitrk2 forced expression determined impairment in differentiation reducing tubulogenesis. In both the above-mentioned mammary cell lines, immunofluorescence analysis showed that Slitrk2 localization is mainly intracellularly as seen in PC12 cells.

Significance: In this work we performed: i) the transcriptional analysis of Slitrk2 in neuronal and non-neuronal systems; ii) the bioinformatic analysis of Slitrk2 topology, phosphorylations and three-dimensional structure and iii) the functional analysis of Slitrk2 in both neuronal and mammary gland cells.

The main findings of this work are related to: i) the *in vivo* expression of Slitrk2 in adult mouse brain; ii) the homology modelling of the three-dimensional structure of Slitrk2 with the mapping of the predicted potential phosphorylation sites, potential N-glycosylation site and predicted ligand-binding sites and iii) the involment of Slitrk2 in the regulation of Intermediate Filament assembly in neuronal cells and the involment of Slitrk2 as a regulator of tubulogenesis in mammary cells.

Future directions: In order to complete this work, we are currently analysing the role of Slitrk2 in embryosgenesis by using *Danio rerio* (Zebrafish) as a model of development. We are also studying more in deep the pathways by which Slitrk2 regulates neuronal cells differentiation. As last, we are now studying at the molecular level the role of Slitrk2 in tubulogenesis not only in mammary gland but also in kidney.

1. REVIEW OF THE LITERATURE: INTRODUCTION

1.1 ANCIENT DISCOVERIES FOR MODERN MOLECULAR MEDICINE: FROM MENDEL TO THE HUMAN GENOME PROJECT

The modern life science research took place from old earthshaking discoveries that needed almost a century not only for their comprehension, but also for their use in the identification of the molecular mechanisms underlying cellular function.

Still to date, it could not be possible to imagine the recent appqroaches used in biochemistry/molecular biology without taking into account the findings from Mendel, Darwin and Miesher at the end of the 19th century.

However even though genetics and biochemistry based on the intuitions of Beadle and Tatum lead to the identification of the first *molecular pathways*, the *Genomic Era* opened after the discovery of the DNA structure by Watson, Crick, and Franklin, had a key role in boosting the knowledge about the role of DNA, RNA and proteins in physiological and pathological conditions.

Only a few decades ago, the possibility to have a complete map of the structural and functional organization of the human genome was considered not as science but as fantasy.

Scientists started to become confident that the human genome could be better characterized only in the 1970s, after Sanger's introduced the technologies for the DNA sequencing [1], that lead to the beginning of the field of *Structural Genomics,* with the launch of two human genome sequencing projects.

The first was publicly funded and was called International Human Genome Project (HGP) lead by Francis Collins, and the latter organized by a private company directed by Craig Venter. Both projects gave a similar map of the Human Genome in 2001 [2, 3].

During the years in which the human genome sequencing projects took place, a large number of Open Reading Frames (ORFs) and Expressed Sequence Tags (ESTs) were annotated in the databases contributed by the European Bioinfomatics Institute

(EBI), the National Center for Biotechnology Information (NCBI), and the Center for Information Biology and the DNA Data Bank of Japan (DDBJ). However, after sequencing and completing the human genome, we lacked even one complete, correct ORF for each human gene locus.

To date, the Ensembl Human Genome Database [4] contains 27,478 contigs [5], but this number is continuously changing due to the iterative nature of the human genome analysis process, illustrating that the efforts to exhaustively catalogue the protein-coding genes in the human genome are still needed and ongoing.

Even though the process of gene discovery and characterization seems to be less topical than other subjects of research, it has to be underlined that the identification of all protein-coding transcripts and the functional role of encoded proteins is still an ongoing process. For this purpose, several gene expression atlases and bioinformatic programs were conceived.

For example, the Human Protein Atlas (the Swedish Human Protein Atlas project, www.proteinatlas.org) [6], is focused on the systematic exploration of the human proteome using Antibody-Based Proteomics. The final aim of the project is to combine high-throughput affinity-purified antibodies with protein profiling in a multitude of tissues and cells assembled in tissue microarrays. In the field of neurobiology, two main atlases are a great source of information for gene expression and function.

These are the Allen Brain Atlas (www.brain-map.org) that containins a genome-wide image database of gene expression (in situ Hybridization and Nissl staining) of developing and adult mouse brain, mouse spinal cord and human brain [7].

The other gene expression atlas of the developing and adult mouse central nervous system is GENSAT (the Gene Expression Nervous System Atlas, www.gensat.org) [8]. The GENSAT project aims to map the expression of genes in the central nervous system of mouse, using both in situ hybridization and transgenic mouse techniques. It is a collection of pictorial gene expression maps of the brain and spinal cord of the mouse. Using the enhanced green fluorescent protein (eGFP) bacterial artificial chromosome (BAC) transgenic technology, this project provides the scientific community with tools to catalog, map, and electrophysiologically record various data of individual cells. The application of Cre (Causes recombination) recombinase technologies allows for cell-specific gene

manipulations, creating transgenic mice that are available to the scientific community.

1.2 REVERSE AND FORWARD GENETICS: NOT EXCLUSIVE APPROACHES TO STUDY A GENE FUNCTION

In the last 10 years, efforts to identify protein-coding genes have been based essentially on five methodologies [9]:

- Analysis of ESTs or mRNAs from cDNA clones;
- Genomic sequence database searches for homologous and/or orthologs genes;
- Comparative genomics: the alignment of genomic sequences from multiple organisms, useful for identifying coding sequences since this approach displays sequence conservation, less applicable to non-coding regions;
- Identification of genes encoding for differentially expressed proteins by *proteomic analysis* (e. g. analysis of proteins from samples of normal and pathological/injured tissues).
- Single genome *de novo* gene identification with *in silico* predictions based on the features of the sequence composition. An example is GenScan [10], a GHMM-based (Generalized Hidden Markov model) computer program for gene discovery.

These methods are not generally mutually exclusive. Given the great number of ESTs and ORFs identified after the completion of the human genome sequence project, the role and the molecular function of the ESTs and ORFs remain to be clearly addressed.

The lack of correlating ESTs/ORFs to specific genes gave rise to *Functional Genomics,* and to date, several "*-omics"* approaches are implemented to understand globally the data obtained the human genome sequences.

The role of the non-coding DNA are essentially not well characterized, and is thought that they only in part represent a class of regulatory genes such as microRNAs [11].

Traditionally, the function of genes has been studied by means of "forward genetics" (from phenotype to gene). In the field of medical genetics the positional cloning of

gene defects underlying hereditary phenotypes was greatly accelerated by advanced maps of the human genome, and similar approaches have also been used to map gene variants underlying mouse phenotypes [12]. In mouse and other model organisms (e. g. Danio rerio, *Zebrafish*) these research efforts were accelerated by the possibility to create random mutations. Analogously, forward genetic approaches are used to manipulate the DNA content of individual cells, followed by identification of the cells displaying the desired phenotype. This is then, again, followed by the identification of DNA changes that induced the phenotype [13].

Examples of cell based "forward genetics" approaches are various two-hybrid screening methods, and expression cloning strategies to identify cell surface receptors [14]. A very recent and interesting approach of Forward Genetics is by the International Mouse Knockout Consortium that generates knockout mouse models for every protein-coding gene [15].

The international genome Projects aimed to analyze genomic sequences led to the identification of a great number of genes without known functions [2]. An example is the identification of the SLITL1 gene [16], recently renamed as SLITRK2 [17], which is the focus of this thesis. SLITRK2 was annotated in the GenBank database in 1999 but its function is still unknown, underlying the necessity of utilizing novel technologies to identify gene function.

A valid approach to address the function of this poorly studied genes is to combine various technologies including cell biology, molecular biology, biochemistry and bioinformatich approaches.

Forward genetics is not the only way to study the function of a given gene. The complementary approach of "reverse genetics" is still required, in order to identify the phenotype associated with an insertion of a gene into a cellular model of choice. This thesis followed essentially this apporach.

1.3 THE LEUCINE-RICH REPEATS PROTEINS

1.3.1 Biological relevance of LRR domains

Leucine-rich repeats (LRRs) are a versatile and evolutionarily conserved protein-ligand interaction motifs found in a large number of proteins of diverse structure, localization and function in bacteria, fungi, plants and animals [18] (Figure 1).

In fact, as is possible to see by searching the UniProtKB database [19], the family of genes encoding signal-peptides containing the LRR motifs seems to have expanded dramatically since the divergence of vertebrate and arthropod lineages.

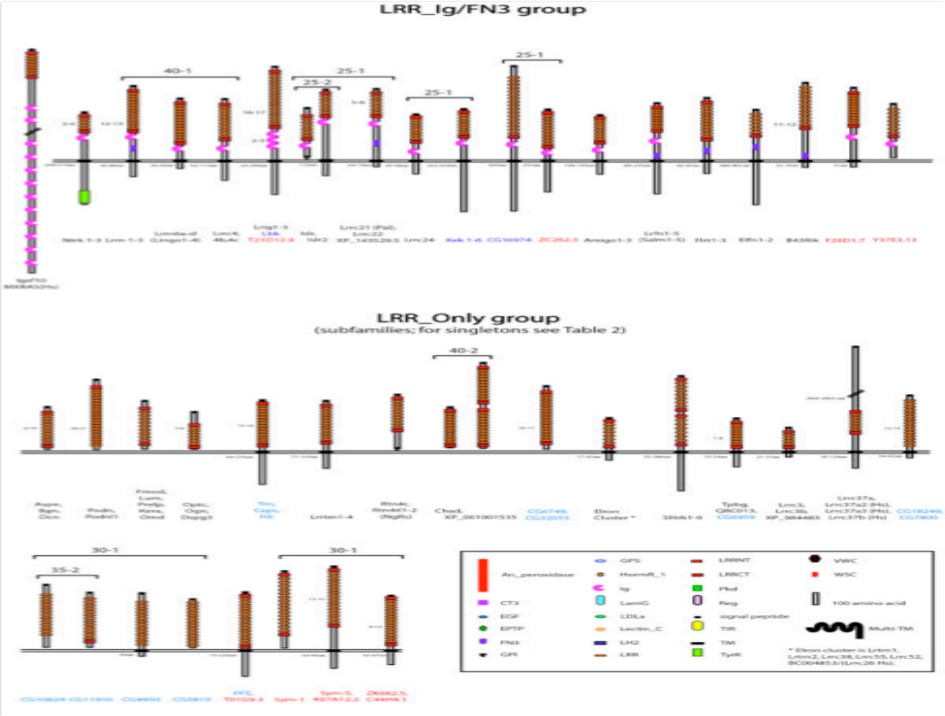

Figure 1. LRR protein predicted architectures. Continued next page.

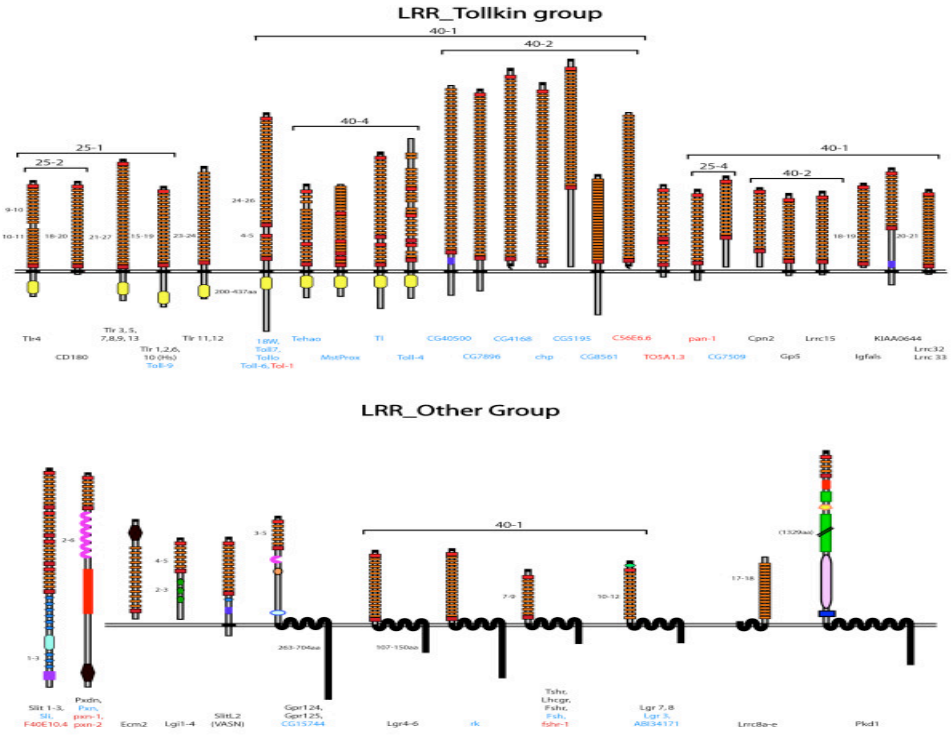

Figure 1. LRR protein predicted architectures. *Consensus architectures for all proteins in the LRR_Ig/FN3 group, LRR_Only group, LRR_Tollkin and LRR_Other groups. Protein names are shown below the corresponding structures (black, mammalian; blue, fly; red, worm) (Adapted from 20).*

Recently, a comprehensive study of proteins containing extracellular LRRs (eLRRs) motifs from worms, flies, mice and humans showed that most are still uncharacterized, even though many are expressed as discrete patterns in the developing mouse brain, such as in the thalamus and cortex [20]. These eLRRs proteins are functionally related in the diverse species analyzed, and, interestingly, there is an important diversification and expansion of the subfamilies identified from worms to human, suggesting that this domain plays a role that is correlated with the complexity of the organisms (Figure 2).

LRR-containing proteins, both transmembrane and secreted, have well-characterized functions that are similar from plants to mammals. In both transmembrane and secreted proteins, the nature of the LRR motif is important for generating a diversity of interactions, with exogenous factors in the immune system and with a large

number of different cell types in the developing neuronal system. The extracellular LRR proteins in mammals include the Toll-like receptors (TLRs), a family of transmembrane proteins characterized by an LRR region, a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain. In flies, the TLR family has also a direct role for some aspects of embryonic and nervous system development [21-24].

Literature screening on these fruit fly proteins shows that many possess a transmembrane (TM) region, in addition to the LRR domain and a signal peptide, and possibly an IgG-like domain. Well characterized examples are: the *capricious* and *tartan* family members, involved in regulating cell adhesion, or the *kekkon* family members, necessary for EGF receptor signal regulation [25-27].

Moreover, in plants, LRR-containing receptor-like kinases (RLKs) compose a large family of proteins (for instance, more than 200 members in *A. thaliana*). Each RLK encompasses an extracellular LRR domain, a transmembrane segment, and a cytoplasmic serine/threonine protein kinase domain. RLKs have diverse roles in signal transduction of extracellular signals to cells [28].

Recent reports showed that TLRs could have a similar role also in mammals [29, 30]. Indeed, in mammals, many of these functions either as transmembrane receptors or secreted proteins with well-known functions have been characterized both in the innate immune system [31] and the nervous system development [32]. The importance of the LRR class of proteins for the nervous system in humans is apparent from the large number of examples implicated in neurological and/or psychiatric disorders including epilepsy [33], Tourette's syndrome [34], schizophrenia [35] and possible Alzheimer's disease [36].

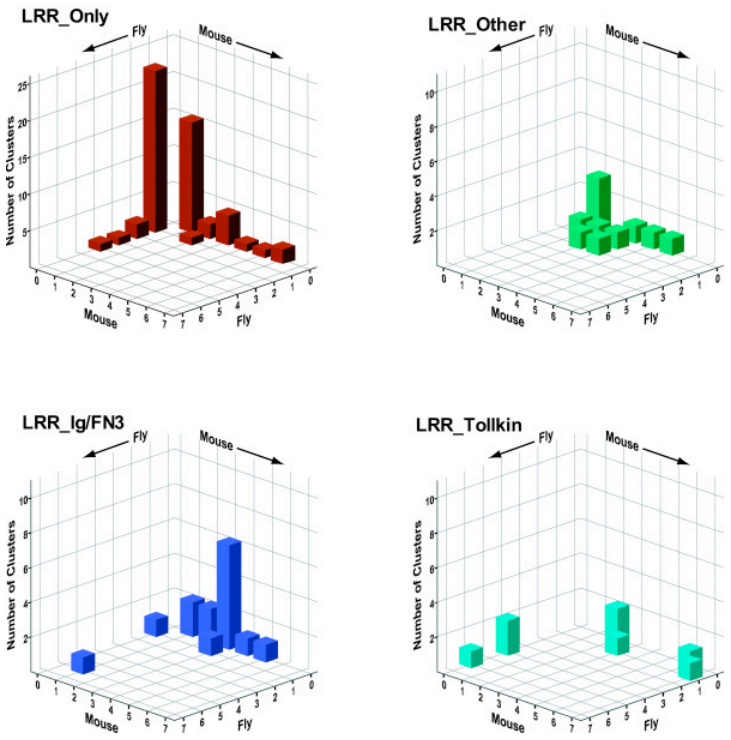

Figure 2. LRR families expansion and diversification. *Species-specific clustering categorization of LRR families in mouse and fly. (Adapted from 20).*

A large number of LRR proteins have been implicated in various aspects of neural development, in genetic studies of flies [37-39] and in assays of neurite outgrowth [17, 40-42], fasciculation [43] and/or synapse formation in mammals [44-45]. Some of these proteins contain, in addition to the extracellular LRR domain, immunoglobulin (Ig) or Fibronectin type-3 (FNIII) domains. In some cases, the functions of LRR proteins are mediated by homophylic interactions [46-48]. In other cases they are mediated by binding of other proteins *in cis* [49-51] and *in trans* configurations [52-54]. Several LRR proteins have been found to modulate the signaling of various growth factor pathways.

Despite the growing number of LRR proteins implicated in nervous system development or disease, this family of proteins has received far less attention as a class than the immunoglobulin [55-56] and cadherin [57] superfamilies.

It is estimated that the nearly 20% of the human genome encodes repeat proteins which are made up of 20–40 amino acid repeats, but this number may represent the low end of estimation since the current database (InterPro) generally used for these searching purposes, does not cover exhaustively the entire human transcriptome [58]. Notably the majority of LRR proteins do not contain transmembrane segments or GPI-anchors, and are either secreted or intracellular in location. In the LRR protein subfamilies, a great diversity of other domains occurs in tandem with LRR domain (Figure 2). For this reason, it is clear that the LRR proteins have a key role in regulating a wide range of cell mechanisms from simple to complex eukaryotes.
Selected examples of human LRR proteins are shown in **Table 1**.
Since certain leucine-rich repeat (LRR) proteins play a key role in the biology of the nervous system, they are attractive as developmental targets for the treatment of certain neuropathologies [32].

Gene Family	*Expression pattern*	*Subcellular localization*	*Protein structure*	*Biological function*
Toll-like receptor family	*Immune cells*	*PM, endosomes*	*LRR domain, TM segment, intracellular domain*	*Innate immunity*
Polycistin 1	*Wide*	*PM, ER*	*large extracellular region with several domains, 11 membrane-spanning segments, cytoplasmic G protein binding site*	*Forms Ca-ion channels*
LRR-containing-G protein coupled receptors	*Wide*	*PM*	*extracellular LRR domain-containing GPCRs*	*Glycoprotein hormone receptors*
Small LRR proteoglycans	*differs between family members*	*EM*	*LRR domains plus glycosaminoglycan moieties*	*Cell growth, adhesion and migration*
Scribble	*Wide*	*Cytoplasm, Nucleus*	*LRR domains, PDZ domains*	*Cell polarity*

Platelet Glycoprotein Ib alpha	*Megacaryoblasts, platelets*	*PM*	*LRR domains, TM region, intracellular part*	*Platelet adhesion*
Slit	*Predominantly neuronal*	*EM*	*complex architecture*	*Roundabout (Robo) receptors, involved in cell migration*
LRRK	*Predominantly neuronal*	*Cytoplasm*	*LRR domains, GTPase, COR, kinase, WD40 domains*	
LGI	*Predominantly neuronal*	*secreted*	*LRR domains and EPTP repeats*	*Enhances AMPA receptor-mediated synaptic transmission*
Neuronal Type I TM or GPI-anchored	*Predominantly neuronal*	*PM, secretory pathway*	*see Table 2*	*see Table 2*

Table 1. *Examples of human proteins with the LRR domain. LRRK, leucine-rich repeat kinase; COR, C-terminal of Roc; EPTP: Epitempin domain; PM, plasma membrane, ER, endoplasmic reticulum; EM, extracellular matrix; TM, transmembrane.*

Examples of well-known LRR-containing proteins that play a role in human neuronal development include: SLIT and Amphoterin-induced gene and ORF (AMIGO) that function in axon guidance [32, 59], NogoReceptor (NgR) and Oligodendrocyte-myelin glycoprotein (OMGp) that regulate axon regeneration [14, 60, 61], Leucine-rich repeat protein kinase (LRRK) that is still functionally uncharacterized but was found frequently mutated in autosomal dominant Parkinson's disease [62], LGI that enhance α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated synaptic transmission [63], NGL-1 that is involved in Netrin G1-mediated growth of embryionic thalamic neurons [41], and LRRTMs that play a role in instructing presynaptic differentiation [64].

It is possible to conclude that LRRs play important roles in regulating neuronal system development, plasticity, architecture and homeostasis. The important role of the LRR proteins is achieved with different molecular strategies since some LLR-

containing proteins serve as ligands (NGL-1), some as receptors or co-receptors (Lingo-1) some as both (OMgp).

1.3.2 Structure of the LRR domain

Leucine-rich repeats are repetitive stretches of 20-30 amminoacids characterized by a consensus sequence and a variable segment. The known consensus sequences are LxxLxLxxNxL or LxxLxLxxCxxL, in which "L" is a branched-chain amminoacid like Val, Leu or Ile, "N" is Asn, Thr, Ser or Cys, and "C" is Cys or Ser [18, 65]. A complete LRR domain is made by a variable number of LRR stretches, generally 2 to 45. The variable segment accounts for different LRR subfamilies. The LRR domain has a unique structure, generally referred as a "horseshoe" shape. This is a banana-like or arc-like shape derived from a curve around a single axis (Figure 3). Rarely, a LRR domain is generated from twisting around another axis. This particular folding offers opportunities for molecular, protein-protein interactions [18].

The supersecondary structure of a LRR domain consists of several parallel β-strands, each one followed by an α-helix or other disordered regions, connected by loops. In this organization, the β-strands reside in the concave side of the LRR domain, whereas the α-helices are in the convex side. Among these substructures, the less conserved are the α-helices, which are responsible for the sequence determinants of the different LRR subtypes. Some LRR subtypes are characterized by amino-terminal and carboxy-terminal "cysteine caps" made by four Cys linked by two disuplhide bridges [18]. These "cysteine caps" seem to be necessary for the proper folding of the LRR domain, and are found mostly in LRR proteins destined for the extracellular/secretory pathway. The role of the capping modules is particularly intriguingly [66]. The capping motifs are thought primarily to ‘‘cover’’ hydrophobic surfaces to prevent aggregation.

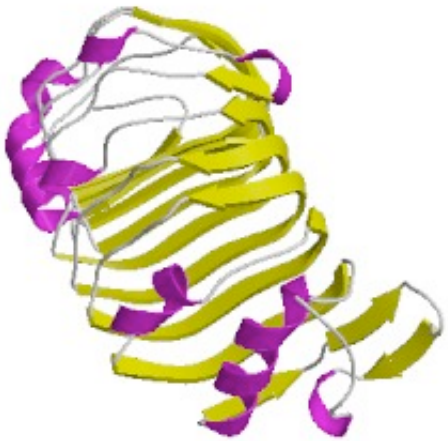

Figure 3. LRR domain topology. *Representation of the three-dimensional structure of the leading member of the LRR topology family according to the CATH domain database (Topology 3.80.10, Ribonuclease Inhibitor, PDB ID 1oznA00).*

Folding of helical domains generally proceeds much more quickly than the β-sheet domains and therefore domain topology controls the protein folding. In fact, without this capping motif, LRRs would probably fold slowly and via multiple routes, as is the case for the β-trefoil domains. Barrick and coworkers [67] showed that folding of InternalinBeta (InlB) is nucleated with the N-terminal capping domain. Mutations in the N-terminal capping domain and the LRR domains 1 and 2 of InlB slowed folding, but the unfolding rate was unaffected. Mutations in the fourth through seventh LRR domains of InlB had the opposite effect: the folding rate was unaffected whereas unfolding proceeded more quickly, consistent with the destabilizing nature of the mutations. Mutations in the third LRR domain slowed the folding rate and increased the unfolding rate. These results clearly define a "nucleation-propagation folding mechanism" starting at the N-terminal capping domain and proceeding towards the C terminus. As with other repeat domains, therefore the transition state may consist of a minimal stable unit of three repeats [68]. Generally, the LRR domain is referred as a "sticky" domain, giving to LRR proteins the ability to form complexes of homo- or hetero-multimeric units, by

homophylic or heterotypic adhesion among family members, as shown for example, with Lingo-1 and NgR ectodomain crystal structures [14, 58, 46, 69, 70].

1.4 NEURONAL LRR PROTEINS: ON OVERVIEW

Literature analysis of vertebrate genes encoding proteins with the signal peptide, TM region/GPI anchor and LRR domain shows that in most known cases, these genes are highly expressed in the nervous system [32].

A list of the more interesting LRRTM (LRR-transmembrane)-protein encoding genes, for which both the neuronal localization and the presence of the LRR domain and/or TM/GPI anchor was verified, is shown in **Table 2**.

Neuronal LRRTM proteins typically contain from 5 to 15 LRRs, with other domains commonly occourring in concert with the LRR such as IgG and FNIII. The order of the domains in the polypeptide chain is defined. If other domains than LRR are present, then the IgG-like domain(s) lie carboxy-terminal to the LRR, and the FNIII repeat lies carboxy-terminal to the IgG-like domain(s). The role of the IgG-like domains and FNIII repeats occourring in these proteins has not been studied extensively. In the case of NGL-1, the IgG-like domain was found not to have a role in Netrin-G1 binding [41].

In LINGO-1 the IgG-like domain was noted to cause a sharp, close to 90° angle between the LRR and the IgG-like domain, placing the latter in opposition at the back of the convex side of the LRR [70]. This induced turn in the LINGO-1 polypeptide chain orientation could be critical for presenting the associated LRR domain in a correct orientation in relation to the plasma membrane.

Only OMgp (Oligodendrocyte myelin glycoprotein) contains some additional extracellular elements [71], such as an unique serine-threonine rich region between the LRR domain and the GPI anchor. OMgp, NgR and Nycaloptin family members are GPI-modified proteins. Some LRRTMs share a relatively small intracellular region after the TM portion. None of these proteins show intrinsic enzymatic activities in their intracellular tail, suggesting that they play a role as a part of a multimeric complex in signal transduction activities, but while this is clearly proven, the partners involved in these multimerizations are unknown.

Several type I transmembrane LRR proteins contain a carboxy-terminal sequence that could serve as a binding site for PDZ (PSD-95/Discslarge/ZO-1)-proteins. PDZ-

domain containing proteins are involved in diverse physiological processes. Importantly, many have scaffolding functions in synapses [72, 73]. Synaptic Adhesion-like Molecules (SALM) 1 and 2, and Netrin G-Ligand (NGL)-2 associate with PSD-95, as well as with other PDZ-domain containing proteins present in the synapse [44, 45, 74]. The PDZ-domain binding motif, which is present in several members of the neuronal LRR membrane protein family, play a role in regulating their protein-protein interactions at least in some cases, and could thus control the intracellular localization of these PDZ domain-containing proteins [45, 51].

Subcellular localization of neuronal LRR membrane proteins has been typically assessed by protein overexpresson assays. Almost all LRR proteins are predicted to localize to the plasma membrane, but for most of them this localization was not confirmed other than *in silico*. For example, Pal (photoreceptor-associated LRR protein, a new poorly characterized retina-specific protein) is known to localize predominantly along a pathway related to secretion of proteins [75], as seen for LRRTM1 [76].

No studies addressing subcellular localization of any endogenous neuronal LRR membrane protein using well-characterized antibodies have been performed. Only NgR1 has been shown to be localized at least partially to the plasma membrane [51-52]. This is an indication that the *in silico* prediction as Transmembrane protein is not a strong indication of the real subcellular localization of these proteins. Another molecular feature to take into account in order to have an accurate estimation of the localization of a predicted LRRTM protein is the presence/absence of Cysteine cappings at both the carboxy- and amino-terminal ends of the LRRs.

A relevant portion of neuronal LRR membrane proteins has been suggested to be involved in regulating neurite outgrowth [17, 41, 44, 52, 69, 77-79] either when overexpressed in neurons or presented as soluble or surface-bound exogenous proteins.

Family name	*Subcellular localization*	*LRRs*	*IgG-domains*	*FNIII*	*Others*	*TM or GPI*	*Intracellular region*	*Biological function*
LRRTM	*ER, other*	*10*	*none*	*none*	*none*	*TM*	*Y*	*Presynaptic differentiation*
SLITRK	*TGN, PM*	*11*	*none*	*none*	*none*	*TM*	*Y*	*Neurite outrgrowth* ***(?)***
Amigo	*PM*	*6*	*1*	*none*	*none*	*TM*	*Y*	*Neurite outgrowth*
NGL	*PM*	*9*	*none*	*none*	*none*	*TM*	*Y*	*Synapse formation*
LINGO	*PM*	*12*	*none*	*none*	*none*	*TM*	*Y*	*Negative regulator of myelination*
FLRT	*PM*	*10*	*none*	*1*	*none*	*TM*	*Y*	*Neurite outgrowth*
NLRR	*PM*	*12*	*1*	*1*	*none*	*TM*	*Y*	*Modulation of EGF signaling*
LRIG	*PM*	*15*	*3*	*none*	*none*	*TM*	*Y*	*EGFR internalization*
SALM	*PM*	*6*	*1*	*1*	*none*	*TM*	*Y*	*Neuronal differentiation*

Lib/LRRC	*PM*	*15*	*none*	*none*	*none*	*TM*	*Y*	*Link to ß-amyloid processing*
Pal	*ER*	*5*	*1*	*1*	*none*	*TM*	*Y*	*Retina-specific expression*
Nyctalopin	*PM*	*11*	*none*	*none*	*none*	*GPI*	*none*	*Involved in normal vision*
OMgp	*PM*	*8*	*none*	*none*	*S/T*	*GPI*	*none*	*Neurite outgrowth*
NgR	*PM*	*8*	*none*	*none*	*none*	*GPI*	*none*	*Negative regulator of myelination*

***Table 2**. Neuronal leucine-repeat transmembrane proteins. LRRTM, Leucine-rich repeats transmembrane neuronal protein; SLITRK, Slit and Trk-like; Amigo, Amphotherin-induced gene and ORF; NGL, Netrin-G1 ligand; LINGO, LRR and Ig domain-containing, Nogo Receptor-interacting protein; FLRT, Fibronectin-leucine-rich transmembrane; NLRR, Neuronal leucine-rich repeat protein; LRIG, leucine-rich repeats and immunoglobulin-like domains protein; SALM, synaptic adhesion-like molecule; LRRC, leucine-rich repeats containing; OMgp, oligodendrocyte myelin glycoprotein; NgR, Nogo-66 receptor; FNIII, fibronectin-type III-like domain, TM, transmembrane GPI, Glycosyl-phosphatidyl inositol anchor; PM, plasma membrane, ER, endoplasmic reticulum; TGN, trans-Golgi network; EM, extracellular matrix. (Modified from 76).*

This is consistent with the LRR domain's role in protein-protein interactions, since LRRs could affect the adhesion to neurons to substrates. Alternatively, intracellularly localized pools of proteins could regulate the transport of other proteins needed for neurite outgrowth. However, it should be noted that experiments measuring neurite outgrowths are likely to be the first *in vitro* assays to be

performed with a neuronally expressed gene of unknown function, and therefore observed effects are likeky not representative of a full spectrum of functions that neuronal LRR serve. Furthermore, out of all the proteins mentioned above, the *in vivo* physiological role in regulating neurite growth has been established only for OMgp [80]. Evidences suggesting the same role in axonal growth and guidance was proven also for NGL-1, NgR1, NLRR-3 and SALM [41, 44, 81, 82].

In summary, several neuronal LRR membrane proteins have important roles in regulating neuronal shape and function by acting as ligands or receptors or both.

Most members of the neuronal LRR membrane proteins seem to function in a non-cell autonomous manner, but their intracellular localization suggest that some could have important cell-autonomous functions.

These findings, in the light of the functions identified for the various members of this superfamily, suggest that LRR proteins collectively contribute to the complexity of connectivity of the mammalian brain and may have been important in brain evolution.

1.4.1 Relevant LRR membrane proteins: NGL family

The netrins (whose name means “one who guides” in Sanskrit) are a family of laminin-related secreted proteins that provide axon guidance. The first netrin characterized, UNC-6 of the nematode *C. elegans*, was identified as the product of a gene that when mutated, leads to defects in cell migration and axon guidance [83]. In neuronal development, netrin-G1 is predominantly expressed by floor plate cells as commissural axons extend toward the ventral midlineNetrin-G1 is an axonal giudance cue, identified by a signal sequence trapping screening, with an unique expression pattern compared to the other Netrins. Netrin-G1 expression is relatively brain-restricted and is enriched in brain regions including olfactory bulb mitral cells, the thalamus and the deep cerebellar nuclei cells. A most important distinction between Netrin-G1 and the other Netrins is that the latter bind neither the *Deleted in colorectal cancer* (DCC) or the UNC-5 receptor complexes [32]. Netrin-G1 is also expressed outside the neuronal system and its transcript was found in pancreas, kidney, lung, bowel, bone and mammary gland. In these organs or tissues, Netrin-

G1 has different roles such as invasion of epithelial cells, leukocytes migration, cell adhesion or angiogenesis.

NGL-1 was identified as an high-affinity ligand for Netrin-G1 [41] from an expression cloning screening. The NGL family accounts for three members, structurally characterized by nine LRRs, an IgG-like domain, a TM fragment and a short intracellular region containing a PDZ-domain binding site (Figure 4).

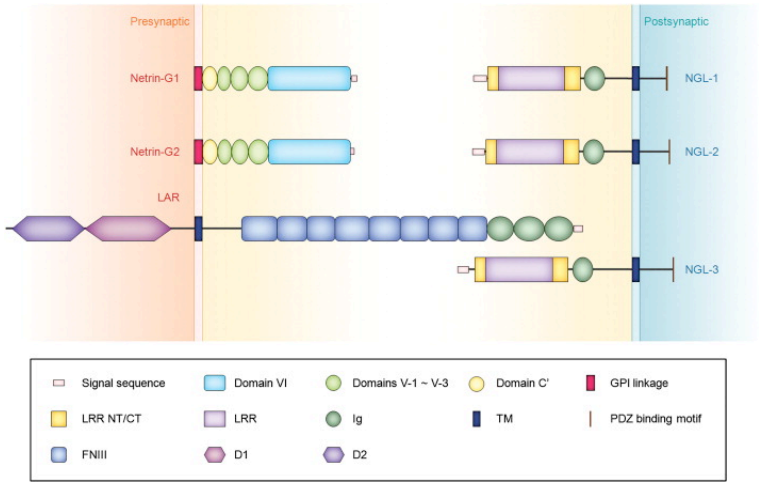

***Figure 4*. Domain structures of NGLs and their ligands (netrin-Gs and LAR).** *Domain VI, laminin N-terminal domain; Domains V-1–V-3, laminin-type EGF-like (or LE) domains 1–3; FNIII, fibronectin type-III; GPI, glycosyl phosphatidyl-inositol; Ig, immunoglobulin; LRR, leucine-rich repeat; LRR NT/CT, N- or C-terminal LRR; D1 and D2, membrane-proximal and membrane-distal tyrosine phosphatase domains of LAR; PDZ, PSD-95/Dlg/ZO-1; TM, transmembrane. (From 83).*

NGL-1 is a 640 aminoacid type I transmembrane protein that exerts two opposite functions: when localized at the cell membrane it promotes the outgrowth of embryonic thalamic axons, conversely, when secreted, has an inhibitory function. An interesting feature of the NGL-1 expression pattern in the brain underlies its funcional interaction with Netrin-G1. Since NGL-1 is expressed in mouse thalamic neurons, and Netrin-G1 is expressed in the striatum and cerebral cortex, it is was hypothesized that NGL-1 could be a chemo-attractant for thalamocortical neurons [41].

In adult mice, it was shown that NGL-2 plays a role as a regulator of neuronal morphology and excitatory synapses [45]. NGL-2 overexpression leads to dendritic protrusion formation by interacting with PSD-95. On the contrary, NGL-2 gene knockout decreased the number of exctatory synapses in cultured hippocampal neurons.

Both NGL-1 and NGL-2 were found expressed in adult human brain [41], even though its role remains elusive, one recent finding on NGL-1 suggests it has a notable neuropathological significance since the carboxy-terminus of NGL-1 interacts with a PDZ domain of *Whirlin,* whose gene mutation underlie the phenotype of the human autosomal recessive deafness locus, DFNB31 [84, 85].

1.4.2 Relevant LRR membrane proteins: AMIGO/Alivin family

AMIGO-1 was the first member of the AMIGO family discovered in a study using differential-display screening for the identification of transcrips upregulated in hippocampal neurons from E18 rat treated with the neuronal growth-promoting factor amphoterin [58]. The other members AMIGO-2 and -3 were identified *in silico*, whereas AMIGO-2 was identified independently, and named *Alivin* (Activity-dependent leucine-rich repeat and Ig superfamily survival-related protein) using differential display screening for genes involved in depolarization and NMDA-dependent survival of cerebellar granule neurons [86] and also hippocampal neurons and the granule cells of the dentate gyrus where it is expressed. It is known that all the three members co-immunoprecipitate *in vitro.* The ectodomain of AMIGO, when expressed as a heavy-chain (Fc) fusion protein and substratum-attached, promoted neurite extension of cultured hippocampal neurons, and, conversely, the same AMIGO ectodomain-Fc fusion protein inhibited fasciculation of neurites [60].

The AMIGO family member share the same architecture (Figure 5), with six LRRs, a single IgG-like domain located next to the transmembrane region, followed by an intracellular part. They are all expressed almost exclusively in the neuronal system but AMIGO-1 has a more brain-enriched accumulation than AMIGO-2 and -3, both of which have an expression pattern that is more diffused [58].

Interestingly, AMIGO-2 was found to be a differentially expressed gene (DEG) in approximately 45% of tumors versus normal tissues from gastric adenocarcinoma

patients. The antisense-mediated knockdown of AMIGO-2 in gastic adenocarcinoma cell lines give rise to morphological and genetic changes that are suggestive of a potential etiologic role for AMIGO-2 in gastric adenocarcinoma, such as promoting the survival of cancer cells [87].

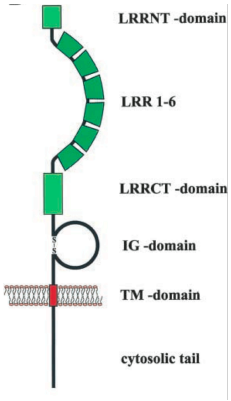

Figure 5. Schematic view of the motifs in the AMIGO proteins. *LRRNT: Leucine Rich Repeats N-terminal Domain; LRRCT: Leucine Rich Repeats N-terminal Domain; IG:Immunoglobulin-like (Adapted from 58).*

1.4.3 Relevant LRR membrane proteins: Nogo-A Receptor and LINGO

The Nogo/OMgp/MAG-NgR signaling network has a major role in the inhibition of neuronal regeneration in spinal cord and brain injury.

Nogo is a member of the ubiquitously expressed Reticulon family that has four members in mammals, RTN1, RTN2, RTN3 and RTN4/Nogo. Among these genes, the expression of Nogo is instead, predominantly nervous-system restricted. Three different protein products are encoded by the RTN4 gene, termed Nogo-A, -B and –C all resulting from alternative splicing and differential promoter usage. Structurally, these three protein products share the same architecture, with common carboxy- and amino-terminal tails and a 66-aminoacid long loop. Nogo-A can adopt multiple topologies, and can be located in the ER, in the Golgi and in the plasma membrane, with an expression pattern spreading throughout the CNS (oligodendrocytes) and the PNS (Dorsal Root Ganglia neurons and Schwann cells)

[88]. Nogo-A acts as a neurite outgrowth inhibitor by coupling to its cognate receptor NgR.

NgR was identified as a receptor that can bind Nogo-66 and is required for Nogo-66 function in neurons [14]. Interestingly, MAG and OMgp were found to also exert their inhibitory function by binding to NgR [88]. The importance of NgR in regulating neuronal function come also from the observation that the NGR gene is located in the 22q11 region, a key susceptibility site in schizophrenia. Furthermore, a recent report [89] confirmed previous findings [35] that demonstrated the association between two NgR gentic variants (R119W and R196H) and schizophrenia cases.

NgR is a GPI-anchored protein, and functions through a membrane spanning co-recepror in order to transduce growth-inhibitory signals generated by its interaction with myelin-associated inhibitors. The first of these NgR coreceptors idenfied was $p75^{NTR}$ [51]. The engagement of $p75^{NTR}$ and by the myelin-associated inhibitors results in the activation of RhoA [90] in susceptible neurons (Figure 6), since in reconstituted non-neuronal systems, the coexpression of NgR and $p75^{NTR}$ does not lead to the activation of RhoA, suggesting the existence of additional neuronal cell-restricted coreceptors. Blocking the $p75^{NTR}$/NgR interaction inhibits the activity of Nogo-A, MAG and OMgp. In cultured neurons, the ectopic expression of NgR can induce growth cone collapse and make neurons responsive to myelin inhibition [14, 51]. Direct evidences for a role of NgR in inhibiting axon regeneration *in vivo* come from the observation that in models of brain or spinal cord injuries, both the intrathecal (or subcutaneous) delivery of the *Nogo-66 competitive binding peptide* (NEP1-40), or of the *ligand-binding soluble NgR1 chimeric protein* (NgR310) improved axon growth and sprouting, leading to an enhanced functional recovery in injured animals [91-94].

Recent reports demonstrated that NgR has a more widespread role than previously hypotesized, being involved not only in modulating axonal plasticity but also in the regulation of the inflammatory response in the CNS. In fact, it is now known that NgR expression is rapidly lost from injured axons undergoing Wallerian degeneration, but is unexpectedly seen in phagocytic macrophages where it modulates the movement of the phagocytic cells to terminate inflammation at the end of Wallerian degeneration. It was estimated that 7 days after nerve injury, about

75% of macrophages expressed NgR, whereas in normal conditions only 12% expressed NgR. The role of NgR in this mechanism is also related to RhoA activation. Indeed, in macrophages, RhoA activation reduces the adhesiveness of macrophages from their substrate and increases their motility [95]. These observations opened the question about the role of NgR in neuroinflammatory diseases such as Multiple Sclerosis and Alzheimer's Disease.

LINGO-1, the neuronal cell-specific coreceptor needed for the activation of RhoA [96] (Figure 6) by the complex $p75^{NTR}$/NgR, was identified in a sequence database search for homologs to the guidance molecule *Slit* [97]. LINGO-1 is a type I transmembrane protein with 12 LRRs and one IgG-like domain. Its cytoplasmic tail has a canonical EGFR-like tyrosine phosphorylation site. Its expression has a rostral-to-caudal gradient in the CNS with high levels in the lymbic system and in neocortex [97], and low levels in the spinal cord. Its transcript is strongly upregulated (while NgR is downregulated) in the dentate gyrus of rat brain after Brain-Derived Neurotrophic Factor (BDNF) or kainate delivery in the CNS, and after spinal cord injury in the PNS [98].

In COS cells, coexpression of human NgR, $p75^{NTR}$ and LINGO-1 conferred responsiveness to the myelin-associated inhibitor and NgR ligand OMgp, as measured by RhoA activation. This effect is mediated by the tetramerization of LINGO-1. In fact, the buried surface of this tetramer serves as an efficient scaffold to facilitate the assembly of the NgR/LINGO-1/$p75^{NTR}$ receptor complex. Upon ligand binding, the NgR/LINGO-1/$p75^{NTR}$ receptor complex triggers intracellular signaling cascades that change the Rac1/RhoA equilibrium in growth cones. RhoA, Rac 1 and Cdc42 are the three most widely expressed members of the small GTPase family, acting coordinately as molecular switches to regulate F-actin dynamics and microtubule assembly during cell morphological changes. Rac1 and RhoA play antagonistic roles in growth cone dynamics through their effector kinases PAK1 and *Rho Kinase* (ROCK), to stimulate growth cone mobility or induce collapse, respectively [51, 88]. However, the molecular links between ligand-receptor activation and RhoA/Rac1 regulation are not clear [88].

Overexpression of LINGO-1 in neurons enhanced responsiveness to myelin-associated inhibitors, while expression of a dominant-negative LINGO-1 construct (with a truncated cytoplasmic tail) attenuated myelin ihnibition in transfected

primary neuronal cultures [96-99]. LINGO-1 also interacts with and mediates the action of TAJ/TROY, an orphan receptor belonging to the TNF receptor family [49, 50]. However, the importance of TAJ/TROY as a signal-transducing receptor for myelin inhibitor-mediated axonal growth arrest still awaits further evaluation, since its expression has not been consistently demonstrated in adult CNS [98].

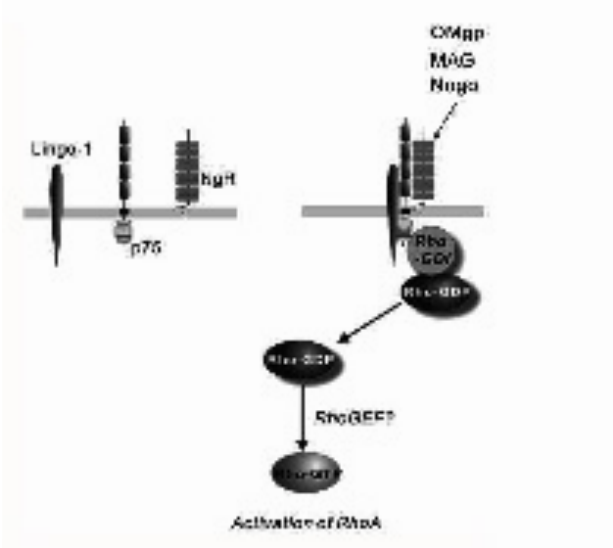

Figure 6. Mechanisms of axon growth inhibition by the Lingo-NgR-p75NTR complex*. In the absence of MAG or Nogo-66, growth and regeneration occurs. The Lingo-NgR-p75NTR complex promotes the dissociation of Rho-GDI from RhoA, allowing RhoA to activate through the exchange of guanine diphosphate for guanine triphosphate. Activated RhoA then interacts with its signaling molecules to elicit axon growth inhibition in some neurons. (Adapted from [99]).*

A more recent report had expanded the CNS function of LINGO-1 [101]. In fact, LINGO-1 is expressed also in oligodendrocytes, where it plays a role in modulating oligodendrocyte differentiation and myelination. Accordingly, dominant-negative LINGO-1, silencing RNA-mediated LINGO-1 knockdown, or LINGO-1-Fc all enhanced differentiation and increased myelination of primary oligodendrocytes in culture. As in inhibition of neurite outgrowth LINGO-1 appears to act through the activation of RhoA, since the overexpression of LINGO-1 activates RhoA and inhibits oligodendrocyte differentiation and myelination.

Oligodendrocyte-neuron co-cultures when treated with LINGO-1-Fc formed well-developed myelinated axons *in vitro*, with distincly defined nodes and internodes. Analysis of LINGO-1 knockout mice revealed that spinal cords from P1 pups have more myelinated axons fibers compared to wild type littermates, and oligodendroglia cultured from these mice had a larger percentage of mature oligodendrocytes than wild type [32].

1.4.4 Relevant LRR membrane proteins: LRRTM family

The human and mouse genes part of a four-membered family named LRRTM (leucine-rich repeat transmembrane neuronal), encoding the LRR-containing type I transmembrane proteins, were recently identified using bioinformatics tools while looking for proteins sharing sequence similarity to the Slit family of axon guidance molecules in an attempt to find novel genes encoding for LRR-containing proteins with similarity to the LRRs of the Slit proteins [42]. All four family members are expressed in mouse brain from Postnatal day (P)0 or earlier through adult. Each LRRTM family member displays the ability to instruct excitatory presynaptic differentiation [102]. The LRRTM proteins are structurally characterized by 10 external leucine rich repeats, with no additional extracellular domains, and a short intracellular tail containing a conserved C-terminal domain with a consensus sequence for interaction with PDF motifs. LRRTMs are all expressed in adult brain and in other tissues with different expression profiles. In situ hybridization expression analysis in adult mouse brain (Figure 7) showed that LRRTMs are widely expressed in brain, with some different specific expression [42]. All four LRRTM mRNAs are predominantly expressed in the neurons of the CNS, with specific expression patterns in different brain regions, and are developmentally regulated.

Interestingly, the expression profile of LRRTMs varied, with LRRTM2 having the most widespread pattern of expression and LRRTM3 the most limited expression profile. The LRRTMs are likely to play a role in the development and maintenance of the CNS, since human LRRTM1 mRNA was highly expressed in brain (mainly cortex) and in salivary gland [42]. Intermediate level of expression was detected in cerebellum, small intestine, spinal cord, stomach, testis, and uterus. LRRTM2

mRNA was also prominently expressed in brain but prominent expression was also detected in many other tissues analyzed and some level of expression was seen in all tissues except bone marrow. LRRTM3 mRNA exhibited a more limited expression profile with expression in brain and particularly in cerebellum. LRRTM4 mRNA was also prominently expressed in the CNS, including cerebellum and spinal cord, and intermediate levels of expression were seen in lung, muscle, prostate, salivary gland, small intestine, stomach, and testis. LRRTM4 expression was seen also in other tissues [42]. During mouse embryogenesis, LRRTM1 is initially detected at 9 days post coitum (dpc) where it is found in the neural tube, otic vesicle, apical ectodermal ridge, forebrain and midbrain up to a sharp central boundary.

LRRTM1 expression at 10 and 11 dpc is restricted to the apical ectodermal ridge. LRRTM2 is not observed until approximately 10 dpc when it is expressed in a subset of progenitors in the neural tube. Moreover, LRRTM2 localized specifically to glutamatergic and not GABAergic synapses in hippocampal cultures [101]. LRRTM3 is present at 8.5 dpc in the neural progenitors of the anterior neural plate, and at 9 dpc is expressed in a half somite wide stripe in the presomitic mesoderm adjacent to the boundary with the most recently formed somite with persistent expression until 11 dpc when the presomitic mesoderm has contracted to the tail bud stripes of expression can still be seen at the boundary with the somites and in the most recently formed somites with additional expression in the epithelium of the hindgut diverticulum. Additional expression is seen in the neural tube, forebrain and hindbrain. LRRTM4 expression is initially seen in the caudal lateral mesoderm of 8.5 dpc embryos and is expressed in the limb mesenchyme, neural tube, caudal mesoderm and in three distinct regions of the head [103].

It was shown that LRRTM1 instructs excitatory presynaptic differentiation in contacting axons, similarly to NGLs and SALMs, and mediates excitatory postsynaptic differentiation in dendrites, localizes to excitatory postsynaptic sites, and is required for normal Vesicular glutamate transporter (VGLUT1) distribution in vivo. These results provide a cellular basis for the linkage of LRRTM1 to handedness and schizophrenia [101].

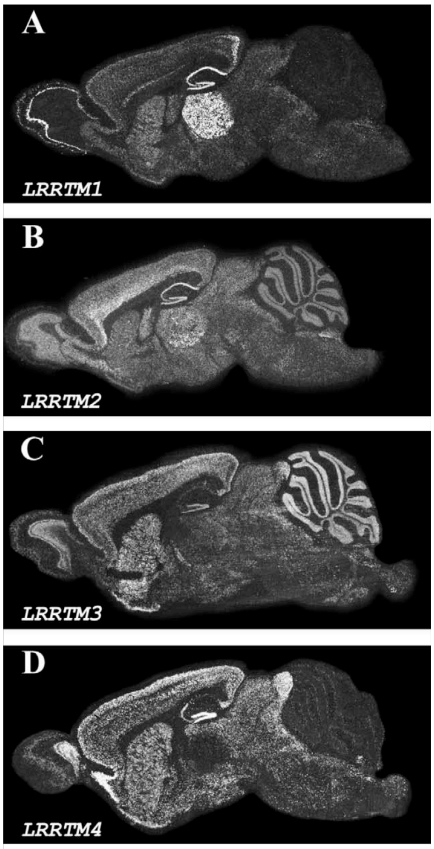

Figure 7. In situ hybridization analysis of mRNA expression of the LRRTM family in the adult mouse brain*. Shown are dark-field emulsion autoradiographs: (A) LRRTM1, (B) LRRTM2, (C) LRRTM3, and (D) LRRTM4. (Adapted from [42]).*

1.5 SLITRK FAMILY MEMBERS IDENTIFICATION

Ther first member of the Slitrk family was identified in a study focused on the organization of the transcriptional map of the entire q27 band of the Human X Chromosome [104]. The interest of this region came from the observation that this X-chromosome portion contains a small number of genes all of which are putatively involved in several X-linked mental disorders, including the gene FMR1, responsible for the Fragile X syndrome. These include susceptibility loci for prostate cancer [105], testicular germ-cell tumors [106], X-linked progressive cone dystrophy [107], and various mental retardation syndromes [108]. Initially, an EST sequence [109] corresponding to the 3' end of the cDNA clone 27500 from a

normalized infant brain cDNA library [110] was mapped to the Xq27.3 region, between loci DXS8028 and DXS1200, based on the Xq27 physical and transcriptional maps generated by this lab [111].

The first sequence analysis performed on this novel gene predicted a protein with similarity with other members of the LRR protein superfamily such as Slit. For this reason, the gene was initially annotated as SLITL1 [16].

In a study focused on the searching for genes that are deregulated in mutant mice with neural tube defects [17, 112], a family of LRR proteins were identified by searching in the the human and mouse nucleotide sequence databases. Sequence comparisons among human Slit1, Slit2, Slit3, and these new proteins revealed that the homology between them and Slit proteins spans the entire N-terminal domain. Moreover, the tyrosine residues in the carboxy-terminus of these new proteins was suggested to be homologous to the carboxy-terminal domains of the Trk neurotrophin receptor proteins. For these reasons, this new family of proteins was named Slitrk, and it was clear that Slitrk2 gene was the same of SLITL1. To date, both the first and the current names for these proteins seem to be misnomers, since the LRRs of Slitrks are no closer to Slit than to other LRRs, and the similarity to Trks is limited to only 4 residues that still are not present in all the member of the Slitrk family (Figure 8).

The slitrk family is a large six-membered subfamily of LRR-containing proteins. These proteins are encoded from genes localized in human chromosomes X (Slitrk 2 and Slitrk 4), 3 (Slitrk 3) and 14 (Slitrks 1, 5 and 6) [112]. All these proteins are characterized by large LRR domain repeats, a short hydrophylic fragment hypothesized to be a transmembrane domain, and a short so-called intra-cytoplasmatic tail. The length of the intracellular tail is the most variable fragment among the member of this family.

The expression profile of the Slitrk genes seems to be unique to each gene even though all are preferentially expressed in the brain and spinal cord [17, 113].

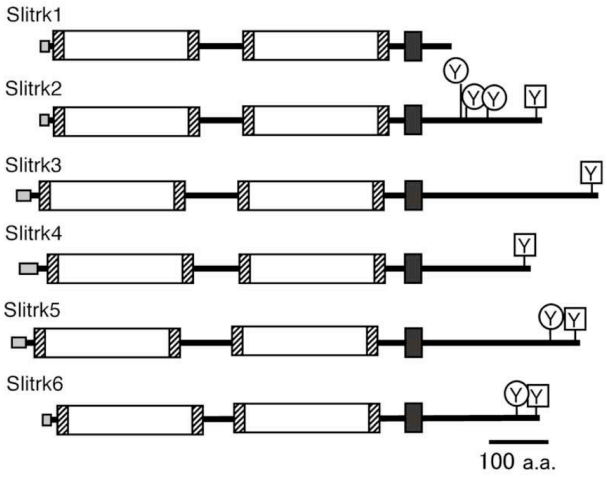

Figure 8. Structure of the Slitrk protein family. *Open box, leucine-rich repeat domain; Hatched box, cysteine-rich region; Closed box, putative transmembrane domain; Shaded box, potential signal peptide region; circled Y, conserved tyrosine residues (potential phosphorylation sites); boxed Y, a tyrosine residue conserved between neurotrophin receptor and Slitrk proteins (Adepted from [17]).*

The exact function of Slitrks remains largely unknown, and their brain-enriched expression suggests that they have a role in the regulation of the development of neuronal system. However, since some Slitrk family members were found expressed outside the neuronal system, it was hypotesized a role for these proteins also is in the modulation of physiological mechanisms outside the neuronal compartment.
For example, Slitrk4 and 5 transcrips were detected in undifferentiated hematopoietic stem cells with a strong downregulaton after differentiation commitment [114].

1.5.1 Slitrk family and neuronal biology

The development of the nervous system requires a wide variety of processes to take place including cellular differentiation, cellular migration, axonal guidance, and synapse formation and refinement. A large number of Type 1 transmembrane proteins containing leucine-rich repeat domains (LRR) are highly expressed in the nervous system and have been implicated in regulating various phases of neuronal development [20, 32, 115].

These proteins have been shown to affect neuronal outgrowth. Several Type I transmembrane proteins (plasma-membrane anchored protein with the N-terminal located outside the cell) with LRR have been identifed, and most are expressed predominantly in the nervous system. Functionally, most of these proteins are related to neurite outgrowth modulating processes, re-myelination inhibition, endo/exocytosis and neuronal patterning. It is clear that Leucine rich-repeats are often found in neurite development-controlling proteins, including not only *Slit* but also some neurotrophin receptors such as Trk family members. Neurites are key morphological features of neurons, and are classified into axons and dendrites. Axons are long slender processes of uniform diameter, whereas dendrites are short tapering processes. Vertebrate neurons typically have only a single axon and multiple dendrites. The temporal development of neurites is divided into two molecular processes: an early phase of initiation, and a later stage of elongation. These processes are strongly regulated by key molecules that are important players in neuritogenesis and/or axonal guidance [116]. The early phase of neurite growth requires membrane expansion at the growth cones and is accomplished by the incorporation of trans-Golgi network (TGN)-derived exocytic vesicles into the plasma membrane [117]. The initiation process of neurite outgrowth requires specifically intensified and oriented exocytosis, whereas axonal guidance/elongation is controlled by positive and negative cues that cause growth, collapse, attraction and repulsion of the growth cones. Often, molecules involved in these processes contain LRR domains. For all these reasons, Slitrks are relevant candidate players in these mechanisms. Evidence in support of this hypothesis came from several avenues.

For example, the Slitrk5 gene is expressed predominantly in neural tissues. Using the Velocigene technology, Shmelkov and collagues [118] replaced the entire encoding exon of Slitrk5 with the lacZ gene to detect Slitrk5 expression *in vivo*. The expression analysis in Slitrk5-knockout-LacZ-knockin mice showed that Slitrk5 is widely expressed throughout the central nervous system, including the cortex and striatum, and restricted to NeuN positive neurons. At a behavioural level, it was shown that starting at 3 months of age, Slitrk5-KO (knockout) mice developed facial hair loss and severe skin lesions that over time produced ulcerations with hemorrhaging (Figure 9). This observation was attributed to self-grooming and not

to skin defects. This compulsive overgrooming behavior with increased anxiety was previously observed in mice deficient for the Synapse-Associated Protein 90/Postsynaptic Density-95-Associated Protein (Sapap)-3 gene, which encodes a postsynaptic scaffold protein. Interestingly, fluoxetine feeding of Slitrk5-KO mice led to a significant reduction in the duration of grooming compared to pretreated mice. These behavioral deficits were also correlated with FosB upregulation in the orbitofrontal cortex of Slitrk5-KO mice. The orbitofrontal cortex is a brain region whose activity is higher in individuals with obsessive-compulsive disorders (OCD).

Regarding neurite extension in Slitrk5KO mice, Sholl analysis revealed a decrease in dendritic arbor complexity and a significant decrease in dendritic complexity of striatal neurons with protein amounts of glutamate receptor subunits NR2A, NR2B, GluR1, and GluR2 decreased by 20–60%, with no significant changes in PSD95 amounts. This study then linked Slitrk5 to the core symptoms of OCD: self-injurious repetitive behavior and increased anxiety [118].

Another strong evidence for a role of Slitrks in regulating neuronal system development comes from the analysis of Slitrk6KO mice [119].

In these mice, Slitrk6 transcripts were first detected at embryonic day (E) 8.5 in the otic placode, which invaginates to form the otic vesicle and then at E10.5, otic vesicle Slitrk6 transcripts were strongly expressed in the ventromedial and laterodorsal regions which give rise to the cochlear and vestibular sensory epithelia. After E15.5, Slitrk6 expression marked the presumptive organ of Corti. Slitrk6 was also detected in vestibular ganglion neurons near the sensory epithelium from E11.5 to E14.5, comparable to what was observed in spiral ganglion neurons. At these time points, Slitrk6 was previously seen expressed in the gastrointestinal tract. In a coronal section of the diencephalon at E13.5, Slitrk6 was found complementary expressed with another uncharacterized LRR protein called Nllr3. The expression of both genes was overlapping from this stage to the adulthood in all neuronal and non-neuronal compartments in which Slitrk6 was detected [120].

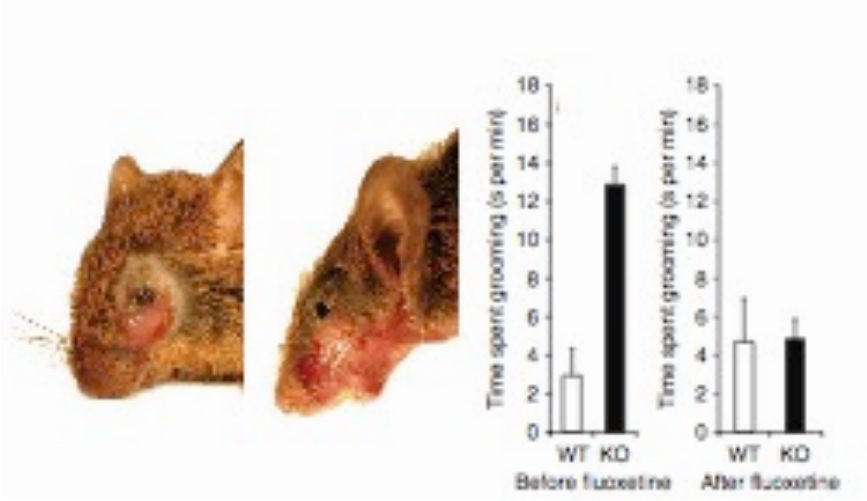

Figure 9. Facial lesions, OCD-like behavior and its alleviation with fluoxetine treatment in Slitrk5-knockout mice*. Phenotypic characteristic of Slitrk5KO mice: excessive grooming leads to severe facial lesions (Left panel). Time spent grooming in Slitrk5KO mice compared to their wild- type littermates before and after treatment with fluoxetine (Right panel). (Adapted from [119]).*

In the postnatal mice, Slitrk6 transcripts were detected strongly in supporting cells of cochlea, weakly in both inner and outer hair cells and in vestibular sensory epithelia, including ampullary cristae, saccular and utricular maculae. Slitrk6 transcripts were densely located at the lumenal surface of the sensory epithelium, where hair cells localize. Interestingly, in Slitrk6KO mice a correlation with both neurotrophin expression and neurotrophin signalling was found since in the inner ear they showed a significant decrease in the expression level of both BDNF and Neurotrophin-3 (NT-3), a downregulation of both TrkB and TrkC at the protein level,, and a decrease of the amount of phosphorylated form of Trk receptors. All these phenotypic changes were limited to the auditory system, since no other behavioral or cognitive changes were displayed by the Slitrk6KO mice. In the explant co-culture experiments, cochlear sensory epithelia from Slitrk6KO mice were found to have less activity in promoting neurite outgrowth of spiral ganglion neurons (Figure 10), indicating that Slitrk6: i) plays an important role in sensory neural development of the inner ear by regulating the expression of molecules that promote survival and neurite outgrowth of sensory neurons and ii) is involved in the

survival and innervation of sensory neurons in the inner ear, at least in part by modulating neurotrophin–Trk signaling.

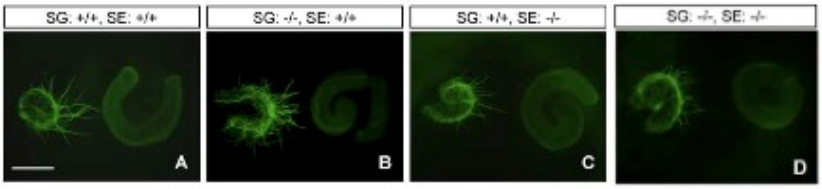

Figure 10. Cochlear sensory epithelia of Slitrk6-knockout mice have less activity in promoting neurite outgrowth of spiral ganglion neurons*. Explant cultures of spiral ganglia (SG) and sensory epithelia (SE) dissected from E14.5 wild-type and Slitrk6-deficient mice. Neurites were visualized by neurofilament immunostaining (green). There were hardly any detectable processes at the beginning of the explant culture. Spiral ganglia of wild-type (A) and Slitrk6-deficient (B) mice can strongly extend their neurites to sensory epithelia obtained from wild-type mice, whereas sensory epithelia of Slitrk6-knockout mice weakly attract neurites of spiral ganglia obtained from both wild-type (C) and knockout mice (D). (Adapted from [119]).*

The observation that Slitrk6-deficient mice do not show any appreciable behavioral/neurological defects is quite surprising since Slitrk6 is expressed in some areas of the embryonic and adult central nervous system [120].

Beside these evidences, however, there is not yet a correlation between any of the Slitrk family members and any human neuronal pathology. In fact, none of the studies in the NHGRI catalog of published Genome-Wide Association (GWA) studies (www.genome.gov/gwastudies) [121] take into account a Slitrk family member as a candidate subsceptibility gene for brain-related diseases.

Moreover, among the diseases not compiled in the NHGRI catalog, a literature search for GWAS fail to find any correlation between Slitrks and neurological pathologies such as, for example, mental retardation or schizophrenia [122-124].

A correlation between a Single Nucleotide Polymorphism of Slitrk2 and Bipolar Disorder susceptibility was found by Smith and collagues [125], although this association appears to be very weak.

1.5.2 Role of Slitrk-1 in neurons: a link with Tourette's Syndrome

Tourette's syndrome (TS, OMIM#137580) is a potentially debilitating neurobehavioral disorder characterized particularly by motor and vocal tics and associated with behavioral abnormalities that affects as many as 1 in 100 individuals [126, 127]. Tics are sudden, brief, intermittent, involuntary or semi-voluntary movements (motor tics) or sounds (phonic or vocal tics). They typically consist of simple, coordinated, repetitive movements, gestures, or utterances that mimic fragments of normal behavior. Males are affected about three times more often than females, and onset usually occurs between 3 and 8 years of age. By age 18 years, more than half of affected individuals are free of tics, but they may persist into adulthood. A substantial portion of clinically referred TS patients also suffer from obsessive-compulsive disorder (OCD), attention deficit hyperactivity disorder (ADHD), or depression [128]. Although the cause of TS is unknown, it is well established that both genetic and environmental factors are involved. Some forms of Tourette's may be genetically linked to a obsessive-compulsive disorder while the relationship between Tourette's and attention-deficit hyperactivity disorder is not yet fully understood. Genetic studies have proven that the overwhelming majority of cases of Tourette's are inherited, although the exact mode of inheritance is not yet known. Tics are believed to result from dysfunction in the thalamus, basal ganglia, and frontal cortex of the brain [129] involving abnormal activity of the brain chemical, neurotransmitter dopamine. Non-genetic factors can influence the severity of the disorder. Sequence variants in Slitrk1 have been suggested to be associated with TS (Table 3). Abelson's group studied Slitrk1 as a candidate gene for TS because of its proximity to a de novo chromosomal inversion in a child with the syndrome and no family history [130] and since Slitrk1 was demonstrated to have a high relative expression in brain regions previously implicated in TS. Although they found no mutation in the child, they identified two different mutations in the Slitrk1 gene among 174 unrelated probands with TS, one non-sense mutation in the Open Reading Frame (ORF), and one variant in two unrelated affected individuals in the 3' UTR of the gene (var321).

Analysis	Results
Sequencing, SNP genotyping	Identified one frameshift mutation and two var321 alleles in 174 unrelated probands; these variants were absent from 3600 and 4296 control chromosomes, respectively.
SNP Genotyping	Out of 1048 GTS- or CT-affected individuals, only one had var321 present, but did not transmit it to the affected offspring.
Sequencing	Sequenced 82 Caucasian patients with TS from North America. No var321 alleles found. Novel Ile236Ile variant found in one GTS patient.
Sequencing	No var321 alleles found in 160 Taiwanese children with GTS.
SNP Genotyping	No var321 alleles found in 307 Costa Rican patients. Five var321 alleles found in 515 Ashkenazi patients, two of whom transmitted var321 to affected children. One in 256 Ashkenazi control individuals also had var321, suggesting overrepresentation of this variant in this population.
SNP Genotyping	No var321 or frameshift mutation found in 208 affected children. Haplotype analysis found significant association with GTS, making this the first study to support the original study that found *SLITRK1* association with GTS.
Sequencing	No var321 alleles or frameshift mutations found in 92 Austrian patients with GTS. One female patient and two affected patients were found to carry a variant within 3′ untranslated region, which was absent from 192 controls.

Table 3. Summary of the Slitrk1 gene variations associated with the TS*. Review of the main studies related to the genetic assessment of the Slitrk1 variants and Single-Nucleotide Polymorphysm related to TS genome association studies. (Adapted from [128]).*

The base change corresponds to a highly conserved nucleotide within the predicted binding site for a microRNA, hsa-miR-189. This miR has an overlapping expression pattern in brain regions previously implicated in Tourette syndrome. Wildtype Slitrk1, but not the frameshift mutant, enhanced dendritic growth in primary neuronal cultures. However, there is controversial evidence about whether variation in the Slitrk1 gene plays a role in TS. In fact, because var321 does not segregate with disease any contribution of SLITRK1 to Tourette syndrome etiology is likely to be small [127]. Moreover, two independent analyses [131, 132] failed to find the var321 change or any other potentially pathogenic changes in the Slitrk1 gene in 82 Caucasian and 160 Taiwanese patients with TS, respectively. However, a relationship between Slitrk1 and TS is possible since Slitrk1 is located on 13q31.1, a region linked strongly with panic disorder, schizophrenia, bipolar disorder and recurrent depressive disorder [133]. A very recent study showed a genetic association between Slitrk1 and TS but not with previously reported DNA variants [134]. Slitrk1 is a 696 amino acids long protein highly expressed in adult and fetal brain and in all specific adult brain regions examined. Slitrk1 protein contains a N-terminal signal peptide, followed by 2 LRR domains, a C-terminal transmembrane domain, and a short cytoplasmic tail. Each LRR domain is flanked by cysteine-rich regions [17]. Slitrk1 function is still unclear even though it was suggested a role in promoting neurite extension.

Slitrk1 was ubiquitously detected in the nervous system and was abundant in the olfactory bulb, frontal cortex, hippocampus and amygdala. Expression was intermediate in pancreas and lung, low in kidney, ovary, heart, and fetal liver, and nearly undetectable in skeletal muscle, spleen, testis, and adult liver.

To further unravel the role of Slitrk1 in TS, Aruga's group generated a knockout mice in order to analyze its phenotype at both the biochemical and behavioral level [135]. Slitrk1-deficient mice did not display any external abnormalities except the males' reduced body weight. Since previous studies have indicated that noradrenergic neurotransmission is altered in some TS patients, and treatment with clonidine, an α2-adrenergic agonist, is sometimes successful [136], they studied the effect of clonidine treatment in Slitrk1-KO mice finding that administration of clonidine attenuated the anxiety-like behavior of Slitrk1-KO in the elevated plus-maze test, whereas clonidine treatment did not alter the locomotor activity of Slitrk1-deficient mice. Therefore, specific behavioral abnormalities in these mice are unlikely to reflect general health effects. Anatomic and histologic analyses of adult Slitrk1-deficient brains did not reveal any obvious abnormalities, even if these mice consistently showed a pronounced decrease in locomotor activity and behavioral abnormalities that may be related to anxiety and depression-like behavior. These behavioural abnormalities could be related to the observation that norepinephrine and its metabolites tended to be higher in the prefrontal cortex and nucleus accumbens of Slitrk1KO mice.

The results taken together suggest that, the behavioral phenotypes of Slitrk1-knockout mice are only partially consistent with those of TS patients. Interestingly, these mice share with TS patient the characteristic of anxiety and depressive disorders. In agreement with the behavioral abnormalities of Slitrk1-deficient mice, Slitrk1 mutations in humans seems to be associated with anxiety or mood-related disorders.

Moreover, dendrites formed by Slitrk1 wild type-expressing cortical pyramidal neurons were were significantly longer than those expressing the frameshift mutant detected in TS patients [130], suggesting that both Slitrk1 may promote dendritic growth and the frameshift mutation likely results in a loss of function. For these reasons, Slitrk1 is still considered as a candidate gene important in neuropsychiatric disorders such as TS and Trichotillomania, another compulsive pathology

characterized by the need to pull out own hairs.

1.5.3 Slitrks as potential Stem Cell Markers

Slitrk proteins were recently suggested as markers for stem and progenitor cells, including embryonic stem cells and hematopoietic stem/progenitor cells. Also, Slitrks were proposed as markers for endothelial cells and cancer cells such as leukemia and lymphoma cells [114] (Figure 11).

Recently, a patent was filed that relates to methods for targeting therapeutic agents to cells expressing slitrks and to methods for identifying drugs that act on slitrks or on slitrk-expressing cells [137]. This patent came from a search for new markers of primitive hematopoietic, leukemic and lymphoma cells. The authors previously observed differential expression of Slitrk5 on CD34+ leukemic cell lines [114]. Results from this study revealed that of 13 leukemic cell lines, four acute lymphoblastic leukemia (ALL), five out of seven acute myelogenous leukemia (AML) and two chronic myelogenous leukemia (CML) cell lines express one or more members of the Slitrk family. Comparison of two closely related cell lines, KG1 and KG1a revealed a cell-specific expression pattern for Slitrk4. Expression was limited to the undifferentiated variant KG1a, but not to the parental KG1 cell line. Of the leukemic cell lines studied only two did not express any of the Slitrks, the myeloid leukemic cell lines NB4 and HL60. It was observed that lymphoblastic leukemic cells expressed more Slitrk1 and Slitrk6, while myeloid leukemic cells express more Slitrk4 and Slitrk5.

In the lymphoma cell lines, a distinct expression pattern was seen. For example, the Daudi and GA10 lines showed no expression of any of the Slitrks, while BC1, BC3 and BCBL1 showed expression of five out of six Slitrks. Slitrk4 and Slitrk5 were widely expressed by the leukemic cell lines whereas, Slitrk1, Slitrk2 and Slitrk6 were quite unexpressed. Slitrk3 instead is not detected in any of the tested leukemic cell lines. Out of four lymphoblastic leukemic cell lines, namely CCRF and 697, express Slitrk1, none express Slitrk2 or Slitrk3, one (CCRF) expressed Slitrk4, three (Jurkat, CCRF and BV173) express Slitrk5 and two express (697 and BV173) Slitrk6. Four cell lines (Raji, BC1, BC3 and BCBL1) expressed Slitrk1, two (BC-1 and OCI-Ly8) expressed Slitrk2 very weakly, three (JD38, BC3 and BCBL1)

expressed Slitrk3, five expressed Slitrk4 (Raji, JD38, BC1, BC3 and BCBL1), four expressed Slitrk5 (JD38, BC1, BC3 and BCBL1) and five expressed Slitrk6 (Raji, JD38, BC1, BC3 and BCBL1). Most notably, the cell lines expressing five of the six slitrk members were all primary effusion lymphomas (PEL), a distinct clinical entity of diffuse body cavity-located lymphomas with no single tumor mass as well as a poorer prognosis in comparison to other lymphomas.

Out of the seven studied myeloid leukemic cell lines, none express Slitrk1 and Slitrk3, cell line, THP1 expresses Slitrk2, KG1a, THP1 and R81 express Slitrk4, HEL, KG1, KG1a, THP1 and R81 express Slitrk5 and HEL expresses Slitrk6. Among the chronic myeloid leukemic cell lines, none express Slitrk2 or Slitrk3, while K562 expresses Slitrk1, and only EM3 expresses Slitrk4, Slitrk5 and Slitrk6. Only two leukemic cell lines do not express any of the Slitrks: myeloid leukemic cell lines NB4 and HL60.

The expression patterns of slitrks by primary cells was studied also on human mononuclear cells (MNC) isolated from peripheral blood, human umbilical vein endothelial cells (HUVEC), human fetal bone stroma (BS), human foreskin fibroblast (HFF), human umbilical vein stromal cells (HUVSC) and human Miz-hES5 embryonic stem cells (ESCs) [137]. All six members of the Slitrk family are expressed by human ESCs and fetal bone stroma, indicating the potential importance of the Slitrks for the identification of these cells. This study suggested that expression of Slitrks is essential during mammalian development. Analysis of Slitrk expression on vascular cells revealed a highly contrasting expression pattern to that of fibroblasts. RT-PCR analysis showed that HUVEC expressed only Slitrk4 and Slitrk5. Treatment of HUVEC with Interleukin1 (IL1) or Phorbol-myristate-acetate (PMA) (chemical used for the activation of HUVEC cells through the expression of molecules that prompte leukocyte adhesion) had no major effect on the expression pattern of Slitrk1-Slitrk6 except for a slight increase in the expression of Slitrk5. A difference in Slitrk expression between leukemic and lymphoma cell lines and normal MNC was observed and may possibly due to reactivation of Slitrks during malignant transformation. This was also proposed for brain tumors [17] although the correlation between brain cancer and Slitrks expression is not convincing since no any clear pattern of up- or down-regulation of Slitrks in brain tumor cells was found.

Human embryonic stem cells expressed all six members of the slitrk family, similarly to human fetal BS, ESC and HUVSC. On the contrary, adult HFF expressed only Slitrk1 and Slitrk6, suggesting that Slitrks are mainly expressed in embryonic and fetal tissues in contrast to differentiated cells.

The initial finding of slitrks being expressed in CD34+ leukemic cells prompted the authors in the study mentioned above–to investigate the expression of slitrks on hematopoietic stem and progenitor cells. Using cell sorting-purified hematopoietic cells, it was shown that human CD34+ hematopoietic progenitor cells isolated from umbilical cord blood express both Slitrk4 and Slitrk5, while the CD34− population express significantly lower levels of Slitrk4 and Slitrk5. Interestingly, this pattern was reproduced in the CD34+ and CD34− populations purified from healthy donors. The robust expression of Slitrk4 and Slitrk5 in the undifferentiated CD34+ cells is drastically downregulated upon differentiation into CD11b+ myeloid, CD41a+ megakaryocytic or CD235a+ erythroid. These data indicate that these Slitrks are predominantly expressed on immature human hematopoietic stem and progenitor cells suggesting that Slitrks could be involved in normal as well as malignant hematopoiesis. Slitrks may also play a role in modulating hemangiogenesis and neo-angiogenesis. Since HSCs and vascular progenitor cells have been studied for their potential in reconstituting hematopoiesis and vascular beds as well as for their promise as targets in the treatment of hematological malignancies and angiogenesis-dependent diseases. Unfortunately, identification and purification of hemangiogenic stem and progenitor cells have been hampered by lack of common markers expressed by primitive endothelial and hematopoietic cells. For this reason, data coming that previous sentences were used by Rafii et collagues to file a patent in which Slitrks were proposed as innovative stem cells/progenitor markers as like as early hemangiogenic cell markers (CD34, CD133 and c-Kit) and other biochemical markers (e.g. lineage specific surface markers or dye efflux capacity markers). Moreover, since undifferentiated pluripotent cells, including human ESC and fetal bone stroma expressed all six members of the Slitrk family, the potential importance of these proteins for the identification of these cells is important.

Although there is a clear expression pattern restricted to Slitrk4 and Slitrk5 in normal hematopoiesis, this is not the case in malignant hematopoiesis, as leukemic and lymphoma cell lines express a wide range of Slitrks. Most notably, the

expression pattern of slitrk4 and slitrk5 changes during differentiation of hematopoietic CD34+ cells, with both markers being significantly downregulated in terminally differentiated myeloid, megakaryocytic and erythroid cells. This suggests a role for slitrk4 and slitrk5 in the differentiation of hematopoietic cells, and as such they could be used phenotypically to identify the more pluripotent hematopoietic stem and progenitor cells.

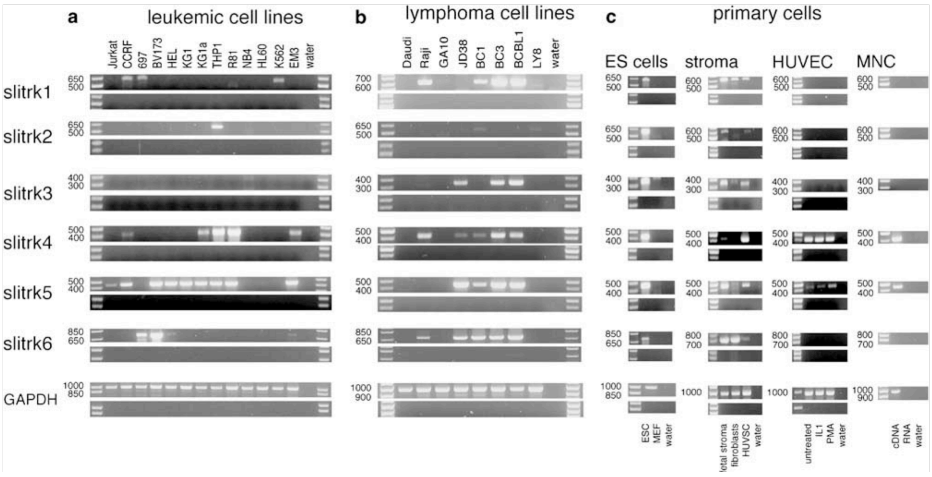

Figure 11. Expression of slitrk1–slitrk6 by primary cells, leukemic and lymphoma cell lines. *RT-PCR analysis in myeloid and lymphoid subsets of leukemic and lymphoid cells (a, b). RT-PCR on cDNA from embryonic stem cells, fetal BS, adult HFFs and HUVSC, HUVEC and MNC (c). (Adapted from [114].*

All of the data taken together suggests that the family of Slitrks may represent important membrane proteins for targeting with agents modulating the function of Slitrks or their (still unknown) ligand(s) may be the next anti-leukemia or lymphoma drugs, as agents for inhibiting over-proliferation of stem or progenitor cells, or as agents useful in regenerative medicine (useful in maintaining stem cells in culture, or for facilitating differentiation of stem cells into differentiated cells types) [137].

2. AIM OF THE STUDY

The general aim of this work was the study of the *in vivo* tissue distribution and the functional characterization of Slitrk2. Moreover, the structural characterization of Slitrk2 with the bioinformatic prediction of its three dimensional structure was achieved.

The specific aims about the *in vivo* tissue distrubution were:

- the transcriptional profile of Slitrk2 during mouse embryogenesis;
- the analysis of Slitrk2 expression in both human and mouse fetal and adult tissues;
- the Slitrk2 transcript distribution in the adult mouse brain.

To achieve these goals, we performed some general analysis such as Northern Blot and PCR, coupled with more selective and refined techniques such as *in situ hybridization.*

Since the knowledge about the comprehensive molecular blueprint of Slitrk2 is still lacking, we perfomed several bioinformatic analyses in order to get more detailed informations about:

- Slitrk2 domain distribution and predicted subcellular organization;
- identification of "signal" sequences to better explain *in vitro* results about its subcellular localization;
- identification of potential phosphorylation sites with biological likelihood;
- the three-dimensional Slitrk2 structure to better define potential ligand (s) binding site (s) and their functional determinants.

We also started to characterize Slitrk2 functionally. This was done by using mainly a neurotrophin-responsive cell line in order to define the role of Slitrk2 in neuronal-cell differentiation. Some biochemical details about this task were unraveled. These studies took into account the phenotypic effect of Slitrk2 transcript ectopic modulation by forced expression or silencing. Our research focused also on the analysis of Slitrk2 overexpression effect during the differentiation of cells belonging to non-neuronal system (mammary gland). Lastly, we discussed the previous knowledge about Slitrk2 in brain cancer and its link with cell stemness.

3. RESULTS AND DISCUSSION

3.1 SLITRK2 TRANSCRIPTIONAL ANALYSIS

Slitrk2 transcript distribution during mouse development was reported in several regions of the developing Central and Peripheral Nervous system including Olfactory Bulb, Basal Layer of the Ventricular zone in the Cerebral Cortex, Trigeminal Ganglia and Inner Granular Layer of the Cerebellum [113]. Another report showed also that the Slitrk2 transcript is particular abundant in the cerebral cortex [112].

In order to identify the developmental kinetics of Slitrk2 expression during mouse embryonic development, we performed Northern Blot analysis on samples obtained from mouse embryos at different stages of development. We detected Slitrk2 gene expression starting from Embryonic Day (E) 11, with an increase in expression level from E11 to E17 (Figure 12). This finding is in agreement with the previous report showing Slitrk2 as being differentially expressed by screening on a database of Expressed Sequence Tags for sequences related to neural tube closure defects during mouse embryo development [17]. The re-expression of Slitrk2 transcript at E11 posits that this gene could play a role both in blastocyst and post-gastrulation embryonic development and later stages of neuronal development.

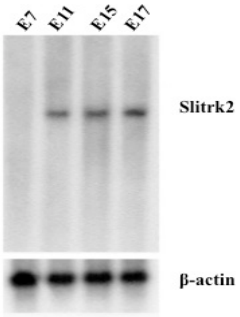

Figure 12. Northern blot analysis of Slitrk2 expression during mouse embryo development. *Northern analysis of polyA mRNAs obtained from mouse embryonic tissues revealed that Slitrk2 is expressed after the day 7.*

In fact, the development of the nervous system proceeds in three main phases. First, nerve cells are generated through cell division of the neuroectodermal precursors

until about E9. Then, after the closure of the neural plate (E10.5), these precursor cells send out axons and dendrites to form profuse synapses with other, remote cells so that communication can begin. Finally, the system of synaptic connections is refined and remodeled according to the pattern of electrical activity in the neural network. Neurulation, such as the generation of neuronal stem/precursor cells, takes place mainly before neural plate closure. After this stage, neural cell specification occurs. At this point we hypothezise that Slitrk2 could be responsible for cell fate determination rather than neural stem/precursor cells generation and renewal since its expression is particularly abundant in the later stages of mouse embryo development.

As shown in Figure 12 and in previous studies, Slitrk2 transcript distribution in developing mouse is already known. For this reason, we focused our attention on its expression in adult mice and on the distribution in adult mouse brain.

By using commercially available mRNA blots of different human and mouse adult tissues, we found that Slitrk2 has two transcripts; 4.4Kb and 7.7Kb in mouse, and 4.4 Kb and 6Kb in human and, primarily detected on brain samples being more weakly expressed in kidney, heart, skeletal muscle and pancreas (Figure 13A).

To further specify the expression of Slitrk2 in brain, we performed Northern Blot also on samples obtained from different human brain anatomical regions. With this analysis, we found Slitrk2 transcript throughout the brain with particular abundance in Hippocampus, Hypothalamus and Substantia Nigra (Figure 13B).

This expression is particularly intriguingly since the physiological function of these brain areas.

The hippocampus is the major component of the mammalian brain and is the site where long-term memory is stored [138]. Long term memory is regulated by a mechanism known as Long-Term Potentiation (LTP), that is required for neuronal plasticity and formation of memory-related synapses. For this reasons, hippocampus functional defects are correlated to neuronal dysfunction, giving rise to memory loss as seen in Alzheimer's disease [139].

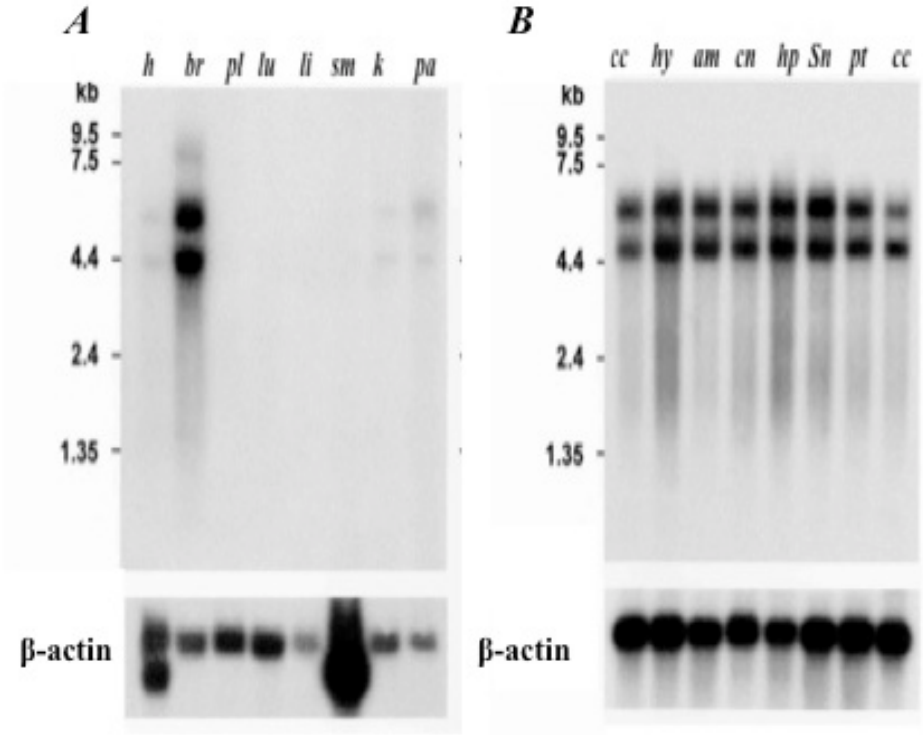

Figure 13. Slitrk2 expression in human adult tissues. *Northern analysis of the polyA mRNA from different human tissues (panel A), and from human brain areas (Panel B). Panel A: h, heart; br, brain; pl, placenta; lu, lung; li, liver; sm, skeletal muscle, k, kidney; pa, pancreas. Panel B: cc, cerebral cortex; hy, hippocampus; am, amygdala; cn, caudate nucleus; hp, hypothalamus; Sn, substantia nigra; pt, putamen; cc, corpus callosum.*

In the hippocampus, *N-methyl-D-aspartic* acid (NMDA) receptor-dependent responses are involved in maintaining neuronal survival, and this survival-promoting effect is frequently regulated by several proteins belonging to the LRR superfamily. For example, it was shown that NMDA-mediated activation of the *Calcium-Calmodulin-Kinase II* (CaMKII) pathway during long-lasting memory and cortical LTP in the CA1 region of hippocampus requires the *Neuronal Leucine-Rich Repeats protein 4* (NLRR4) [140].

Amyloid-beta plaques (Aβ) play a fundamental role in driving the unhealty state of the hippocampal and cortical neurons during AD progression. However, Aβ deposits are extracellular accumulation of β-sheets proteins that are not correctly removed by

old or injured neurons. As such, the Aβ determines neuronal cell death by activating apopotic pathways. The mechanisms by which Aβ activates these pathways are various, and it has been shown that Amyloid Precursor Protein (APP) could interact with several receptors such as the low-affinity Nerve Growth Factor (NGF) receptor p75-NTR [146] and nicotinic acetylcholine receptors [141-144].

An interesting finding was that the *Nogo66 Receptor* (NgR), a protein that shares with Slitrk2 the same extracellular folding (shown later), physically associates with APP, and this association seems to be related to a decrease in APP processing by secretases with reduction of Aβ plaques formation [145]. Moreover, it was suggested that hippocampal dysfunction could be the reason of the long-term memory deficits observed in schizophrenic patients [146].

The hypothalamus is a very small portion of the brain dedicated to the neuroendocrine control of several activities of the autonomic nervous system such as body temperature management, fatigue, sleep and circadian cycles [147]. The hypothalamus receives several input stimuli coming from both the external environment and the internal body that must be integrated into behavioral and humoral responses through outputs exerted by both neural projections and endocrine hormones. Hypotalamic neuron function is tightly regulated by several molecular mechanisms in which also LRR proteins are involved. As an example, the *Extracellular signal-Regulated Kinase* (ERK1/ERK2) activation pathway induced by depolarization in rat hypothalamic suprachiasmatic nucleus, is mediated by the *suprachiasmatic nucleus circadian oscillatory protein* (SCOP) [148].

The substantia nigra (SN) is the brain region deputed to the management of body movement, reward and addiction. It is a symmetric component of the brain since the brain has two SNs, one on each side of the brain midline. Neurons that account for SN are mainly GABAergic and dopaminergic. In these neurons, the *Leucine-Rich Repeats Kinase 2* (LRRK2, also known as Dardarin / Park8) is known to interact with several partners such as *Mitogen-activated protein kinase activating kinase* (MKK) 3/6 or MKK4/7, β-tubulin and moesin [149]. Even though the exact function of LRRK2 is still unknown, while it is clear that LRRK2 plays a role in regulating dopaminergic neuron survival in the SN *pars compacta*, a region involved in the pathology of Parkinson's disease (PD). In fact, dominant mutations in the gene

LRRK2 are the most common cause of inherited PD. The most prevalent LRRK2 mutation, G2019S, lies within the $Mg2^{+}$-binding motif (DYG) of the kinase domain, and has been demonstrated to increase the kinase activity of LRKK2, both in heterologous and autophosphorylation assays. This mutation seems crucial in neurite outgrowth, because forced expression G2019S-LRRK2 in primary cortical neurons leads to a dramatic reduction in neurite length and branching by inhibiting Rab5a [150], a small GTPase required for endocytosis in both the axonal and the somatodendritic domains of polarized neurons [151]**.** For all these reasons, Slitrk2 could be a candidate as one of the players of these mechanisms.

In order to have a complete *in vivo* transcriptional map of the Slitrk2 transcript distribution, we performed an *in situ hybridization* (ISH) on specimens obtained from adult mice (Figure 14). We performed the analysis on sagittal sections of adult mouse brain (sagittal level 16, according to the Brain Atlas) finding that Slitrk2 transcript was detected, following the antero-posterior axis, in the Olfactory Bulb, on the CA1-2-3 regions of the Hippocampus, in the second layer of the cerebral cortex, in the Dentate Gyrus and Cerebellar Cortex. Among these positive regions, the hippocampal compartment showed highest levels of Slitrk2 expression.

The stainings are of particular interest for at least two reasons. Firstly, it has been clearly shown that the Dentate Gyrus plays a key role in the establishment of the associative memory. Loss of memory is one of the features of AD. All the pathological changes that contribute to AD are known to occur in the Dentate Gyrus. In fact, Tau protein-pathology, Aβ-deposits, neuronal loss and modifications of the excitatory–inhibitory neurochemistry all take place during AD establishment and progression particularly in this hippocampal formation [152]. The evidence of Slitrk2 expression in the Dentate Gyrus has to be taken into account since the Dentate Gyrus, together with the Subventricular Zone of the lateral ventricle, is one of the two well-established brain sites in which adult neurogenesis associated with Neural Stem Cells regeneration is activated [153]. For this reasons, Slitrk2 could be potentially considered as one of the mediators in the pathways surrounding one of the mechanisms described above (e.g. Long-Term memory, regulation of synaptogenesis etc.).

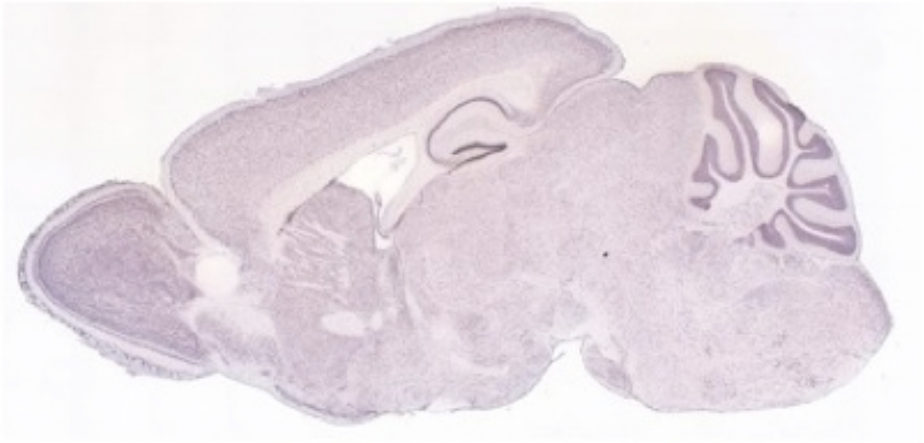

Figure 14. mRNA expression of Slitrk2 in the mouse adult brain. *In situ hybridization of brain sagittal sections of the brain with antisense cRNA probes for Slitrk2. Slitrk2 is expressed in the Olfactory bulb (Ob), in the inner granular layer of the cerebellum (IGL), in the Dentate Gyrus (CA1, CA3) and in the second layer of the cerebral cortex (Cc).*

Slitrk2 RT-PCR expression analysis was performed in human samples to confirm the results obtained by Northern blot analysis. We confirmed that Slitrk2 is expressed in brain and weakly detected in mammary gland, prostate, testis, and pancreas with no amplification in skeletal muscle (Figure 15).

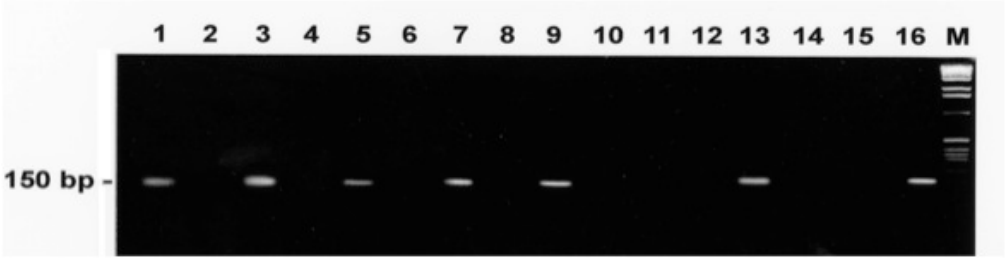

Figure 15. RT-PCR expression analysis of Slitrk2 in human tissues. *Lanes 1,3,5,7,9,11,13: cDNA from mammary gland, brain, pancreas, prostate, testis, skeletal muscle and fetal brain respectively. Lanes 2,4,6,8,10,12,14: mRNA of mammary gland, brain, pancreas, prostate, testis, skeletal muscle and fetal brain after DNase treatment before retro-transcription reaction. Lane 15: PCR negative control with no template. Lane 16: PCR-positive control.*

From Figure 15 we conclude that Slitrk2 could have a wider role also outside the nervous system, for example in regulating morphogenesis/organogenesis. The

expression in mammary gland is intriguingly since this system is constituted by tubular structures, leading to the possibility that Slitrk2 is involved in tubulogenesis (as shown later).

3.2 SLITRK2 STRUCTURAL ANALYSIS AND BIOINFORMATIC PREDICTIONS

In a study focused on the mapping of the Xq27 band it was shown that on the genomic context at the cytogenetic band Xq27.3, spanning between the markers DXS8028 and DXS1200 were located two genes previously unknown [16]. The first gene was found to contain 5 exons, with exon 5 containing a coding sequence for a protein of 845 aminoacids. The second gene *CXorf2*, was contained in the first intron of the first gene. Currently the role of the putative CXorf2 protein is uncharacterized. The gene encoding for the 845 aminoacid-long protein was annotated in 1999 in the EMBL database as *SLIT-L1* [16]. The name SLIT-L1 was proposed because the predicted encoded protein was found to share the same domain distribution seen with the SLIT protein family SLIT (e. g. Leucine-Rich Repeats).

More recently a gene family was identified in a screen for down-regulated genes in mice with neural tube closure defects [17]. One member of this gene family was discovered to be SLIT-L1. The gene family was designated as *Slit-and-Trk like (Slitrk)* because of their similarity with both SLIT and Trk (Tropomyosin-related kinase, the so-called "high-affinity" neurotrophin receptor) family members, and SLIT-L1 was renamed as Slitrk2.

However, the analyses introduced here suggests that the Slitrk family bear little homology with the Trks family. The aminoacidic sequence of the Slitrk familiy proposed to be similar to Trks, the YLDxL consensus, is shown in Figure 16.

In fact, the YLDxL sequence of Trks is similar to the YLxVL sequence in Slitrks and in particular to the YLEVL in Slitrk2, However, the consensus YLxVL and YLDxL, are found in many other proteins as a common binding motif for SH_2-containing proteins, indicating that the similarity between Slitrk2 and TrkA proposed by Aruga *et al.* is not convincing.

Figure 16. Alignment of the intracellular tail region showing similarity between Trks and Slitrks according to Aruga et al. *In purple, Tyrosine can be phosphorylated by PLCγ in Trks. Adapted from [17].*

3.2.1 Slitrk2 domains and Post-Translational Modification (PTM) Analysis

According to UniProt database [19], and some previous studies [17], Slitrk2 is thought to be a putative type I transmembrane protein having a signal peptide (aa 1-21), an N-terminal extracellular domain (aa 22-621), a transmembrane region (aa 622-642) and a short intracellular tail at the C-terminal (aa 643-845). In the N-terminal ectodomain, Slitrk2 contains 14 Leucine-rich Repeats.

3.2.1.1 Signal peptide and predicted subcellular localization of Slitrk2

The initial prediction of the subcellular localization of Slitrk2 was done by looking to the hydropathy profile of its amminoacidic sequence [17]. In this search, Slitrk2 was found to have an hydrophobicity peak between residues from 600 to 690, that corresponds to the 622-642 region of Slitrk2. Based on this aminoacidic profile, Slitrk2 was referred as a transmembrane protein.

As other neuronal predicted transmembrane LRR proteins were found to be not integral to the plasma membrane, we performed comparative analysis using protein subcellular localization prediction databases.

The main topological features of Slitrk2 was carried out using the *PredictProtein* server. The main output from *PredictProtein* relies on secondary structure analysis, PROSITE sequence motifs, low-complexity regions, nuclear localization sites, disulfide bonds, transmembrane regions and directionality [154].

Slitrk2 was found to have a $N_{lumenal}$-$C_{cytosol}$ orientation, with the first 619 aminoacids being outside the cell. After the transmembrane helix (residues 620-640), the

sequences from 641-845 is predicted to be intracellular. No kinase activity domain was found in the intracellular fragment, and no KDEL (Lys-Asp-Glu-Leu) retention consensus and Nuclear Localization Sequences were also found. Apart from its transmembrane domain, Slitrk2 could enter the secretory pathway as a secreted molecule. Also WolfPSORT [155] and HSLPred [156] predicted plasma membrane localization for Slitrk2 with a commitment for the secretory pathway. The signal peptide in the pro-sequence was confirmed by PrediSi [157] and SignalP [158] (cleavage site at 21-22).

Apparently, Slitrk2 has two main features characterizing plasma membrane proteins: a signal peptide and an hydrophobic α-helix of about 20 residues. However, in some cases, proteins showing this topological blueprint could skip membrane localization being retrieved by the Trans Golgi Network (TGN). This retrevial has been shown to correlate with some consensus sequences near to both the transmembrane region and the C-terminus that are recognised by the COP-I coatomer. Among these sequences, the most known are those of the KxKxx family (KKxx) (K=Lys, x=any aminoacid) and RxR (R=Arg, x=any aminoacid). Some of these sequences were found on both Type I and Type II transmembrane proteins [159]. Type I and Type II transmembrane proteins are different in their orientation, with the Type I being directed in a $N_{lumenal}$-$C_{cytosol}$ way, and the Type II having a $N_{cytosol}$-$C_{lumenal}$ directionality [160].

For example, it was reported that some NMDA receptor subunits contain RxR sequences that inhibit their translocation to the plasmalemma. TGN retention is strictly dependent on the activation of the full activity of the integral-membrane anchored NMDA subunits [161]. Other Golgi-retrieving sequences such as KKxx and RxR were found in some Cluster of Differentiation (CD) family members, such as CD8 and CD4 [162]. Interestingly, it was reported that to exert their retrieval activity these KKxx and/or RxR sequences require a transmembrane domain and a particular localization (RxR must be near to the transmembrane domain and KKXX must be near to the C-terminal side) [163].

A detailed screening of the primary sequence of Slitrk2 reveals the presence of some of these targeting signals (Figure 17).

In particular, an evident RK stop-anchor sequence is proximal to the transmembrane region, followed by a KKXX consensus that is C-terminal. In most of the Golgi-

retained proteins, the KKXX sequence is close to the last 10 residues of the protein, as seen in the Isoform 2 (according to UniProt output for Slitrk2 search) of Slitrk2. Given the heterogeneity of these retention sequences, that do not act as consensus but rather as an "aminoacidic environment" that is still poorly characterized, it is likely that the KKXX sequence in the cytoplasmic tail of Slitrk2 act as a Golgi-retention signal. Another consensus was found in Slitrk2 primary protein, KRRK. The KRRK sequence is a consensus accounting for nuclear localization, but such nuclear positioning for Slitrk2 is unlikely even if predicted due to the presence of an hydrophobic transmembrane region.

3.2.1.2 Potential phosphorylation site predictions

The possibility that Slitrk2 has residues that can be phosphotylated have been suggested by Aruga [17] but was suggestion comes from analysis performed without any database screening. Only recently it was shown that Slitrk1 is a phosphorylable protein [164], even if Aruga's work failed to underly any potential phosphorylation site in this protein, suggesting that the phosphorylation status of this family is lacking. According to UniProt database two phosphorylation sites are suggested at Phosphothreonine 588 and Phosphotyrosine 756. We therefore, performed in depth analysis by integrating the outputs from PredictProtein with that of NetPhosK, ScanSite and ELM [165-167]. Several potential phosphorylation sites were found in the Slitrk2 sequence using three parameters: stringency of the result (to obtain statistical value), server cross-matching (validity value) and solvent-accessibility (biochemical value) (Figure 18). We reduced the list of potential phosphorylation sites to only two hot-spots phosphorylation sites. It is to be noted that in the UniProt listing, high-lighted additional sites that could not be cross matched and not introduced here.

It is to be stressed that the phosphorylation observed on Threonine 588 annotated in UniProt comes from a screening for phosphorylated pepides with a kind of high-throughput screening in HeLa cells, that found a phosphopeptide from the reconstitued Slitrk2 sequence [168]. Since this region of Slitrk2 is predicted to be outside the cells, this means that i) this finding is wrong, or ii) the predicted extracellular part of Slitrk2 could effectively reside inside the cell.

>Q9H156|643-845

VLKR**RK**GVPSVPRNTNNLDVSSFQLQYGSYNTETHDKTDGHVYNYIPPPVGQMCQNPIYMQK
EGDPVAYYRNLQEFSYSNLEE**KKEE**PATPAYTISATELLEKQATPREPELLYQNIAERVKEL
PSAGLVHYNFCTLPKRQFAPSYESRRQNQDRINKTVLYGTPRKCFVGQSKPNHPLLQAKPQS
EPDYLEVLEKQTAISQL

>Q9H156-2 |643-733

VLKR**RK**GVPSVPRNTNNLDVSSFQLQYGSYNTETHDKTDGHVYNYIPPPVGQMCQN
PIYMQKEGDPVAYYRNLQEFKTSLENIWRPCLH**KK**

Figure 17. Slitrk2 retention site in Isoforms 1 and 2. *The aminoacidic sequence of the cytoplasmic tail of the two Slitrk2 isoforms is shown. In both Slitrk2 isoforms the RK stop-anchor sequence (in green) is very close to the beginning of the end of the transmembrane region of the protein. Distal from the RK consensus, the KKXX retention sequence is near to the C-terminus (in red). Upper sequence: Slitrk2 reference intracellular tail (from UniProt); Lower sequence: Slitrk2 Isoform 2 intracellular tail (from UniProt).*

The latter modification in Tyrosine 756 was hypotesized by similarity, but the stringency of our approach seemed to exclude any reliability of this suggestion.

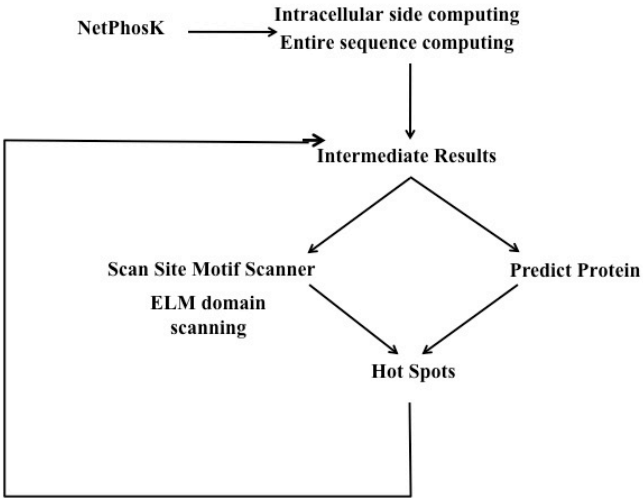

Figure 18. Phosphorylation predictions by computational approaches. *NetPhosK analysis was carried out on both the complete Slitrk2 sequence and on only the intracellular portion of the protein. The results were integrated with those of the Scan Site Motif Scanner and then remapped on the ELM outputs. The list of potential phosphorylation sites was refined according to residue's solvency and accessibility (Predict Protein) and reviewed based on*

available literature on verified functional phosphorylation sites observed in neuronal LRR proteins.

Threonine 806 is located in the consensus **LYGTPR**. According to the ELM scanning, this is a consensus sequence, located in a WW domain (InterPro IPR002349), for Cyclin-Dependent Kinase phosphorylation. Interestingly, this sequence is recognised also as a 14-3-3 protein binding site, raising the question if regulated cell-cycle related phosphorylation could change the availability of this sequence for other binding partners, such as for the 14-3-3 protein, regulating Slitrk2 activity and/or degradation. Among the member of the Slitrk family, Slitrk1 activity and cleavage by secretases has been shown to be regulated by phosphorylation in a 14-3-3 binding site [164].

The other putative phosphorylation site is at the Tyrosine 832, in the PDZ domain (IPR001478) **PDYL**.

As discussed in the introductory section, the PDZ-domain could regulate protein-protein interactions by controlling the intracellular localization of some neuronal LRR proteins. Looking to the ELM results, this site can play a role in protein sorting as Tyrosine-based sorting signals are responsible for the interaction with μ-subunit of the "Adaptor Protein complex". Adaptor protein (AP) complexes are cytosolic heterotetramers that mediate the sorting of membrane proteins in the secretory/endocytic pathway. Tissue-specific sorting events such as the basolateral sorting in polarized epithelial cells, the biogenesis of specialized organelles including melanosomes and synaptic vesicles are all regulated by members of AP complexes [169]. This mechanism of sorting regulation by phosphorylation was demonstrated, for example, in the dynamic control of NMDA receptor membrane insertion by regulated release of NMDA channels from the ER. NMDA receptor activity in neurons is also regulated by Protein Kinase C (PKC)-mediated suppression of ER retention through potential PDZ interactions [161].

After this these analysis we can conclude that Slitrk2 has two main potential phosphorylation sites in their C-terminal side (T806 and Y832), and this suggests that Slitrk2 function, stability and localization could be regulated by post-translational modification (Figure 19).

This stability and regulation control by phosphorylation or that Slitrk2 has phosphorylation sites must be verified *in vivo,* but specific antibodies against the candidate phospho-residues are not commercially available for further immediate analysis.

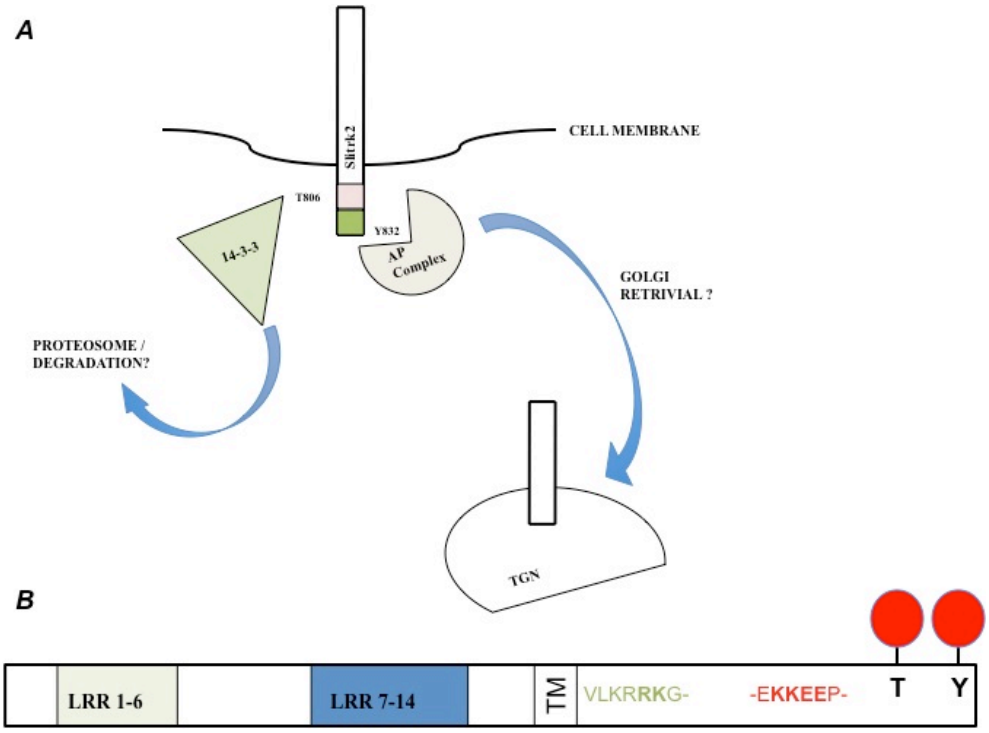

Figure 19. Putative Slitrk2 protein recycling and domain distribution. *Slitrk2 cisternal progression and degradation according to biological-driven predictions (Panel A). Overview of the main computed structural features of Slitrk2 (Panel B; Green: Stop-anchor sequence; Red: c-terminal Golgi-retrieval sequence; T: Threonine 806; Y: Tyrosine 832).*

3.2.2 Three-dimensional structure prediction and comparison

The three-dimensional structure prediction of Slitrk2 was carried out since for functionally uncharacterized proteins, 3D structure analysis is a good way to make strong predictions concerning their functions. The most successful approach for predicting 3D structure is by comparing known structures to the protein of interest (homology modeling) since the number of foldings in nature is limited [170]. Different remotely homologous protein sequences, indeed, adopt remarkably similar

structures. If a folding homolog can be found, an alignment of two or more sequences can generate a 3D model of the sequence of interest.

Slitrk2 3D structural modeling was carried out using Phyre, a pure fold-recognition service and I-TASSER, a fold recognition server site generating results that are refined with *ab initio* calculations [170-171].

The complete and the extracellular and cytoplasmic portions of Slitrk2 were all used as input for predictions, but significant outputs were only obtained for the extracellular ectodomain of Slitrk2. For the cytoplasmic tail, both the prediction servers gave results with poor statistical values (see Materials and Method section).

In detail, the structures with highest predicitions values from Phyre obtained using the complete Slitrk2 sequence were all for the extracellular domains of protein involved in ligand-binding or protein-protein interaction. Members of the Toll-like Receptor family (TLR3, 4, 2) and neuronal proteins such as Lingo-1 were found to be best templates. The same results and templates were obtained with the prediction on the extracellular part only when modeling was performed using only the first 620 residues of Slitrk2 or extracellular domains. No significant predictions were obtained with the sequence spanning the cytoplasmic tail, and no significant correlation with the Trk family or other related kinases were obtained.

I-TASSER produced 5 different models for the various sequences submitted. The 5 models obtained using the complete sequence and the extracellular part were quite similar, since the prediction obtained with the entire sequence was related solely to the residues belonging to the ectodomain. All these models were found to have an horse-shoe shape folding, typical of proteins enriched in LRR. Also in this case, the templates for comparison purposes used by I-TASSER are involved in protein-protein interactions or ligand-bindings. The two principal hits used by I-TASSER to elaborate the model were the PDB records 1ziwA and 2z64A. The first represents the extracellular domain of the Toll-like Receptor 3, and the latter is the Nogo Receptor extracellular part.

A 3D I-TASSER model from the prediction of the extracellular part is shown in Figure 20 with the secondary structures distributions agreeing with those predicted by Predict Protein. The program PDB viewer was used to determine that the domain distribution of the 3D model was in agreement with the LRR distribution obtained with the secondary sequence analysis.

Only one of the five potential N-glycosylation sites (Asn 84) predicted by Net-N-Glyc [172] is solvent-accessible and located in the convex side of the LRR1. This kind of location is often seen in glycosylated, LRR-enriched proteins [70].

The I-TASSER model for Slitrk2 showed similarity with a feature seen in both the Lingo-1 ectodomain and NogoR crystal structure [70, 173] and showed evident "capping" at both the N-teminal and C-terminal sides of their LRRs. In particular, Cysteines 29 and 35 appear to be close, in a disulphide-bond favoring distance. The same was observed for the Cysteines 27-33 and 3-9 of NogoR and Lingo1, respectively. The vicinity of Cys 533-Cys 558 and Cys 535-Cys 578 of the Slitrk2 3D model was recapitulated by that of Cys 334-Cys 357 and Cys 336-Cys 382 of Lingo1. This means that Slitrk2 LRRs could be "capped" at both the amino- and the carboxy-terminus. This capping, as previously discussed is often found in LRR proteins involved in exo/endocytosis mechanisms.

At the convex side of the generated 3D model there are three hydrophobicity clusters exposed: Leu73-Leu171, Phe198-Leu249 and Phe364-Tyr434, suggesting a direct involvement of Slitrk2 in ligand-binding. The last hydrophobic patch falls into a low-complexity region, making it attractive as a candidate for characterizing the ligand-binding properties of Slitrk2.

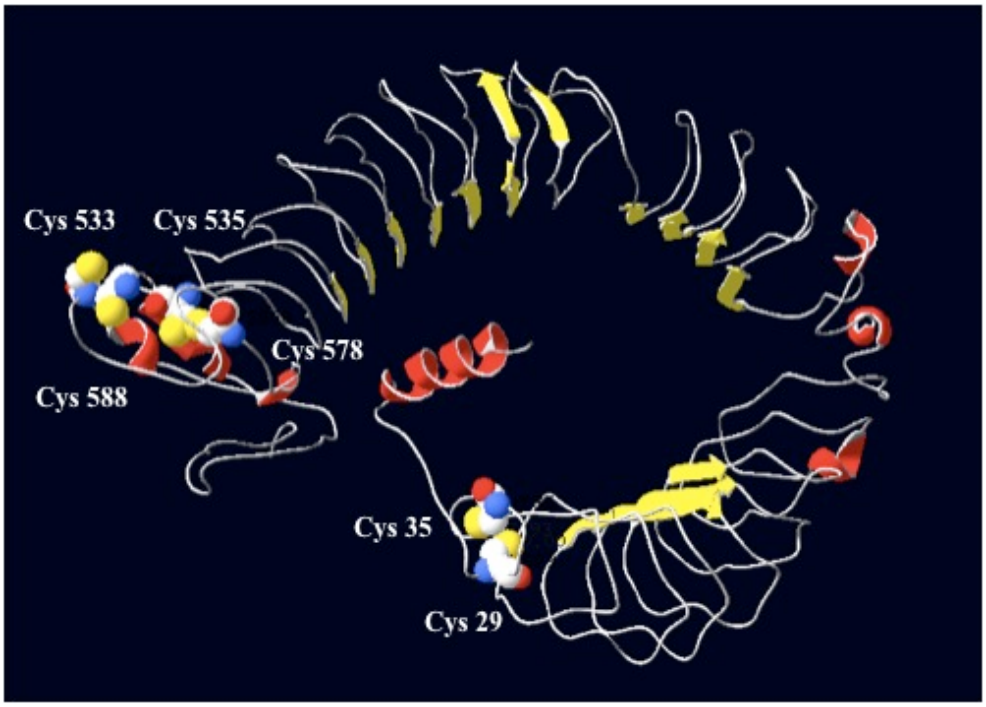

Figure 20. I-TASSER model of the Slitrk2 ectodomain. *N- and C-caps are high-lighted with Cysteines side chains. Yellow: LRR beta sheets.*

3.3 SLITRK2 FUNCTIONAL STUDIES

3.3.1 Role of Slitrk2 in rat PC12 cell differentiation

The role of Slitrk2 in neuronal cell differentiation is unknown. A presumptive role of Slitrk2 in inhibiting neurite extension was previously shown but not fully characterized [17]. We performed immunofluorescence analysis to detect Slitrk2 localization in PC12 cells during NGF-mediated differentiation. PC12 is a cell line derived from a rat pheochromocytoma that acquires a neuronal-like phenotype, as seen in primary sympathetic neurons, after exposure to NGF with decrease in cell proliferation and extension of neurite processes [174]. We chose this cell line as a system to study sympathetic neuron differentiation.

By immunofluorescence (IF) analysis, we found that Slitrk2 subcellular localization is mainly intracellular in PC12 naive cells, and displayed polarized expression along

one side of the perinuclear boundary. During NGF-mediated differentiation, Slitrk localization did not change, but decreased in expression in a time-dependent manner. Cells being treated for 7 days with NGF showed very poor Slitrk2 staining. In differentiation-committed cells, Slitrk2 seems to be localized at the growth cone of nascent neurites (Figure 21A).

To quantitate the extent of the time-dependent decrease in Slitrk2 staining detected by IF, we performed quantitative Real Time PCR (qRT-PCR) analysis in order to confirm whether the Slitrk2 transcript was down-regulated during neurotrophin-mediated differentiation (Figure 21B). After 24hrs of NGF stimulation, Slitrk2 expression levels dropped significantly with respect to the level of naive cells and this level was maintained for 7days of stimulation.

This suggested that Slitrk2 is regulated by NGF in a direct or indirect manner. We therefore checked Slitrk2 expression levels in NGF-treated cells for 3 days and collected the cells at various time points after NGF withdrawal. As shown in Figure 22, the Slitrk2 levels were up regulated after NGF removal. Slitrk2 transcript enrichment was linear during the first 6 hours after NGF withdrawal, with expression stabilizing at 48hrs.

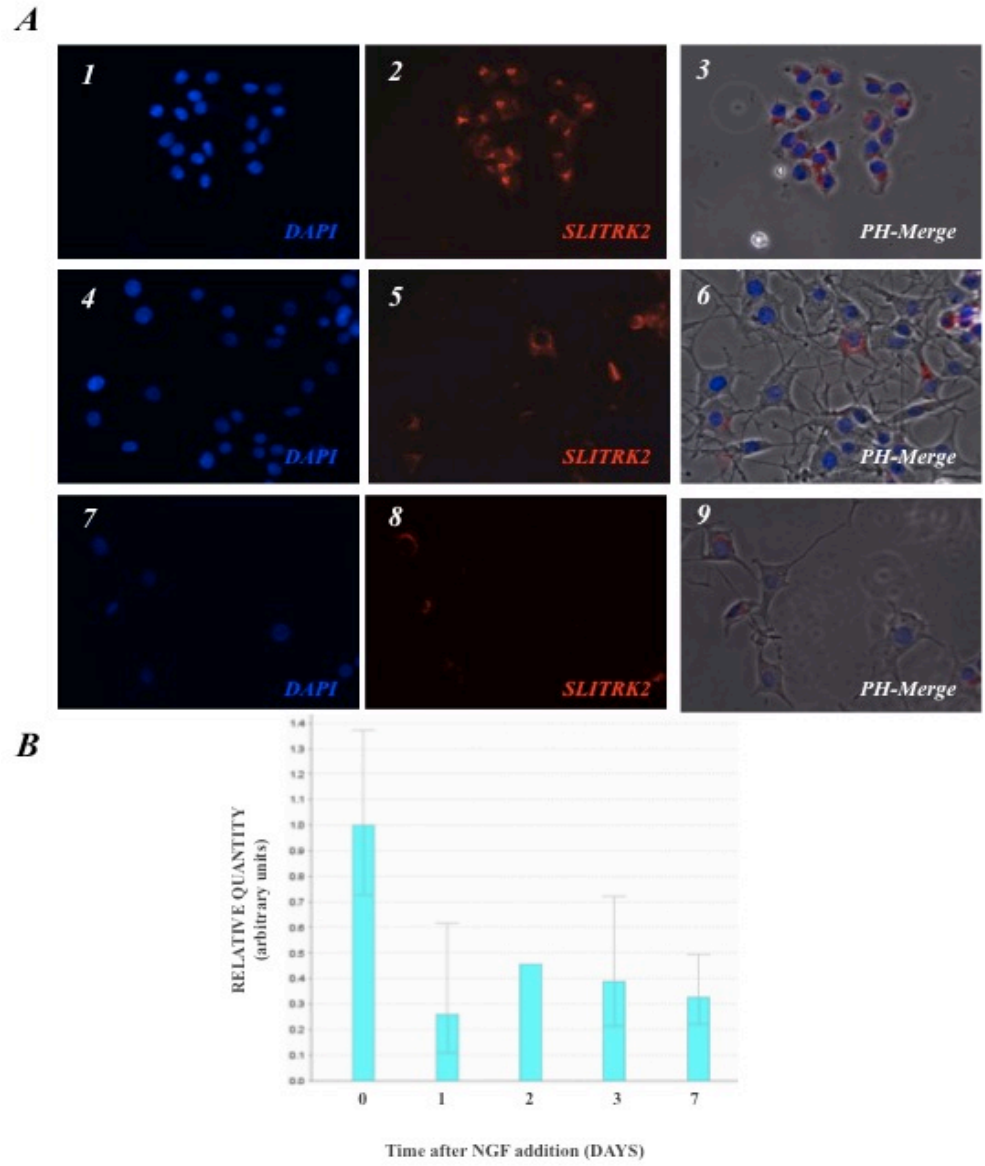

Figure 21. Slitrk2 protein localization and mRNA expression analysis in PC12 cells. *(Panel A) Immunofluorescence staining for Slitrk2 in PC12 cells: naive (1-3) and NGF-treated for 3 days (4-6) or 7 days (7-9). Magnification 40X. (Panel B) Quantitative PCR analysis of the Slitrk2 transcript levels in PC12 cells during NGF stimulation.*

The analysis was not carried out after 48 hours because NGF-committed PC12 cells deprived of NGF and maintained in serum-free conditions undergo cell death. Also,

NGF withdrawal does not induce a full plasma-membrane translocation of Slitrk2 (Figure 23).

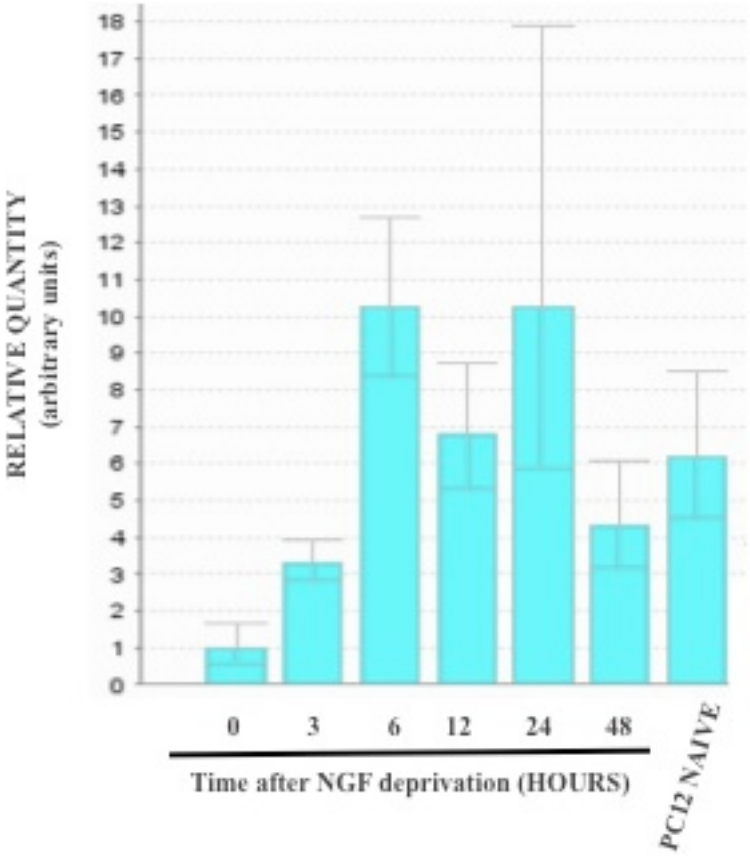

Figure 22. NGF withdrawal induced Slitrk2 mRNA upregulation. *qRT-PCR on RNA obtained from NGF-differentiated PC12 cells after NGF withdrawal showing a time-dependent increase of Slitrk2 transcript.*

We can hypotesize that Slitrk2 is a gene whose transcription is repressed by NGF stimulation, at least in PC12 cells, and that Slitrk2 could play a role during neurotrophin-depletion cell injury.

It is known that NGF activated signalling during differentiation is mainly related to the activation of the "high affinity" neurotrophin receptor TrkA. One of the main downstream targets of TrkA activation leading to neuronal cell differentiation is the

c-Fos transcription factor [175]. However, in a screening for potential promoter sites upstream the Slitrk2 genomic locus, we failed to find any c-Fos binding sites, suggesting that NGF regulates Slitrk2 transcription only in an indirect manner. In support, another study focused on the identification of Immediately Early Genes (IEGs) regulated by NGF in PC12 cells failed to identify Slitrk2 as a gene whose transcription is directly linked to NGF stimulation [176].

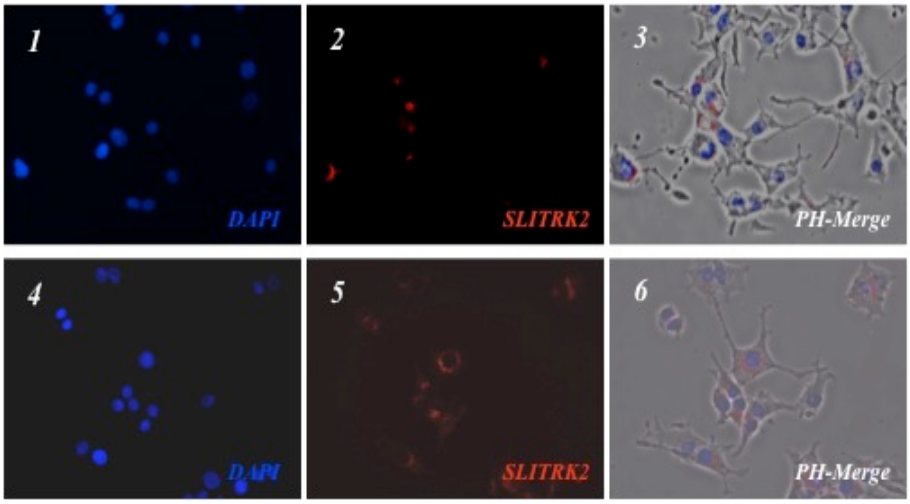

Figure 23. Immunofluorescence analysis of Slitrk2 in PC12 cells after NGF deprivation. *Immunostaining analysis showed that the upregulation of Slitrk2 detected by qRT-PCR after NGF-withdrawal for 24hours (4-6) is due to two contributions: an increase in Slitrk2 expression at at the single-cell level and an increase of the total number of Slitrk2-expressing cells compared to control-differentiated cells (1-3). Magnification 40X.*

These findings suggested that Slitrk2 is downregulated with NGF-mediated differentiation of PC12 cells, but this downregulation is not directly regulated by NGF-activated downstream signaling.

Two further questions came up after these experimental efforts. The first is if i) Slitrk2 down-regulation could be a mechanism that by itself is able to commit PC12 cells to differentiate, and the second ii) whether Slitrk2 may be involved in neurite retraction and/or cell stress mediated response after NGF withdrawal in NGF-differentiated PC12 cells. Neurite retraction, in fact, is often seen in NGF-treated PC12 cells after NGF removal.

To test the first proposal, we analyzed the effect of the *in vitro knock down* of the Slitrk2 transcript in PC12 by using short-interfering RNA (siRNA). As shown in Figure 24A, Slitrk2 siRNA treatment without NGF did not induce any differentiation. Furthermore, since PC12 cell differentiation is accompanied by a decrease in cell proliferation, we checked the cell proliferation profile of Slitrk2-siRNA treated cells, and we observed that PC12 cell proliferation was not affected (Figure 24C).

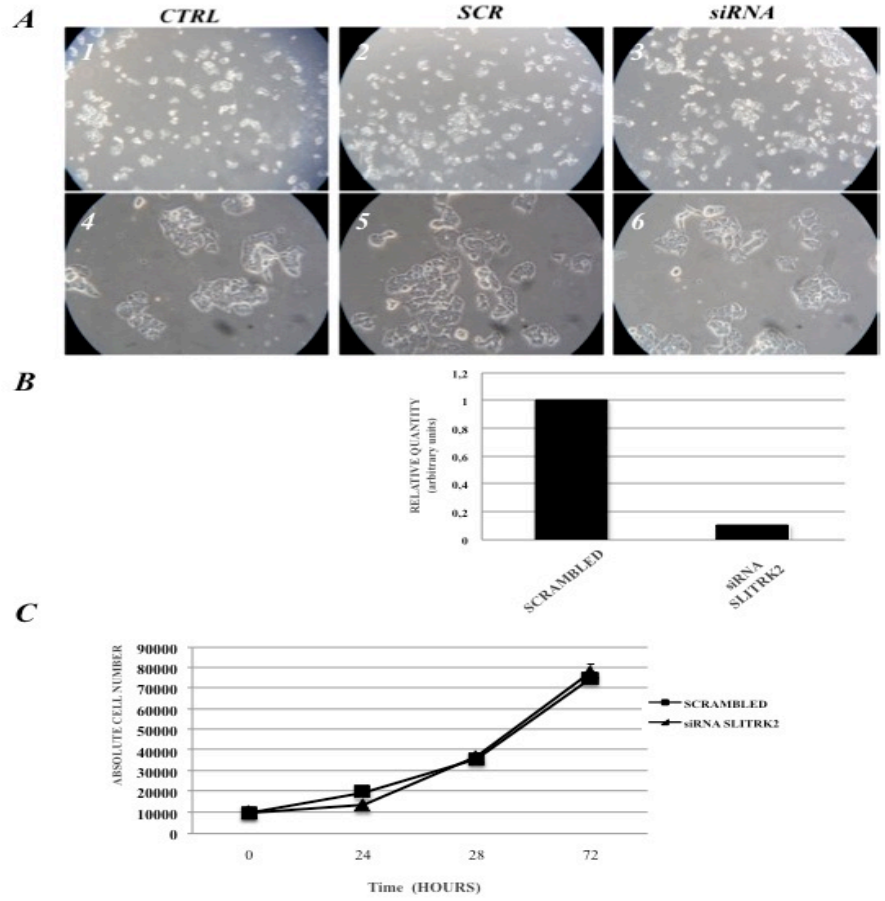

Figure 24. Slitrk2 down regulation is not sufficient to induce differentiation of PC12 cells. *Treatment with 5μM of Slitrk2-siRNA for 72hours does not promote neurite sprouting (Panel A; 1,4: untreated; 2,5: scrambled-siRNA-treated cells; 3,6: Slitrk2 siRNA treated cells; 1-3: 10X magnification, 4-6: 20X magnification) or alter cell proliferation (Panel C). Efficacy of Slitrk2 transcript silencing was assessed by qRT-PCR (Panel B).*

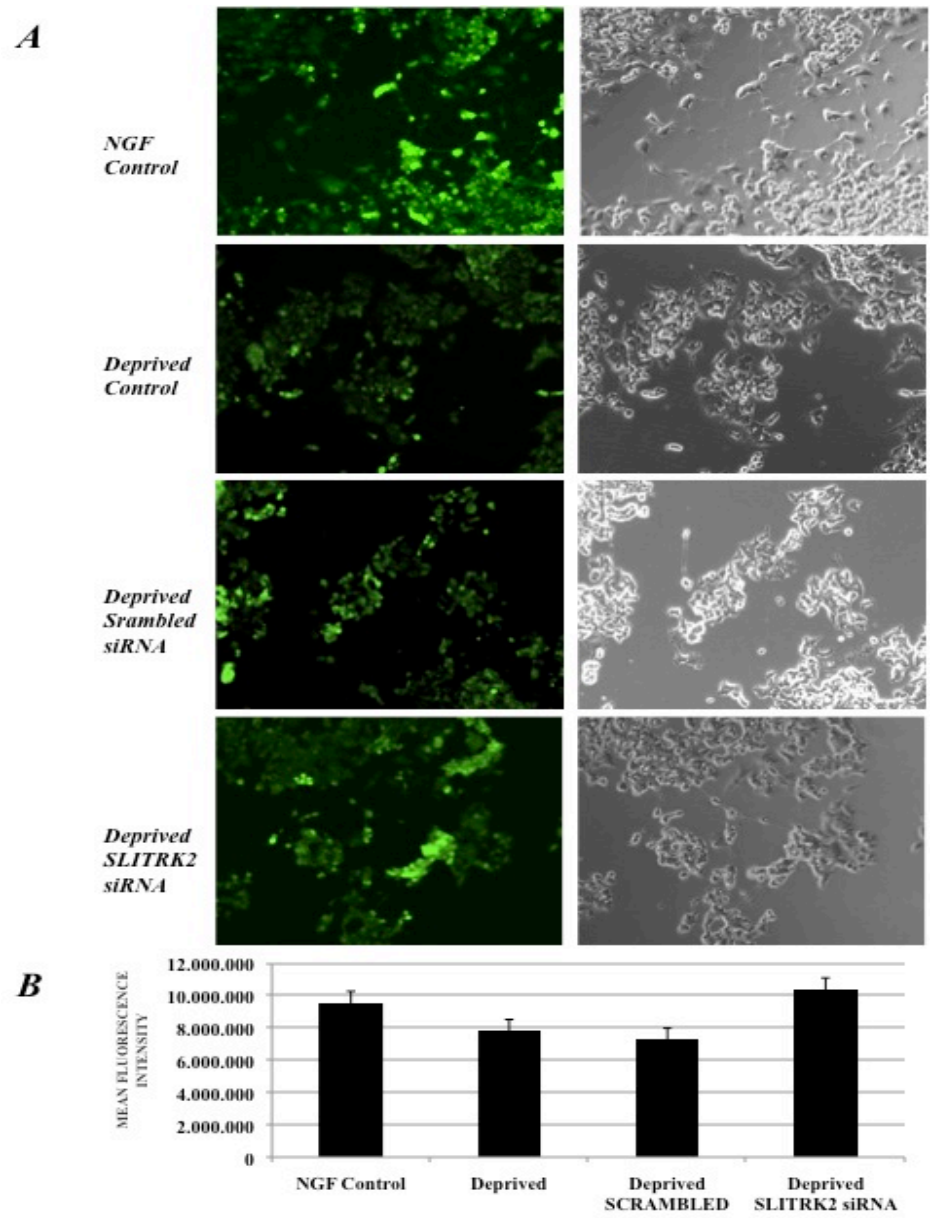

Figure 25. Slitrk2 down regulation prevents NGF withdrawing induced cell injury. *Cell Tracker Green staining of PC12 cells subjected to NGF deprivation showed a reduction in the metabolic activity that is rescued by Slitrk2 silencing. Panel A: fluorescence microscopy analysis, magnification 20X; Panel B: densitometric analysis of cell mean fluorescence intensity (expressed in arbitrary values) carried out with the NIH-ImageJ software.*

Taken toghether, these results showed that Slitrk2 downregulation is a mechanism necessary but not sufficient for PC12 cell differentiation.

The second question not addressed so far was if Slitrk2 could be involved in cell induced damage after NGF withdrawal. In order to address the second question, we performed a cell viability assay by evaluating the intensity of staining of NGF-differentiated cells subjected to NGF depletion in presence or absence of Slitrk2 siRNA with the Cell Tracker Green dye assay. The Cell Tracker Green assay is a cell-permeable dye used to evaluate cell viability [177] since the intensity of the green fluorescent signal evaluated by, for example, fluorescence microscopy, is directly related to the metabolic state of a healthy cell. With this approach, we found that inhibiting re-expression of Slitrk2 (with Slitrk2 siRNA) after NGF removal from differentiated cells did not results in a concomitant decrease in the number of cells with fluorescent staining and the intensity of fluorescence which was instead observed in both Scrambled-treated and withdrawed control samples (Figure 25). To see if Slitrk2 is involved in general in cell-damage, we checked Slitrk2 expression levels in PC12 cells subjected to radical oxygen species (ROS)-mediated stress. For this purpose, qRT-PCR analysis of the Slitrk2 transcript abundance in cells treated with Hydrogen Peroxide (HP) revealed that HP treatment does not change significantly its relative quantity (Figure 26).

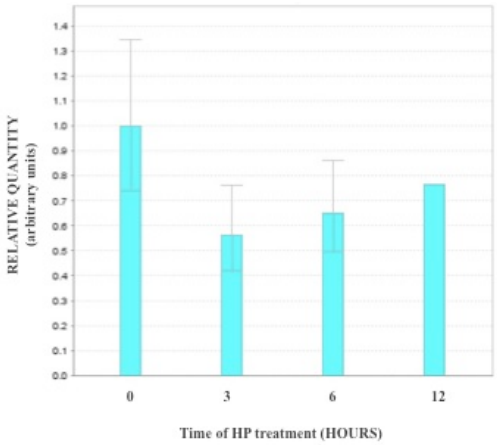

Figure 26. Slitrk2 transcript levels are not influenced by radical oxygen species induced stress in culture conditions. *qRT-PCR was carried out on PC12 cells treated with hydrogen peroxide for different times.*

Our findings that Slitrk2 transcript downregulation occurs during treatment of PC12 cell with NGF without being the driving force for cell differentiation and previous observation that Slitrk2 forced expression limits the differentiation properties of PC12 cells [17], leaded us to try to understand the molecular mechanisms that correlate Slitrk2 and PC12 cells differentiation. To understand the molecular mechanisms that correlate Slitrk2 and PC12 cells differentiation, we generated a Slitrk2-encoding vector containing the human Slitrk2 coding sequence [178].

The efficient subcloning of the human Slitrk2 coding sequence into a mammalian cell-expression vector was verified by both sequencing and digestion with specific restriction endonucleases (Figure 27).

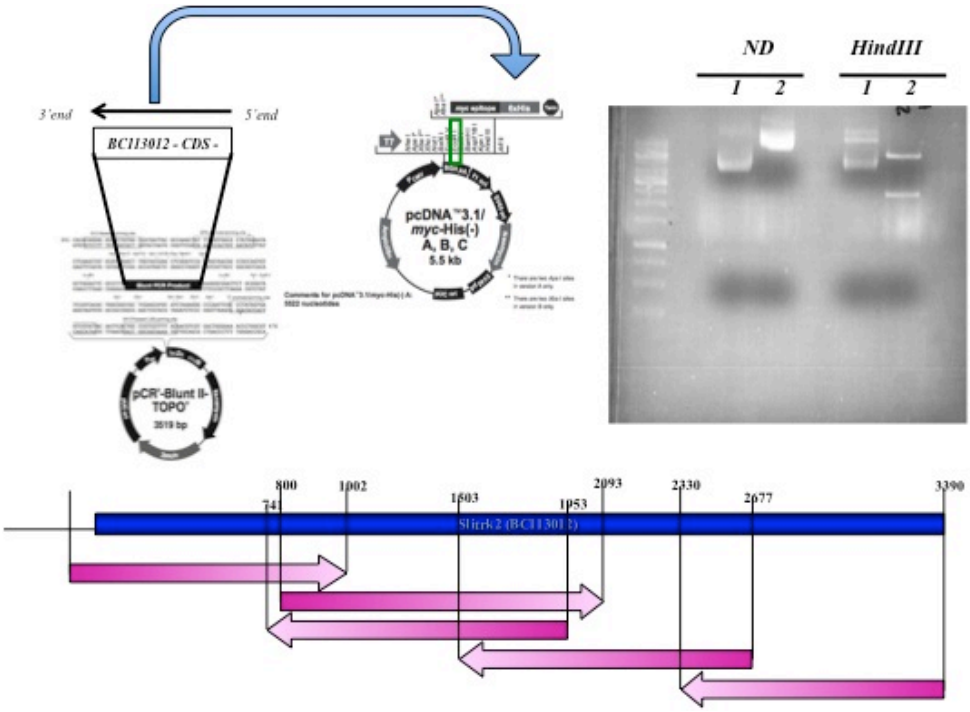

Figure 27. Slitrk2 expression vector construction strategy. *The Slitrk2 coding sequence was obtained from commercially available IMAGE clones and then subcloned in the pcDNA3.1(-) plasmid for expression in mammalian cells. Insert directionality and sequence verification were carried out by both enzymatic digestion and sequencing with appropriate primers.*

The transient forced expression of Slitrk2 into PC12 cells was in effect able to reduce the ability to induce neurite sprouting in the presence of NGF (Figure 28). This mechanism was found to be related to an inhibition of the correct architecture of Neurofilaments but not to some general inhibition of needed transcriptional events needed for differentiation.

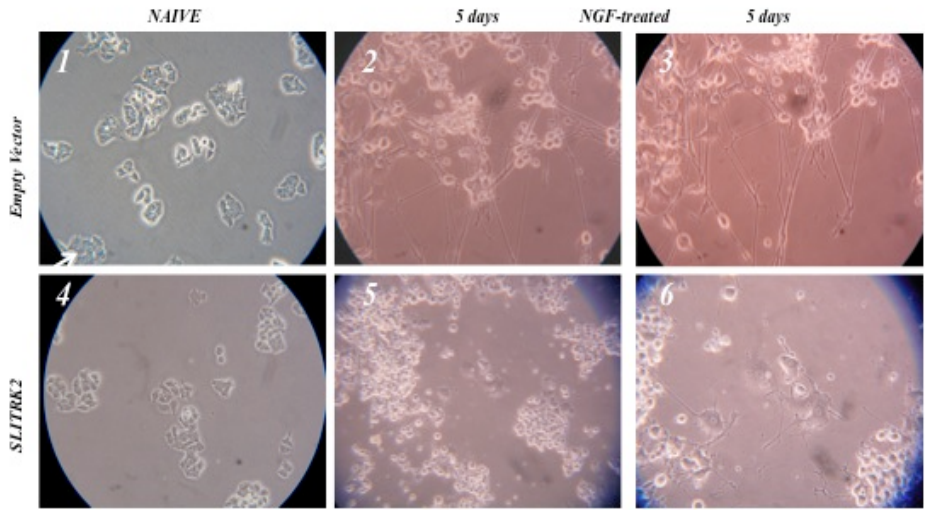

Figure 28. Slitrk2 forced expression reduces PC12 cells responsiveness to NGF induction. *While the overexpression of Slitrk2 does not change the morphology of naive cells Slitrk2 inhibits neurite sprouting and synapse formation (white arrows) after NGF treatment for 5 days. Magnification 20X (1,2,4,5); 40X (3,6).*

The main intermediate filament protein in differentiated PC12 cells, Peripherin, seems to be unable to spread into the nascent neurites in Slitrk2-transfected cells (Figure 29A). However, the transcription factors DLX5 and Isl-1 are neither excluded from the nucleus by or downregulated by the overexpression of Slitrk2, indicating that this mechanism does not account for the Slitrk2-related inhibition of differentiation (Figure 29B-C). We chose DLX5 and Isl-1 since these two molecules are shown to be expressed in PC12 cells and play a key role in neuritogenesis and neuronal precursor migration [179-181]. The previously shown Slitrk2 localization (Figure 22) asked us if this molecule could exert its function as a regulator of exo/endocytosis, since they are, toghether with microtubule reorganization, the main

mechanisms by which a cell completes its differentiation program. In fact, axon/dendrite outgrowth requires not only actin remodeling but also a very tight regulated re-cycling of molecules at the neurite tips [116]. For this reason, we checked the effect of Slitrk2 overexpression also in mediating Syntaxin1 (HPC-1) granule accumulation and localization. This analysis failed to reveal any different profile of cytosolic granulation and neurite end tips localization for HPC-1 in Slitrk2-transfected cells compared to empty vector-transfected cells (Figure 29D).

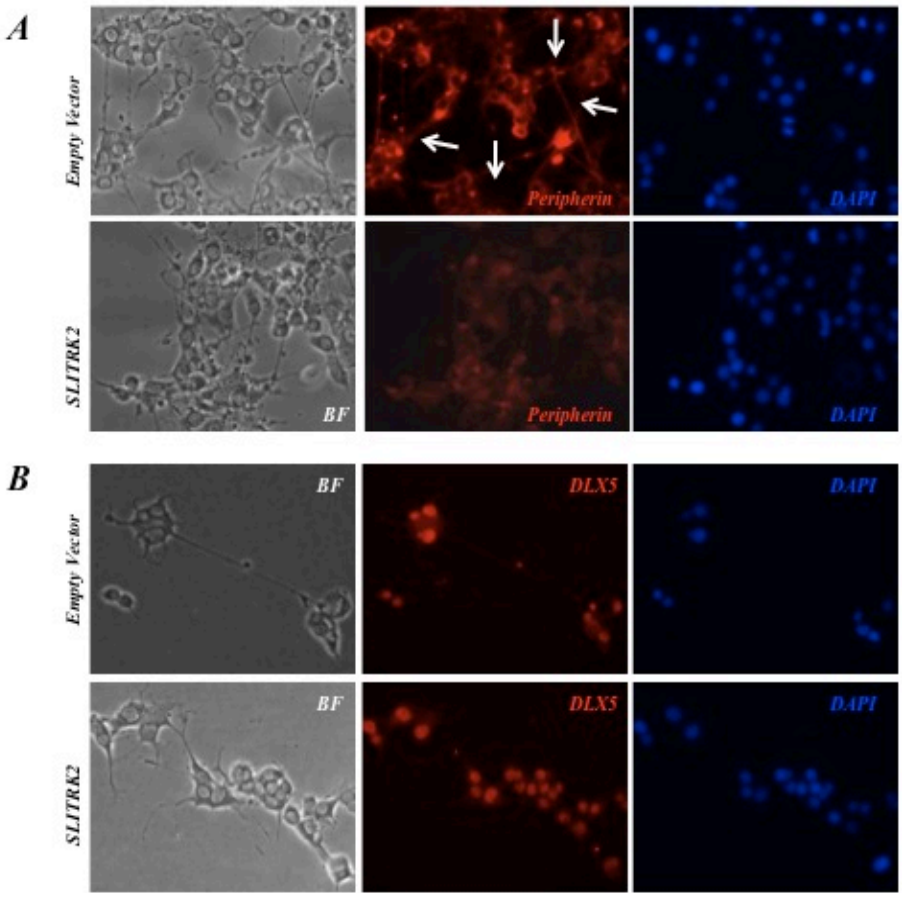

Figure 29. Slitrk2 impairment of cell differentiation is related to poor Neurofilament assembly. Continued next page.

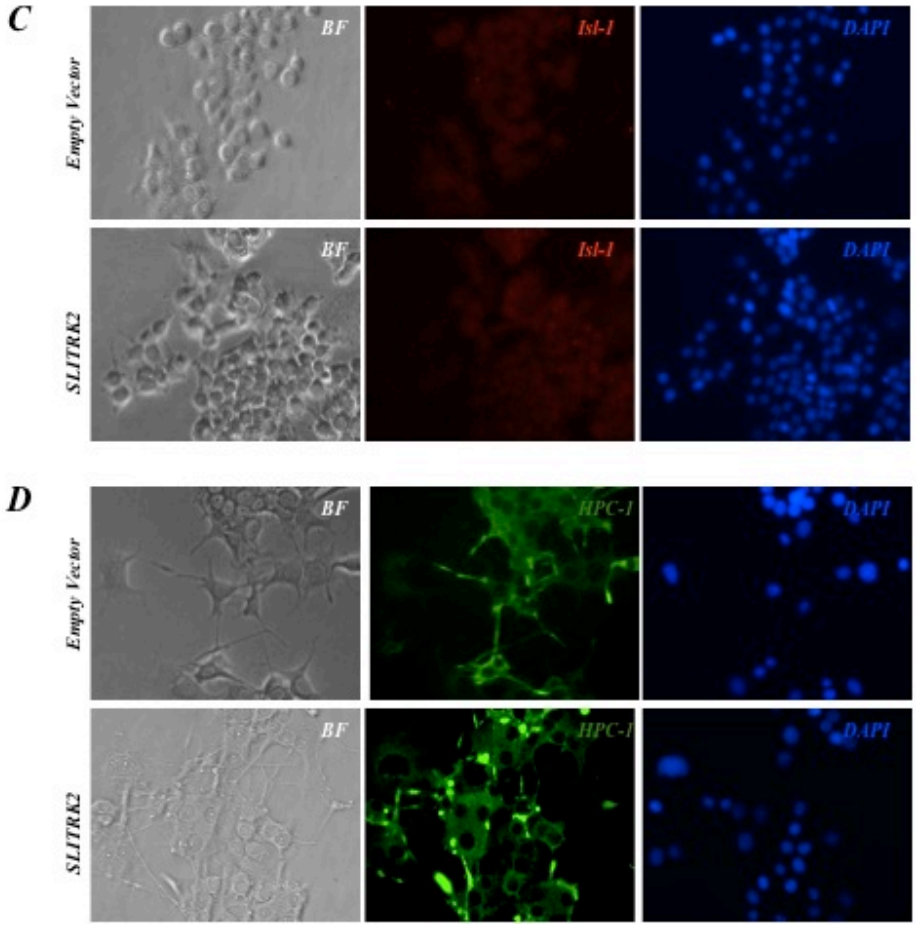

Figure 29. Slitrk2 impairment of cell differentiation is related to poor Neurofilament assembly.. *In Slitrk2-transfected treated with NGF for 3days, Peripherin does not assemble in a neurite-functional manner, limiting their efficient sprouting (Panel A). This inhibition of differentiation does not correlate with transcription DLX5 or Isl-1 mislocalization (Panel B and C, respectively). In some Slitrk2-transfected cells, a certain number of cells with undifferentiated phenotype showed reduction in Syntaxin granulation at the growth cones (Panel D). Magnification 40X.*

After these observations, we can conclude that Slitrk2 is involved in NGF-mediated differentiation, being downregulated during neuronal phenotype commitment of PC12 cells. Its function is likely to be related to an impairment of differentiation and to a cell damage state, not in general but rather linked exclusively to the depletion of

neurotrophins. This inhibitory effect is related to a mechanism involved in a successful neurofilaments sprouting and assembly.

3.3.2 Expression in brain cancer and relationships with stemness

As underlined in the introductory part of this thesis, Slitrk family members in general were proposed to be differentially expressed in brain tumors and to be related to a stemness state of hematopoietic cells [17, 117]

In order to unravel this issue in the neuronal tissue, we performed qRT-PCR analysis on RNA samples obtained from brain cancer cell lines. We used cell lines derived from tumors of both neuroepithelial (neuroblastoma, SK-N-BE) and glial origin (astroglioma, U87).

Levels of Slitrk2 in the cells cultured in standard adherent conditions were found to be down regulated in all the cell lines assayed compared to the human brain RNA reference (Figure 30).

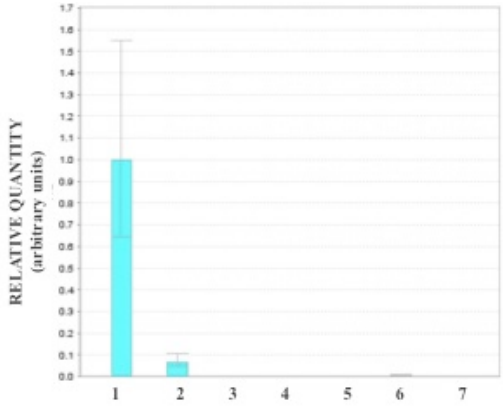

Figure 30. Slitrk2 is downregulated in brain cancer cell lines. *qRT-PCR showing the Slitrk2 transcript down regulated in glial (2-5) and neuroepithelial tumor cells (6-7). 1: whole human brain; 2: U87; 3: U373; 4: primary glioblastoma; 5: clinical recurrence of 4; 6: SKNBE, 7: SKNMC.*

Among these cell lines SK-N-BE and U87 contain a side population of cells with stem cell properties [182, 183] and may be cultured as “neurospheres". Neurospheres (NS) are compact structures obtained from and contain cells with stem cell properties when cultured in non-adherent conditions.

In samples from 7 days-old NS from U87 cells we found a slight down-regulation of Slitrk2 expression compared to its adherent counterpart altough this is not a direct correlation between Slitrk2 expression and stemness (Figure 31).

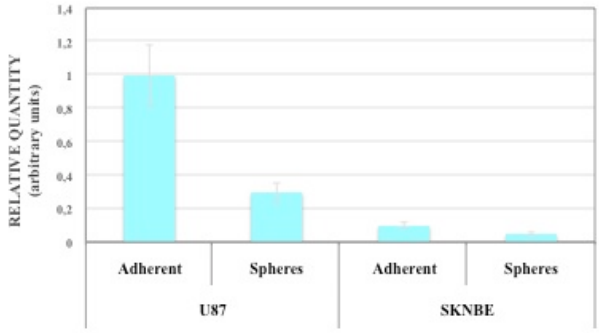

Figure 31. Slitrk2 expression levels are weakly downregulated in neurospheres. *Neurospheres cells showed an weak decrease in Slitrk2 expression, appreciable only in U87 cells but not in SK-N-BE cells.*

The sphere generation is considered a differentiation assay so we would expect less stem/progenitor cells in spheres than in adherent cultures for stem cell (SC) lines that evolved as adherent cultures. Down regulation or low levels of a SC marker in spheres in normal. Then, this result correlate with that obtained in PC12 cells, indicating that Slitrk2 is downregulated in differentiation assays.

Furthermore, Slitrk2 knocking-down with siRNA Slitrk2 failed to modify the neurosphere-formation properties of U87 cells compared to scrambled-siRNA control (Figure 32).

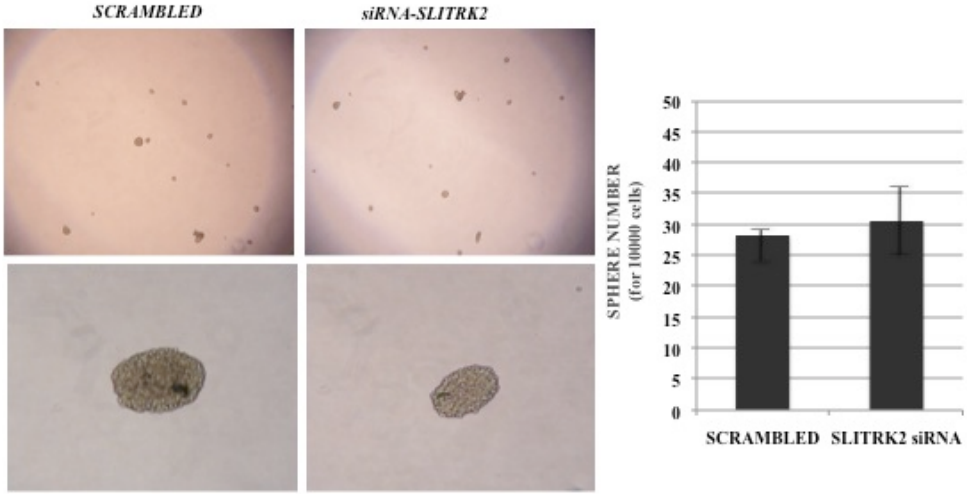

Figure 32. Slitrk2 expression does not improve the sphere numbers of both glioblastoma and neuroblastoma cells. *Phase-contrast pictures of 7days-old neurospheres obtained from 3-days pretreated (with Slitrk-2 siRNA or Srcambled siRNA) U87 cells (Upper panel: 10X magnification; Lower panel: 40X magnification; Right panel: manual sphere counting).*

3.3.3 Slitrk2 as a general regulator of cell differentiation: lessons from non-neuronal cell systems

As seen until this section, we provide evidence that Slitrk2 is a negative regulator of neuritogenesis in PC12 cells. This role, as seen in SK-N-BE and U87 cells, seems to be unrelated to a stem-cell phenotype induction of Slitrk2, leading to the hypothesis that it acts as a general regulator of cellular morphogenesis required during axon sprouting.

Cell morphogenesis is not an unique feature seen during neuritogenesis, and this event does not take place only during neuronal cell differentiation. Indeed, this is fundamental to many aspects of cellular function in a developmental context, including cell division and differentiation, response to physiological stimuli and motility. For example, both angiogenesis and epithelial tubulogenesis are clear examples of cell morphogenesis. In all these cases, actin remodeling and microtubules reorganization and membrane dynamics are known mechanisms regulating the morphological changes that account for morphogenic transitions, that

in most cases are accompanied by changes in total cell surface area, as illustrated in epithelial morphogenesis and neuritogenesis [184].

Several molecular players of these cellular events are commonly needed by cells of different origing during cell morphogenesis. In the previous chapters, we showed that Slitrk2 is expressed, even at low levels, in tubuli-forming organs such as, for example, mammary gland.

The organization of epithelial cells into tubular structures (Epithelial tubulogenesis), is crucial for the development of several organs. In vertebrates, tubular organs often contain many different cell types with localized distribution and specialized functions (such as fluid secretion or gas exchange). Tubulogenesis needs dynamic and interdependent cellular processes, namely cytoskeletal reorganization, assembly of intercellular junctional complexes, and cell polarization. This process, *in vivo,* involves the interaction of different cell types and various environmental cues. In vertebrates, tubules come from two main mechanisms: the invagination of cells from an epithelial sheet, as occurs in the formation of the neural tube or through the organization of initially unpolarized cells into cord-like structures that invade the surrounding mesenchyme, forming branched hollow tubules lined by polarized cells [185**,** 186].

It is quite easy to expect then that several molecules play similar role in both neuritogenesis and mammary gland tubulogenesis. An unexhaustive example of them are, as an example related to different mechanism commonly underlying tubulo- and neuritogenesis, Slit and Semaphorin proteins (as guidance cues), RhoA (small GTPase involved in cytoskeletal remodeling and polarity), Connexins (gap junctions), Rab5 (endocytic trafficking controller) and Wnt family members [187-194].

Driven from these evidences, we attempted to evaluate the role of Slitrk2 in mammary gland tubulogenesis.

For this purpose, we used two different cell lines, one with normal (non tumorigenic) phenotype, MCF10A [195], and one with tumorigenic properties associated with a stem-cell molecular signature [196].

A preliminary qRT-PCR screen showed that in both cell lines Slitrk2 expression is quite undetectable (Figure 33), suggesting that it is not linked to a cancer phenotype in breast cells.

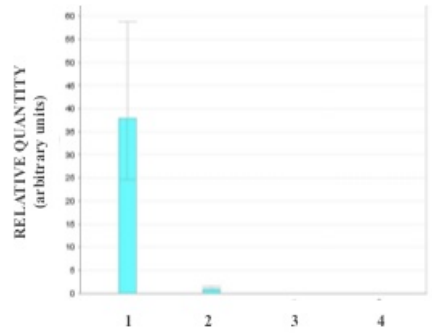

Figure 33. Slitrk2 expression in normal and tumoral brest cell lines. *1:Brain reference control; 2: Human mammary gland; 3: MCF10A cells; 4: LA7/3F12 cells.*

To unravel the effect of the ectopic expression of Slitrk2 in these cells, we performed a "tubuli assay" by embedding the cells in a rat tail collagen matrix. This system enables breast cells with tubulogenic potential to grow as organized, three-dimensional, branched structures.

Despite an unchanged morphological aspect in adherent growing conditions, both Slitrk2-transfected LA7 and MCF10A cells showed a reduction in tubuli formation and branching, with cells organized in rounded-alveolar-like structures (Figure 34).

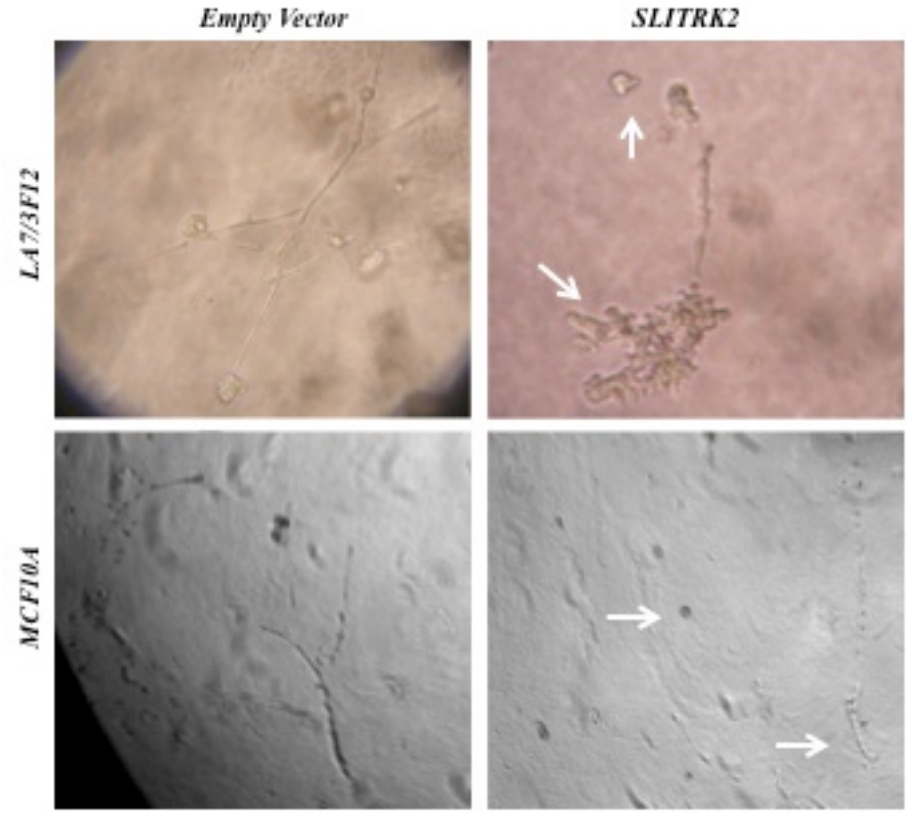

Figure 34. Slitrk2 ectopic expression inhibits tubulogenesis in mammary epithelial cells. *Mammary epithelial LA7/3F12 and MCF10A cells transfected with a Slitrk2-encoding plasmid are inhibited in their tubuli formation properties in collagen (white arrows). Magnification 10X.*

By analyzing the levels of the stem-cell transcription factor T-box3 (TBX3), we confirmed that this inhibition of differentiation is unrelated to a stemness state of the cells, as previously shown in SK-N-BE, since the levels of TBX3 are unaffected in Slitrk2-transfected cells compared to empty vector-transfected cells (Figure 35).

In both LA7 and MCF10A cells, moreover, Slitrk2 localization is similar to that seen in PC12 cells, indicating that it is mostly localized intracellularly rather than at the plasma membrane (Figure 36) independently from any protein folding-impairment mechanism, sometimes responsible for the intracellular accumulation of plasma-membrane resident protein in transformed, tumoral cells [197].

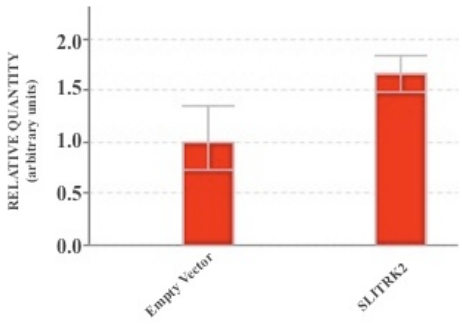

Figure 35. Tbx3 expression levels in La7/3F12 cells are not influenced by Slitrk2. *Relative transcript quantity of Tbx3 showing that the rdcuction in tubuli formation in Slitrk2-transfected cells are not related to a stem-cell promoting effect of Slitrk2.*

According to these findings, we can conclude that Slitrk2 act as general inhibitor of differentiation. This function is not achieved by a direct relationship with cell stemness, but it acts downstream as a partner of the complex molecular systems required for the morphological cellular changes that take place during cell differentiation and specialization.

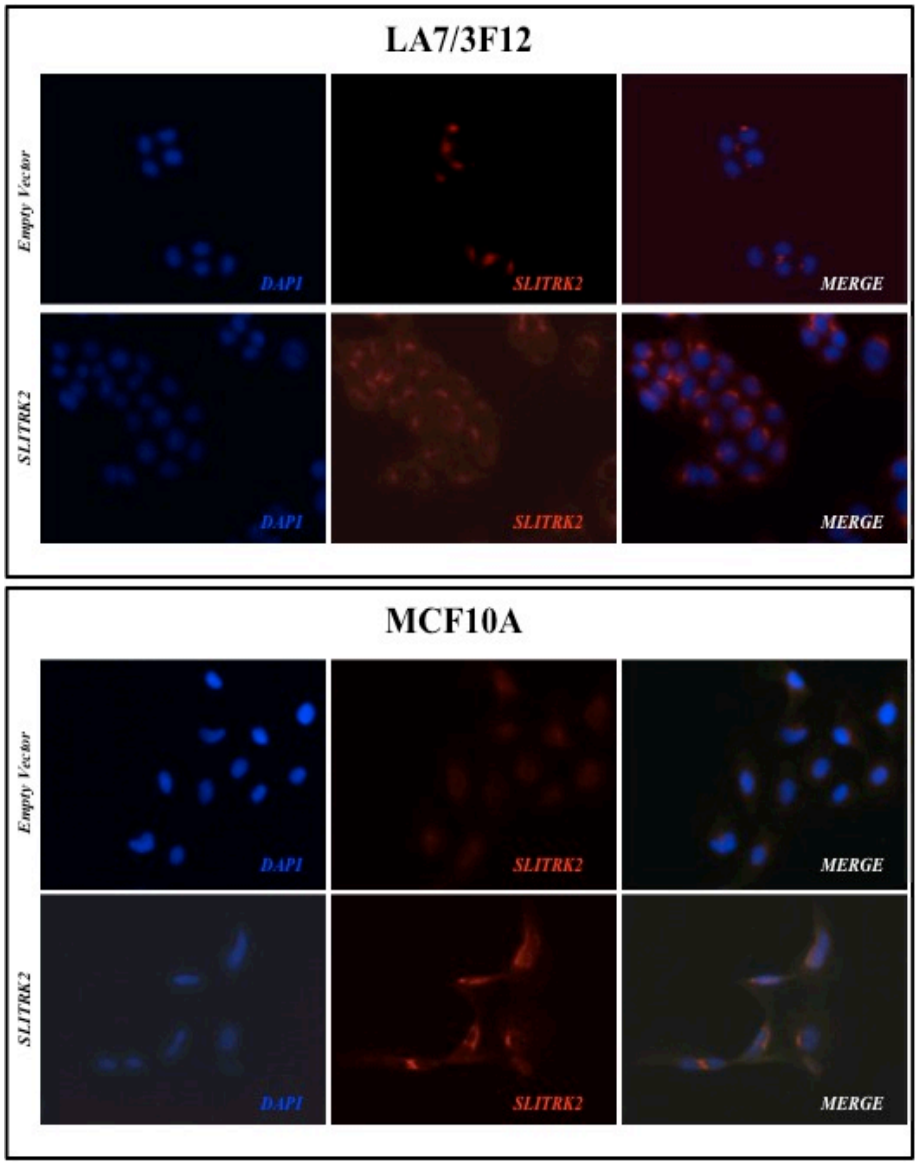

Figure 36. Intracellular localization of Slitrk2 in mammary epithelial cells. *Slitrk2-transfected LA7/3F12 and MCF10A cells showed an intracellular localization of Slitrk2, as seen previously in PC12 cells. Empty-vector transfected cells showed a very weak staining, according to qRT-PCR quantitation. Magnification 40X.*

4. CONCLUDING REMARKS AND FUTURE DIRECTIONS

This study unraveled some unknown features of the Slitrk2 gene product.

We focused our attention on three main points:

1. The characterization of Slitrk2 expression with a transcriptional analysis approach, performed with Northern Blots, PCR and *In situ hybridizations.*

2. The *in silico* characterization of the Slitrk2 protein, from the sequence to the folding.

3. The functional characterization of Slitrk2 in neuronal and non-neuronal systems with both differentiation assays and transcript analysis done by quantitative Real time PCR.

Slitrk2 gene encode for a protein belonging to the neuronal Leucine-Rich Repeats family.

It is a 844 residues long protein whose precursor contains a 21 aminoacid long signal peptide for the plasma membrane anchorage. Plasmalemma seemed to be its preferred localization, thanks to a 20 residues long hydrophobic transmembrane α-helix. Near to this transmembrane region, a "stop-anchor" sequence seems to drive correctly this plasma-membrane anchorage during the sorting in the *cisternal* proceeding.

With our *in silico* approach we identified two main phosphorylation sites: Threonine 806 and Tyrosine 832. These post-translational modifications could regulate both its retrivial into the ER-Golgi compartment and its recognition by auxiliary proteins such as the 14-3-3, regulating its function and degradation. An intuguing chapter is those related to the *in vivo* localization of Slitrk2. As mentioned above, Slitrk2 has all the features of an integral plasma-membrane protein. Our data, however, showed that this localization accounts only for a little percentage of the Slitrk2 molecules in both neuronal (PC12) and non-neuronal (LA7/3F12 and MCF10A) cells. This finding is not so suprising since also other LRR-Transmembrane Proteins were found to be located mainly intracellularly. This could raise the question if Slitrk2 is a component of the wide complex of protein that regulate neuritogenesis at the exo/endocytic control level. We found that Slitrk2 main function is to impair cell differentiation. In fact, its function is particularly detectable under differentiation-

promoting conditions. In our differentiation assays, we found that Slitrk2 forced expression inhibit both neuritogenesis in PC12 cells and tubulogenesis in epithelial mammary LA7/3F12 and MCF10A cells.

Our observation posit that Slitrk2 could be involved in brain cancer, altough at the moment we don't know exactly how, since in neuronal cancer cells it is down-regulated compared to control normal brain cells. With overexpression assays, based on the utilization of replication-defective lentiviral particles, we are now trying to further analyze the effect of Slitrk2 ectopic expression in brain cancer cell lines. For this purpose, we chose to perform our experiments by using two similar cell lines that share an homogeneous *in vitro* behavior related to retinoic acid responsiveness, proliferation and *in vivo* tumorigenicity in NOD/SCID mice, and of course Slitrk2 expression, but charaterized by two different molecular profiles related to stemness. These cell lines are SK-N-BE, known as a "brain cancer stem cell line", and SK-N-MC, a brain cancer cell line with no published stemness properties, that we verified to be unable to form neurospheres.

At the transcriptomic level, we performed a detailed analysis of Slitrk2 expression in neuronal and non-neuronal systems.

We found that Slitrk2 expression started at day 11 during mouse embryonic development, being expressed also in the adulthood. During this lifetime, with an *in situ hybridization* approach, we observed that Slitrk2 transcript is particularly abundant in some selected brain areas such as Olfatory Bulb, Cerebral Cortex, Cerebellar Inner Layer and, most of all, in the Dentate Gyrus of the hippocampus.

This expression, together with its differentiation-inhibitory activity, lead to the hypothesis that Slitrk2 could be involved with neuronal defects such as those seen in Schizophrenia, Bipolar Disorders and Alzheimer's Disease. However, until now any Genome-Wide Association Study or Linkage Study found a correlation between Slitrk2 altered expression or mutations and neurological diseases.

In general, it is now well known the relevance of the neuronal LRR family members in the regulation of mechanisms such as neurite outgrowth, synapse formation and growth factor signalling.

To better evaluate the role of Slitrk2 in these processes, we recently focused our research on the study of the phenotypical / behavioral effects of Slitrk2 expression modulation in a simple and manageable animal system: Zebrafish (*Danio rerio*).

With this model, we programmed to analyze Slitrk2 function at both the embyonic and adult level with:

PCR analysis of Slitrk2 expression;

Whole-mount *in situ hybridization;*

Developmental analyses done with *in embryo* forced expression (mRNA injection in oocytes);

Developmental analyses with *in embryo knock-down* with the use of the Morpholynated-antisense oligonucleotides.

While this thesis was being completed, a preliminary PCR screening of Slitrk2 expression during Zebrafish development was completed in collaboration with Dr. Sangiorgi L. and Prof. Cotelli F. (University of Milan). Our initial results showed that, as seen in mouse, Slitrk2 is not expressed in early stages of embryo develompment, since its expression started to be detectable at the stage of 5 somites. This result, taken toghether with the expression analysis we done in mouse, support our hypothesis for a role of Slitrk2 during post-gastrulation embryonic development. Also in Zebrafish, Slitrk2 expression levels are quite flat from the later stages of development to the fish adulthood.

it is noticeable that one member of the Slitrk family, Slitrk1, was found to be linked to neurological disorders such as Tourette's Syndrome and maybe Trichotillomania. Although also the function of Slitrk1 is currently unclear, is it obvious the need of research in the characterization of these proteins. A recent work, moreover, showed that Slitrk1 could be processed by secretases. These class of proteins are involved also in the ethiology of Alzheimer's disease. Since we found that Slitrk2 ectodomain share the same three-dimensional architecture of important neuronal-development and survival regulators such as Lingo1 and NogoR, it is possible an involvment of Slitrk2 in these above-mentioned processes. The observation that Lingo1 and NogoR could interact with p75NTR to regulate the amyloid protein downstream signal, could open a new field of research focused on the identification of new partners of these macromolecular complexes involved in the modulation of the amyloid pathway. Our observations that Slitrk2 *in vitro knock out* with short-intefering RNAs (siRNAs) could prevent the drop in cell viability after neurotrophin deprivation in NGF-differentiated PC12 cells, and that during this stressogenic treatment Slitrk2 expression is strongly up-regulated, raise the possibility that

Slitrk2 is directly involved in the modulation of neuronal cell death following neurotrophin withdrawing. This point is currently under examination in our laboratory.

We found also that Slitrk2 expression is linked in an inderect way to NGF signalling. In PC12 cells, its transcript decreases in a time-dependent manner during NGF stimulation. On the contrary, in NGF-differentiated PC12 cells, the neurotrophin withdrawal leads to an important time-dependent increase of Slitrk2 mRNA.

We are now analyzing this indirect relationship since a direct transcriptional regulation of NGF on Slitrk2 appears to be unlikely.

In summary, with this work we gave a blueprint of Slitrk2 related to:

1. expression during mouse embryogenesis,
2. fetal and adult tissue distribution;
3. in vivo expression in adult mouse brain;
4. protein subcellular localization in neuronal and non-neuronal cell lines;
5. role in regulating differentiation of neuronal and non-neuronal cell lines;
6. relationships with brain cancer and stemness;
7. transcriptional dynamics related to neurotrophin signalling.

Our research is then focused on:

1. Phenotypic effects mediated by Slitrk2 up/down regulation during Zebrafish development:
2. Analysis of Slitrk2 relationships with brain cancers;
3. Identification of potential inducers of Slitrk2 plasma-membrane localization;
4. Expansion of the molecular interactors of Slitrk2 by which it exerts its differentiation-inhibitory effect.

In conclusion, our research suggested that Slitrk2 is a negative regulator of both axonal sprouting in neurons and tubulogenesis in mammary gland.

In PC12 cells this action seems to be related to an impairment of Intermediate Filament (Peripherin) assembly.It can acts as a brake that maintains the cells in an undifferentiated phenotype. The observation that Slitrk2 is upregulated during neurotrophin withdrawal in NGF-differentiated PC12 cells reinforce this hypothesis, since after neurotrophin removal NGF-differentiated PC12 cells are subjected to neurite retraction. This mechanism is also associated with neuronal cell death,

leading to the hypothesis that Slitrk2 in differentiated neuronal cells is one mediator of cell death mechanisms. These two sentences are not contradictory since other molecules (e. g. CyclinD1) have two opposite effects in naive and post-mitotic, differentiated neuronal (PC12) cells.

5. MATERIALS AND METHODS

5.1 Cell Cultures and Treatments

5.1.1 Neuronal Cell culture

PC12 cells [174] were grown in Dulbecco's Modified Eagle's Medium with Glutamax (DMEM-Glutamax, Gibco) supplemented with 10% heat-inactivated Horse Serum (HS, EuroClone), 5% Fetal Bovine Serum (FBS, Gibco) and 1% Penicillin-Streptomycin solution (PS, Gibco). For differentiation assays, cells were plated onto rat-tail collagen [198] coated plates (Falcon) or chamberslides (Nunc) in complete medium. After 24 hours, cells were washed twice with DMEM and maintained in reduced-serum medium supplemented with 50 ng/ml of 2,5S Nerve Growth Factor (NGF, Promega) for at least three days before treatments. In NGF-depletion assays, cells differentiated for 5days were washed three times with DMEM and then maintained in reduced-serum medium without NGF for the indicated times. Hydrogen Peroxide (HP) was added at the concentration of 250 μM directly to the culture media.

U87 and SK-N-BE cells were grown in DMEM-Glutamax, supplemented with 10% FBS and 1%PS. For generation of neurospheres, cells were plated in low-attachment plates (Bibby Sterilin) at clonogenic density by using DMEM media supplemented with 1X B27 (Invitrogen), 20 ng/ml EGF (Sigma), 20 ng/ml basic fibroblast growth factor (Sigma), and 4 μg/ml heparin (Sigma). [182, 183].

5.1.2 Mammary Cell culture

LA7/3F12ms cells [196] were maintained in DMEM-Glutamax supplemented by 10%FBS, 1% PS and 50ng/ml of each insulin (Ins) and hydrocortisone (HC) (Sigma).

MCF-10A cells [195] were grown in a mixture of 1:1 DMEM-Glutamax and F12 nutrient medium (Gibco) supplemented with 5% HS, 20 ng/ml EGF, 10 μg/ml Ins, 0.5 μg/ml HC, 2mM di-butyryl-cyclic AMP (dbcAMP, Sigma) and 1% PS.

For differentiation in collagen (Tubuli assay), monolayer cultures were detached by trypsinization, mixed with collagen from rat tails (prepared according to [198] and plated at a concentration of 10000 cells/well in 24-well plates. After the collagen had solidified, 500µl of the medium previously described was added.

5.1.3 Ectopic Expression and gene silencing

For the forced expression of Slitrk2, the human Coding Sequence (NCBI accession BC113012) was subcloned into a pcDNA3.1 expression vector from the commercial cloning vector pCR-BluntII-TOPO (OpenBiosystem, clone ID 40028693). Successful orientation and sequence validation of the obtained pcDNA3.1-SLITRK2 vector were verified by both restriction endonuclease digestion and sequencing. All the cell lines were transfected with the Amaxa Nucleofector II Device (Lonza) with the following settings: plasmid DNA: 2µg, cell number $1x10^6$, Solution: V, 100µl, Programs: P020 (LA7/3F12ms), X013 (MCF10A), U029 (PC12). The empty vector-transfected cells were used as experiental controls.

The *in vitro* knock-down of Slitrk2 in PC12 or U87 cells was performed using the commercial Mission siRNA (Sigma, ID SASI_Rn02_00230305 [Rat Slitrk2], ID SASI_Hs01_00047045 [Human Slitrk2]). The efficiency of silencing was evaluated 72hours after treatment with qRT-PCR relatively to Slitrk2 amplification in scrambled-siRNA-treated cells (Universal Negative Control, Sigma). siRNA treatments were carried out by using the InterferIN reagent (PolyPlus) according to the manufacturer's specifications.

5.2 Transcriptional Analyses

5.2.1 In situ hybridization

In sith hybridization was carried out according to standard protocols [199]. In brief, cRNA probes were synthesized from *Xba*I- (antisense) or *HindIII*-linearized (sense) Slitrk2-containing plasmid pBS-SK. The transcription reaction was incubated at 37°C for 2 hr with one microgram of linearized plasmid mixed with T7 or SP6 RNA polymerase (New England Biolabs) in transcription buffer and nucleotides. The RNA transcript was purified by incubation with 2 µl DNaseI (RNase-free,

Promega) followed by purification with RNAse-free G-50 sephadex columns (GE Healthcare).

Brain sagittal cryostat sections were thaw-mounted and air-dried fixed for 10 min in 4% Paraformaldehyde, washed in PBS, acetylated in 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine, permeabilized in 1% Triton X-100/PBS, and washed in PBS. The sections were then blocked for 6 hr in hybridization buffer [50% formamide, 4 × SSC (0.6 M NaCl, 60 mM sodium citrate, pH 7.0, 4 mM EDTA] and 1× Denhardt's solution. The sections were hybridized with 1 mg/ml digoxygenin-labeled cRNA in hybridization buffer for 16 hr at 65°C. The sections were then washed in 0.2× SSC for 1 hr at 72°C, washed 2 × 10 min in PBS, blocked with 10% heat-inactivated lamb serum in PBT, and then incubated with a 1:2000 dilution of Dig-specific antibody (Roche) for 2 hr. The sections were then washed 3 × 10 min in PBS, equilibrated in NTMT buffer (0.1 M Tris, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$, 0.1% Tween 20, 2 mM Levamisol) for 5 min, and then reacted with 0.45 mg/ml 4-Nitroblue tetrazolium chloride (NBT, Roche), 0.175 mg/ml 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP, Roche) in AP buffer for 16 hr at room temperature.

5.2.2 Northern Blot

Northern blot analyses were performed using commercially obtained human and mouse mRNA blots with different adult and fetal tissues, according to the manufacturer's recommendations (Clontech). A PCR product corresponding to a portion of the Slitrk2 coding region, was generated using synthetic complementary primers designed between nucleotides 5388 and 6446 of the genomic sequence and was labeled with α^{32}P-dCTP using RediPrime (GE Healthcare). Pre-hybridization and hybridization were performed according to standard protocols [199], autoradiography was done for 28-36 h.

5.2.3 RNA extraction, RT-PCR and qRT-PCR

RNA was extracted using Trizol Reagent (Invitrogen) according to manufacturer's specifications. Reverse transcription for RT-PCR was performed using 1μg of total human RNA from different tissues (Clonthech) after RNA treatment with DNAse I (Invitrogen), and SuperScript II RT according to the manufacturer's directions. RT-PCR was then carried out using 50 ng of cDNA as template with

1U of Taq Polimerase (GeneSpin). HPRT amplification was used to normalize reactions. RT-PCR reaction for Slitrk2 was performed using the primers 27500A (5'AATCCTGACTGAGGTGGCTT3') and 27500B (5'ACCAGCCTGTCATCAAATGA 3') which amplified a fragment of 150 bp.

Quantitative RT-PCR (qRT-PCR) was performed with templates obtained from 1μg of RNA being retrotranscribed after DNAse I treatment with the RNA-to-cDNA Master Mix (Applied Biosystems). 4.5 ng of cDNA were subjected to amplification with Sybr-Green Master Mix (Applied Biosystems) using the AB 7500 real-time PCR System (Applied Biosystems) with the following thermal protocol: i) DNA melting: 94°C for 10mins; ii) amplification: 94°C, 15sec; 60°C, 30sec.

The primer sequences for the amplification of Slitrk2 and HPRT (as endogenous control) were:

Rat HPRT1 FW: 5'-TCCATTCCTATGACTGTAGATTTTATCAG-3'

Rat HPRT1 REV: 5'-AACTTTTATCTCCCCCGTTGACT-3'

Human HPRT1 FW: 5'-TTTGCTGACCTGCTGGATTACA-3'

Human HPRT1 REV:5'-GGTCATTACAATAGCTCTTCAGTCTGAT-3'

Rat SLITRK2 FW: 5'-CCTCCTGATGTGTTTGCCTGTAA-3'

Rat SLITRK2 REV: 5'-CAGACGCCGCTCAGCATT-3'

Human SLITRK2 FW: 5'-ACCAGCCTGTCATCAAATGA-3'

Human SLITRK2 REV: 5'-AATCCTGACTGAGGTGGCTT-3'

5.3 Biochemical Assays

5.3.1 Immunofluorescence analysis

For Slitrk2 immunostaining were used three methods of fixation/permeabilization: with absolute Methanol (WWR) for 10mins at -20°, with Paraformaldehyde (PFA, Sigma) and with PFA plus Triton 0,2% (Sigma) for 10mins at RT. For Syntaxin1 and DLX5 staining, cells were fixed in Methanol. After fixation/permeabilization, all the Immunostainings were performed by blocking the aspecific antibody binding with Blocking Buffer (BB, 10% FBS, 2% Bovin Serum Albumin Faction V, in PBS) for 1hour at RT. Incubation with primary antibodies (diluted in BB) was carried out for 2 hours at RT. After extensive washing, cells were probed with specific

AlexaFluor (Invitrogen)-conjugated secondary antibodies for 45mins at RT. Nuclei were stained with DAPI. Images were taken at 40X magnification with Olympus microscopy coupled to CCD camera and dedicated software (CellF) . Post-acquisition analysis was performed with the Image J software (NIH). The following antibodies were used: anti-Slitrk2 (ab67305 and ab67305, Abcam, rabbit polyclonal), anti-Syntaxin (clone HPC1, Sigma, mouse monoclonal), anti-DLX5 (AV32867, Sigma, rabbit polyclonal), anti-Peripherin (MAB1527, Chemicon, mouse monoclonal).

5.3.2 Cell Tracker Green staining

Cell Tracker Green (Invitrogen) stock solutions were made 1mM in DMSO. Cells were stained for 30mins with working dilutions (1μM) made in PC12 differentiation medium prior to analysis. After loading, dye excess was removed with extensive washes in DMEM. Cell metabolism-dependent dye fluorescent activation was assessed with the Olympus microscopy. Images were taken at 20X and 40X magnification. Post-acquisition analysis was performed with the Image J software (NIH).

5.4 Bioinformatics

5.4.1 Preliminary BLAST, PSI-BLAST and Pfam

Initial PSI-BLAST and BLAST searches were made on the NCBI website http://www.ncbi.nlm.nih.gov using the default threshold E-value against PDB, Swissprot and non-reduntant sequence database. Human Slitrk2 sequence (UniProt Q9H156) was used for the calculations. A default value fo 1.0 was used for Pfam runs.

5.4.2 Topological features and secondary structure prediction

Secondary structure prediction was obtained from PredictProtein [154] using default parameters. Additional searches control were done on the 3D-Jury metaserver [200]. For hydrophobicity searches and transmembrane sequences SignalP [158], PrediSi [157], WolfPSORT [155] and HSLPred [156] were used. Each calculation was carried out with default parameters.

Phosposites in the Slitrk2 sequence were predicted with the following strategy. The initial mapping of potential kinase-recognition sites was done with NetPhosK [165]. The list were cleaned from all the sites located on the extracellular side. The remainings were scanned with ScanSite and ELM [166, 167] and the outputs were crossed in order to have a list of the most likely predicted sites. The final list was refined by looking at the solvent-accessibility of the residues highlited by PredictProtein an then revised with a data-mining approach with a PubMed search for experimentally verified phosphorylation sites in (neuronal) LRR protein.
N-glycosilations were predicted with NetNGlyc [172]. The five potential N-glycosylated sites were verified by accessibility and domain distribution. The two most likely N-glycosylation sites were mapped on the 3D structure obtained from I-TASSER.

5.4.3 3D structure predictions

The Slitrk2 sequence was submitted entirely or in separate pieces (extracellular and cytoplasmic) to 3D-jury, Phyre and I-TASSER [170, 171] in order to exclude sequence limit submission and worse recognitions. I-TASSER 3D models are built based on multiple-threading alignments and iterative simulations. Default parameters were used. The best model obtained for the modeling of the extracellular Slitrk2 domain had a C-score of -1,52, improving the prediction carried out on the whole sequence (C-score of -2,08). The run with the intracellular tail gave a model with C-score of -3,02. The model built by Phyre using as query both the extracellular or the whole sequence of Slitrk2 had E-values of $5{,}1e^{-41}$ and $3{,}9e^{-40}$, respectively. The intracellular tail model is characterized by an E-value of 79.
Figure 21 was built with the PDBwiewer software (http://www.expasy.org/spdbv) [201]

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