CEREBELLAR CORTICOGENESIS IN THE LYSOSOMAL ACID PHOSPHATASE (ACP2) MUTANT MICE: PURKINJE CELL MIGRATION DISORDER

BY

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Abstract

In a mutant mouse called nax as the result of mutation in Lysosomal Acid phosphatase (Acp2), layers of the cerebellar cortex are impaired and monolayer Purkinje cells (Pcs) turn to multi-layered Pcs that ectopically invade the molecular layer. We investigated reelin-Dab1 signaling as an important pathway for Pcs migration and monolayer formation in cerebellum. ERK1/2 is a member of mitogen activated kinases family and suggested to be a downstream of reelin signaling. We hypothesize that the establishment of mono-layered Pcs rely on reelin through ERK1/2 pathway. Acp2 mutant mice were used for this study and molecular expression and distribution were assessed by immunohistochemistry, RT-PCR, western blotting, and cell culture. Results suggest that reelin may modulate the ERK1/2 expression, thus lower expression of reelin and higher phosphorylation of Dab1 leads to over expression of the ERK1/2 that causes the Pcs to over migrate and form multilayer in nax cerebellar cortex.

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LIST OF ABBREVIATIONS

Acp5: acid phosphatase-type 5

AP: anterior–posterior

ApoER2: Apolipoprotein E receptors 2

bHLH: basic-helix-loop-helix

Bmp: Bone morphogenetic protein family

BSA: Bovine Serum Albumin

Calbindin: Calcium binding protein

CCAC: Canadian Council on Animal Care

CDK: Cyclin Dependent Kinase

CN: Cerebellar Nuclei

CNS: central nervous system

DAB: Diaminobenzidine

Dab1: Disabled 1

DV: dorsal–ventral

EGZ: external germinal zone

Ephrin-B: EphB tyrosine

ERK1/2: extracellular signal-regulated kinase 1 and 2

FBS: fetal bovine serum

Fgf : Fibroblast growth factor family

gcl: granule cell layer

GSK3: Glycogen Synthase Kinase-3 beta

HH: Hedgehog family

IP3R: inositol phosphate 3 receptor

IsO: isthmic organizer

JNK1-3: c-Jun N-terminal kinases 1-3

LAP: Lysosomal acid phosphatase

LCH: lissencephaly with cerebellar hypoplasia

LDLR: Low Density Lipoprotein Receptors

LIMK: LIM kinase

MAPK: Mitogen activated protein kinases

ME: mercaptoethanol

ml: molecular layer

nax: naked-ataxia

NMDA receptors: N-methyl-D-aspartate receptors

Pcl: Purkinje cell layer

PCR: Polymerase Chain reaction

Pcs: Purkinje cells

PFA: paraformaldehyde

PFA: paraformaldehyde

PI3K: Phosphatidylinositol-3-Kinase

PIPs: Pax2-positive interneuron progenitors

PKB: protein kinase B

RCM: reelin conditioned medium

RL: Rhombic lip

Sepp1: Selenoprotein P

UBCs: Unipolar Brush Cells

VLDLR: Very Low Density Lipoprotein Receptor

VZ: ventricular zone

Wnt: Wingless-Int protein family

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CHAPTER 1: INTRODUCTION

The purpose of this study was to increase our knowledge of cerebellar corticogenesis with a focus on the migration of Purkinje cells (Pcs) in mice. Purkinje cells are originated from ventricular zone (VZ) during early embryonic development and after migration form a monolayer of cells in cerebellar cortex postnatally. The mechanism of cerebellar Pcs migration and monolayer formation have been investigated using a mouse model called nax that shows severe defects in Pcs migration and monolayer formation during development.

1.1 Cerebellum

The cerebellum is an important organ which plays a vital role in motor coordination and cognition (1). Cerebellar motor functions are involved with balance, posture, coordination, and balanced muscular activity. However increasing body of evidence are confirming the role of the cerebellum in cognitive functions including emotions, attention, sleep, language, and also the non-somatic visceral responses (2). The cerebellum is made of a vermis at the midline and two hemispheres on each side. Avian and mammalian cerebellum is divided into 3 lobes and subdivided into 10 lobules (1-10). Due to well-defined cerebellar neuronal organization, numerous researchers have used this organ to investigate neurodevelopmental processes including cell fate specification and stem cell maintenance (3).

An adult cerebellum has a three-layer cortex including; the molecular layer (ml), Purkinje cell layer (Pcl), and granule cell layer (gcl). Ml which is the most superficial layer located beneath the pia matter contains dendrites of Purkinje cells (Pcs), axons of granule cells (gcs), basket cells, stellate cells, and Bergmann glial cell processes. The second layer positioned at the middle consists of a single layer of Pc

bodies and the third layer includes gcs, Golgi cells and Unipolar Brush cells (UBCs) (4-6). Cerebellum receives afferents and efferent to process the information about motor programing. There are two major types of the afferents in the cerebellum called climbing fibers and mossy fibers. Mossy fibers synapse on the cerebellar gcs. Axons of the gcs then give rise to the parallel fibers ascending toward the ml. The parallel fibers then relay the excitatory synapse on the dendrites of the Pcs. The Pcs project the output of the cerebellum to cerebellar nuclei. Climbing fibers carry information from inferior olivary nucleus and synapse with the Pcs directly (7).

1.2 Development of central nervous system

Formation of the neural tube is the basis of central nervous system (CNS) development which occurs during neurulation process. Neurulation is a morphogenetic event, which takes place in early embryogenesis at the Carnegie stage 13 (28 days post fertilization) in human (8) and E7-7.5 in mouse (9). It initiates with ectoderm thickening and neural plate formation, in which a flat sheet of neuroepithelial cells roll up and form the neural tube (10). Initially, the cortical plate consists of neuroepithelial, mantle, and marginal layers (11). The neural tube is formed under the control of genes and molecules that define the basal plate, the alar plate, roof plate and floor plate. The neural tube development is patterned along dorsal–ventral (DV) and anterior–posterior (AP) axes, some vesicles that are generated at the rostral part of the neural tube will produce prosencephalon (forebrain) comprising the telencephalon and the diencephalon, the mesencephalon (midbrain), the rhombencephalon (hindbrain) (which is relevant to this study) comprising the metencephalon and myelencephalon, and the spinal cord at the caudal part (12, 13). There are four basic morphogenetic signals participating in neural tube formation and differentiation including: Wingless-Int protein family (Wnt), Hedgehog family (HH),

Bone morphogenetic protein family (Bmp) and Fibroblast growth factor family (Fgf) (14). The patterning of the neural tube relies on ventralizing factors of SHH and dorsalizing factors of BMP which are produced from notochord/floor plate and roof plate, respectively (15, 16). In general, the basal plate produces all motor neurons and related interneurons, while the alar plate is the source of sensory component of the CNS.

1.3 Development of the cerebellum

Development of cerebellum occurs during both embryonic and postnatal stages. In human embryology development of different layers of the cerebellum were identified by Rakic and Sidman spanning from 7-40 week gestation and into one year after birth (17). Cerebellar primordium is formed at stage 14 (32 days) and migration of neuronal progenitors occurs at stages 18 and 19 (44–48 days) (2) however, in the mouse it starts at embryonic day 9 and continues up to postnatal day 18. The hindbrain or rhombencephalon segmentation occurs after neural tube closure and leads to the production of rhombomere 1-8. The alar plate of the rhombomere 1 is developing cerebellum territory that is called cerebellar anlage (primordium). Development of the cerebellar anlage depends on the signals from the isthmic organizer (IsO), a neural tube narrowing between mesencephalon and rhombencephalon. IsO maintains structural polarity to the neighboring parts and arranges the cellular composition of the rhombomer 1 and mesencephalon (6, 18). FGF8 which is specified in the most anterior hindbrain is an essential molecule to a developing cerebellum (19). *Gbx2* in hindbrain and *Otx2* in midbrain are two genes with opposite but complementary developmental roles. Since their action is on the site of Iso they play a role in regulating FGF8 expression (20). Differential development

and cellular survival in developing cerebellum is highly dependent on the gradient of FGF8 signals in Iso (18, 21).

1.4 Specification of cerebellar germinal zones

Formation of different subtypes of neurons in the cerebellum is determined by the spatial and temporal specification of cerebellar primordium germinal zones. Ventricular zone (VZ) and Rhombic lip (RL) are the main germinal zones located at the ventral and dorsal regions of the cerebellar primordium that will generate distinct neuronal subtypes including cerebellar glutamatergic and GABAergic neurons. The identities of these cerebellar progenitors are derived from important components including basic-helix-loop-helix (bHLH) proteins, ATHO1 (also called MATH1) and PTF1a (22, 23). RL germinal zone expresses ATOH1 resulting in the production of glutamatergic neurons and Ptf1a expressed in VZ targets the production of GABAergic neurons (23, 24).

1.4.1 Patterning of the rhombic lip-derived phenotypes

Granule cells, UBCs, and large cerebellar nuclei neurons are categorized as cerebellar glutamatergic neurons. They are generated from rhombic lip and use glutamate as their neurotransmitter.

1.4.2 Granule Cells

Granule cells are located at the most inner layer of cerebellar cortex. Each cell contains 4 to 5 dendrites and a thin unmyelinated axon that splits and forms a “T” shape parallel fiber and synapse with the Pc dendrites in the molecular layer. Excitatory inputs from mossy fibers and inhibitory inputs from Golgi cells are received through the enlarged structures at the end of dendrites (25). Granule cells formation, migration, and differentiation starts from embryonic days E12.5- E16 in

mouse and at embryonic stage 23 in human (8 weeks), and progenitors of granule cells which are newly born from RL, migrate rostrally in a sub-pial stream to form an external germinal zone. When ATOH1 is turned on, granule cell precursors start their migration from RL. Expression of ATOH1 which is the main factor to produce gcs precursors is influenced by signaling of BMP at choroid plexus and roof plate (26). During postnatal stages, gcs are proliferated by the major signaling mechanism of SHH that is secreted by Pcs. (5, 27, 28). Differentiation of gcs happens after formation of the external germinal zone when the cells exit the cell cycle. Migration of the gcs is facilitated by the formation of a neuron-glial adhesion underneath the cell surface and a leading process containing lamellipodia and filopodia covering the glial fiber (29).

1.4.3 Unipolar Brush cells

Unipolar Brush cells are categorized as a group of interneurons in cerebellar cortex that has excitatory functions. They are found abundantly in the areas that regulate the sensorimotor signals, control position of the eyes, head, and body. A UBC with a typical phenotype contains one thick dendrite that ends in a brush-like structure that synapses with terminals of mossy fibers (30, 31). Regarding their function and chemical composition, UBCs are categorized as type I and type II (32). During the cerebellar development, UBCs are; 1) generated by their progenitors in RL (E10.5–13.5) controlled by the expression of WNT1, however, WNT1 will be downregulated when UBCs start migrating from their germinal zone (33). The newborn neurons remain 1-2 days in RL before moving toward their destination and then they dorsally migrate through white matter and by p10 they reach the granular layer. The ultimate differentiation of these cells happens during P2-P28.

1.4.4 Development of the Cerebellar Nuclei

Development of Cerebellar Nuclei (CN) takes place in accordance with the cerebellar cortex and uses almost the same molecular mechanisms. Lineage mapping of ATOH1 and PTF1a has proven that excitatory and inhibitory neurons of CN are generated from the RL and VZ. Thus both ATHO1 and PTF1a genes will give rise to formation of CN. The ATHO1+ CN neurons which are large excitatory projection glutamatergic neurons are determined from the first stages in RL. CN neurons that are produced from PTF1a zones are the first set of inhibitory neurons intended to be positioned in superficial layers (23, 34). In fact, Pax2-positive interneuron progenitors (PIPs) which are coming from $Gsx1^{+}$/PTF1a domain reach white matter and participate in CN construction.

1.4.5 Rhombic lip-derived Neurons Are Glutamatergic

Glutamatergic neurons are the major excitatory neurotransmitters found in CNS. Glutamate is released from a presynaptic neuron and can be received by three essential receptors within neurons including N-methyl-D-aspartate receptors (NMDA receptors), kainate receptors, and AMPA receptors. Activation of NMDA receptor leads to long-term potentiation and requires glycine as well as glutamate to mediate calcium flux into the neuron and trigger the downstream signaling cascades. AMPA receptors regulate fast excitations meaning that ion channels of the receptors respond quickly to glutamate. The proper function of Kainate receptors needs formation of a complex of its subunits (GluK1-GluK3) (35, 36). These receptors have a role in regulating the release of inhibitory GABA transmitters. In developing cerebellum glutamatergic neurons are produced from RL germinal zone. Although the precise mechanism to identify the cerebellar glutamatergic neuronal subtypes has not been

fully understood yet, expression of PAX6 from RL-derived cells for instance CN, gcs and UBCs indicate excitatory neurons in the cerebellum (37).

1.5 Patterning of the ventricular zone-derived phenotypes

Purkinje cells, Golgi cells, and interneurons are categorized as cerebellar GABAergic neurons. They are generated from different progenitors of ventricular zone.

1.5.1 Purkinje cells

Purkinje cells are large-sized neurons with a branched dendritic tree. Pcs are arranged in a monolayer which is crossed by the extended axons of gcs that create parallel fibers. It has been estimated that a single Pc synapses with 150 000 to 200 000 parallel fiber. Thus dendritic formation and differentiation is a crucial process during cerebellar development (38, 39). Two excitatory afferent pathways from climbing fibers and mossy fibers are received and modulated by Pcs. Pcs are considered as central neurons of cerebellar cortex because these are the only neurons that project the output of cerebellar cortex to CN (40, 41).

Development of Pcs in human occurs during an extended period of time, Pcs plate is established by 13 week pregnancy. In terms of morphology, they are considered to be multipolar up to the 28 week pregnancy when they start forming a monolayer. Ultimate dendritic tree differentiation extends well into the first postal year (2, 42). However in mouse, they are born at E10-12 and then form embryonic clusters at E14-P1 followed by cluster dispersal at P1-4 and finally, a monolayer of Pcs is formed by P4-7 (2).

Calcium binding protein (Calbindin) and inositol phosphate 3 receptor (IP3R) are the proteins that specifically expressed in axon, soma, and dendrite of Pcs (43).

Calbindin-D28k is a fast calcium buffer protein being expressed once Pcs mitosis is terminated and they are about to migrate and differentiate. The complex function of this protein has not been fully understood, however it is known that plays role in neuronal survival through modulation of calcium channels and calcium sensor proteins (44). Differentiated Pcs establish inhibitory neurotransmission in order to take part in configuring different zones of the cerebellum (45, 46).

1.5.2 Golgi Cells

Golgi cells are considered as slow firing interneurons spanning throughout the gcl. Their long and numerous processes extend in different cortical zones. Their dendrites receive inputs from mossy fibers-gcs-parallel fibers and their axons toward gcs dendrites in the cerebellar glomeruli. Using glycine and GABA neurotransmitters they deliver feed-backward inhibition to gcs (44).

1.5.3 Molecular layer Interneurons

The role of GABAergic interneurons is to adjust the stability and dynamics of neural circuits. Pax-2 expression indicates GABAergic interneurons including stellate cells, basket cells, and Golgi cells in the developing cerebellar cortex. Is has been suggested that Pax-2 accept this role as a transcription factor for these specific cell types after E12 (47). The interneurons including stellate cells and basket migrate toward the ml without penetrating into the population of gcs precursors at that stage (48).

1.5.6 Ventricular zone-derived neurons are GABAergic

Among various amino acid that play either inhibitory or excitatory roles in CNS, γ-aminobutyrate (GABA) is considered principal inhibitory neurotransmitter. GABA is not able to cross the blood-brain-barrier thus it is synthesized within the

neurons through the metabolic pathway in which glucose is used as a precursor to produce GABA. GABA-A and GABA-B are two major receptors for this neurotransmitter and binding of ligand to these receptors will lead to hyperpolarisation of the membrane and reduces the firing of the action potential (49, 50).

Particularly during cerebellar development, GABAergic neurons are classified as Pcs and PAX-2^{+} interneurons. These neuron precursors are produced from progenitors that highly express PTF1a in VZ (51). In addition, Pc development and cell cycle is also regulated by neurogenin 1 and 2 as the other downstream factors of PTF1a (52). From E10-12 they produce Pcs, interneurons in cerebellar nuclei and from E13.5 they produce Golgi cells, lead to stellate and basket cells in later stages (53).

1.6 Neuronal Migration in Brain

Neuronal migration is developmental process in which neurons travel from their germinal zones to their final position (54). There are two modes of migration in the developing central nervous system including somal translocation and glia-dependent locomotion. Somal translocation happens in newly-born neurons where the distance between birthplace and destination is short, thus, migration is mediated through leading process of the migrating cell. At later stages of migration, neurons use radial glial fibers to move from one place to another, this mode of migration is called glia-dependent locomotion. Radial glial cells facilitate the neuronal migration by having their long radial process extended toward the cortical surface while their cell bodies are located at VZ (55, 56). Reelin signaling pathway plays an essential role in migration of neurons in developing brain. Expression of reelin from Cajal-Retzius

neurons starts from embryonic stages. Although most of the population of Cajal-Retzius neurons will be degenerated throughout the first postnatal week, secretion of reelin continues well into adulthood (57). During the early postnatal stage (both mice and human), reelin is expressed by the gcs of the cerebellum, interneurons of the hippocampus, and a subset of GABAergic interneurons in the cerebral cortex (58). The signaling pathway and its components will be discussed in detail in the following sections. Numerous studies have exhibited the role of reelin deficiencies in the patients with schizophrenia, bipolar disorder, autism, and major depression. In reelin-deficient rodents, ataxia is the most noticeable symptom and affected animals die around the first postnatal month of their life. Ataxia is not the prominent symptom in humans with a mutation in *RELN* gene, but neurodevelopmental delay, hypotonia (low muscle tone), and language deficit make the most critical symptoms (59). Mutation in *RELN* gene in humans that can be inherited as an autosomal recessive disorder causes a severe deficiency in the cerebral cortex, brain stem, hippocampus, and cerebellum. The form of this malformation is categorized as lissencephaly with cerebellar hypoplasia (LCH). In addition, it is important to consider that reelin signaling is the same in the cerebellum as the other parts of developing cerebral cortex (59).

1.6.1 Neuronal migration in cerebellum

The migration process of the cerebellar neurons mostly takes place through interaction with radial glia cells. The connection between these neurons and radial glia is mediated through detection of extracellular factors by neuronal receptors. reelin is the most important extracellular factor to trigger embryonic Pc clusters (E14) to migrate in the cerebellar cortex (60). All rhombic lip-derived cells in the cerebellum express reelin during development. Reelin is co-localized with other important

developmental transcription factors such as ZIC1, PAX6, and TBR2 in a subset of the cells derived from RL (61). During early postnatal development, reelin is expressed from gcs precursors in external germinal zone (EGZ) and aggregates in their extracellular matrix during Pcs spread from cluster phase. When the formation of the monolayer is completed reelin expression will be downregulated from EGZ (62).

1.6.2 Purkinje cells Migration and Monolayer Formation

During perinatal development in mice, Pcs which are derived from VZ migrate to the core of the developing cerebellum and establish Purkinje cell cluster. However; it has been shown that Pcs that are born at posterior periventricular zone around E10.5 exhibit tangential migration to the anterior direction (5).

Embryonic Pcs that are born at E10, use radial migration to move from VZ of fourth ventricle toward the caudodorsal direction and form Pcs plate. Primarily, the Pcs plate is a thick layer made of several cells known as Pc cluster, but the Pcs consequently spread out and form a monolayer during perinatal development (2). During radial migration phase, Pcs express a high amount of reelin receptors in addition to Dab1 protein and mRNA. There are two basic approaches currently being adopted in research into the role of reelin on Pcs migration. One approach suggests that reelin plays a role when migrating Pcs tend to detach from radial glia fibers. In the absence of reelin (reeler mutant), numerous adherence junctions between Pcs and radial glial fibers have been observed. The second approach recommended indirect effect of reelin on migrating Pcs. This approach was formed based on the abnormal curves and disorganized processes of radial glial fibers in the absence of reelin (59). Monolayer formation of Pcs is a very critical step during development, firstly because it defines cerebellar boundaries as well as folia formation and secondly, cerebellar

output depends on the appropriate synapse of Pcs with the molecular layer input to generate cerebellar circuit (63). However, Kim et al. used synchrotron X-ray microscopy to show 3-D images of Pcs dendrites and reported that unlike the direct role of reelin in Pcs migration it does not directly contribute to dendrite formation (64).

1.7 Structure and chemistry of reelin

Reelin protein was first discovered by the end of 20th century. This large glycoprotein with 388 kDa of molecular weight is encoded by *RELN* gene, which is localized on chromosome 7 in human and chromosome 5 in mice (65). The structure of the molecule includes an N-terminal region, reelin domain, and a highly conserved C-terminal region. N-terminal part of the molecule is composed of a signaling peptide, a domain similar to F-spondin (an extracellular protein that promotes neurite growth), and a small region containing CR-50 epitope. This epitope is critical for reelin signaling cascade (66). Reelin domain contains 8 repeats and each repeat contains two sub-repeats that are separated by an EGF (Epidermal Growth Factor) - like domain. The C-terminal region is comprised of several positively charged amino acids (67). When the protein is synthesized in the cytoplasm, it moves toward the extracellular matrix. At the beginning, the secreted protein has a molecular weight of ~450 kDa; however, it undergoes several cleavages between its 8 repeats and consequently, divides into 3 fragments. Cleavage of N-terminal sites is facilitated by a metalloprotease while C-terminal cleavage is carried out by a protease that is yet unknown (59). Jossin *et al*., 2007 have shown that the central fragment with 190 kDa is a principal determining factor to trigger reelin signaling pathway (68). This protein has proteolytic function and acts as a serine protease in signaling cascades. Proteolytic

activity of reelin involves with appropriate nerve-muscle connection, neuromuscular junction development, and maturation of motor end-plate (57).

1.8 Receptors of reelin

The molecular cascades that regulate the development of cortical structures are not well understood. The development of cortical layers requires the neurons to exit their cell cycle at some exact periods of time, adhere to and transport along glial fibers, then separate from the fibers, and locate into distinct layers. Receptors on the surface of neurons control these interactions and play important roles in these events (69). Apolipoprotein E receptors 2 (ApoER2; which is also identified as LRP8) and VLDLR (Very Low Density Lipoprotein Receptor), are two reelin receptors that belong to the family of Low Density Lipoprotein Receptors (LDLR). This family of receptors is comprised of multifunctional proteins that are ubiquitously expressed on the surface of all cells and play critical role in cholesterol hemostasis. Cholesterol facilitates the communication and signaling of neurons in brain (70). All the members of this family have five structural domains including an EGF domain, ligand binding domain in the N-terminal region, a transmembrane domain, cytoplasmic tail with NPxY motif, and an O-linked sugar domain (71, 72). Regulation of signaling and endocytosis occurs by the interaction of NPxY motif with the proteins containing phosphotyrosine binding (PTB) domain. It has been demonstrated that VLDLR and ApoER2 have 50% of sequence homology. Upon binding of reelin to receptors, a specific lysine amino acid in reelin extracellularly interacts with the first binding repeats of the receptors (71). Among reelin fragments, the one containing C-terminal residue has the highest affinity to ligand the ApoER2 and VLDLR (73).

1.8.1 Very Low Density Lipoprotein Receptor (VLDLR)

Very low density lipoprotein receptor contains 837 amino acid residues, which the initial 27 of them are hydrophobic and considered to play a role as a signal peptide. Several studies have compared VLDLR amino acids in human and mouse and found that they are essentially identical up to 97% (74). mRNA transcripts of VLDLR are in their highest expression in brain specifically in the cerebellum and cerebral cortex. Skeletal muscles and heart are the other two parts of the body with high VLDLR expression (75). Iwata *et al.*, showed that over-expression of VLDLR in rat leads to a significant hyperactivity accompanied by impaired memory function. They concluded that expression levels of this protein play a role in motor function and cognition (76).

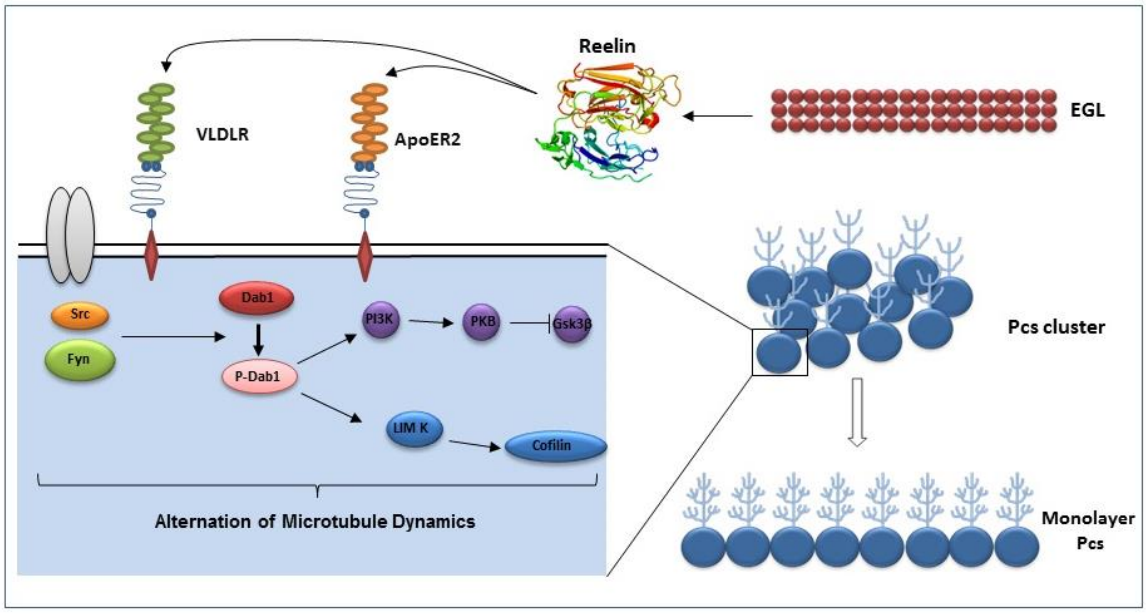
1.8.2 Apolipoprotein E Receptor 2 (ApoER2)

The ApoER2 protein is comprised of 870 amino acids. The basic structure of the ApoER2 is similar to VLDLR; however they differ significantly in the size of their O-linked glycosylation domains, which is more than double in size in ApoER2 (71). It has been reported that this glycosylation is associated with the stability of the receptors, nevertheless, little is known about its mechanism of action on receptors and their functions (77). The expression level of ApoER2 mRNA is high throughout the CNS with higher expression specifically in Pcs of the cerebellum. mRNA transcripts of this receptor have been measured to be high in testis and placenta as well (78). Similar to VLDLR, expression of ApoER2 starts from E12 (mouse) in preplate and it is considerably high in Pcs from E16.5 / E17. In contrast, VLDLR expression is strong at E18 and continues into postnatal day P0-P6 in Pcs (71, 79).

1.9 Reelin signaling

During the development of brain, binding of reelin to either one of the ApoER2 or VLDLR initiates the downstream signaling cascade. Clustering of the receptors then activates two families of kinases including Fyn kinase family and Src tyrosine kinase family. This activation phosphorylates Dab1 (Disabled 1) on its four tyrosine residue. Dab1 interacts with the intracellular regions of reelin receptors (65, 80). Phosphorylation of Dab1 results in the induction of several pathways. One of the major cascades is activation of Phosphatidylinositol-3-Kinase (PI3K), which consequently increases the phosphorylation level of protein kinase B (PKB) and inhibits Glycogen Synthase Kinase-3 beta (GSK3). In normal condition, GSK3 effects on tau protein and mediates microtubule stability; however, in this pathway inhibition of GSK3 alters the dynamic of microtubule formation and leads to neuronal migration (80). Alternation of the microtubule dynamics, which occurs following Dab1 phosphorylation, is influenced by LIS1 Protein. Termination of the signaling cascade happens when a specific population of Dab1 is phosphorylated and degraded via proteasome system (81). reelin signaling is a very complex cascade that can activate and regulate many other intracellular pathways, therefore, in this section only molecules that have been the focus of recent researches are being addressed (Figure 1).

Figure 1. Reelin signaling pathway in migration and monolayer formation of cerebellar Purkinje cells.



Three basic functions have been reported to occur as the result of reelin-Dab1 signaling during brain development including cellular adhesion, dendritic process growth, and neuronal positioning and migration (82). In the context of neuronal migration, which is also considered as a critical issue in cerebellar development, reelin signaling regulates the formation of the cytoskeletons for migration of neurons.

It has been recently reported that reelin-Dab1 signaling through ApoER2 activates LIM kinase 1 (LIMK), which then leads to the phosphorylation of a protein called cofilin at the leading processes of migrating neurons as well as the growing branches in dendrites and axons. Cofilin can depolymerize F-actines and consequently facilitate the formation of new actin filaments. Phosphorylation of cofilin inhibits its ability to depolymerize and leads to cytoskeleton stability of the leading process toward the marginal zones under the cortical surface (83). In addition, reelin is

considered as a signal to stop migrating neurons via stabilizing the cell cytoskeleton (84).

EphB tyrosine (Ephrin-B) kinases are the other critical regulators for cell adhesion in developing brain. Bouche *et al.*, demonstrated that reelin is able to activate EphB signaling in neurons independent from the classical pathway mediated through ApoER2 and VLDLR. This signal integration is facilitated by direct binding of reelin to the EphB transmembrane extracellularly. Additionally, they have confirmed their data by reelin treatment that led to auto-phosphorylation and consequent proteolytic degradation of EphB proteins, signifying that the reelin-EphB interaction can induce EphB signaling in responsive cells (85). On the other hand, if the EphB is impaired the reelin-Dab1 does not function properly while activation of EphB is able to rescue the neuronal disorganization in the absence of reelin in reeler mouse (reelin mutant will be discussed in section 1.10) (86).

CDK5, a member of the family of Cyclin Dependent Kinase (CDK), is a Serine/Threonine protein kinase principally expressing in neurons and Cdk5 mutant exhibits the similar neuronal positioning in its cortex to reeler mouse (87). Together with previous studies in this field, Fuchigami *et al.*, confirmed that CIN85, a protein that interacts with Dab1 is phosphorylated by CDK5 activation. CIN85- Dab1 signaling can modulate the actin formation and stability in neuronal structures (88, 89).

Integrin's are family of transmembrane receptors that connect the cytoskeleton to the extracellular matrix and play role in cell adhesion and migration. The function of α3β1 integrin that mediates adhesion of neurons to the radial glia fibers has suggested the potential reelin- α3β1 integrin interactions. Embryonic analysis revealed co-expression of α3β1 integrin with Dab1 (90). It has been suggested that

α3β1 integrin binding to Dab1 in response to reelin, can trigger their degradation and consequently facilitate the detachment of neurons from Radial glia fibers (91).

1.9.1 Reelin and ERK signaling

Mitogen activated protein kinases (MAPK) mediate the phosphorylation and activation of nuclear transcription factors that regulate cell proliferation, differentiation, migration, stress responses, apoptosis, and survival. This family of kinases includes the extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinases 1-3 (JNK1-3) and P38. In the process of the migration, cells may apply different types of kinases in this family depending on the cell type, extracellular stimulus, and/or phosphorylation of the distinct substrates. Variety of mitogens and growth factors can stimulate the Ras_Raf-1_MEK-1/2_Erk-1/2 (main components of Erk1/2 signaling) signaling module. In this signaling pathway Thr and Tyr residues within the Erk1/2 Thr-Glu-Tyr motif is phosphorylated and eventually results in alteration of cell adhesion dynamics and cell migration.

Recently there have been some studies which link the reelin signaling pathway to the MAP family of kinases. ERK1/2 in association with reelin formed the central focus of a study by Lee *et al.*, in which the authors demonstrated that full-length reelin moiety can activate the ERK 1/2 signaling, however, this activation is not mediated through the canonical signaling pathway of reelin. reelin-ERK1/2 signal transduction can significantly regulate learning, memory, and synaptic plasticity in postnatal and adult brain (92). C-Jun N-Terminal kinase (JNK) is another member of the MAPK family that plays a role in cell trafficking, adhesion, morphogenesis in neurons, neurotransmission, and cell response to stress. Studies suggest that ApoER2 is important in the recruitment of this kinase (93). In this context it is necessary to

mention that MAPK family are activated by various factors for instance the ERK pathway that is activated by number of cell matrix proteins including vitronectin, collagen and fibronectin, in additions other stimuli such as EGF, FGF, VEGF, and urokinase plasminogen activator (uPA) (94). Brain-derived neurotrophic factor (BDNF) is another factor that has been reported to play a role in maturation of cerebellar gcs. It is suggested that MEK is modulated by BDNF (95).

1.10 Animal Models of reelin /Dab1/ Vldlr/ ApoER2

Reeler mouse is the result of an autosomal recessive mutation on *RLN* gene. Many regions of the brain are affected by this mutation including the hippocampus, neocortex, inferior olive, olfactory bulb, substantia nigra, and cerebellum (96). All these structures in the brain are interconnected together but in this section, we will focus on the malformations that influence the cerebellum. There are a number of important differences between reeler mutant and a normal mouse. According to Mariani *et al*., the cerebellum size of the reeler mouse is 25-33% less than a normal mouse, exhibits no foliation, and has neuronal disorganization in most areas of the cortex, accompanied by the decreased number of UBCs and gcs. The first sign of a cerebellar defect in reeler mouse appears by malformation of Pcs clusters around E14 (97). In addition to abnormal Pc migration, these mutants reveal no significant amount of foliation in their cortex, while wild-type embryos exhibit initial evidence of foliation by E17. In spite of the severe cerebellar neurons disorganization, 7% of the Pcs manage to form a monolayer and consequently, normal cytoarchitecture can be observed in small cortical regions. 10% of the Pcs are positioned in the granular layer and the rest (around 80%) are in the form of cellular masses (96, 98). In addition, axon growth and connections are distorted in cerebellar neurons, which can be the consequence of altered migration of Pcs. Electrophysiological studies of Pcs in reeler

mouse indicated that these neurons have normal sodium and calcium-dependent spikes (99). Based on neurobehavioral tests performed on heterozygous and homozygous reeler mice, it has been suggested that heterozygous reeler is an appropriate model to study the psychiatric disorders specifically schizophrenia (100). In contrast, Krueger *et al.*, reported that heterozygous reeler can be considered as a model to study general concepts of psychiatric disorders but not schizophrenia (101).

Deficiencies in downstream molecules of reelin signaling pathway such as Dab1, ApoER2/ Vldlr, and Src/Fyn indicate approximately identical phenotype and cerebellar malformations (102). Spontaneous autosomal recessive mutation in Dab1 gene located on chromosome 4 results in scrambler mutant phenotype. Observation of adult cerebellum revealed hypoplasia accompanied by the lack of foliation in this mutant. Scrambler mutants are distinguishable from the wild type at around age P8 by their ataxic gate and tremor (59). Histological studies confirmed decreased number of gcs by 80% as well as significant reduce in the number of Pcs (96).

Double Vldlr/ApoER2 knockouts show precisely the same phenotype in the reeler or scrambler (103). Single knockouts of Vldlr and ApoER2 exhibit smaller size cerebellum with decreased foliation and cortical lamination, however, this phenotype is more evident in the cerebellum of Vldlr mutant (71).

ApoER2 regulates the endocytosis of ligand called Selenium. This transport is mediated by Selenoprotein P (Sepp1) transported from the liver to the brain. The phenotype of ApoER2 mutant mice is similar to the Sepp1 knockout showing lower levels of selenium at the brain, which is the site of ApoER2 expression (104).

1.11 Lysosomal acid phosphatase 2 (Acp2) mutant mice

The animal model used in this study called nax (naked-ataxia) is the result of a spontaneous mutation of lysosomal acid phosphatase 2 (Acp2/LAP) in mouse. ACP2 is one of the many soluble luminal hydrolases responsible for lysosomal catabolic functions. It is an enzyme ubiquitously expressed in all cells of the body. There are two lysosomal acid phosphatases expressed in the lysosomal compartments. The first is sensitive to inhibition by tartrate (LAP/Acp2) and the second is acid phosphatase-type 5 (Acp5), which is resistant to tartrate. LAP is a transmembrane protein that is comprised of alpha and beta subunits. In humans, the beta and alpha subunits are encoded by the Acp2 and Acp3 genes, respectively (105). The Acp2 gene is located on the short arm of human chromosome 11, and on chromosome 2 in the mouse. ACP2 is a transmembrane protein containing a single transmembrane domain, a highly glycosylated luminal domain, and a carboxy-terminal cytoplasmic tail. This isoenzyme is responsible to hydrolyze orthophosphoric monoesters into alcohol and phosphate. In addition, Acp2 contributes in the removal of the M6P recognition marker from proteins entering into the lysosomes. A spontaneous autosomal recessive mutation in Acp2 (when a replacement of guanine with adenine at exon 7 happens and results in the conversion of a glycine into glutamic acid at position 244 of the protein) is responsible for cerebellar and skin malformations in nax mice.

1.12 Acp2 expression in cerebellum

Expression of the acp2 in mouse tissue has its highest levels in testis and brain. In brain, it is exclusively expressed in choroid plexus epithelial cells, pyramidal neurons, and Pcs of cerebellum. Our laboratory has already shown that regulation of the expression of ACP2 in the cerebellum of C57B/6 mouse is dynamic during development. In neonatal mice, ACP2 is expressed in the cerebellum, caudal

midbrain, and choroid plexus of the 4th ventricle. In the cerebellum, until P4, ACP2 is expressed in most of the neuronal types of the cerebellum. However, by around P8, ACP2 expression of is limited to the somata of Pcs in the posterior lobe vermis and hemispheres. By P10, an increase in the expression level was observed in the vermis and hemispheres of the entire cerebellum, while the enzyme was still detected in soma of the Pcs, but also weakly in the dendrites. By P13, ACP2 expression was clearly observed in the soma and dendrite of a subset of Pcs.

1.13 Cerebellum abnormalities in nax mice

Nax mice can be distinguished from normal siblings by their phenotypic characteristic, which includes short stature, delayed coat hair appearance or whole body alopecia, and ataxic gait. In addition, the size of the whole brain is smaller (0.28g) compared to the wild type (0.47 g) based on the examination of the fixed whole brains at P19. Cerebellum of the nax mutant is also smaller in size with underdeveloped characteristics such as partially hypo-plastic or absent vermis. The average weight of the nax mutant is around 50% less than the wild type sibling and a significant reduction of their weight occurs at ~ P17 and the mutant death happens shortly after that at around P18-P26 (1).

Performing macroscopic observation of the adult nax mouse brain revealed a substantial reduction in the cerebellum size when compared with the wild type siblings. Histological analyses indicated that in nax mice the cerebellum cytoarchitecture is disrupted compared with the organization of the classical cerebellar cortex (including three layers of molecular. Precursor in the external granule cells fail to migrate to form EGZ, the Pcl is disorganized, and the Bergmann fiber structure is disrupted. The EGZ, which is composed mainly of very densely

packed soma of gcs, is lacking in nax cerebellum and there is a significant decrease in the number of the gcs.

In the human, a disorder called Gomez Lopez Hernandez syndrome (GLHS) shows similar symptoms of the nax mutant including alopecia, rhombencephalon-synapsis, and trigeminal anesthesia.

1.14 Rational of the Study

Purkinje cells are normally aligned into a monolayer that separates the ml from the gl. Immunohistochemically staining with Calbindin reveals that the soma of these cells are ectopically placed in a multilayer manner and do not develop organized dendritic trees, but rather extend their dendrites in a completely uncoordinated fashion, appearing shorter and clearly disoriented. On the contrary to the wild type sibling, there is not a clear boundary between the molecular and Pc layer and it seems that Pcs have not followed a normal pathway to migrate, stop and form a monolayer however they invaded the molecular layer. In this study we investigate which molecular signaling pathway controls the Pcs migration process in Acp2 mutant mouse that leads to mal-positioning of Pcs in cerebellar cortex.

Hypothesis:

In this project I will test the hypothesis that reelin-ERK1/2 signalling pathway plays a critical role in the formation of monolayer Pcs in mouse cerebellar cortex

Aims:

1. To investigate differential expression of reelin pathway in the cerebellum of the wild type and nax mutant mouse

2. To determine whether reelin-Dab1 affects the ERK 1/2 signaling pathway in developing cerebellum

CHAPTER 2: MATERIAL AND METHODS

2.1 Animal Model and Maintenance

Regulations of the Canadian Council on Animal Care (CCAC) were applied for the animal husbandry for all the animals used in this study. Nax mutant was obtained by transferring the nax mutant embryos from Institute of Human Genetics in the University Medical Center, Georg-August University, Gottingen, Germany followed by establishing the colony in the Genetic model center of University of Manitoba. Initially mice (C57 BL/6) heterozygous were bred to achieve nax mutant (homozygote/heterozygote/wild type ratio was 25%:50%: 25%, respectively). The mice colony was kept at room temperature with relative humidity (18–20°C, 50–60%) on a 12:12 light and dark cycle. Based on the phenotypic characteristics including smaller size, alopecia (lack of hair), and ataxia nax mutant can be distinguished from the wild type sibling. Since there are a number of animals in this study that were used at the early ages such as P1 and P3, PCR was performed to confirm the genotype of the animals.

2.2 Mouse Genotyping

Genotyping of the mutant mouse was performed using AccuStart^{TM} II Mouse Genotyping Kit (Quanta, Biosciences).

2.2.1 DNA Extraction

Tail samples were added to a 70µl of Extraction Reagent. Then samples were heated to 95°C for 30 minutes followed by cooling them in room temperature. An equal volume of Stabilization Buffer was then added to the extracts. DNA extracts can be directly used to run PCR.

2.2.2 Polymerase Chain reaction (PCR)

Extracted DNA's were used for PCR and all the PCR reactions were performed in 25µl of PCR master mix solution.

Table 1: Reagents for PCR

PCR Reaction Setup Component	Volume for 25-µL rxn.
AccuStart II GelTrack PCR SuperMix (2X)	12.5 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
Nuclease-free water	9 µL
DNA Extract	2.5 µL
Final Volume (µL)	25 µL

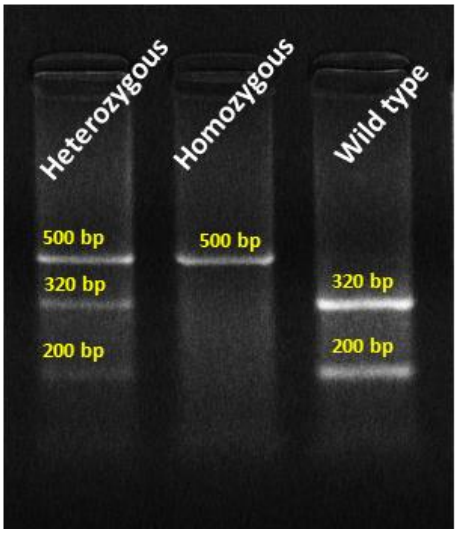
Transcripts were amplified from *nax* mutant colony. The PCR cycle comprised of an initial step including 5 cycles of denaturation step at 95˚C for 4 min, 32 cycles of annealing step at 56 for 45 ˚C sec was done followed by 1 min at 72˚C extension and final extension at 72 ˚C for 10 min. Annealing temperature was selected based upon the GC and AT content in each primer pair. Acp4F (5' GCACTCTGTGCCTTCTCCAT-3') and Acp4R (5'-CTGGGAGATTTGGGCAACTA-3') primers were utilized in PCR reaction.

2.2.3 Restriction digestion with *Bam*HI enzyme

Master mixture for restriction digestion for each sample contained 2.5 µl of Buffer and 1.66 µl of *Bam*HI enzyme. Then 4.16 µl of reaction mixture was added to each tube of 25µl PCR product. PCR products were incubate at 37°C for 2 hrs for the digestion and run on a 2.5% agarose gel prepared in 1x TAE buffer with 1µg/ml

ethidium bromide. The bands were visualized using a gel documentation system Fluorchem-8900 gel imager (Alpha Innotech Corp, San Leandro, CA, USA). Fragments of 525 bp were indicative of homozygous genotype, two fragments of 320 bp and 200 bp were indicative of wild type genotype.

Figure 2. Agarose gel showing different genotypes of nax mutant mouse



2.3 Perfusion and Sectioning

All mice were deeply anesthetized with 20% isoflurane, USP (Baxter Co. Mississauga, Ontario, Canada) in propylene glycol (Sigma-Aldrich Canada Co., Ontario, Canada) using a desiccator. The mice were transcardially perfused with 15 ml of 0.1 M phosphate buffer saline (pH 7.4) and 30 ml of 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS). The brains were removed and post-fixed in 4% PFA at 4°C for at least 24 hours. The cerebellum was removed and cryoprotected using 10% (2 h), 20% (2 h) and 30% (24 h) sucrose solution in PBS. The cerebella were then frozen in clear frozen section compound (VWR, Mississauga, Ontario, Canada) at -80°C for 30 min. Transverse sections of the cerebellum were serially cut at a 20 µm thickness using a -20°C cryostat and collected in PBS for free-floating immunohistochemistry.

2.4 Immunohistochemistry

Peroxidase immunohistochemistry was performed on cerebellar sections by washing the sections thoroughly with PBS followed by stopping the endogenous peroxidase activity using 0.3% H2O2 for 20 min. Then sections were blocked with 10% normal goat serum and then incubated in 0.1M PBS buffer containing 0.1% Triton X-100 and the primary antibody for 16–18 h at room temperature. Secondary incubation in horseradish peroxidase (HRP) –conjugated goat anti-rabbit or HRP-conjugated goat anti-mouse antibody lasted 2 h at room temperature. Diaminobenzidine (DAB, 0.5 mg/ml) was used as the chromogen to visualize the staining. Sections were dehydrated through an alcohol series, cleared, and cover-slipped with mounting medium. For negative control samples, similar as mentioned above, addition of primary antibody was replaced by a suitable IgG isotype. Sections were washed with PBS and peroxidase activity was measured by using DAB.

2.5 Immunofluorescence

For double immunofluorescence, samples were co-incubated in primary antibodies overnight (at room temperature) and then incubated in Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG for 1 hat RT. Samples were then washed with PBS and cover slipped with Fluorsave.

Table 2: Primary antibodies used in immunohistochemistry and immunofluorescence

Primary Antibody	Dilution	Source
reelin (Mouse monoclonal)	1:250	Hybridoma bank, University of IOWA
Dab-1 (Rabbit polyclonal)	1:300	#117055, SIGMA-ALDRICH
CaBP (Mouse monoclonal)	1:1000	#300, CEDARLANE
CaBP (Rabbit polyclonal)	1:1000	#CB-38a CEDARLANE
Erk1/2 (Rabbit polyclonal)	1:100	#4370, Cell Signaling
PAX6 (Mouse monoclonal)	1:50	Hybridoma bank, University of IOWA

Table 3: Secondary antibodies used in immunohistochemistry and immunofluorescence

Secondary Antibody	Dilution	Source
Goat anti rabbit IgG, HRP conjugate	1:500	#12-348,Millipore
Goat anti mouse IgG, HRP conjugate	1:500	#AP308P,Millipore
Alexa Fluor 488 goat anti-mouse IgG	1:1000	#A32727,Thermo Fisher
Alexa Fluor 594 goat anti-rabbit IgG	1:1000	#A11037,Thermo Fisher

2.6 Image Acquisition

In order to perform bright field microscopy, Zeiss Axio Imager M2 microscope (Zeiss, Toronto, ON, Canada) was used and images were analyzed with Zeiss Microscope Software (Zen Image Analyses software; Zeiss, Toronto, ON, Canada). Florescent microscopy of the cerebellar sections was performed using Zeiss Lumar V12 Fluorescence stereomicroscope (Zeiss, Toronto, ON, Canada). High

magnification fluorescence microscopy was performed using a Zeiss Z1 and Z2 Imager and a Zeiss LSM 700 confocal microscope (Zeiss, Toronto, ON, Canada). Adobe Photoshop CS5 Version 12 was used to edit, crop, and correct contrast and brightness of the images.

2.7 Western Blotting

After cerebellar tissues were collected from the animal they were added to the appropriate amount of lysis buffer (based on the tissue size) containing protease inhibitor cocktail (Life Science, cat# M250) and phosphatase inhibitor (Sigma Aldrich, cat# P5726) and samples were kept in -80 until used for western blotting. Concentrations of the protein were measured using BSA kit (Bio-Rad cat#5000121) and loading samples were prepared by adding loading buffer (Tris-Hcl 60mM, glycerol 25%, SDS 2%, mercaptoethanol (ME) 14.4Mm, bromophenol blue 0.1%, H2O). In order to perform electrophoresis on samples they were first heated at 90 ˚C for 5 min and 15 µl of each sample was loaded on a 6-15% polyacrylamide gel based (percentage of the gel was selected based on the molecular weight of the protein) 10 µl of precision plus protein were used as marker standard (Thermo Fischer Scientific, ON, Canada). After proteins were separated using electrophoresis they were transferred onto a PVDF membrane using a transfer buffer (500nM glycine, 50mM tris-HCl, and 20% methanol) for 1 h, at room temperature, 100 volts. Blocking of the membrane then performed using 5% milk for ERK1/2 antibody and 3% Bovine Serum Albumin (BSA) for Anti Dab-1 antibody in 1x TBST (Tris-buffered saline/0.01% tween 20) at room temperature overnight. Then membranes were incubated overnight at 4˚C with suitable primary antibodies (see Table 4) in 5% milk and 5% BSA for in 1x TBST. After enough incubation with primary antibody, membranes were washed with 1xTBST 3x20 min and incubated with secondary

antibodies (HRP) (see Table 4) for 1hr at room temperature. Membranes were washed 3x20 min and incubated with ECL reagent (Pierce, ON, Canada) for 2-3 min.

Table 4: Primary antibodies used in western blotting

Primary Antibody	Dilution	Source	Molecular Size
reelin	1:1000	Hybridoma bank, University of IOWA	388kD
Dab-1	1:1000	#SAB4503448 ,Sigma-Aldrich	59 kD
P-Dab1	1:1000	#SAB4504377 ,Sigma-Aldrich	59 kD
Total ERK1/2	1:1000	# 9102S,Cell Signaling	42,44 kD
P-ERK1/2	1:1000	#4370,Cell Signaling	42,44 kD
VLDLDR	1:1000	#sab1303099, Sigma-Aldrich	95 kD
Beta(β)- Actin	1:5000	# A5441, Sigma-Aldrich	40 kD

Table 4: Secondary antibodies used in western blotting

Secondary Antibody	Source	Dilution
Goat anti rabbit IgG, HRP conjugate	#12-348,Millipore	1:1000
Goat anti mouse IgG, HRP conjugate	#AP308P,Millipore	1:1000

2.8 Densitometry Analysis of Western Blots

The signal intensity of each protein band was measured by using Alphaease software. For each protein, the band was measured and normalized to the total protein band or the β-Actin/GAPDH.

2.9 Cell Culture

2.9.1 PC12

PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla. Since this cell line has an embryonic origin from the neural crest it is considered as an appropriate model for in vitro studies. Based on the previous studies (106-110) this cell line was used to explore the association of ERK1/2 phosphorylation with DAB1 phosphorylation. PC12 cells were maintained in DMEM-high glucose (Invitrogen) with 10% fetal bovine serum (FBS), 100 U/mL Penicillin and 100 μg/mL streptomycin in a 5% CO2 incubator at 37°C.

2.9.2 Preparation of reelin Condition Medium

Reelin is also expressed from other tissues such as kidney during the development. HEK-293 (Human Embryonic kidney) cells were used to provide reelin condition medium for this study. In addition it should be mentioned that since the genomic structure of the *Reln* gene is highly conserved, this gene has the same structure in mouse and human tissue (111). Cells were grown in 10-cm plates until they reached 80% confluence in a 5% CO2 incubator at 37°C. They were washed once with PBS and incubated for 24 h with serum-free medium (DMEM-high glucose, 100 U/mL penicillin and 100 μg/mL streptomycin) in a 5% CO2 incubator at 37°C. Later, the cell medium was collected and centrifuged at 1,000 rpm for 5 min, and the supernatant was stored at 4°C. The remaining plates with cells were filled

again with serum-free medium and incubated for 24 h in a 5% CO2 incubator at 37°C. The collecting procedure was repeated a total of three times (72 h), and then the supernatant was concentrated 10 times using Amicon Ultra-15 centrifugal filter units with a 100 kDa membrane cut-off, according to the manufacturer's protocol (Millipore)(112).

2.10 Gene expression analysis

Total RNA was extracted using a kit (TRIzol® Plus RNA Purification Kit, Life Technologies, NY, USA) according to manufacturer's instructions. Quality and quantity of RNA were determined by measuring the absorbance at 260 and 280 nm using NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All samples had an absorption ratio A260/A280 between 1.8 – 2.2. RNA (1 μg) from each sample was treated with RQ1 RNase-Free DNase® (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions, to remove genomic DNA contamination. Reverse transcription was performed using SuperScript VILO cDNA Synthesis Master Mix (Invitrogen, Grand Island, NY, USA), according to the manufacturer's instructions. Real-time PCR (RT-PCR) reactions were performed in a Roch lightCycler 96 Real-Time System using Power SYBR green master mix (Life Technologies) in a final volume of 20 μl reactions. All samples were tested in triplicates. Differences in the threshold cycle (ΔCt) number between the target genes and the housekeeping gene TATA Box binding protein (TBP), which was used to calculate differences in expression. Primers sequences are provided in table 4.

Table 4. Primer sequences used in gene expression analysis

Gene	Forward Primer	Reverse Primer
reelin	TCGCCTTTCTTTTTCCTGTG	TTCCTGTCCCGGTACGTAGT
DAB1	CAGCGAAGCCACTTTGATAA	TAACTTGTCTCCCCGAGCTG
VLDLR	TCGGGCTTTGTTTACTGGTC	AGTAGAGGCGGCTTTTGACA

2.10.1 Statistical analysis

The results were compared and analyzed using a student t-test to compare between two groups and one-way/two-way analysis of variance (ANOVA) to compare between more than two groups. The significance level was adjusted at $P < 0.05$. All the experimental conditions were repeated at least GraphPad Prism 6 (GraphPad Software, Inc. La Jolla, CA, USA) was used for statistical procedures and graph plotting.

2.10 Migration assay

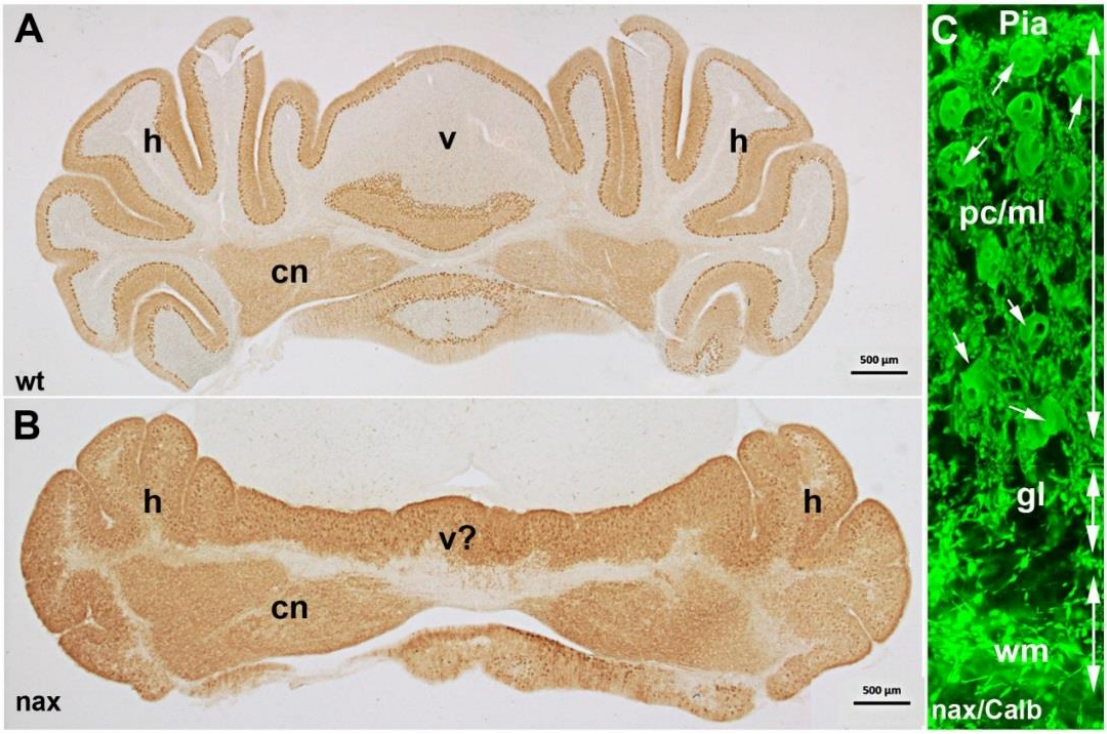
Two well silicone insert with a defined cell-free gap was used to performed migration assay. The culture inserts were placed in 12 well culture plate and 5000 cells were added to each chamber. Before the day of migration the cells were cultured under starved condition for 24 hours. Then inserts were removed and cells were treated with reelin condition medium for 72 hours to migrate. Untreated cells were used as control. ERK1/2 inhibitor U0126 (Cell signaling Cat#9903S) used to co-treat the cells with reelin condition medium. 10µM concentration of ERK1/2 inhibitor was used in this assay.

CHAPTER 3: RESULTS

3.1 Purkinje cell are mal- positioned and disorganized in nax mutant mouse compared to the wild type sibling

Initially to study the difference in distribution and localization of the Pcs in wt and nax mutant we applied the immunostaining of Calb1 antibody as a specific marker for Pcs in P17 wt and mutant mouse. P17 mice were chosen because at this stage the development of the lobules can be clearly seen and also migration and monolayer formation of the cerebellar Pcs is completed, thus the differences of the wt and nax is more clear to investigate. In addition to the smaller size (1) and underdeveloped lobulation of nax cerebellum, Calb1 revealed a significant difference in organization of the Pcs. The lobules with two distinct hemispheres and well-developed vermis are observed in the wild type sibling cerebellum. Purkinje cells are localized in a monolayer manner beneath the molecular layer with no sign of ectopic Pcs. CN is located intact in the cerebellum.

Figure 3. Transverse sections through the wild type and nax mutant cerebella at P17 immunostained with Calb1

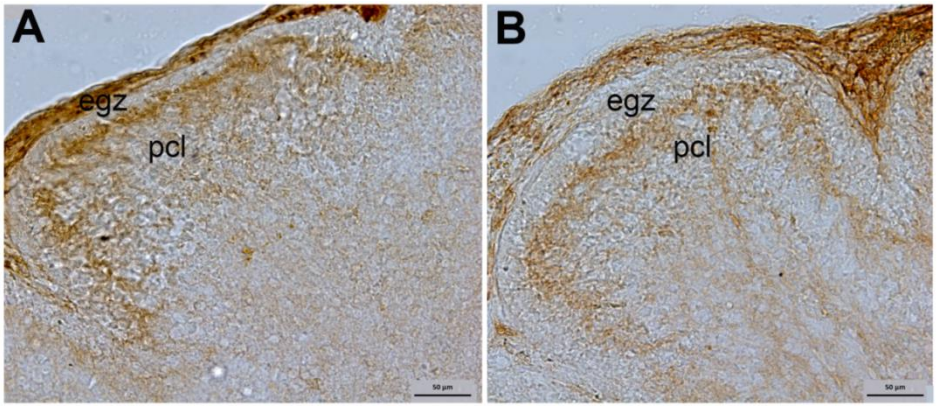


(A) Transverse section of P17 wt mouse stained using Calb1 show that Pcs are arranged in monolayer. Cerebellum has a developed vermis with two hemispheres on both sides. **(B)** Examination of nax cerebellum reveals that the size of the cerebellum is smaller and lobules are not fully developed. Hemispheres are not divided by an underdeveloped and hypoplastic vermis. Purkinje cells are arranged in a multilayer manner instead of a monolayer. Examination of the Pcs in nax cortex immunoflourscence staining with Calb1 indicates disorientation and the lack of the proper migration of the Pcs. No distinct boundary is observed between molecular layer and the Pcl and Pcs have moved toward the ml and reach to pia surface. Arrows indicate the Pcs soma **(C)**. ml, molecular layer; h, hemisphere; V, vermis; cn, cerebellar nuclei; gl, granular layer; wm, white matter. Scale bar = 500 µm in **(A, B)**; 50 µm in **(C)**.

3.2 Reelin in expressed by the cells of external germinal zone in wt and nax mutant mouse at P1

To determine if reelin is present in nax mutant and also observe the localization and distribution of this protein in developing cerebellum the immunohistochemistry with anti-reelin antibody was performed at P1 wt and nax mouse. reelin is expressed by the precursors of the granule cells localized in the external germinal zone (egz).and staining revealed the similar expression and distribution in nax and wt. This data is in accordance with the level of mRNA expression (that will be discussed in figure 10) and protein expression shown by western blot (in figure 7) confirms that the reelin as a critical protein to stimulate the Pcs migration cannot be the only determinant factor to the differential migration seen in Pcs of the *nax* mutant mouse.

Figure 4. Sagittal sections of P1 wt and nax immunostained with reelin

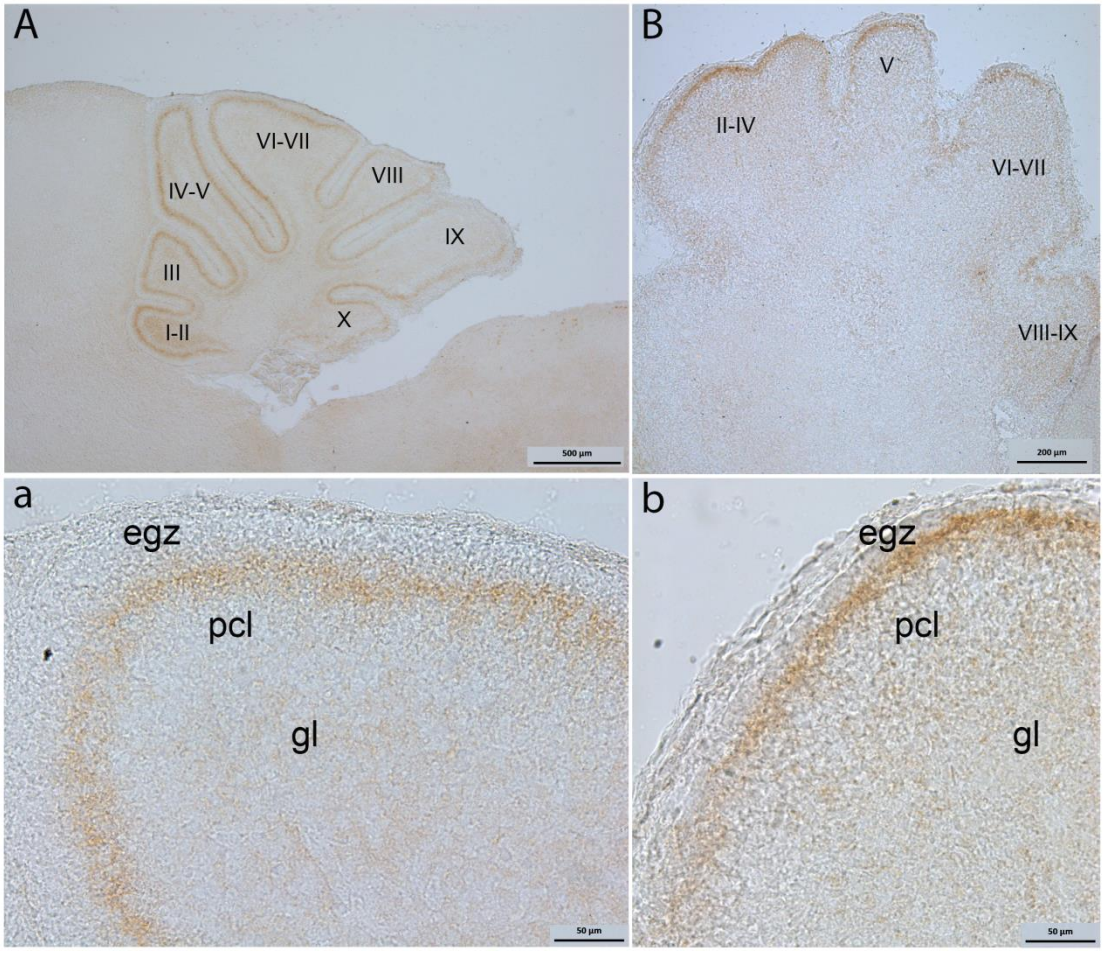


Sagittal sections immunostained with Calb1 antibody at P1 specify uniform expression of reelin protein in the nax and wt sagittal cerebellar (A) wt (B) nax showing that there is no significant difference in the pattern of expression at this age. egz, external germinal zone; pcl, Purkinje cell layer. Scale bar = 50 μm in (A) and (B).

3.3 Reelin in expressed by the cells of egz in wt and *nax* mutant mouse at P3

Since proteins may play different roles during development they might dynamically be expressed in different regions thus we further investigated the reelin expression and distribution in wt and nax mouse by immunostaining of reelin antibody on the sagittal sections of P3 mouse. Smaller cerebellum of the nax reveals almost the same pattern of the expression of reelin in egz compared to the wt sibling.

Figure 5. Sagittal sections of P3 wt and nax immunostained with reelin



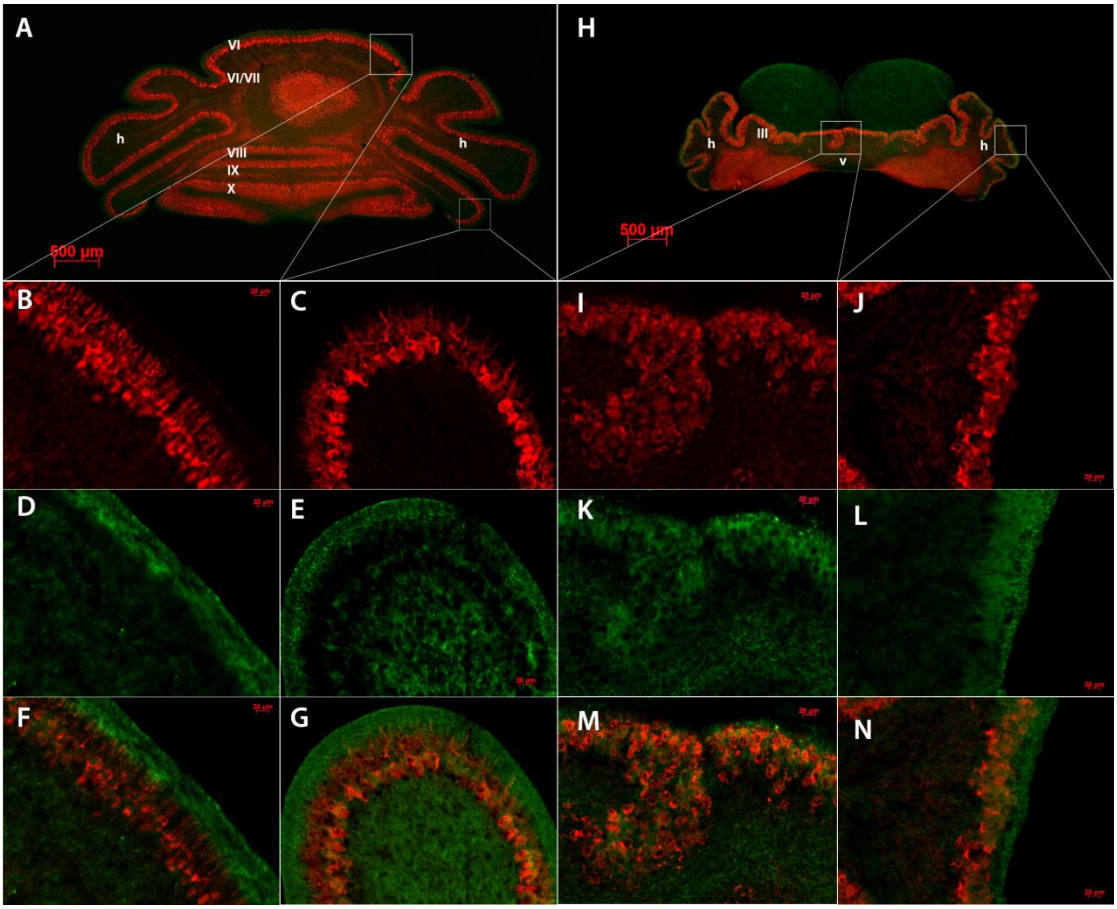
Immunostaining of the sagittal sections of P3 wt and *nax* mutant cerebellum revealed the similar pattern of expression and distribution of reelin in egz in both wt and nax sections **(A, B)**. However, the underdeveloped cerebellum and egz in nax mutant (**B**) compared to the wt **(A)** is accompanied by slightly less expression of reelin.(a) Shows

the higher magnification of **(A)** and **(b)** is higher magnification of **(B)**. egz, external germinal zone; pcl, Purkinje cell layer; gl, granule layer. Scale bar = 100 μm in **(A)** and **(B)** and 50 μm in **(a)** and **(b)**.

3.4 Reelin and Calb1 expression in P8 wt and nax mutant mouse

To further characterize the expression of reelin during the postnatal stages of mouse life span, expression of reelin in Pcs at P8 mouse cerebellum was investigated. We found that egz of the wt section is well developed and reelin expressing cells can be clearly observed in the lateral and medial cerebellar cortex. As our lab recently reported the anteromedial cerebellum in nax mutant is not properly developed compared to posterolateral cerebellum thus reelin expression in the antromedial part of the nax cerebellar cortex is observed to be weaker; however reelin expression in lateral parts is almost comparable to the wt.

Figure 6. Double immunostaining of reelin (green) and Calb1 (red) on frontal sections of P8 wt and nax mouse.



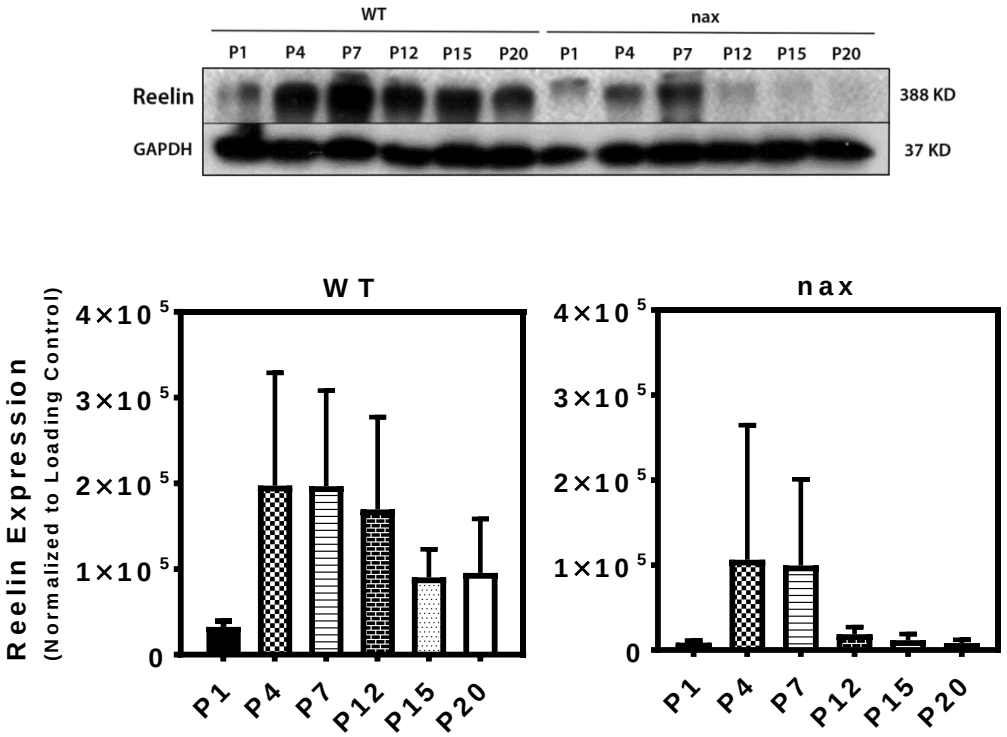
The wild type **(A)** shows reelin expression in the well-developed egz in the medial **(B-F)** and lateral **(C-G)** cerebellar cortex. **(H)** The nax cerebellum shows reelin expression in the lateral cerebellar cortex **(J-N)** but lack/weak in anteromedial part **(I-M)**. Scale bar= 500 µm in **(A)** and **(H)** and 20 µm in **(B-G)** and **(I-N)**

3.5 Reelin expression is showing a similar trend in wt and nax mutant mouse

Western blot analysis of reelin expression during cerebellar development performed at P1, P4, P7, P15 and P20. Due to different roles of reelin during development we chose wide range of ages from P1 to P20 in order to have a better understanding of reelin expression in nax. Immunoblots of total cell lysate from wt sibling and *nax* mouse cerebellum indicate an up-regulation of reelin from P1 to P4 in

both wt and mutant followed by a down-regulation from P12 to P20 in wt and nax samples. It is observed that the trend of reelin expression is the same in both wt and nax samples during Purkinje cell monolayer formation that is started at P4 and is continued by P7.

Figure 7. Western blotting of the cerebellar samples of wt and nax mouse at P1, P4, P7, P12, P15, and P20 with reelin.

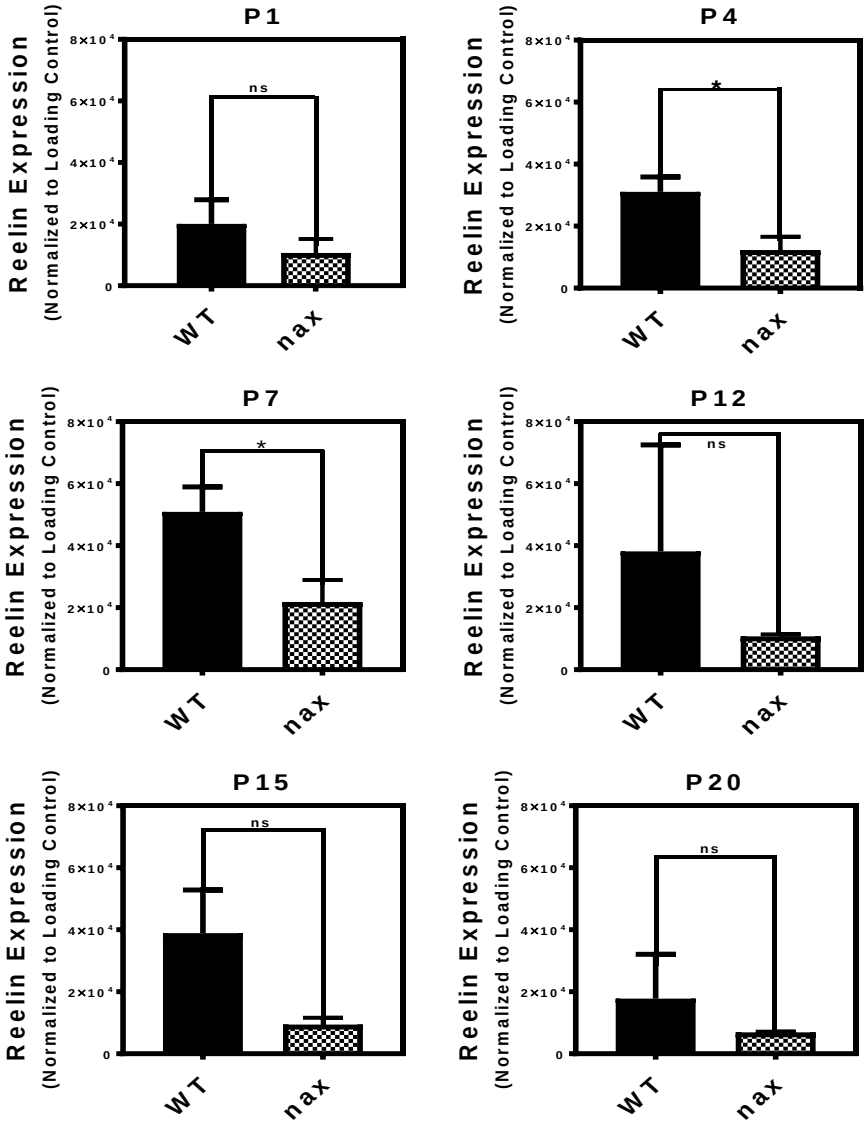


Western blotting of the cerebellar samples of wt and nax mouse at P1, P4, P7, P12, P15, and P20 shows the same trend but lower expression of reelin in nax compared to the wt. This experiment was repeated over three different litters for each postnatal days in wt and nax siblings (wt; n=3 and nax; n=3). The data in the bar graph are presented as the mean ± SEM, and statistical analysis was performed using one-way ANOVA.

3.6 Reelin expression is lower at P4 and P7 in wt compared to the nax mutant

To have a better understanding of reelin expression we compared its expression in wt and nax separately. As it is observed in the following graphs expression of reelin is significantly lower at P4 and P7. These two days are considered critical during Pcs development, since spreading of Pcs clusters and Pcs monolayer formation occurs during this period from P4 to P7. However expression of reelin is lower in all days in this study but they are significant.

Figure 8.Comparison of the reelin expression level in wt and nax

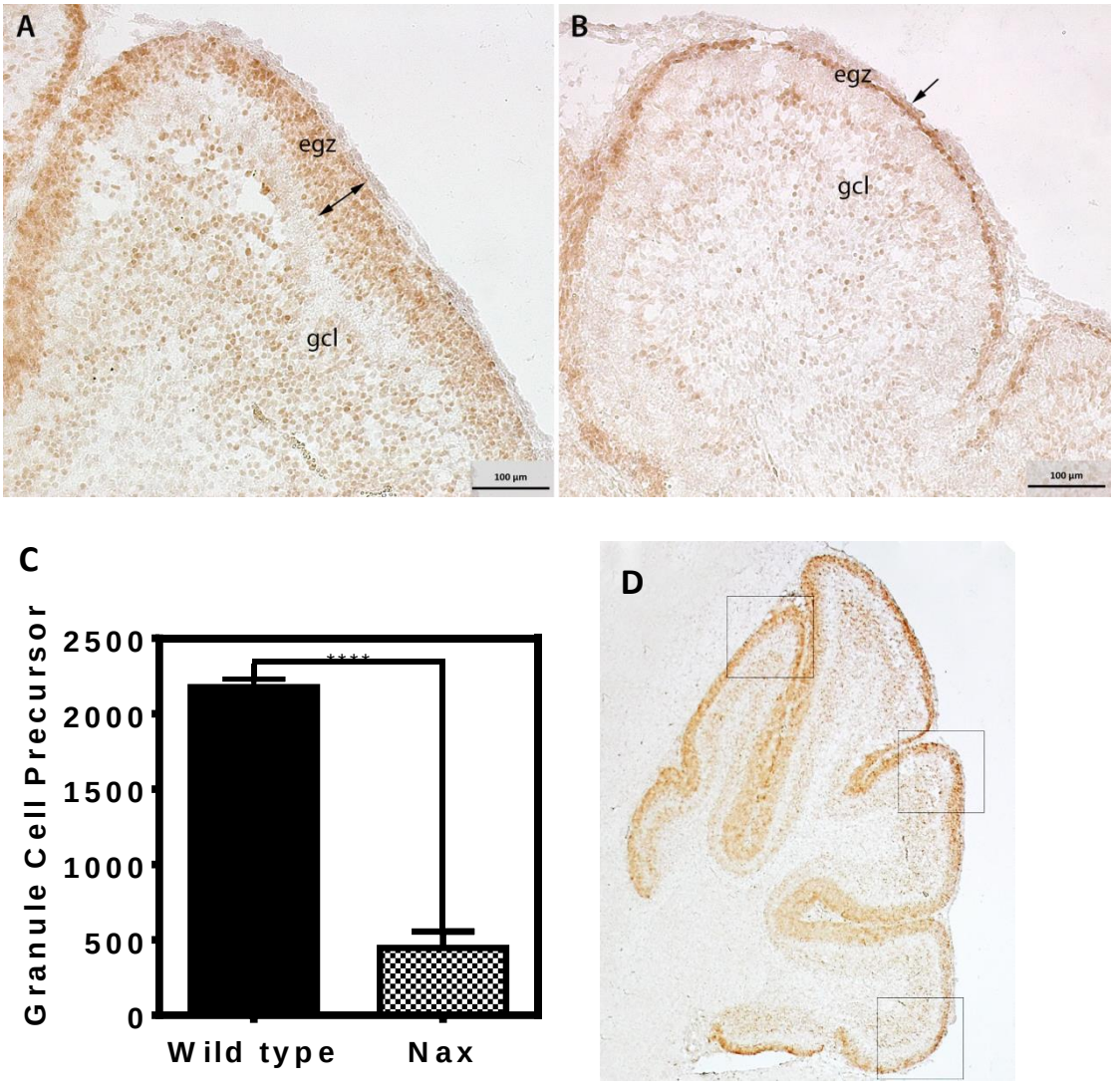


Comparison of the reelin expression between wt and nax shows lower expression of this protein in the nax mutant during the P4 (B) and P7 (C) which are the most critical days in development of the Purkinje cells and monolayer formation in mouse. The data in the bar graph are presented as the mean ± SEM, and statistical analysis was performed using unpaired t-test (P* <0. 05).

3.7 Proliferation of Granule cell precursors is severely affected in nax mutant mouse

Granule cell precursors that establish egz are main cells that produce Reelin. These cells are located at the outer layer of developing cerebellar cortex at this stage. To determine the probable reason that caused the reduction in Reelin expression, we performed the immunohistochemistry to investigate whether the egz formed normally in nax mutant mice or not. Sagittal sections of a P6 mouse cerebellum immunostained with PAX6 as a marker for gc precursor shows a significant reduction in number of gc precursors in nax mutant mouse compared to the wt sibling.

Figure 9.Immunostaining of P6 cerebellar sections of wt and mutant with Pax6

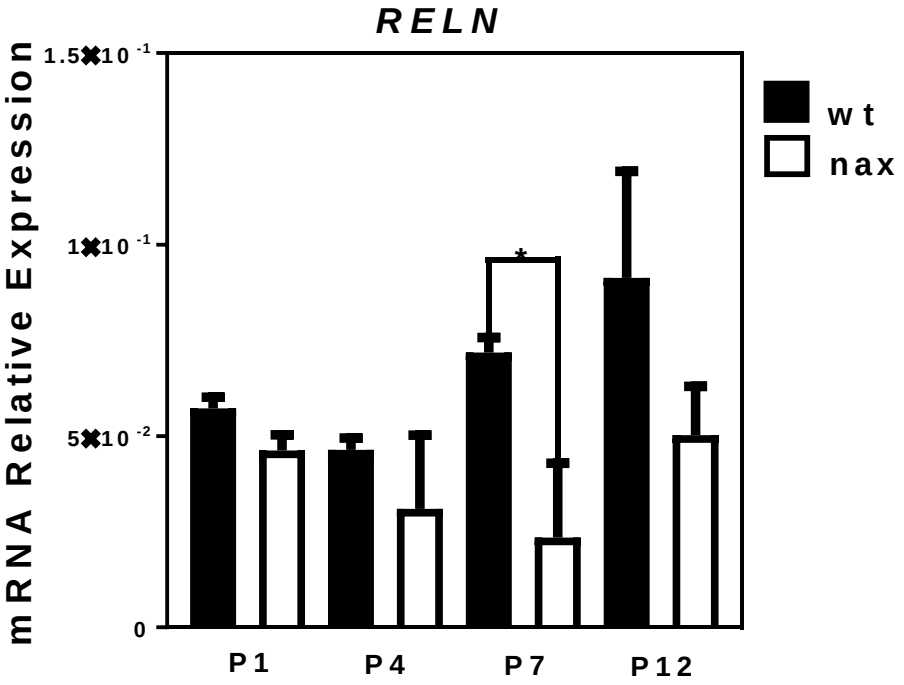


Immunostaining of P6 cerebellar sections of wt and mutant with Pax6 revealed **(A)** thick layer of granule cell precursor that is indicated by double headed arrow in egz, **(B)** Number of the granule cells precursor is reduced in the egz that is shown by arrow. Scale bar= 100 μm in **(A)** and **(B)**. Cell counting of the sagittal sections of P6 samples revealed a significant difference in the number of granule cell precursors in wt and nax**(C).** The bars represent the average cell counts at P6 wt and nax siblings (wt; n=3 and nax; n=3) and shown that granule cell precursors are less in nax mutant compared to wt. The data in the bar graph are presented as the mean ± SEM, and statistical analysis was performed using unpaired t-test ($P<0.05$).**(D)** A sample of the section showing three fields were selected on each sagittal section and cells were counted from each field.

3.8 Lower mRNA transcription levels of the *Reln* was observed in nax

Gene expression level of the Reelin was performed to investigate the differences at gene level in wt and nax mutant. We observed that the level of mRNA expression is downregulated in both wt and nax however it starts increasing from P7 in wt and P12 at nax. Transcription level of mRNA is significantly lower at P7.This confirms the western blotting data showing that lysosomes do not play role in degrading protein however they could have an essential role in their transcription level. Unlike the protein analysis with western blot for Reelin that was performed with mice samples up to P20, all the RT-PCR analysis in this study was performed with mice samples up to age P12. The reason for this selection is that unfortunately recently many litters in our nax colony do not survive for long time (up to P20) and we were not able to maintain older mice for this part of the experiments.

Figure 10. *Reln* mRNA expression was analyzed by RT-PCR

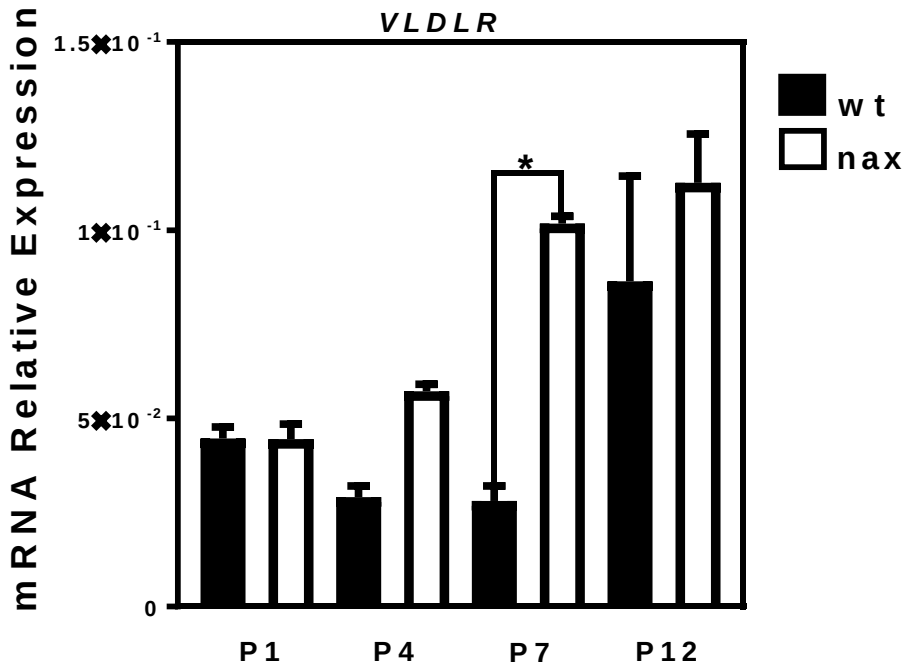


Transcription levels of Reelin by semi-quantitative RT-PCR from wt and nax cerebellum at P1, P4, P7, P12 shows that a lower transcription level of Reelin is observed in nax mutant compared to the wt sibling and it is significantly lower at P7. 2 sets of mice samples were used to perform this experiment. The data in the bar graph are presented as the mean ± SEM, and statistical analysis was performed using one-way ANOVA and statistical analysis for P7 was performed using unpaired t-test ($P^*<0.05$).

3.9 Higher transcription levels of the *Vldlr* observed in nax compared to the wt sibling

After observation of the reelin molecule as an important component of the reelin signaling pathway, transcription level of *Vldlr* as the receptor of the pathway was analysed. mRNA expression level downregulated from P1 to P7 and upregulated afterward in wt while in nax transcription level has increased steadily from P1 to P12. At P7 the transcription level is significantly lower in wt compared to nax.

Figure 11. *Vldlr* mRNA expression was analyzed by RT-PCR

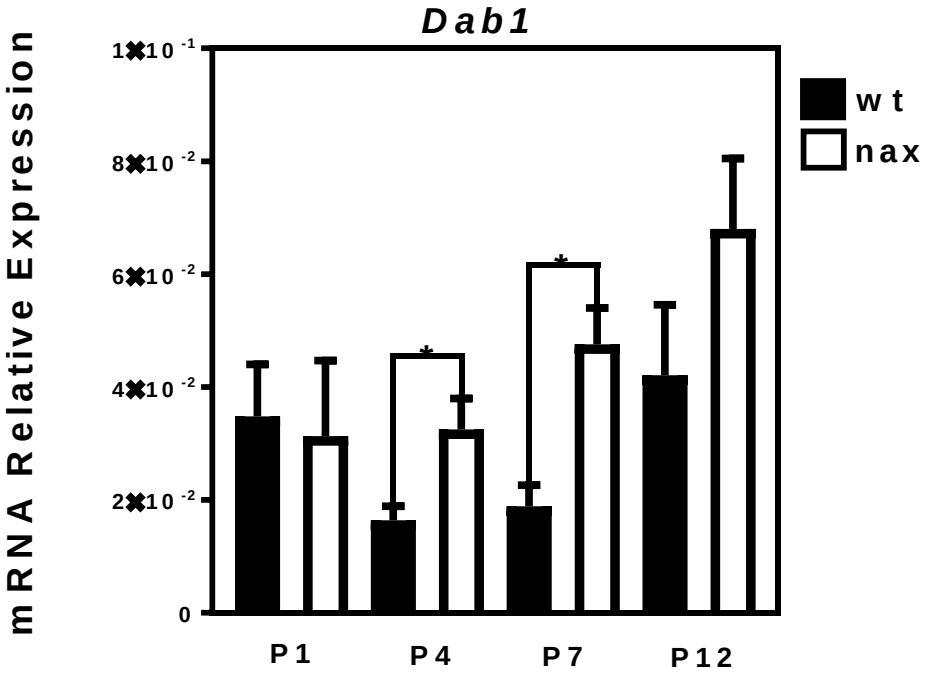


Higher mRNA expression level of *Vldlr* in nax is observed compared to the wt siblings. P7 shows a significant increase in the transcription level of *Vldlr* gene. Two sets of mice cerebellar samples used to perform this experiment. The data in the bar graph are presented as the mean ± SEM, and statistical analysis was performed using one-way ANOVA. Statistical analysis for P7 wt and nax was performed using unpaired t-test (P* <0.05).

3.10 Higher expression level of *Dab1* was observed in nax compared to the wt sibling

Dab1 is a critical downstream of the reelin signaling pathway and mRNA transcription level of the *Dab1* is shown that at P1 is higher in the wt than the nax cerebellum. While increasing the level of transcription is observed in nax afterward. Significant increase is observed at P4 and P7 in wt. This data is in accordance with the lower level of reelin expression at P7 while higher level at P7 for *Vldlr* and *Dab1* gene.

Figure 12. *Dab1* mRNA expression was analyzed by RT-PCR



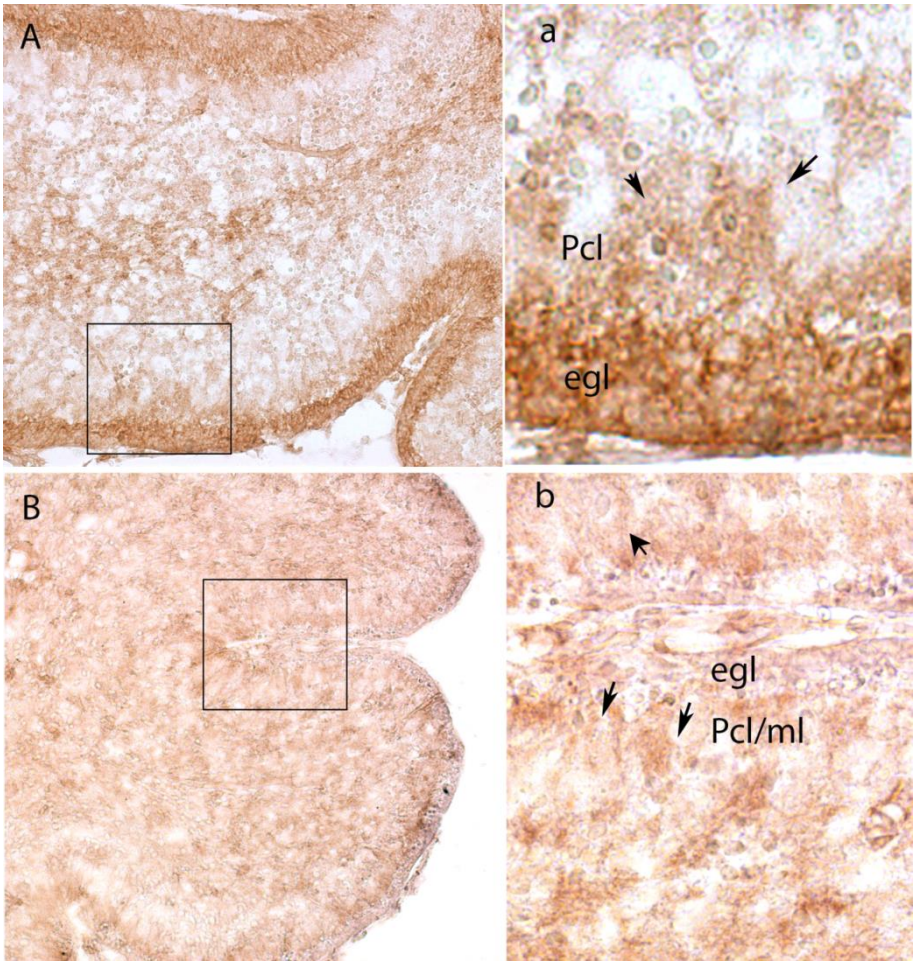
Higher mRNA expression level of *Dab1* in nax is observed compared to the wt siblings. P7 and P4 show a significant increase in the transcription levels of *Dab1* gene. Two sets of sample used to perform this experiment. The data in the bar graph are presented as the mean ± SEM, and statistical analysis was performed using one-

way ANOVA and statistical analysis for P4, P7 graphS was performed using unpaired t-test.

3.11 Similar expression of P-Dab1 at P3 wt and nax mutant mouse cerebellum

To determine whether P-Dab1 is present in Pcs of nax and to investigate its distribution and localization, the immunohistochemistry was performed using anti P-Dab1 antibody on P3 wt and nax mutant mouse cerebella. Expression of P-Dab1 is observed uniformly in Pcs of both wt and nax cerebellum of P3 mice.

Figure 13. Sagittal sections of wt and nax mouse at P3 immunostained with P-Dab1



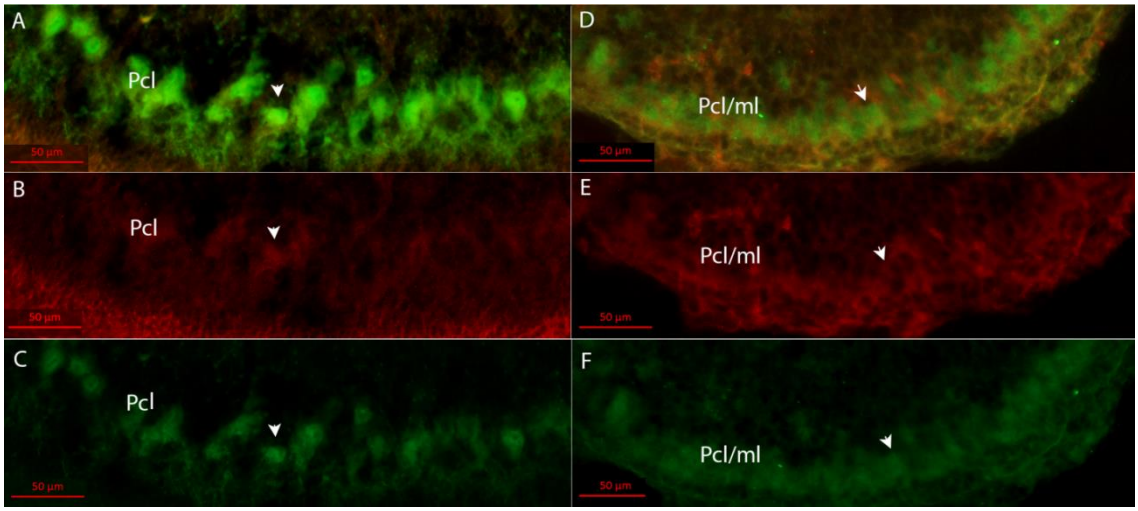
Sagittal sections of wt and nax mouse at P3 immunostained with P-Dab1 showed a similar pattern of expression. (A) wt and (B) nax mutant sections of P3 mouse

immunostained with P-Dab1.(a) higher magnification of (A) and (b) higher magnification of (B). Arrowheads indicate localization of P-Dab1in Pcs. Scale bar= 500 µm (A) and (B), 50 µm (a) and (b). Pcl, Purkinje cell layer; egl, external granule layer; ml, molecular layer

3.12 P-Dab1is expressed in Pcs of P3 wt and nax mice

To further confirm if the phosphorylation of Dab1 is localized in Pcs, double immunostaining of P-Dab1 (red) and Calb1 (green) was performed. In both wt and nax, P-Dab1 is co-localized with Calb1, the uniform distribution of P-Dab1 is observed in the pcl and their processes in the molecular layers are shown.

Figure 14. Double immunostaining of P-Dab1 and Calb1 was performed on midsagittal sections of P3 wt and nax mutant cerebellum

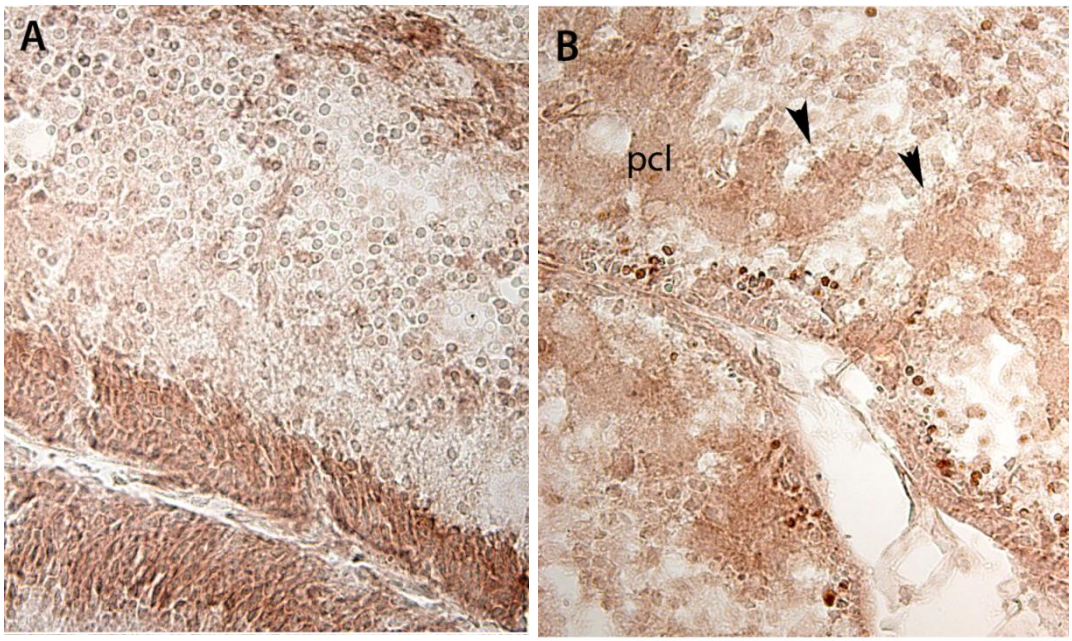


Double immunostaining of P-Dab1 and Calb1 shows that Calb1 (green) and P-Dab1 (red) were uniformly co-localized in Pcs (**A,B)**. Arrowheads in **(A-C)** are showing Pcs and arrowheads in **(D-F)** show the Pcs that migrated toward the ml. Scale bar= 50 µm in **(A-F)**. pcl, Purkinje cell layer; ml, molecular layer.

3.13 P-Dab1 expression is decreased at P6 in wt while is continued in the nax mutant

To further characterize P-Dab1 expression we performed immunohistochemistry of P-Dab1 antibody at P6 cerebellar wt and nax sections. The P-Dab1 immunopositive Pcs were observed in nax mutant mouse. wt sections showed the lack of obvious P-Dab1 expression in Pcs soma; this could suggest that by this age which is close to completion of the Pcs monolayer formation, wt Pcs stop phosphorylation of Dab1 while nax Pcs continuously phosphorylate of Dab1.

Figure 15 .Sagittal sections of wt and nax mouse cerebellum at P6 immunostained with P-Dab1

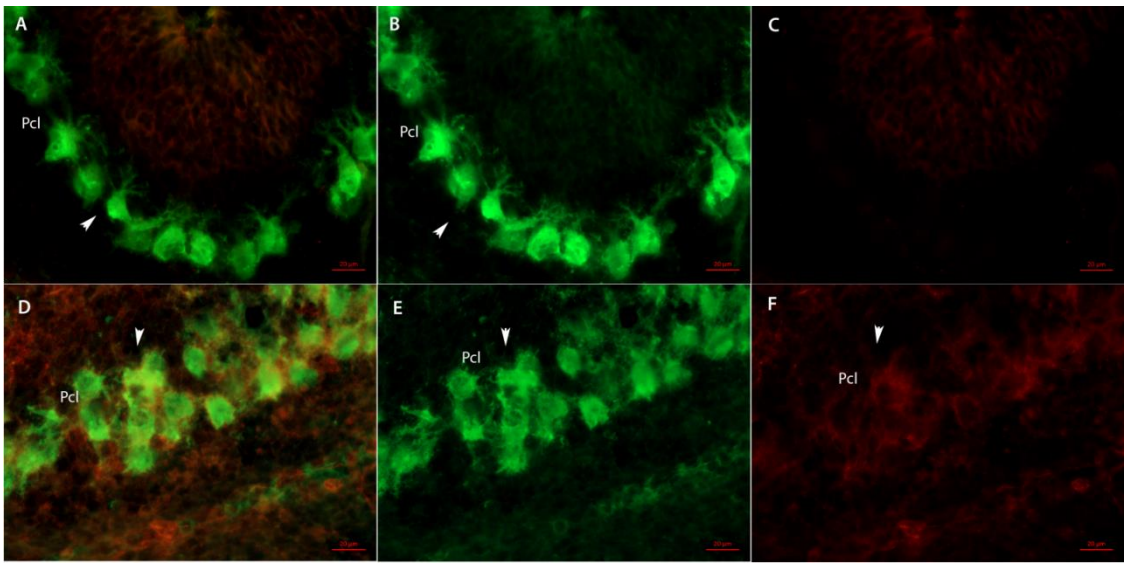


Sagittal sections of wt and nax mouse cerebellum at P6 (A) wt and (E) nax mutant sections of P6 mouse immunostained with P-Dab1. P-Dab1 expression is not observed in wt (A) however Dab-1 expression in showed by arrowhead in Pcs of *nax* mutant (E). Scale bar= 50 μm in (A) and (B).pcl, Purkinje cell layer.

3.14 P-Dab1 is co-labelled with Calb1 in Pcs of nax mutant at P6 but not in wt sibling

Confirmation of the previous experiment was performed using double immunofluorescent staining of P-Dab1 (red) and Calb1 (green) of P6 wt and *nax* mice to investigate if Dab1 is continuously phosphorylated in Pcs. Similar to the single staining P-Dab1 is not present in the wt however still expressing in the nax sibling.

Figure 16. Double immunostaining of P-Dab1 (red) and Calb1 (green) was performed on sagittal sections of P3 wt and nax mouse

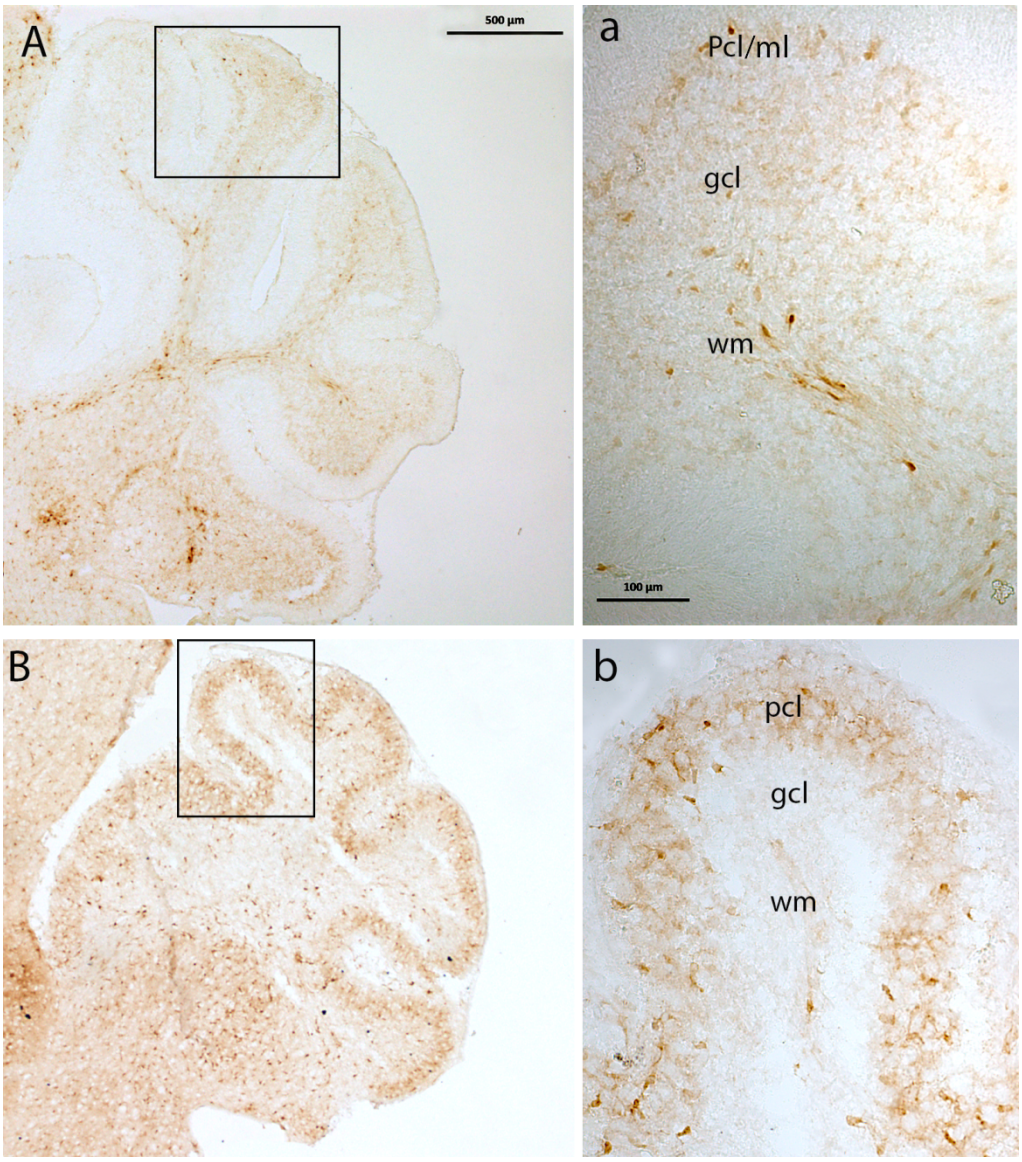


Double immunostaining of P-Dab1 (red) and Calb1 (green) was performed on sagittal sections of P3 wt and nax mouse. Calb1 was uniformly expressed in wt **(A, B)** and nax **(D, E)**, however P-Dab1 phosphorylation is not observed in wt **(C)** and it is present in nax **(F)**. Arrowheads indicate Pcs and the co-localization of Dab-1 and Calb1 in Pcs is shown in **(D)**. Scale bar= 20 μm in **(A-F)**. pcl, Purkinje cell layer.

3.15 Different expression pattern of P-ERK1/2 in wt and nax mouse cerebellum

To examine whether monolayer formation of Pcs through the reelin signaling pathway depends on activation of downstream signaling molecules, we performed in *vivo* and *in vitro* analysis of ERK1/2 which is a kinas regulating cell migration, proliferation and differentiation. ERK1/2 can also play a role as the downstream of Dab1 signaling. Firstly, the immunohistochemistry performed to observe the distribution and localization of P-ERK1/2 in the cerebellar sections of wt and nax mutant mouse at P6. Results revealed that in wt, Phosphorylated ERK1/2 is mostly present at white matter and some scattered gcs and in the nax, high expression of P-ERK1/2 is observed in Pcl/ml layer.

Figure 17. Sagittal sections of wt and nax mouse at P6 immunostained with P-ERK1/2.

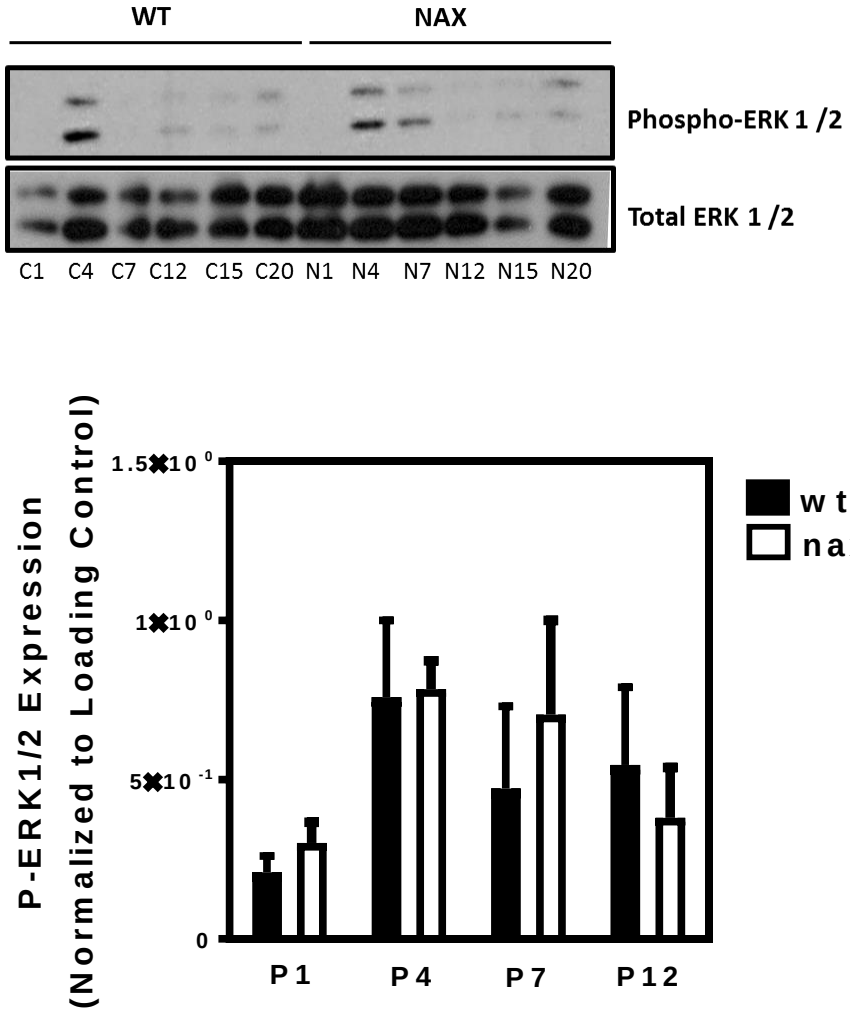


Sagittal sections of wt and nax mouse at P6 immunostained with P-ERK1/2. (A) the P-ERK1/2 immuno-positive cells are located in gc, Pc layer, and neurons of cerebellar nuclei, but not in ml. (a) a higher magnification of the box in A. (B) ERK1/2^{+} cells are shown a higher density of expression and disorganized manner in Pcl/ml and ml. (b) higher magnification of B is indicated. Scale bar= 500 (A,) and 100 (a,b). gcl, granule cell layer; pcl, Purkinje cell layer; wm, white matter.

3.16 Differential expression of ERK1/2 in wt and nax mutant mouse cerebellum

Followed by performing immunostating to observe differential pattern of expression of P-ERK1/2 in *nax* compared to wt sibling, immunoblotting was performed to realise the different amount of P-ERK1/2 expression in cerebellum samples of (P1, P4, P12, P15, P20) mice. Higher expression of P-ERK1/2 is observed in the nax mutant at P4 and P7.

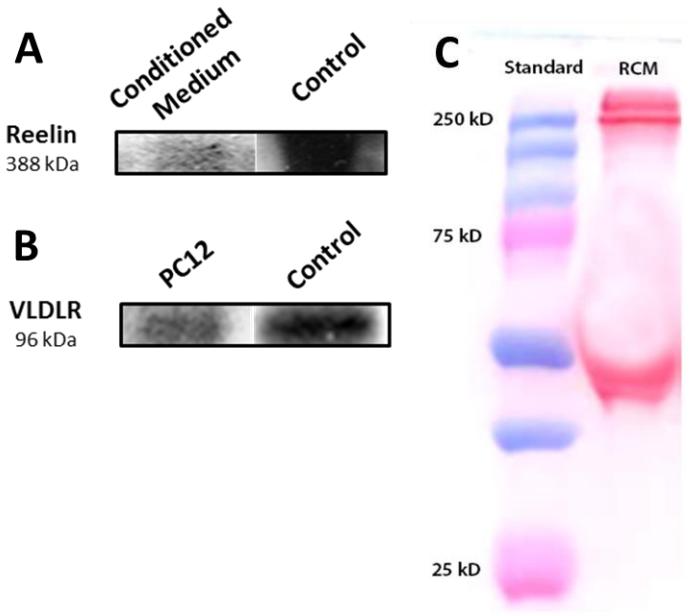
Figure 18. ERK1/2 expression in cerebellum of the wt and nax mice



Total cell lysate from cerebellum of wt and nax mutant mice have been prepared for indicated time points after the birth (P1, P4, P7, P12, and P20) and immunoblots were arranged for expression of ERK1/2 in the cerebellum. Phosphorylation of ERK1/2 is increased in day 4 in wt and decreased afterwards while in nax cerebellum it increases in day 4 and remained active till day 7. 2 sets of sample used to perform this experiment. The data in the bar graph are presented as the mean ± SEM, and statistical analysis for graph was performed using one-way.

3.17 Validation of the presence of reelin and its receptor in the medium

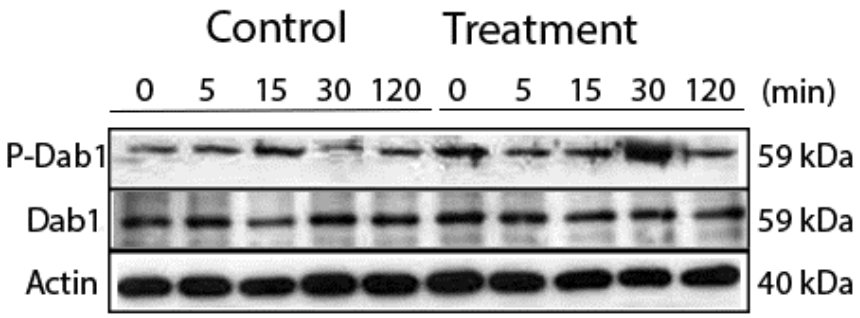
In order to perform the *in vitro* experiments, reelin condition medium was prepared from HEK cells as explained in the methodology. (A) To confirm the presence of reelin protein in the medium, 10 times concentrated reelin conditioned medium was analyzed using western blotting. Cerebellum sample of a P7 wild type mouse was used as the positive control. (B) The presence of the VLDLR in PC12 cell line was also confirmed in using Western blotting. Cerebellum sample of a P7 wild type mouse was used as the positive control. (C) Ponceau staining shows that concentrated medium is filtered properly since there are only proteins with molecular wight higher that 100 kD available after transfer onto the membrane. As shown in the Ponceau stained membrane there is a chance of possible non-reelin band in the medium. It is speculated that these high molecular bands are extracellular matrix proteins secreted by cells, however these factors are unlikely to induce activate ERK1/2 pathway, since most of the ERK1/2 activators have low molecular weight which have been filtered from the medium).

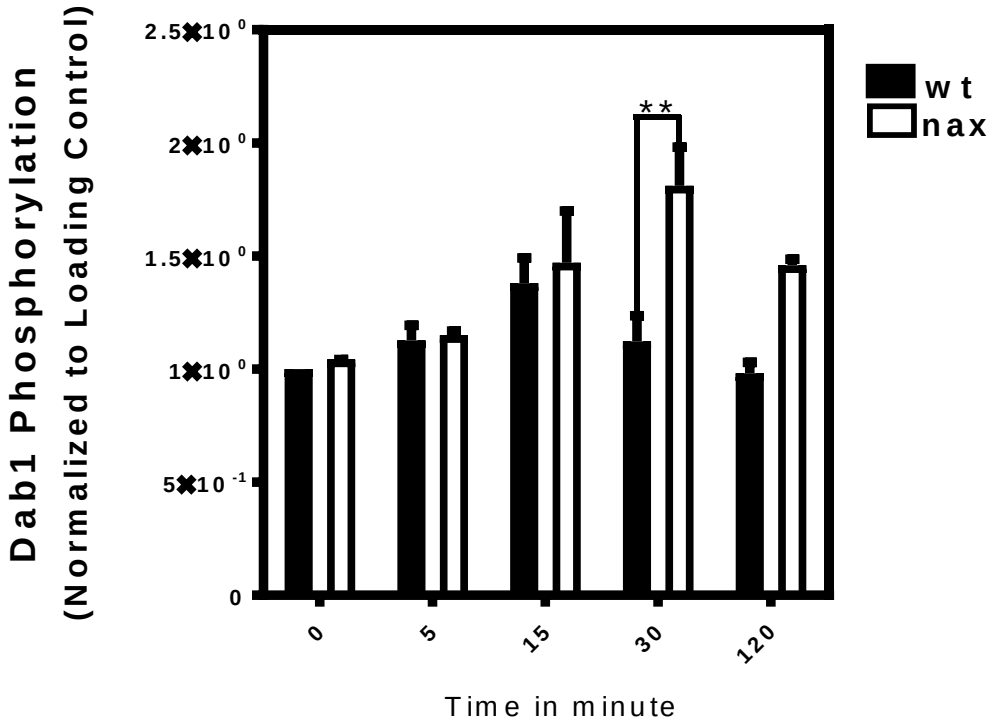


3.18 Reelin induces Dab1 tyrosine phosphorylation in PC12 cells

Tyrosine phosphorylation of Dab1 is mediated by binding of reelin to the lipoprotein receptors (ApoER2 and/or VLDLR). Interaction of the proteins and receptors occurs when protein binds to the NPxY motifs in the cytoplasmic tails of receptors in the cell. We measured the phosphorylation level of Dab1 when reelin is added to the environments of the PC12 cells at different time points (0, 5, 15, 30,120 minutes). Results revealed higher level of phosphorylation from 15 min in treated group compared to the controls.

Figure 19. Expression of Dab1 in PC12 cells after reelin treatment





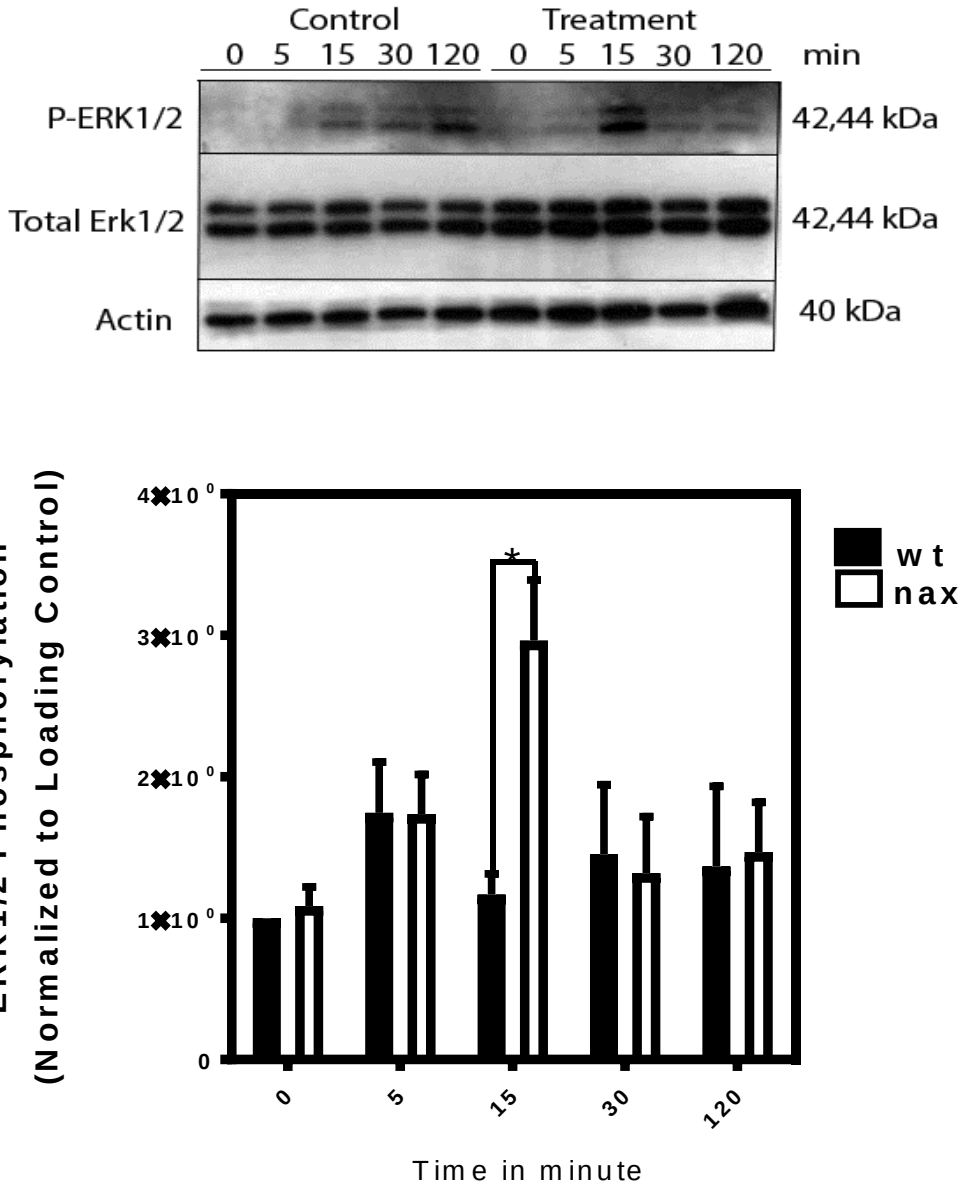
Western blott analysis of PC12 cells treated with reelin conditioned medium in different time points (0, 5 min, 15min, 30min, 120 min) demonstrated an increased levels of Dab1 phosphorylation from 15 mins as compared to control- untreated cells. β-Actin was used as a loading control. Results are expressed as mean ± SEM in 3 independent experiments. Statistical analysis for graph was performed using one-way ANOVA (**$P < 0.05$).

3.19 Phosphorylation of ERK1/2 is in parallel with Dab1 phosphorylation in PC12 cells

We have demonstrated that reelin as an extracellular glycoprotein stimulates Dab1 phosphorylation in PC12 cells. In this experiment, we have observed that ERK1/2 phosphorylation is induced followed by Dab1 phosphorylation in the same cell line. PC12 cells treated with reelin conditioned medium in different time points (0,5 min,15min, 30min,120 min) increased level of ERK1/2 phosphorylation at 15

min is observed in treated cells. Duration of the phosphorylation signal of ERK1/2 can be transient and switch on/off quickly while effective to transduce the signal (113).

Figure 20. Expression of ERK1/2 in PC12 cells after reelin treatment



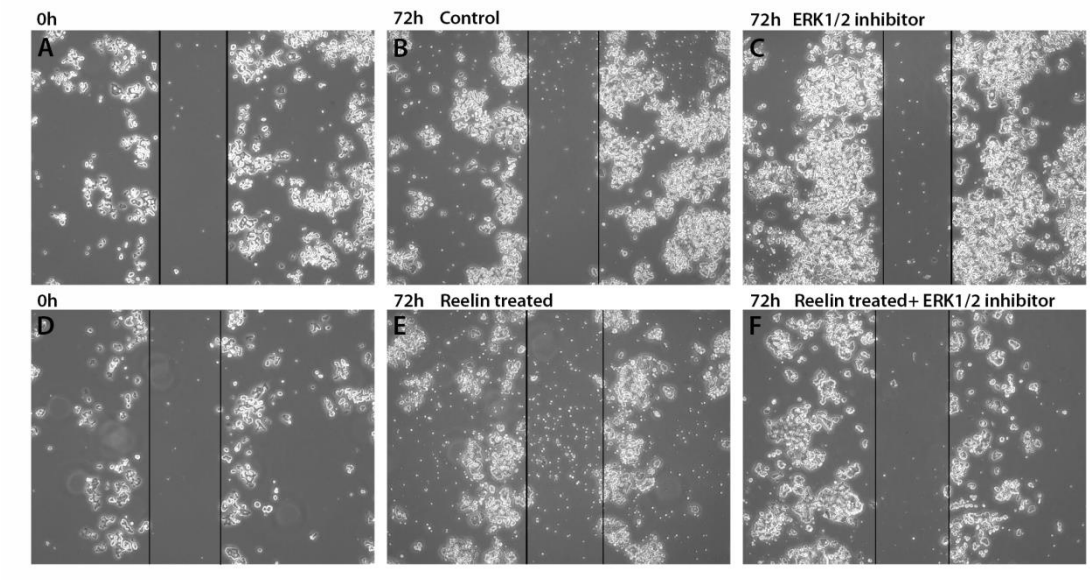
Western blot analysis of PC12 cells treated with reelin conditioned medium in different time points (0, 5 min, 15min, 30min, 120) demonstrated an increased level of ERK1/2 phosphorylation at 15 mins as compared to control- untreated cells. β-Actin was used as a loading control. Results are expressed as mean ± SEM in 3

independent experiments. Statistical analysis for graph was performed using one-way ANOVA (*$P < 0.05$).

3.20. Reelin condition medium induces PC12 cell migration and ERK1/2 inhibitor stops the migration

Based on the data shown in figure 15 and 16 we decided to investigate if the Dab1-ERK1/2 phosphorylation is functional to mediate migration. Effects of reelin treatment on the migration of PC12 cells were assessed by scratch assay. PC12 cells have tendency to grow in clusters with some scattered cells attached cells. Confluent cells were incubated with reelin conditioned medium (RCM) and migration of scratched wounds after 48 hr and 72 hr were assessed. RCM treatment increased the migration capacity of PC12 cells. However, RCM treatment combined with ERK1/2 inhibition had significant effect on the migration of the cells even at 10 µM concentration.

Figure 21. Effect of reelin treatment and ERK1/2 inhibition on PC12 cells migration



Cells were seeded in silicon chambers separated by an insert at the middle. When cells reached 60-80% confluency, the insert was removed resulting in a cell-free zone. Control cells are shown **(A, D)**, cells were remained untreated **(A)** or incubated with

reelin condition medium **(E)**. 10 µM concentration of ERK1/2 inhibitor was added to control **(C)** and reelin treated cells **(F)**.Images were acquired from a bright field microscope at 0 and 72 hours. Original magnification: ×20. This data is representative of three independent experiments.

CHAPTER 4: DISCUSSION

In the nax mutant (Acp2-/-) cerebellum, Pc migration is remarkably impaired and form multilayer disoriented cells positioned from putative Pcl to pial surface. reelin signaling pathway is crucial for Pc migration and monolayer formation. The reelin signalling pathway was examined in nax mutant cerebellum to determine whether the Pcs multilayer formation is due to misregulation of reelin pathway. The trend of reelin expression appeared to be similar in wt and nax cerebellum. However, reelin expression was significantly ($P^{*}<0.05$) lower in nax mutant at P4 and P7. Meanwhile, RNA transcription levels of both VLDLR and adaptor protein Dab1 were significantly increased at P4 and P7. Phosphorylated ERK1/2 is affected in nax cerebellum and expressed in a subset of dispositioned unidentified cells and significantly increased at P7. Migration assay experiment using PC12 cells revealed that reelin can induce Dab1 and ERK1/2 phosphorylation and migration is stopped once ERK1/2 is inhibited. The result of this study suggests that ERK1/2 in association with the reelin-Dab1 signaling pathway can be a regulatory component in Pc monolayer formation and is impaired in nax mutant cerebellum.

4.1 Reelin expression is down regulated in nax mutant mouse

Multilayer Pc formation in nax mutant is probably independent to reelin expression per se. The differential expression of the reelin protein as the key factor in Pcs migration in nax cerebellum can be explained by decrease in the number of gcs, and an impairment in its transcriptional or translational regulatory mechanisms. It is necessary to realize if the protein is degraded due to the malfunction of the lysosomes in the cells (as the result of the lysosomal mutation in Acp2 mutant) or there is a difference in the level of mRNA transcription level of this molecule.

The first cerebellar cells that express reelin are born at around E13 during the development of the cerebellum from gc precursors that are derived from rhombic lip (114, 115). It is shown that *Reln* mRNA expression is exclusively present in cerebellar granular layer (116, 117). In the cerebellum of the reeler mouse the significant decrease in the number of the gc precursors in egz has been considered to interfere with Pcs maturation and high number of the ectopic Pcs through white matter (118). It has been reported in scrambler mutant cerebellum which is identical to reeler in many aspects; the gcs are greatly reduced in number (119). Thus, the number of the granule cells precursors during the development of the cerebellum appears to be a critical factor for secretion of reelin and the Pcs migration and maturation, consequently. However, a normal level of the *Reln* mRNA is detected in the scrambler cerebellum implying that migration defect can be linked to the downstream of the reelin pathway (119). As it has been mentioned earlier, all layers in the cerebellar cortex (gcl, pcl and ml) have been affected as the result of the ACP2 mutation. Our laboratory has previously reported the significant decrease in the number of the granule cells in nax mutant which is the direct result of a lower number of gcs precursors (1). These findings suggest that the lower expression of the reelin is the direct result of the lower number of the gcs precursors in egz that express reelin, however, this amount is only significant at P4 and P7. reelin expression is comparable during the first postnatal day (P1-P4) in both nax and wild type. Given that the clear opposite phenotype of the Pcs positioning in reeler-scm mutant cerebellum (deep in white matter) and nax mutant cerebellum (Pcl and ml), indicate Pcs migration disorders, but independent to reelin expression.

In this study we have shown that as the animal ages from P7 till P20, the reelin expression is upregulated in wt and downregulated in nax. It has been reported that by

the completion of neuronal migration, a pattern of the change in expression of reelin is observed. In this phase, reelin mediates the synaptic plasticity in neurons. It is expressed in GABAergic interneurons of neocortex and hippocampus as well as the granule cells of the cerebellum (120).

4.2 Differential expression of the reelin signaling components

Source of the reelin protein secretion, its receptors, and downstream signaling molecules can be different during cerebellar development. reelin is expressed by gcs while the two receptors and downstream molecules are expressed in Pcs. To study the expression of reelin pathway, three components of the reelin signaling were selected: reelin, VLDL receptor, and Dab1 molecule. However, proper signaling of the reelin is highly dependent on the collaborative function of the two receptors, VLDLR and ApoER2. In this study, VLDLR was considered to be tested because VLDLR knockouts have been shown with a greater number neurons migrating toward the marginal zone (121) meaning that in the cerebellum of VLDLR mutants Pcs invade the molecular layer. This phenotype is similar to the nax mutant cerebellar phenotype and confirms the important role of VLDLR as stop signal for Pcs migration. Moreover, fate mapping analysis revealed that late-born neurons in ApoER2 knockout mice fail to migrate appropriately in the cortex (122) but the early-born neurons of VLDLR knockouts, form an organized layer of the inner cortex, proves that VLDLR cannot have an essential influence on the migration of early-born neurons (123). In our study VLDLR gene expression was revealed to be higher in nax mutant compared to the wild-type sibling suggesting that due to a lower amount of reelin, Pcs of the nax mutant have up-regulated the expression of the receptors through a compensatory mechanism (124-126).

The level of Dab1 gene has been identified as a critical component affecting the response of the neuronal cells to reelin (127). However, Dab1 might have a role in the transduction of the signal by reelin to the target during the neuronal migration (121, 128). reelin acts as a ligand for the transmembrane receptors and induces the tyrosine phosphorylation of Dab1 protein in vivo. Reduction or augmentation in the phosphorylation level of the Dab1 protein can regulate the neuronal positioning and migration (129). In the present study, *Dab1* mRNA level has interestingly revealed a gradual increase compared to the wt sibling which is similar to VLDLR. Immunostaining of the phosphorylated Dab1 reveals the same pattern of expression. Up to postnatal day 3 phosphorylation of the Dab 1 is observed in both wt and nax while Dab1 phosphorylation is decreased significantly in wt at P6, but continued in nax. Phosphorylation of the Dab1 is observed in ml/Pcs and also in egz. In the following section I have provided some possible explanations for this result.

4.3 Higher *Dab1* mRNA and phosphorylation level is observed in nax mutant

Normally, Dab1 is stable in cells; however, it can be degraded through the proteasome activity in response to the reelin signaling. Proper Dab1 degradation is an important factor in limiting the extension and spreading of the reelin molecule signals and it helps the fine-tuning of the reelin response (130). Dab1 and Src Family Kinase (SFKs) act in a positive-feedback loop, implying that tyrosine phosphorylation of Dab1 might continue as long as Dab1 is available (131). The Dab1 ubiquitination and proteasomal degradation are critical mechanisms to regulate the cellular response to reelin and are highly dependent on the SFKs rather that activation of PI3K (as a downstream of Dab1) (132). This fact highlights the role of ubiquitin-proteasome system and Dab1 on neuronal positioning. Cullin5 is a protein playing essential function in Dab1 degradation by binding to the phosphorylated Dab1. Cullin5

knocked down model revealed a significant increase in the level of Dab1 and interestingly the neurons were (similar to the nax cerebellar cortex) invaded the marginal zone of the cortex (133). It is, therefore, concluded that such connections exist between higher levels of Dab1 expression in nax and the system to degrade this protein. Thus, if degradation of Dab1 is not facilitated via proteasome system, reelin machinery stays active as long as Dab1 is available in the cell.

Despite the fact that the exact mechanism of the reelin machinery is yet poorly understood, many studies have highlighted the role of reelin in migration and axon guidance as well as a stop signal or positional signal in migrating neurons (56, 134-136). It is believed that reelin contributes to the stopping process of the neuronal migration which itself depends on two separate essential requirements:1) detachment of the migrating neurons from radial glial cells or Bergmann glial fibers and, 2) stabilization of the cytoskeleton of migrating neurons (137). One of the mechanisms to explain the stop signal role of the reelin is through phosphorylation of n-cofilin in which stabilize the cytoskeleton and stop the neurons migration (84). Real-time imaging revealed that cortical neurons stop migration when their leading processes encounter the reelin-rich marginal zone (138).

Many studies with molecular genetics approaches have provided evidences that reelin signaling is the most important factor that attracts migrating neurons toward marginal zones. For instance, Hammond *et al*, generated the Dab1 mosaic chimeras and showed that, $Dab1^{+/+}$ cells were able to migrate radially toward the reelin-rich marginal zones to form a multilayer super cortex beneath the marginal zone (139). Additionally, in-utero electroporation of Dab1 shRNA at E18 mouse embryos revealed the delay in migration and process formation of the neurons (133).

There is also a growing body of research focused on the detachment role of reelin than a stopping signal role. In a study by Hack *et al,* it has been shown that neuronal precursors in reeler mutant brain are accumulated in a form of clusters in the olfactory bulb indicating that they did not transform from tangential chain-migration to radial individual migration (140).

The stop signal role of the reelin has been supported in an investigation on mutations that are involved with other molecules in reelin signaling pathway. A mutation in an key element of Dab1 phosphorylation, called Fyn (member of the src family kinases), revealed over migration and invasion of the late-born neurons toward reelin-rich marginal zone (141). Similarly, in the Dab1 mutant that lacks C-terminal region, late-born neurons of the cortical plate invade the reelin rich areas of the cortex (142). In addition, real-time imaging techniques have also shown that migration is being stopped when the neurons arrive at the reelin-rich marginal zone (138). Accordingly, in the nax mutant, over migration of the Pcs toward the ml is observed resulting in a multilayer of the cells beneath the reelin-rich zone of gcs precursors. Therefore, it can be concluded that Pcs continued their migration toward reelin-rich zone but due to a significant decrease in the amount of reelin transcription and translation in gcs precursors (reelin-rich zone); they have not received the proper signal to stop migration.

Another possibility to study the over migration and multilayer formation of Pcs in nax cerebellum is to consider the role that radial glial cells may play a role in reelin signaling pathway. Formation of the radial glial cell scaffolds is a critical step in the neuronal migration process. There are a number of studies reported that genes encoding *Dab1*, *Vldlr*, and *ApoER2* are also expressed in radial glial cells (143). In

reeler and/scrambler mutant, normal form of the glial scaffolds are not properly formed in the dentate gyrus and cerebellum (119, 144). Thus, it is speculated that the existence of the reelin might control the distribution and density of adhesive molecules on the surface of the radial glial fibers resulting in migration of neurons toward their destination (137). Thus is can be hypothesized that connections exist between low reelin expression and high expression levels of Dab1 and VLDLR in nax through Bergmann glial fibers. Immunostaining of the P-Dab1 molecule in this study indicates that, in addition to Pcs, P-Dab1 is observed in areas containing migrating granule cells in cerebellar sections. Expression of Dab1 is essential for migration of the Pcs but evidence shows that Dab1 is observed in radial glia precursors whose processes span the entire cortical wall from the ventricular zone to the pial surface (143). Radial glial processes are required for granule cell migration. Moreover, It has been suggested that reelin has a dual role as a differentiation factor for radial glial cells and a positional role for radial fiber orientation and dentate granule cell migration (136) but there is no literature available for this role of reelin in cerebellar gcs migration.

In the brain of reeler embryos, Dab1 is expressed in a higher level but phosphorylated at lower level implying that Dab1 phosphorylation depends on reelin function (145). In the current study, lower level of reelin lead to higher expression and phosphorylation of Dab1, this result implies that there might be another limiting component of tyrosine phosphorylation of Dab1. Based on the literature, this component could be the reelin receptors (VLDLR and ApoER2), the SFKs, or some unknown molecule or cellular compartments acting downstream of the Dab1 signaling (e.g. MAPK).

4.4 ERK1/2 expression and distribution is affected in nax mutant

P-ERK1/2 appears to be affected in nax and it is expressed in subset of dispositioned cells in cerebellum and probably under induction of Dab1 significantly increased at P7. Generally, ERK/MAPK has been associated with migration of various cell types and inhibition of this pathway using PD98059 and/or U0126 inhibitors is implicated to block several factors that induce migration, for instance fibronectin, vitronectin and collagen, growth factors, and fetal calf serum. It has been shown that $MEK^{-/-}$ mutant has affected cell migration (146). Cellular migration of the endothelial cells is enhanced through ERK1/2 and PI3K/Akt pathway (147) and inhibition of Ras/Raf/ERK pathway has significantly increased the migration of endothelial cells (148). It is also reported that the regulation of the ERK1/2 in normal human neuronal progenitor cells is highly correlated to the cell migration (149).

In CNS, ERK1/2 signaling plays a functional role in migration, proliferation, neuronal differentiation, synaptic maturation and activity (150-152). The important role of activated ERK1/2 in proliferation of glial cells in brain development is also reported (153). Recent studies revealed that upregulation of Raf/ERK induce the migration of both neurons in spinal cord and dorsal root ganglia. In addition, Raf/ERK up-regulation inhibits the formation of the dendritic spines in neurons and eventually leading to neural circuit imbalances (154, 155). Interestingly, inhibition of the ERK1/2 resulted in restoration of cell adhesion and affect neuronal migration, proper development, and dendritic spines formation in spinal cord neurons (156). In the cerebellum, *Erk1/2* mRNA in adult rat cerebellar samples (157) and expression of ERK2 protein (158) have been studied and revealed its critical role in regulating cerebellar function and physiology. Nevertheless, the defined pathway in which intracellular signaling pathways organize the stereotypically precise development of

the cerebellum is far from being understood. ERK1 knockout mouse shows a mild defect in development of Tcells and MEK dominant-negative knockout reveals defects in placental vascularization and results in death of the mice (159).In our study, immunostaining of the ERK1/2 revealed a different pattern of expression in nax and wt. In the wt, immunoreactivity of ERK1/2 is observed in the cells located in white matter and in gcl, but not is ml, however in nax cerebellum there are many unidentified scattered cells in Pcl/ml layer. Immunostainings performed in our lab have not confirmed the co-localization of these cells with known cerebellar neuronal markers. These findings are in contrary to previous studies that suggested P-ERK1/2 is co-localized with glial fibrillary acidic protein immunopositive cells and present in maturing Pcs and gcs. Recent studies also reported that activated ERK1/2 is observed in Bergman glia cells on P4, P10, P15 and astrocytes on P10 and P1. However, in adult mice p-ERK reactivity is shown in Pc layer and scattered cells in the cerebellar white matter (150). Overall the wide range of p-ERK immunoreactivity and distribution during cerebellar development indicate its dynamic role regarding differentiation, proliferation, and migration of the neuronal subtypes and glial cells in cerebellum.

In vitro experiments of this study indicate that reelin-Dab1- ERK1/2 pathway might be an important regulatory signal in Pc monolayer formation and is impaired in *nax* mutant cerebellum. ERK1/2 is activated by reelin through Dab1 phosphorylation and SFK.

Moreover, it is shown that the inhibition of the PI3K pathway block reelin-dependent activation of ERK that is confirming a cross talk between PI3K and ERK pathway (160). While another study that focused on different isoforms of the reelin molecule, indicated that reelin is able to activate two signaling transductions through

different receptors and protein domains. The N-terminal fragment, the C-terminal fragment or the un-cleaved full length reelin protein can bind to an unidentified receptor that could result in activation of MEK/ERK1/2 signaling. Since phosphorylation of Dab1 is in a positive loop with SFKs expression, it can be assumed there might be a link between reelin induction and MEK/ERK1/2 activation. In addition, in a reeler or scrambler mouse brain, AKT and ERK 1/2 have shown to be defective (92). PI3K and Akt are the two critical components in both reelin and ERK1/2 that can be considered as the cross-talk between signaling pathways. In addition, a study highlighted that tyrosine phosphorylation of Dab1 is necessary for activation of the PI3K-dependent kinase, Akt, and Src family kinases. However, Fyn is another important regulator of Dab1; its deficiency does not stop the Akt activation by reelin. These results suggest that reelin can induce the MAPK pathway through non-canonical pathway involved with some unidentified molecules (161).

CHAPTER 5: CONCLUSION AND FUTURE DIRECTION

Reelin is an important glycoprotein that plays a dual role in development of the brain. The first phase of its function starts in the embryonic stage and is involved with neuronal migration and proper positioning of neurons in cerebral cortex. Several studies have investigated structure, function, and signaling pathway of reelin protein in recent decades. In this research, stop signal role of the reelin was studied in a mouse model called nax that shows severe migration defect of cerebellar Purkinje cells. During the development of mice, Pcs form a monolayer beneath the ml by P7; however, in nax mutant multilayer of Pcs invade the ml. To investigate this migration disorder different *in vivo* and *in vitro* technique were used to study the downstream of reelin pathway. In vivo studies indicated the lower expression of reelin followed by a higher expression of the VLDL receptor and Dab1 protein in nax mutant compared to

the wild type sibling, in addition ERK1/2 was expressed in some unidentified cells with higher expression in nax cerebellum at p7.To investigate if ERK1/2 is a downstream of Dab1 the *in vitro* experiment carried out by treating the PC12 cells with reelin condition medium. The result of this study suggests the potential role of ERK1/2 in stop signal for Pcs during development. It seems that lower expression of reelin has led to higher expression of VLDLR and DAB1 on one side and higher expression of ERK1/2 as a downstream factor of Dab1 on the other side. Upon evidences provided in this study, it is concluded that reelin can modulate the ERK1/2 expression and control the migration and positioning of the Pcs in cerebellum through reelin-Dab1-ERK1/2 pathway. Investigation of the detailed mechanism is out of the scope of this study, however, in the following paragraph there are some points suggested to further investigate the migration of Pcs.

Purkinje cells are the most important subset of neurons in the cerebellum that their migration is directly regulated by reelin signaling; however, the exact mechanism of this regulation has not been fully explained yet. One source of weakness in this type of research could be the limitations to study the Pcs *in vitro*. Cell-cell interactions, which are necessary for differentiation and survival of Pcs, make it challenging for researches to provide purified cultures of Pcs.

The present study has some limitations that need to be addressed in order to fully understand the mechanism underlying the migration disorder in Pcs. First, it seems necessary to confirm results of the *in vitro* experiments by using cerebellar primary cultures or neuronal cultures to observe the Pcs migration when treating with reelin and inhibiting ERK1/2. In this study PC12 cells were not treated with nerve growth factors to be differentiated into neuronal like phenotype cells. Preparation of

the reelin condition medium has been one of the limitations of this study. In the future studies it is suggested to use the HEK293 cells that stably express the reelin or pharmacological approaches should be taken into consideration. In addition, the PI3K/AKT changes should be analyzed since it is the factor linking reelin and ERK1/2 pathways. Finally, more profound studies should be performed to realize how Acp2 point mutation can affect the reelin signaling and neuronal migration. For instance, one of the factors linking reelin to the Acp2 enzyme is cholesterol metabolism. In hepatocyte cells of the Acp2/Acp5 deficient mice it has been shown that vldlr level has increased. Since vldlr is a member of low density lipoproteins with an important role in cholesterol metabolism it is suggested that the level of cholesterol need to be measured in nax and wt samples.

Finally, we should consider that control of the cell migration is coordinated by all members of the MAPK family and depends on phosphorylation of certain substrates leading to reorganization of microtubules and filamentous actin (94). For example, there is a molecular link between ApoER2 and the JNK signaling pathway. ApoER2 is able to form a protein complex containing Dab1, JNK-interacting proteins, and other binding components on the surface of neurons (162). Since multiprotein complex can contribute to the reelin-ApoER2 signaling it is suggested that the role of other MAPK components can be tested in order to better understand Purkinje cell migration.

References

1. Bailey K, Rahimi Balaei M, Mannan A, Del Bigio MR, Marzban H. Purkinje cell compartmentation in the cerebellum of the lysosomal Acid phosphatase 2 mutant mouse (nax - naked-ataxia mutant mouse). PloS one. 2014;9(4):e94327.
2. Marzban H, Del Bigio MR, Alizadeh J, Ghavami S, Zachariah RM, Rastegar M. Cellular commitment in the developing cerebellum. Front Cell Neurosci. 2014;8:450.
3. Tong KK, Ma TC, Kwan KM. BMP/Smad signaling and embryonic cerebellum development: stem cell specification and heterogeneity of anterior rhombic lip. Development, growth & differentiation. 2015;57(2):121-34.
4. Hashimoto M, Hibi M. Development and evolution of cerebellar neural circuits. Development, growth & differentiation. 2012;54(3):373-89.
5. Butts T, Green MJ, Wingate RJ. Development of the cerebellum: simple steps to make a 'little brain'. Development. 2014;141(21):4031-41.
6. Martinez S, Andreu A, Mecklenburg N, Echevarria D. Cellular and molecular basis of cerebellar development. Frontiers in neuroanatomy. 2013;7:18.
7. Llinas R, Negrello MN. Cerebellum. Scholarpedia. 2015;10(1):4606.
8. Smith JL, Schoenwolf GC. Neurulation: coming to closure. Trends Neurosci. 1997;20(11):510-7.
9. Gray J, Ross ME. Neural tube closure in mouse whole embryo culture. Journal of visualized experiments : JoVE. 2011(56).
10. Wilde JJ, Petersen JR, Niswander L. Genetic, epigenetic, and environmental contributions to neural tube closure. Annual review of genetics. 2014;48:583-611.
11. Teng CS, Teng CT, Cooper G, Rodnitzky RL. Neuroembryology and neurogenesis. Neuroscience in Medicine: Springer; 2003. p. 111-28.
12. Wurst W, Bally-Cuif L. Neural plate patterning: upstream and downstream of the isthmic organizer. Nature reviews Neuroscience. 2001;2(2):99-108.
13. Sadler TW. Langman's medical embryology: Lippincott Williams & Wilkins; 2011.
14. Martinez-Ferre A, Martinez S. Molecular regionalization of the diencephalon. Frontiers in neuroscience. 2012;6:73.
15. Lee KJ, Jessell TM. The specification of dorsal cell fates in the vertebrate central nervous system. Annual review of neuroscience. 1999;22(1):261-94.
16. Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, et al. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell. 1993;75(7):1417-30.
17. Zecevic N. Synaptogenesis in layer I of the human cerebral cortex in the first half of gestation. Cerebral cortex (New York, NY : 1991). 1998;8(3):245-52.
18. Nakamura H, Katahira T, Matsunaga E, Sato T. Isthmus organizer for midbrain and hindbrain development. Brain research reviews. 2005;49(2):120-6.
19. Sato T, Joyner AL. The duration of Fgf8 isthmic organizer expression is key to patterning different tectal-isthmo-cerebellum structures. Development. 2009;136(21):3617-26.

20. Garda A-L, Echevarría D, Martínez S. Neuroepithelial co-expression of Gbx2 and Otx2 precedes Fgf8 expression in the isthmic organizer. Mechanisms of development. 2001;101(1):111-8.
21. Basson MA, Echevarria D, Ahn CP, Sudarov A, Joyner AL, Mason IJ, et al. Specific regions within the embryonic midbrain and cerebellum require different levels of FGF signaling during development. Development. 2008;135(5):889-98.
22. Wang VY, Rose MF, Zoghbi HY. Math1 expression redefines the rhombic lip derivatives and reveals novel lineages within the brainstem and cerebellum. Neuron. 2005;48(1):31-43.
23. Leto K, Arancillo M, Becker EB, Buffo A, Chiang C, Ding B, et al. Consensus paper: cerebellar development. The Cerebellum. 2015:1-40.
24. Hoshino M, Nakamura S, Mori K, Kawauchi T, Terao M, Nishimura YV, et al. Ptf1a, a bHLH transcriptional gene, defines GABAergic neuronal fates in cerebellum. Neuron. 2005;47(2):201-13.
25. Wolf MK, Dubois-Dalcq M. Anatomy of cultured mouse cerebellum. I. Golgi and electron microscopic demonstrations of granule cells, their afferent and efferent synapses. Journal of Comparative Neurology. 1970;140(3):261-79.
26. Machold R, Fishell G. Math1 is expressed in temporally discrete pools of cerebellar rhombic-lip neural progenitors. Neuron. 2005;48(1):17-24.
27. Tichy J, Zinke J, Bunz B, Meyermann R, Harter PN, Mittelbronn M. Expression Profile of Sonic Hedgehog Pathway Members in the Developing Human Fetal Brain. BioMed research international. 2015;2015.
28. Haldipur P, Bharti U, Govindan S, Sarkar C, Iyengar S, Gressens P, et al. Expression of Sonic hedgehog during cell proliferation in the human cerebellum. Stem cells and development. 2012;21(7):1059-68.
29. Gregory WA, Edmondson JC, Hatten ME, Mason CA. Cytology and neuron-glial apposition of migrating cerebellar granule cells in vitro. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1988;8(5):1728-38.
30. Dino MR, Schuerger RJ, Liu Y, Slater NT, Mugnaini E. Unipolar brush cell: a potential feedforward excitatory interneuron of the cerebellum. Neuroscience. 2000;98(4):625-36.
31. Mugnaini E, Sekerkova G, Martina M. The unipolar brush cell: a remarkable neuron finally receiving deserved attention. Brain research reviews. 2011;66(1-2):220-45.
32. Kim JA, Sekerkova G, Mugnaini E, Martina M. Electrophysiological, morphological, and topological properties of two histochemically distinct subpopulations of cerebellar unipolar brush cells. Cerebellum (London, England). 2012;11(4):1012-25.
33. Hagan N, Zervas M. Wnt1 expression temporally allocates upper rhombic lip progenitors and defines their terminal cell fate in the cerebellum. Molecular and cellular neurosciences. 2012;49(2):217-29.
34. Leto K, Carletti B, Williams IM, Magrassi L, Rossi F. Different types of cerebellar GABAergic interneurons originate from a common pool of multipotent progenitor cells. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2006;26(45):11682-94.
35. Barria A. Electrophysiological Tagging of Ionotropic Glutamate Receptors. Ionotropic Glutamate Receptor Technologies. 2016:45-60.

36. Fiszman ML, Erdélyi F, Szabó G, Vicini S. Presynaptic AMPA and kainate receptors increase the size of GABAergic terminals and enhance GABA release. Neuropharmacology. 2007;52(8):1631-40.
37. Englund C, Kowalczyk T, Daza RA, Dagan A, Lau C, Rose MF, et al. Unipolar brush cells of the cerebellum are produced in the rhombic lip and migrate through developing white matter. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2006;26(36):9184-95.
38. Kapfhammer JP. Cellular and molecular control of dendritic growth and development of cerebellar Purkinje cells. Progress in histochemistry and cytochemistry. 2004;39(3):131-82.
39. White JJ, Arancillo M, Stay TL, George-Jones NA, Levy SL, Heck DH, et al. Cerebellar zonal patterning relies on Purkinje cell neurotransmission. The Journal of Neuroscience. 2014;34(24):8231-45.
40. Mishina M, Uemura T, Yasumura M, Yoshida T. Molecular mechanism of parallel fiber-Purkinje cell synapse formation. Frontiers in neural circuits. 2012;6:90.
41. Chaumont J, Guyon N, Valera AM, Dugué GP, Popa D, Marcaggi P, et al. Clusters of cerebellar Purkinje cells control their afferent climbing fiber discharge. Proceedings of the National Academy of Sciences. 2013;110(40):16223-8.
42. Adcock KH, Metzger F, Kapfhammer JP. Purkinje cell dendritic tree development in the absence of excitatory neurotransmission and of brain-derived neurotrophic factor in organotypic slice cultures. Neuroscience. 2004;127(1):137-45.
43. Oberdick J, Smeyne RJ, Mann JR, Zackson S, Morgan JI. A promoter that drives transgene expression in cerebellar Purkinje and retinal bipolar neurons. Science (New York, NY). 1990;248(4952):223-6.
44. Bastianelli E. Distribution of calcium-binding proteins in the cerebellum. Cerebellum (London, England). 2003;2(4):242-62.
45. Takeo YH, Kakegawa W, Miura E, Yuzaki M. Rorα regulates multiple aspects of dendrite development in cerebellar purkinje cells in vivo. The Journal of Neuroscience. 2015;35(36):12518-34.
46. Chen XR, Heck N, Lohof AM, Rochefort C, Morel M-P, Wehrlé R, et al. Mature Purkinje cells require the retinoic acid-related orphan receptor-α (RORα) to maintain climbing fiber mono-innervation and other adult characteristics. The Journal of Neuroscience. 2013;33(22):9546-62.
47. Maricich SM, Herrup K. Pax-2 expression defines a subset of GABAergic interneurons and their precursors in the developing murine cerebellum. J Neurobiol. 1999;41(2):281-94.
48. Weisheit G, Gliem M, Endl E, Pfeffer PL, Busslinger M, Schilling K. Postnatal development of the murine cerebellar cortex: formation and early dispersal of basket, stellate and Golgi neurons. The European journal of neuroscience. 2006;24(2):466-78.
49. Achim K, Salminen M, Partanen J. Mechanisms regulating GABAergic neuron development. Cellular and molecular life sciences : CMLS. 2014;71(8):1395-415.
50. Kelsom C, Lu W. Development and specification of GABAergic cortical interneurons. Cell & bioscience. 2013;3(1):1.
51. Mizuhara E, Minaki Y, Nakatani T, Kumai M, Inoue T, Muguruma K, et al. Purkinje cells originate from cerebellar ventricular zone progenitors positive for Neph3 and E-cadherin. Developmental biology. 2010;338(2):202-14.

52. Florio M, Leto K, Muzio L, Tinterri A, Badaloni A, Croci L, et al. Neurogenin 2 regulates progenitor cell-cycle progression and Purkinje cell dendritogenesis in cerebellar development. Development. 2012;139(13):2308-20.
53. Whalley K. Development: The origins of interneuron diversity. Nature Reviews Neuroscience. 2007;8(9):655-.
54. Nadarajah B, Alifragis P, Wong RO, Parnavelas JG. Neuronal migration in the developing cerebral cortex: observations based on real-time imaging. Cereb Cortex. 2003;13(6):607-11.
55. Gaiano N, Fishell G. The role of notch in promoting glial and neural stem cell fates. Annual review of neuroscience. 2002;25(1):471-90.
56. Cooper JA. A mechanism for inside-out lamination in the neocortex. Trends in neurosciences. 2008;31(3):113-9.
57. Lakatosova S, Ostatnikova D. Reelin and its complex involvement in brain development and function. The international journal of biochemistry & cell biology. 2012;44(9):1501-4.
58. Herz J, Chen Y. Reelin, lipoprotein receptors and synaptic plasticity. Nature Reviews Neuroscience. 2006;7(11):850-9.
59. Fatemi SH. Reelin glycoprotein: structure, biology and roles in health and disease. Molecular psychiatry. 2005;10(3):251-7.
60. Fatemi S. Reelin mutations in mouse and man: from reeler mouse to schizophrenia, mood disorders, autism and lissencephaly. Molecular psychiatry. 2001.
61. Fink AJ, Englund C, Daza RA, Pham D, Lau C, Nivison M, et al. Development of the deep cerebellar nuclei: transcription factors and cell migration from the rhombic lip. The Journal of neuroscience. 2006;26(11):3066-76.
62. Miyata T, Ono Y, Okamoto M, Masaoka M, Sakakibara A, Kawaguchi A, et al. Migration, early axonogenesis, and Reelin-dependent layer-forming behavior of early/posterior-born Purkinje cells in the developing mouse lateral cerebellum. Neural development. 2010;5(1):1.
63. Butts T, Green MJ, Wingate RJ. Development of the cerebellum: simple steps to make a 'little brain'. Development. 2014;141(21):4031-41.
64. Kim J, Park T-J, Kwon N, Lee D, Kim S, Kohmura Y, et al. Dendritic planarity of Purkinje cells is independent of Reelin signaling. Brain Structure and Function. 2015;220(4):2263-73.
65. Folsom TD, Fatemi SH. The involvement of Reelin in neurodevelopmental disorders. Neuropharmacology. 2013;68:122-35.
66. Curran T, D'Arcangelo G. Role of reelin in the control of brain development. Brain research Brain research reviews. 1998;26(2-3):285-94.
67. Ichihara H, Jingami H, Toh H. Three novel repetitive units of reelin. Brain Res Mol Brain Res. 2001;97(2):190-3.
68. Jossin Y, Gui L, Goffinet AM. Processing of Reelin by embryonic neurons is important for function in tissue but not in dissociated cultured neurons. The Journal of neuroscience. 2007;27(16):4243-52.
69. Graus-Porta D, Blaess S, Senften M, Littlewood-Evans A, Damsky C, Huang Z, et al. Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. Neuron. 2001;31(3):367-79.

70. Pfrieger F. Cholesterol homeostasis and function in neurons of the central nervous system. Cellular and Molecular Life Sciences CMLS. 2003;60(6):1158-71.
71. Reddy SS, Connor TE, Weeber EJ, Rebeck W. Similarities and differences in structure, expression, and functions of VLDLR and ApoER2. Mol Neurodegener. 2011;6:30.
72. Beffert U, Stolt PC, Herz J. Functions of lipoprotein receptors in neurons. Journal of lipid research. 2004;45(3):403-9.
73. Hibi T, Mizutani M, Baba A, Hattori M. Splicing variations in the ligand-binding domain of ApoER2 results in functional differences in the binding properties to Reelin. Neuroscience research. 2009;63(4):251-8.
74. Oka K, Tzung KW, Sullivan M, Lindsay E, Baldini A, Chan L. Human very-low-density lipoprotein receptor complementary DNA and deduced amino acid sequence and localization of its gene (VLDLR) to chromosome band 9p24 by fluorescence in situ hybridization. Genomics. 1994;20(2):298-300.
75. Wyne KL, Pathak K, Seabra MC, Hobbs HH. Expression of the VLDL receptor in endothelial cells. Arteriosclerosis, thrombosis, and vascular biology. 1996;16(3):407-15.
76. Iwata K, Izumo N, Matsuzaki H, Manabe T, Ishibashi Y, Ichitani Y, et al. Vldlr overexpression causes hyperactivity in rats. Molecular autism. 2012;3(1):1.
77. May P, Bock HH, Nimpf J, Herz J. Differential glycosylation regulates processing of lipoprotein receptors by γ-secretase. Journal of Biological Chemistry. 2003;278(39):37386-92.
78. Kim DH, Iijima H, Goto K, Sakai J, Ishii H, Kim HJ, et al. Human apolipoprotein E receptor 2. A novel lipoprotein receptor of the low density lipoprotein receptor family predominantly expressed in brain. The Journal of biological chemistry. 1996;271(14):8373-80.
79. Larios JA, Jausoro I, Benitez M-L, Bronfman FC, Marzolo M-P. Neurotrophins regulate ApoER2 proteolysis through activation of the Trk signaling pathway. BMC neuroscience. 2014;15(1):1.
80. Frotscher M. Role for Reelin in stabilizing cortical architecture. Trends in neurosciences. 2010;33(9):407-14.
81. Suetsugu S, Tezuka T, Morimura T, Hattori M, Mikoshiba K, Yamamoto T, et al. Regulation of actin cytoskeleton by mDab1 through N-WASP and ubiquitination of mDab1. Biochemical Journal. 2004;384(1):1-8.
82. Olson EC, Walsh CA. Reelin/Dab1 signaling in the developing cerebral cortex. Reelin Glycoprotein. 2008:89-105.
83. Chai X, Förster E, Zhao S, Bock HH, Frotscher M. Reelin stabilizes the actin cytoskeleton of neuronal processes by inducing n-cofilin phosphorylation at serine3. The Journal of Neuroscience. 2009;29(1):288-99.
84. Chai X, Förster E, Zhao S, Bock HH, Frotscher M. Reelin acts as a stop signal for radially migrating neurons by inducing phosphorylation of n-cofilin at the leading edge. Communicative & integrative biology. 2009;2(4):375-7.
85. Bouché E, Romero-Ortega MI, Henkemeyer M, Catchpole T, Leemhuis J, Frotscher M, et al. Reelin induces EphB activation. Cell research. 2013;23(4):473-90.
86. Sentürk A, Pfennig S, Weiss A, Burk K, Acker-Palmer A. Ephrin Bs are essential components of the Reelin pathway to regulate neuronal migration. Nature. 2011;472(7343):356-60.

87. Ohshima T, Ogawa M, Hirasawa M, Longenecker G, Ishiguro K, Pant HC, et al. Synergistic contributions of cyclin-dependant kinase 5/p35 and Reelin/Dab1 to the positioning of cortical neurons in the developing mouse brain. Proceedings of the National Academy of Sciences. 2001;98(5):2764-9.
88. Fuchigami T, Sato Y, Tomita Y, Takano T, Miyauchi Sy, Tsuchiya Y, et al. Dab1-mediated colocalization of multi-adaptor protein CIN85 with Reelin receptors, ApoER2 and VLDLR, in neurons. Genes to Cells. 2013;18(5):410-24.
89. Sato Y, Taoka M, Sugiyama N, Kubo Ki, Fuchigami T, Asada A, et al. Regulation of the interaction of Disabled-1 with CIN85 by phosphorylation with Cyclin-dependent kinase 5. Genes to Cells. 2007;12(12):1315-27.
90. Dulabon L, Olson EC, Taglienti MG, Eisenhuth S, McGrath B, Walsh CA, et al. Reelin binds alpha3beta1 integrin and inhibits neuronal migration. Neuron. 2000;27(1):33-44.
91. Sanada K, Gupta A, Tsai LH. Disabled-1-regulated adhesion of migrating neurons to radial glial fiber contributes to neuronal positioning during early corticogenesis. Neuron. 2004;42(2):197-211.
92. Lee GH, Chhangawala Z, von Daake S, Savas JN, Yates JR, Comoletti D, et al. Reelin induces Erk1/2 signaling in cortical neurons through a non-canonical pathway. Journal of Biological Chemistry. 2014;289(29):20307-17.
93. Stockinger W, Brandes C, Fasching D, Hermann M, Gotthardt M, Herz J, et al. The reelin receptor ApoER2 recruits JNK-interacting proteins-1 and -2. The Journal of biological chemistry. 2000;275(33):25625-32.
94. Huang C, Jacobson K, Schaller MD. MAP kinases and cell migration. Journal of cell science. 2004;117(20):4619-28.
95. Katz DM. Regulation of respiratory neuron development by neurotrophic and transcriptional signaling mechanisms. Respiratory physiology & neurobiology. 2005;149(1):99-109.
96. Cendelin J. From mice to men: lessons from mutant ataxic mice. Cerebellum & ataxias. 2014;1(1):1.
97. Mariani J, Crepel F, Mikoshiba K, Changeux J, Sotelo C. Anatomical, physiological and biochemical studies of the cerebellum from Reeler mutant mouse. Philosophical Transactions of the Royal Society of London Series B, Biological Sciences. 1977:1-28.
98. Beierbach E, Park C, Ackerman SL, Goldowitz D, Hawkes R. Abnormal dispersion of a purkinje cell subset in the mouse mutant cerebellar deficient folia (cdf). The Journal of comparative neurology. 2001;436(1):42-51.
99. Dupont J-L, Gardette R, Crepel F. Bioelectrical properties of cerebellar Purkinje cells in reeler mutant mice. Brain research. 1983;274(2):350-3.
100. Schmitt A, Turck CW, Pilz PK, Malchow B, von Wilmsdorff M, Falkai P, et al. Proteomic similarities between heterozygous reeler mice and schizophrenia. Biological psychiatry. 2013;74(6):e5-e10.
101. Krueger DD, Howell JL, Hebert BF, Olausson P, Taylor JR, Nairn AC. Assessment of cognitive function in the heterozygous reeler mouse. Psychopharmacology. 2006;189(1):95-104.
102. Kuo G, Arnaud L, Kronstad-O'Brien P, Cooper JA. Absence of Fyn and Src causes a reeler-like phenotype. The Journal of neuroscience. 2005;25(37):8578-86.

103. Herz J, Bock HH. Lipoprotein receptors in the nervous system. Annual review of biochemistry. 2002;71:405-34.
104. Burk RF, Hill KE, Olson GE, Weeber EJ, Motley AK, Winfrey VP, et al. Deletion of apolipoprotein E receptor-2 in mice lowers brain selenium and causes severe neurological dysfunction and death when a low-selenium diet is fed. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2007;27(23):6207-11.
105. Ashtari N, Jiao X, Rahimi-Balaei M, Amiri S, Mehr SE, Yeganeh B, et al. Lysosomal Acid Phosphatase Biosynthesis and Dysfunction: A Mini Review Focused on Lysosomal Enzyme Dysfunction in Brain. Current molecular medicine. 2016;16(5):439-46.
106. Wen J, Li H, Zhang Y, Li X, Liu F. Modification of HSP proteins and Ca2+ are responsible for the NO-derived peroxynitrite mediated neurological damage in PC12 cell. International journal of clinical and experimental pathology. 2015;8(5):4492.
107. O'Neill K, Musgrave IF, Humpage A. Extended Low-Dose Exposure to Saxitoxin Inhibits Neurite Outgrowth in Model Neuronal Cells. Basic & Clinical Pharmacology & Toxicology. 2016.
108. Ferreira RS, dos Santos NAG, Martins NM, Fernandes LS, dos Santos AC. Non-cytotoxic Concentration of Cisplatin Decreases Neuroplasticity-Related Proteins and Neurite Outgrowth Without Affecting the Expression of NGF in PC12 Cells. Neurochemical research. 2016;41(11):2993-3003.
109. Zhang J, Cai S, Li J, Xiong L, Tian L, Liu J, et al. Neuroprotective Effects of Theaflavins Against Oxidative Stress-Induced Apoptosis in PC12 Cells. Neurochemical research. 2016;41(12):3364-72.
110. Zhu L, Cao M, Ni Y, Han L, Dai A, Chen R, et al. Up-Regulation of TAB3 Is Involved in Neuronal Apoptosis After Intracerebral Hemorrhage. Cellular and Molecular Neurobiology. 2016:1-11.
111. Royaux I, Lambert de Rouvroit C, D'Arcangelo G, Demirov D, Goffinet AM. Genomic organization of the mouse reelin gene. Genomics. 1997;46(2):240-50.
112. Larios JA, Jausoro I, Benitez M-L, Bronfman FC, Marzolo M-P. Neurotrophins regulate ApoER2 proteolysis through activation of the Trk signaling pathway. BMC neuroscience. 2014;15(1):108.
113. Ghavami S, Rashedi I, Dattilo BM, Eshraghi M, Chazin WJ, Hashemi M, et al. S100A8/A9 at low concentration promotes tumor cell growth via RAGE ligation and MAP kinase-dependent pathway. Journal of leukocyte biology. 2008;83(6):1484-92.
114. Hevner RF. Reelin and the cerebellum. Reelin Glycoprotein: Springer; 2008. p. 141-58.
115. Sinagra M, Gonzalez Campo C, Verrier D, Moustie O, Manzoni OJ, Chavis P. Glutamatergic cerebellar granule neurons synthesize and secrete reelin in vitro. Neuron glia biology. 2008;4(3):189-96.
116. Schiffmann SN, Bernier B, Goffinet A. Reelin mRNA expression during mouse brain development. European journal of neuroscience. 1997;9(5):1055-71.
117. Pesold C, Impagnatiello F, Pisu MG, Uzunov DP, Costa E, Guidotti A, et al. Reelin is preferentially expressed in neurons synthesizing gamma-aminobutyric acid in cortex and hippocampus of adult rats. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(6):3221-6.

118. Hamburgh M. Analysis of the postnatal developmental effects of “reeler,” a neurological mutation in mice. A study in developmental genetics. Developmental biology. 1963;8(2):165-85.
119. Goldowitz D, Cushing RC, Laywell E, D'Arcangelo G, Sheldon M, Sweet HO, et al. Cerebellar disorganization characteristic of reeler in scrambler mutant mice despite presence of reelin. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1997;17(22):8767-77.
120. Knuesel I. Reelin-mediated signaling in neuropsychiatric and neurodegenerative diseases. Progress in neurobiology. 2010;91(4):257-74.
121. Trommsdorff M, Gotthardt M, Hiesberger T, Shelton J, Stockinger W, Nimpf J, et al. Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. Cell. 1999;97(6):689-701.
122. Hack I, Hellwig S, Junghans D, Brunne B, Bock HH, Zhao S, et al. Divergent roles of ApoER2 and Vldlr in the migration of cortical neurons. Development. 2007;134(21):3883-91.
123. Reddy SS, Connor TE, Weeber EJ, Rebeck W. Similarities and differences in structure, expression, and functions of VLDLR and ApoER2. Molecular neurodegeneration. 2011;6(1):30.
124. Nordberg A. Neuroreceptor changes in Alzheimer disease. Cerebrovascular and brain metabolism reviews. 1991;4(4):303-28.
125. Pokk P, Kivastik T, Sobol D, Liljequist S, Zharkovsky A. Is upregulation of benzodiazepine receptors a compensatory reaction to reduced GABAergic tone in the brain of stressed mice? Naunyn-Schmiedeberg's archives of pharmacology. 1996;354(6):703-8.
126. Ary A. Compensatory mechanisms for GnRH production in Fgf8-deficient mice. 2016.
127. Rice DS, Sheldon M, D'Arcangelo G, Nakajima K, Goldowitz D, Curran T. Disabled-1 acts downstream of Reelin in a signaling pathway that controls laminar organization in the mammalian brain. DEVELOPMENT-CAMBRIDGE-. 1998;125:3719-29.
128. Yaffe MB. Phosphotyrosine-binding domains in signal transduction. Nature reviews molecular cell biology. 2002;3(3):177-86.
129. Hiesberger T, Trommsdorff M, Howell BW, Goffinet A, Mumby MC, Cooper JA, et al. Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. Neuron. 1999;24(2):481-9.
130. Arnaud L, Ballif BA, Cooper JA. Regulation of protein tyrosine kinase signaling by substrate degradation during brain development. Molecular and cellular biology. 2003;23(24):9293-302.
131. Bock HH, Herz J. Reelin activates SRC family tyrosine kinases in neurons. Current Biology. 2003;13(1):18-26.
132. Bock HH, Jossin Y, May P, Bergner O, Herz J. Apolipoprotein E receptors are required for reelin-induced proteasomal degradation of the neuronal adaptor protein Disabled-1. The Journal of biological chemistry. 2004;279(32):33471-9.
133. Feng L, Allen NS, Simo S, Cooper JA. Cullin 5 regulates Dab1 protein levels and neuron positioning during cortical development. Genes & development. 2007;21(21):2717-30.
134. Rakic P, Caviness VS. Cortical development: view from neurological mutants two decades later. Neuron. 1995;14(6):1101-4.

135. Frotscher M. Cajal—Retzius cells, Reelin, and the formation of layers. Current opinion in neurobiology. 1998;8(5):570-5.
136. Zhao S, Chai X, Förster E, Frotscher M. Reelin is a positional signal for the lamination of dentate granule cells. Development. 2004;131(20):5117-25.
137. Zhao S, Frotscher M. Go or stop? Divergent roles of Reelin in radial neuronal migration. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry. 2010;16(4):421-34.
138. Dulabon L, Olson EC, Taglienti MG, Eisenhuth S, McGrath B, Walsh CA, et al. Reelin binds α3β1 integrin and inhibits neuronal migration. Neuron. 2000;27(1):33-44.
139. Hammond V, Howell B, Godinho L, Tan S-S. Disabled-1 functions cell autonomously during radial migration and cortical layering of pyramidal neurons. Journal of Neuroscience. 2001;21(22):8798-808.
140. Hack I, Bancila M, Loulier K, Carroll P, Cremer H. Reelin is a detachment signal in tangential chain-migration during postnatal neurogenesis. Nature neuroscience. 2002;5(10):939-45.
141. Kuo G, Arnaud L, Kronstad-O'Brien P, Cooper JA. Absence of Fyn and Src causes a reeler-like phenotype. Journal of Neuroscience. 2005;25(37):8578-86.
142. Herrick TM, Cooper JA. A hypomorphic allele of dab1 reveals regional differences in reelin-Dab1 signaling during brain development. Development. 2002;129(3):787-96.
143. Luque JM, Morante-Oria J, Fairen A. Localization of ApoER2, VLDLR and Dab1 in radial glia: groundwork for a new model of reelin action during cortical development. Brain research Developmental brain research. 2003;140(2):195-203.
144. Forster E, Tielsch A, Saum B, Weiss KH, Johanssen C, Graus-Porta D, et al. Reelin, Disabled 1, and beta 1 integrins are required for the formation of the radial glial scaffold in the hippocampus. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(20):13178-83.
145. Howell BW, Herrick TM, Cooper JA. Reelin-induced tryosine phosphorylation of disabled 1 during neuronal positioning. Genes & development. 1999;13(6):643-8.
146. Webb DJ, Nguyen D, Gonias SL. Extracellular signal-regulated kinase functions in the urokinase receptor-dependent pathway by which neutralization of low density lipoprotein receptor-related protein promotes fibrosarcoma cell migration and matrigel invasion. J Cell Sci. 2000;113(1):123-34.
147. Wang L, Zhang ZG, Zhang RL, Gregg SR, Hozeska-Solgot A, LeTourneau Y, et al. Matrix metalloproteinase 2 (MMP2) and MMP9 secreted by erythropoietin-activated endothelial cells promote neural progenitor cell migration. Journal of Neuroscience. 2006;26(22):5996-6003.
148. Shi C, Lu J, Wu W, Ma F, Georges J, Huang H, et al. Endothelial cell-specific molecule 2 (ECSM2) localizes to cell-cell junctions and modulates bFGF-directed cell migration via the ERK-FAK pathway. PloS one. 2011;6(6):e21482.
149. Moors M, Cline JE, Abel J, Fritsche E. ERK-dependent and-independent pathways trigger human neural progenitor cell migration. Toxicology and applied pharmacology. 2007;221(1):57-67.
150. Zsarnovszky A, Belcher SM. Spatial, temporal, and cellular distribution of the activated extracellular signal regulated kinases 1 and 2 in the developing and mature rat cerebellum. Brain research Developmental brain research. 2004;150(2):199-209.

151. Yu CG. Distinct roles for ERK1 and ERK2 in pathophysiology of CNS. Frontiers in biology. 2012;7(3):267-76.
152. Ishii A, Furusho M, Dupree JL, Bansal R. Role of ERK1/2 MAPK signaling in the maintenance of myelin and axonal integrity in the adult CNS. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2014;34(48):16031-45.
153. Bajetto A, Barbero S, Bonavia R, Piccioli P, Pirani P, Florio T, et al. Stromal cell-derived factor-1α induces astrocyte proliferation through the activation of extracellular signal-regulated kinases 1/2 pathway. Journal of neurochemistry. 2001;77(5):1226-36.
154. Cao F, Zhang X, Liu T, Li X, Malik M, Feng S. Up-regulation of Ras/Raf/ERK1/2 signaling in the spinal cord impairs neural cell migration, neurogenesis, synapse formation, and dendritic spine development. Chinese medical journal. 2013;126(20):3879-85.
155. Yang K, Cao F, Sheikh AM, Malik M, Wen G, Wei H, et al. Up-regulation of Ras/Raf/ERK1/2 signaling impairs cultured neuronal cell migration, neurogenesis, synapse formation, and dendritic spine development. Brain Structure and Function. 2013;218(3):669-82.
156. Xu D, Cao F, Sun S, Liu T, Feng S. Inhibition of the Ras/Raf/ERK1/2 Signaling Pathway Restores Cultured Spinal Cord-Injured Neuronal Migration, Adhesion, and Dendritic Spine Development. Neurochemical research. 2016;41(8):2086-96.
157. Thomas K, Hunt S. The regional distribution of extracellularly regulated kinase-1 and-2 messenger RNA in the adult rat central nervous system. Neuroscience. 1993;56(3):741-57.
158. Flood DG, Finn JP, Walton KM, Dionne CA, Contreras PC, Miller MS, et al. Immunolocalization of the mitogen-activated protein kinases p42MAPK and JNK1, and their regulatory kinases MEK1 and MEK4, in adult rat central nervous system. Journal of Comparative Neurology. 1998;398(3):373-92.
159. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature. 2001;410(6824):37-40.
160. Simó S, Pujadas L, Segura MF, La Torre A, Del Río JA, Ureña JM, et al. Reelin induces the detachment of postnatal subventricular zone cells and the expression of the Egr-1 through Erk1/2 activation. Cerebral cortex. 2007;17(2):294-303.
161. Ballif BA, Arnaud L, Cooper JA. Tyrosine phosphorylation of Disabled-1 is essential for Reelin-stimulated activation of Akt and Src family kinases. Molecular brain research. 2003;117(2):152-9.
162. Stockinger W, Brandes C, Fasching D, Hermann M, Gotthardt M, Herz J, et al. The reelin receptor ApoER2 recruits JNK-interacting proteins-1 and-2. Journal of Biological Chemistry. 2000;275(33):25625-32.