University of Alberta

Regulation of Dab1 Splicing and Phosphorylation in Developing Neuronal Cells

by

Indrani Dutta

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Dedication

For Ma, Baba and Mani

Abstract

The Reelin signaling pathway is involved in the migration of neurons during brain development. Reelin binding to the receptors VLDLR and ApoER2 leads to Dab1 phosphorylation, thereby activating a cascade of downstream pathways.

Here, we show that the neuronal splicing factor Nova1 is involved in the exclusion of Dab1 exons 9b and 9c. In particular, the expression of Nova1 isoform 2, which excludes exon 4, coincides with exclusion of Dab1 exons 9b and 9c in P19 cells. Nova1 knockdown in P19 cells results in increased inclusion of Dab1 exons 9b and 9c.

We also show that Dab1 phosphorylation is regulated by the Shp-2 phosphatase. Intriguingly, in addition to its role as a phosphatase, Shp-2 can function as an adapter protein to recruit kinases that phosphorylate Dab1 in P19 cells but not HEK293T cells. These results suggest an additional level of complexity to the regulation of Dab1 phosphorylation during neuronal development.

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I am deeply indebted to all my friends in Edmonton who have been a pillar of support through these years. They have stood by me through thick and thin making it so easy for me to start my new life here in a completely new country. In them, I found a home so far away from my home.

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ABBREVIATIONS

μM	micromolar
ApoER2	apolipoprotein E receptor 2
AS	alternative splicing
ASF	Alternative Splicing Factor 1
ATP	adenosine triphosphate
BBP	branch binding protein
bp	base pair
BrdU	bromodeoxyuridine
Cdk	cyclin dependent kinase
ch	chicken
CNS	central nervous system
Crk	CT10 virus regulator of protein kinase
CUGBP	CUG binding proteins
Dab	Disabled
Dab1	Disabled-1
DCX	Doublecortin
DT	DNA transfected
E	embryonic
ED	embryonic day
EGF	epidermal growth factor
ELAV	embryonic lethal abnormal vision
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
FGF	fibroblast growth factor
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factors
GFP	green fluorescent protein
GSK	glycogen synthase kinase

hnRNP	heterogeneous nuclear ribonucleoprotein
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
Jak	Janus kinase
LIS1	Lissencephaly 1
LTR	long terminal repeat
Nckβ	non-catalytic region of tyrosine kinase beta
n-cofilin	non-muscle cofilin
NICD	Notch intracellular domain
NMDAR	N-Methyl-D-aspartate receptor
Nova1	neuro-oncological ventral antigen
nPTB	neuronal polypyrimidine tract-binding protein
nt	nucleotide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
PH	pleckstrin homology
PI/PTB	protein interaction/phosphotyrosine binding
PI3K	phosphatidylinositide-3-kinase
PKA	protein kinase A
PKC	protein kinase C
POMA	paraneoplastic opsoclonus-myoclonus ataxia
pre-mRNA	precursor mRNA
PTB	phosphotyrosine binding/ polypyrimidine tract binding protein
PTK	protein-tyrosine kinase

PTP	protein-tyrosine phosphatase
pTyr	phosphorylated tyrosine
RA	retinoic acid
RIPA	radioimmunoprecipitation assay
RRM	RNA recognition motif
RS	arginine-serine
RT	reverse transcription
S	serine
SFK	src family kinase
SH2	Src Homology 2
Shh	sonic hedgehog
Shp-2	SH2 domain-containing tyrosine phosphatase
shRNA	short hairpin RNA
siRNA	small interfering RNA
SMA	Spinal Muscular Atrophy
snRNP	small nuclear ribonucleoprotein
SOCS	suppressor of cytokine signaling
SR	serine arginine
STAT	signal transducer and activator of transcription
Sxl	Sex lethal
T	threonine
TBS	Tris-buffered saline
TK	tyrosine kinase
TNFR	Tumour Necrosis Factor Receptor
Tyr	tyrosine
U2AF	U2 auxiliary factor
VLDLR	very low density lipoprotein receptor
Y	tyrosine

Chapter 1:

INTRODUCTION

1.1 GENERAL INTRODUCTION

1.1.1 Brain Development

The process of brain development is highly coordinated, dynamic and complex. It begins with the induction of genes that are responsible for the formation of the neural tube which spans the length of the embryo. The neural tube in the head region is destined to form the brain and retina. The developing brain can be divided into 3 main structures: forebrain, midbrain and hindbrain. The rostral part of the forebrain develops into the neocortex which assumes a laminar structure. In vertebrate neocortex, neurons settle into six layers that interact with neuronal axons to establish neuronal network and circuitry [1]. This laminar structure is formed in an inside-out manner with the earliest-formed neurons located in the deepest layers of the neocortex and the later-formed neurons located in the more superficial layers. Formation of the laminated structure of the neocortex occurs in three general steps: (i) establishment of cell identity, (ii) directed neuronal migration and (iii) assembly into neuronal layers [1-2].

Initially, the neocortex is comprised of proliferative precursor cells arranged in a layer known as the ventricular zone. Once the precursors undergo their final mitosis, they undergo a migratory phase. The first wave of post-mitotic neurons forms a preplate which includes the Cajal-Retzius cells [3]. During this first wave, post-mitotic neurons migrate by extending their long leading processes that are attached to the outer pial surface, a process called somal translocation [2, 4]. Subsequent waves of post-mitotic neurons migrate

across the preplate by following tracts that are formed by radial glial cells, a process called radial glial locomotion [4]. [5]. Each wave of migrating neurons goes past their predecessors leading to the formation of the inside-out laminar structure that is characteristic of the neocortex [1].

1.1.2 Proteins Involved in Neuronal Migration

Many proteins are required for neuronal migration. For example, Lissencephaly 1 (LIS1) is a very important protein involved in brain development. Cortical lamination is disrupted in *LIS1* null mice [6]. LIS1 associates with microtubules and is involved in mitotic cell division and chromosomal segregation. LIS1 may also regulate extension of the leading processes of neurons [6]. Doublecortin (DCX) is another protein involved in neuronal migration which also associates with microtubules. DCX and LIS1 work together to increase polymerization and promote neuronal migration. It is hypothesized that DCX and LIS1 may function in concert to facilitate extension of the leading processes of neurons [7].

Cyclin dependent kinase 5 (CDK5) is a kinase that associates with an activator protein p35 [8]. CDK5 and p35 expression is restricted to post-mitotic neurons. There is evidence suggesting that CDK5-p35 plays a role in cell-cell adhesion and binds to β-catenin [8] . The CDK5-p35 complex is also believed to be involved in neuronal-glial interaction and migration of neurons [8]. Another extensively studied protein that plays a role in neuronal migration

and neuronal development is Reelin. This protein will be reviewed in detail later.

SH2 domains are phospho-tyrosine binding domains that promote protein-protein interaction. SH2 domains are an integral part of cell signaling transduction and there have been many SH2 domain proteins implicated in brain development including Src, Shp-2 and SHIP2. Hence, to understand the process of cell signaling in the brain, it is important to understand the concept of SH2 domain and tyrosine phosphorylation.

1.1.3 Tyrosine Phosphorylation

Cell signaling is a complex and tightly regulated event that ensures rapid and specific propagation of cellular signals. There are many types of post-transcriptional modifications such as phosphorylation and ubiquitination which are used by the cell to propagate specific and accurate signal transduction. One of the major post-transcriptional modifications that play key roles in cell signaling and protein activity is tyrosine phosphorylation. Phosphotyrosine (pTyr) mediated signaling plays an essential role in regulating the key pathways that are involved in cell growth, proliferation, differentiation and migration [9]. pTyr signaling depends on three signaling molecules: (i) protein-tyrosine kinases (PTKs) which add a phosphate group to tyrosine, (ii) protein-tyrosine phosphatases (PTPs) which remove a phosphate group from tyrosine, and (iii) proteins with modular (protein) interaction domains which recognize and bind to the tyrosine-phosphorylated

substrate and recruit other proteins with the same or different domain(s), thereby regulating downstream signaling events [10]. The interaction between the substrate tyrosine and the collection of available PTKs and PTPs regulate the timing, localization and longevity of phosphorylation events.

1.1.4 SH2 Domains

SH2 domain-containing proteins are the largest family of proteins that are involved in recognition of phosphorylated tyrosine (p-Tyr). SH2 domains are the conserved non-catalytic modules that regulate both target activity and interaction with other proteins. SH2 domains are found in many different types of proteins such as protein tyrosine kinases, transcription factors and molecular adaptors [11]. There are over 100 proteins that contain at least one SH2 domain. These proteins contain a diverse range of additional interaction domains such as SH3, PTB (phosphotyrosine-binding) and PH (Pleckstrin homology), and catalytic domains such as those found in TK (tyrosine kinase) and phosphatases. These combined domains increase the substrate specificity and diversity of signaling function of the protein [12-13]. SH2 domain proteins can be adaptors, kinases, phosphatases, transcription factors and ubiquitination regulators.

The most important property of the SH2 domain is its selectivity for pTyr. The pTyr plays the most important role in determining the binding specificity of the protein to the SH2 domain [11]. However, the surrounding residues also play an important role [11, 14-19]. For example, the residues

adjacent to the binding pocket of the SH2 domain bind to the residues that are C-terminal to the pTyr of the partner protein; hence, these C-terminal residues also play a role in determining the binding specificity. There are many pTyr-containing short linear motifs that bind to SH2 domains and are conserved across species.

Tyrosine phosphorylation acts as a signal to recruit SH2-containing proteins. These SH2-containing proteins can be activated once they bind to the target by juxtaposing next to them. Another activation mechanism is that the SH2 domain of the protein is initially bound to its catalytic domain which renders the protein enzymatically inactive, but when the SH2 domain binds to the pTyr residue of the target, it relieves the constraints on the catalytic domain making it active. Moreover, the SH2 domain once bound can itself become a substrate for phosphorylation resulting either in conformational changes or creating docking sites for additional SH2 proteins [20].

SH2 domain proteins can be classified into the following functionally different groups of proteins (Figure 1.1).

Enzymes: In enzymes, the SH2 domain is linked to a catalytic domain such as protein/lipid kinase, phosphatase, phospholipase C, RasGAP domain or Rho-family GEF enzymes may also contain SH3 and PH domains that can aid the interaction of the SH2 domain protein with the target protein juxtaposing the enzyme to its substrate [21].

Adaptor proteins: The adaptor proteins are generally comprised of one SH2 domain and multiple SH3 domains. The adaptor protein increases the option

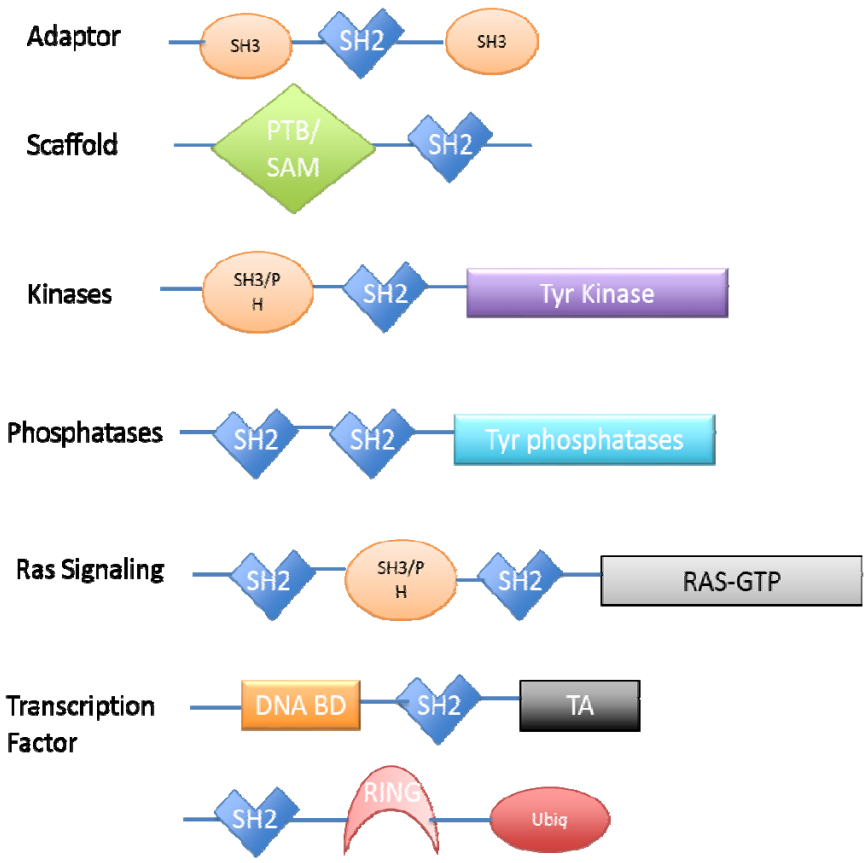

Figure 1.1. Structure of the different SH2 domain proteins.

for negative and positive regulation. The presence of multiple SH3 domains allows the recruitment of several downstream targets via a single phosphotyrosine site [20]. For example, Grb2 binds to an activated receptor using its SH2 domain and uses its SH3 domain in turn to bind to the Ras guanine exchange factor SOS. SOS enables the production of GTP- bound Ras followed by activation of MAPK pathway [22-26].

Docking proteins: Many SH2 proteins are involved in the formation of multi-protein complexes. Typically, a docking protein with an SH2 domain can bind to a receptor with the help of domains like PTB. Docking proteins can recruit multiple SH2 domain containing proteins through their multiple tyrosine phosphorylation sites and help in the formation of multiprotein complexes. [20, 27-28].

Transcription Factors: Transcriptional regulators like STAT that are found in the plasma membrane contain an SH2 domain. STATs are recruited by activated receptor tyrosine kinases through their SH2 domains which in turn leads to their tyrosine phosphorylation. This phosphorylation induces interaction between two STAT molecules and promotes their dimerization [29-30] and activation as a transcription factor.

Growth Factor Receptors: The SH2 domain on receptor tyrosine kinases recruits many signaling molecules once the receptor has been autophosphorylated. An illustration for this is PDGF (Platelet Derived Growth Factor). Studies have shown that SH2 domain proteins bind to the activated PDGF receptor and induce cell growth and cell division [31-32].

Cytoplasmic signaling molecules and kinases: The activated receptor molecules can recruit adaptor proteins. As already discussed, these adaptor proteins bind to membrane receptor proteins with their SH2 domains. This results in the recruitment of proteins with other binding domains like SH3. For example, receptor tyrosine kinases like EGFR (Epidermal Growth Factor Receptor), when activated can recruit Gbr2 which can in turn use its SH3 domain to bind to the guanine exchange factor SOS which facilitates the activation of Ras and activation of the MAPK pathway [19, 28, 33-34]. Kinases play a crucial role in cell signaling and are locked in an inactive state when not required. Kinases can be effectively activated by post-translational modification, their specific localization at signaling complexes and selective targeting of substrate by interacting with secondary recruitment sites in the substrate [35].

Hence, SH2 domains play diverse and fundamental roles in cell signaling and other cell processes. One example of a vital SH2 domain protein involved in cell signaling is the Disabled 1 (Dab1) protein which plays a crucial role in migration and position of neurons during brain development.

1.2 REELIN-DAB1 SIGNALING PATHWAY

The development of the brain entails coordinated migration of neurons and their precise positioning into distinct layers that leads to the laminated structure of the brain. The Reelin signaling pathway is crucial for neuronal development and neuron migration and positioning in the developing brain.

During development, binding of Reelin to its receptors Very Low Density Lipoprotein Receptor (VLDLR) and Apolipoprotein ER2 (ApoER2) leads to the activation of Src family kinases (SFK) and phosphorylation of the adaptor protein Dab1 which in turn phosphorylates downstream effectors. The end product of this cell signaling pathway is the proper migration and positioning of neurons in the laminated structures of the brain (Figure 1.2) [36-38]. Mutations in *Reln*, *Dab1* or *ApoER2/VLDLR* genes result in extensive migration defects and layer inversion in the cerebral cortex and lamination disruption in the cerebellum and hippocampus [39-43]. Defects in the Reelin-Dab1 signaling pathway have also been implicated in many human diseases such as lissencephaly, epilepsy and schizophrenia [44-45].

Dab1 is a cytoplasmic protein that is a key member of the Reelin signaling pathway [46]. Dab1 was first identified in Drosophila where it was found to be involved in eye development and formation of axons [47-48]. In vertebrates, Dab1 has been shown to be primarily an adaptor protein and predominantly expressed in the developing CNS. Both the human and mouse *Dab1* genes extend over 1.1 Mb and consist of 14 main exons. The *Dab1* gene undergoes multiple alternative splicing events which result in the production of over 11 isoforms with the major isoform consisting of 555 amino acids. The 555-amino acid Dab1 protein is comprised of 3 major parts: an N-

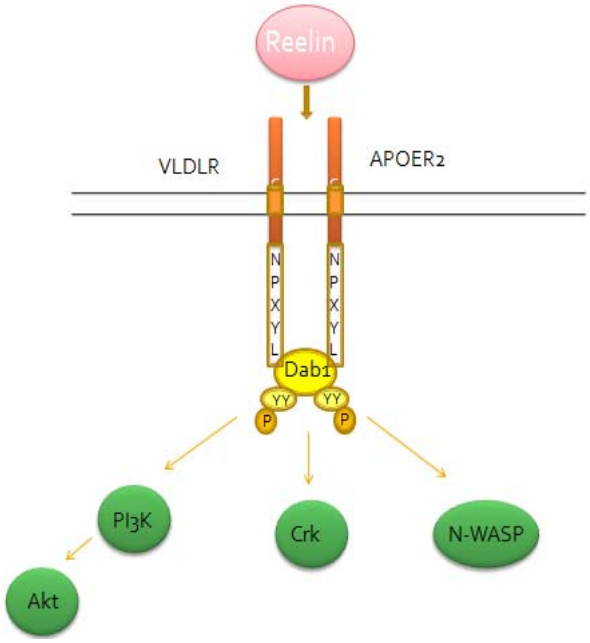

Figure 1.2 Reelin-Dab1 signaling pathway in developing brain. The Reelin ligand binds to the receptor ApoER2 and VLDLR which leads to phosphorylation of Dab1. Phosphorylated Dab1 then transmits the signal to downstream pathways (adapted from Zhang *et al*., 2008).

terminal PTB/PI domain that binds to the Reelin signaling receptors, an internal tyrosine-rich region and a C-terminus serine/threonine rich region.

Mutation of the *Dab1* gene leads to many defects in the developing brain. Mice with mutation in the *Dab1* gene are known as *scrambler* [49-50]. In these mice, the cerebellum is malformed with laminar defects and a reduced number of Purkinje cells. Furthermore, the layers in the cerebral cortex are inverted. *Scrambler* mice undergo ataxia, seizures and cognitive delays. These abnormalities are very similar to the *reeler* (Reelin knockout mice) phenotype suggesting that Dab1 and Reelin function in the same signaling pathway [50].

As mentioned earlier, the Dab1 PTB domain is an important interacting domain which comprises amino acid sequence Asn-Pro-x-Tyr (NPxY) [51]. This domain helps Dab1 bind to other proteins with the same domain. VLDLR and ApoER2 are two binding partners of Dab1 [51-53] which activate SFKs and relay the Reelin-Dab1 signal by phosphorylating Dab1 [52]. The PTB domain also binds to the phosphoinositides in the plasma membrane lipid bilayer. This helps Dab1 interact with other proteins while still attached to the plasma membrane [26].

The central part of Dab1 is comprised of a group of tyrosine residues. As discussed earlier, phosphorylation is a crucial regulator of signaling pathways. Dab1 has been widely reported to be tyrosine phosphorylated. There are five conserved tyrosine residues in the C-terminus of Dab1 (Y185, Y198/Y200, Y220, Y232) that resemble the phosphotyrosine binding site for

many receptor and non-receptor tyrosine kinases [54]. These five tyrosines represent four tyrosine phosphorylation sites [YQXI sequences (Y185 and Y198/Y200) and YXVP sequences (Y220 and Y232)]. When Reelin binds to the receptors VLDLR and ApoER2, Src kinase is activated and phosphorylates the tyrosine residues in Dab1, thereby activating Dab1 [55-58]. The activated Dab1 can now recruit other SH2 domain proteins to activate a cascade of signaling pathways. Mutation of all five Dab1 tyrosine residues results in a phenotype very similar to that of *reeler* and *scrambler* mice, highlighting the importance of tyrosine phosphorylation in signal relay [54].

The four tyrosine phosphorylation sites act as docking sites that promote protein-protein interaction and enable Dab1 to act as an adaptor protein. However, the four tyrosine phosphorylation sites relay distinct signals. The first two tyrosine phosphorylation sites interact with SFKs. These two sites are conserved among all vertebrates indicating their functional relevance [59]. Phosphorylation of SFK recognition sites Y185 and Y198 leads to recruitment of PI3-kinase and activation of Akt which is involved in cortical development [60]. PI3K activation leads to phosphorylation of n-cofilin which in turn contributes to neuronal migration. The Y185 and Y198 phosphorylation sites also bind to SOCS (Suppressors of Cytokine Signalling) SH2 domains [61]. The last two tyrosine phosphorylation sites (Y220 and Y232) are Abl recognition sites which recruit Crk/Crk-L-C3G and Nckβ [59, 62-66]. Crk is essential for cell migration, cell adhesion and immune cell response [67].

The Y185 and Y198 phosphorylation sites have also been shown to be responsible for ubiquitination of Dab1. It has been reported that Cullin5 (Cul5), an E3 ubiquitin ligase component, forms a complex with SOCS proteins that is bound to the phosphorylated Y185/Y198 Dab1 and targets it for polyubiquitination. Knocking down Cullin 5 leads to accumulation of active phosphorylated Dab1 in the migrating neurons and abnormal migration of these neurons resulting in disrupted laminar structure. [61].

It is clear that tyrosine phosphorylation of Dab1 is a complex process that plays a key role in activating various downstream pathways. Therefore, it is very important to have a clear understanding of how Dab1 phosphorylation is regulated. This thesis focuses on the regulation of Dab1 phosphorylation by alternative splicing and SH2 domain phosphatase Shp2

1.3 SPLICING

Transcription of a gene leads to the production of pre-mRNA. In a pre-mRNA transcript, the exons (coding regions) are interrupted by non-coding introns. Splicing involves the removal of introns and joining of exons leading to the formation of a mature mRNA that codes for a protein.

1.3.1 Mechanism of Splicing

Splicing is performed by a macromolecular complex known as a spliceosome [68]. The spliceosome comprises 5 small nuclear ribonucleoprotein (snRNP) particles (U1, U2, U4, U5 and U6) and other

associated auxiliary factors [69-70]. The snRNPs are small nuclear RNAs that exist in a complex with different proteins. snRNPs mainly have two roles: (i) they, along with other factors, bind to the pre-mRNA sequentially and define the exon-intron border, and (ii) catalyse reactions involving the spliceosome complex with the pre-mRNA by bringing reacting sites in close proximity with one another. These reactions consist of radical formation and breaking RNA-RNA base pairing [62-64].

The intron has 4 major sites that are required for splicing: (i) the **donor site** near the 5' end of the intron which includes a GU dinucleotide at the intron end within a larger, less conserved consensus sequence, (ii) an **acceptor site** which consists of the dinucleotide AG at the 3’ end of the intron, (iii) a **branch point** 20-50 bp upstream of the acceptor site, and (iv) a **polypyrimidine tract** between the branch point and the acceptor site (Figure 1.3) [60].

The first step in splicing is the binding of the U1 snRNP and associate factor SF1 to the donor site. One subunit of U2AF (U2 auxiliary factor) binds to the PY tract and the other subunit to the 3’ splice site. The PY binding subunit interacts with the BBP (Branchpoint Bridging Protein) and helps it to bind to the branch point. This arrangement is known as **complex E** (Early) [69]. The next step is the binding of the U2 snRNP to the branch site replacing the BBP. This is aided by the U2AF. The base pairing between the U2 snRNP is such that the A in the branch point is extruded out and it now stands as a single unpaired nucleotide. This is known as **complex A**. The next step is the

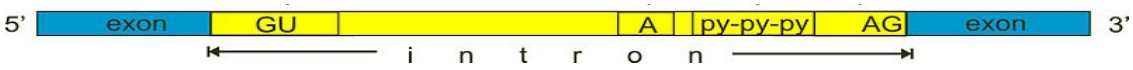

Figure 1.3 Different components of RNA splicing (Wikipedia).

recruitment of the trimer U4, U5 and U6. U6 replaces U1 in the 5' splice site. This is known as **complex B**. Then the U4 is released from the complex enabling the U6 to interact with U2. This is known as **complex C** (Figure 1.4) [69, 71]. This final rearrangement forms a lariat structure and is the active site which juxtaposes the 5' splice site and the branch site. This enables the reaction to proceed further with two transesterification reactions [69]. The OH group of the A in the branch site acts as a nucleophile and attacks the phosphate group of G (of the AG) which leads to the cleavage of the phosphodiester bond between the sugar and phosphate [69]. The free 5' end of the intron is joined to the A in the branch site and the exon is now released. In the second transesterification reaction, the free OH group of the 5' exon now becomes the nucleophile and attacks the phosphoryl group in the 3' splice site. This leads to the attachment of the 3' and 5' exons. This reaction also releases the intron lariat [69].

1.3.2 Alternative Splicing

Alternative splicing is a phenomenon whereby more than one transcript can be produced from a single gene. This process involves rearrangement and assembly of different combinations of exons. Alternative splicing plays a pivotal role in increasing the diversity and complexity of a proteome. Up to 95 percent of the mammalian genes may undergo alternative splicing [71].

Alternative splicing can determine the molecular interactions, subcellular localization or function of a protein. The different isoforms formed

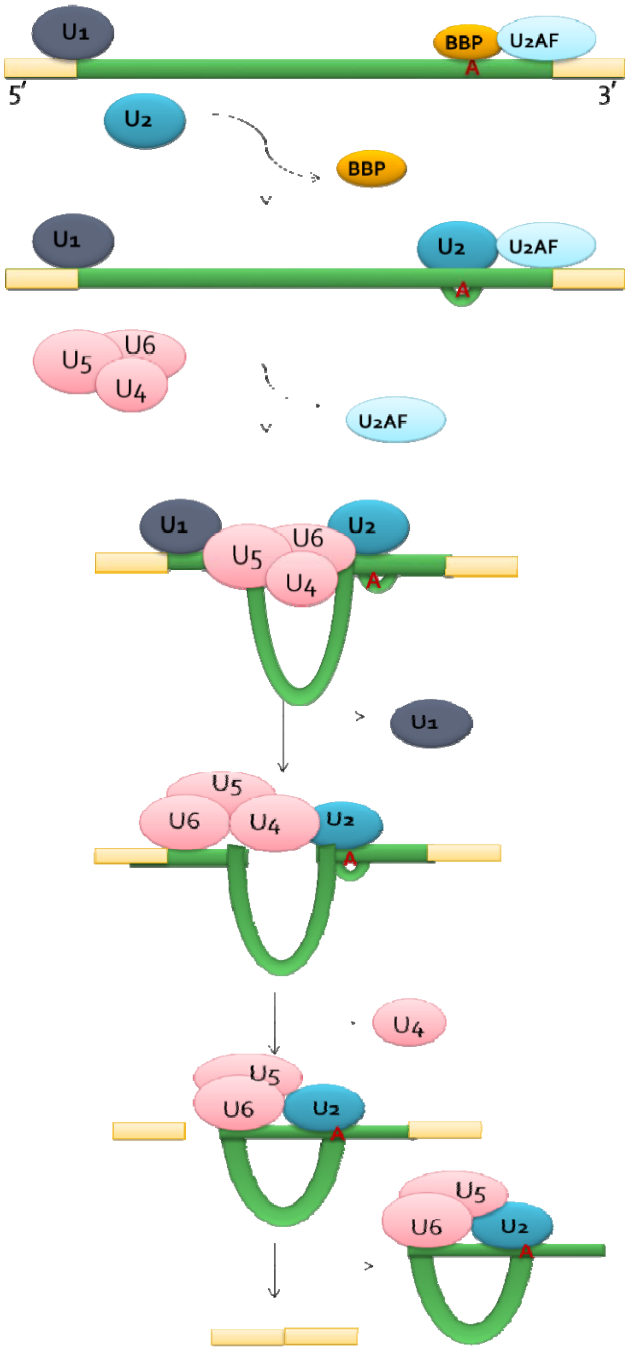

Figure 1.4. Stages of splicing (adapted from the book Molecular biology of the gene by Watson *et al*).

may have similar or distinct functions. One of the isoforms may act as dominant negative of the other. Alternative splicing can also act as a switch for turning a gene on or off by production of isoforms which include or exclude stop codons. Alternatively, inclusion of introns may prevent a transcript from being transported out of the nucleus and hence never get translated [72].

The most prominent examples that illustrate the importance of alternative splicing are the Slx and Tra/Tra-2 proteins in Drosophila. Tra/Tra-2 and Slx are RNA-binding proteins that are involved in splicing regulation. Slx inhibits splicing by competing with splicing factors such as U2AF whereas Tra/Tra-2 enhances splicing by recruiting general splicing factors to weak splice sites. Slx and Tra/Tra-2 are essential and sufficient to determine the sex of *Drosophila* [73-76]

1.3.3. Types of Alternative Splicing

There are many ways in which an exon and an intron can be spliced. These are depicted with some examples shown in Figure 1.5 [69]. The term **cassette exons** refers to either inclusion or exclusion of an exon into the mature mRNA. **Mutually exclusive splicing** is the selection and inclusion of a single exon from an array of two or sometimes more exons. As the name suggests, **intron retention** is the retention of an intron in the mature mRNA. This intron can code for amino acids, contain a stop codon or lead to a frameshift which can in turn lead to modified or non-functional protein [77]. **Competing 3' and 5' splice sites,** also known as **exon modification,** refers

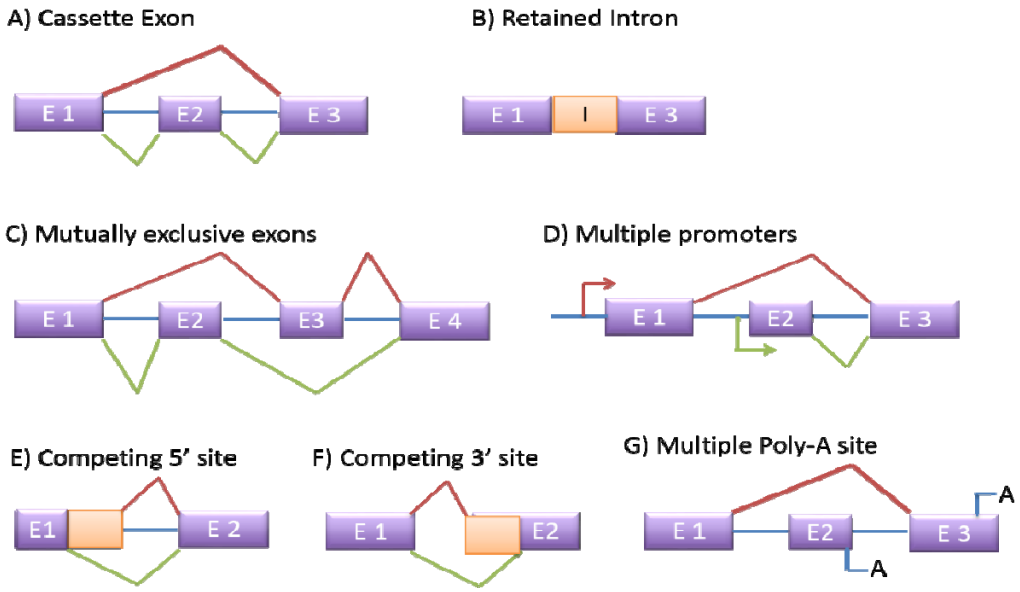

Figure 1.5 Different types of alternative splicing.

to the presence of an alternate splice site within an exon or intron which results in addition or deletion of a few base pairs in the mature mRNA. Two additional modes of splicing which fall under the category of transcriptional regulation rather than alternative splicing are: (i) **alternative promoter of transcription** whereby the initiation of transcription starts upstream of different alternative exons 1 and (ii) use of **different 3' polyadenylation sites** which results in different 3' ends in the mature mRNAs [69, 71].

1.3.4 Regulation of Alternative Splicing

1.3.4.1 Splicing Elements and Factors

Alternative splicing is regulated by both *cis* and *trans* factors. *Cis* factors comprise the pre-mRNA sequence whereas *trans* factors include RNA molecules other than the pre-mRNA and protein factors. Along with the constitutive splice sites, there are many auxiliary sequences or elements that are mainly involved in the regulation of alternative splicing. These elements are broadly divided into silencers and enhancers of splicing, which are subdivided into exonic splicing enhancers (ESE), exonic splicing silencers (ESS), intronic splicing enhancers (ISS) and intronic splicing silencers (ISS) based on their location in the pre-mRNA. Exon inclusion and skipping is modulated and determined by a fine balance between these elements [75, 78]. Moreover, the major sequences determining splicing in a constitutive pre-mRNA may deviate from their normal pattern in alternative splicing. For example, the branch point of a constitutive pre-mRNA is located 40 nt away

from the 3' splice site, but in some alternatively spliced pre-mRNAs, the distance between the branch point and 3' splice site may be as much as 400 nt and hence result in a weaker splice site and a weaker recognition of splice site. Also, the high level of evolutionary conservation in the sequences of many introns suggests that additional regions may also contribute to splicing regulation [79-80].

The other components of splicing regulation are the splicing factor proteins. There are many families of splicing activator proteins such as SR proteins and splicing repressor proteins such as hnRNP that bind to enhancer and silencer regions of the pre-RNA and modulate RNA splicing [81].

1.3.4.2 SR Proteins

SR proteins are a family of RNA binding proteins with a high serine and arginine content. These proteins play a very important role in both constitutive and alternative splicing. SR proteins have one or two RNA-binding domains at the N-terminus and an arginine/serine-rich domain at their C-terminus called the RS domain. SR proteins play many roles in splicing. In many cases, they can act as both splicing factors and regulators of splicing. As well, SR proteins are known to mediate protein-protein interactions. In fact, SR proteins alone are sufficient to activate enhancer-dependent splicing based on an in vitro assay involving binding of the RS domain to the pre m-RNA [82].

There is strong evidence showing that SR proteins bind to the 3' splice site and enhance the splicing out of the adjacent intron. SR proteins are believed to carry out their function by either recruiting U2AF to a weak polypyrimidine tract or by negating the effect of the splicing inhibitors [76, 83]. There is also evidence for binding of SR proteins to the 5' splice site and promoting its recognition probably by interacting with U1 snRNP. Studies have shown that SR proteins can bind constitutively to the 3' and 5' splice sites and help in defining the exon-intron boundaries [84-85].

1.3.4.3 Splicing Activation

There are different models formulating the mechanism of splicing activation. One of the earlier models proposed that splicing is activated by SR proteins recruiting the U2AF65 subunit of U2AF to the pyrimidine tract. Enhancers such as SRm160 and SRm300 subsequently enhance the interaction between the spliceosome and the ESE elements [86]. A more recent model suggests that the SR proteins bind to the branch point and promote the formation of complex A [87]. Although hnRNPs are mainly known to be splicing repressors, they may sometimes function as splicing activators as well. For example, hnRNP L binds to an ISE and enhances the weak 5' splice site [88].

1.3.4.4 Splicing Repression

There are numerous examples of splicing repression. For example, the SLX protein in *Drosophila* represses splicing of genes by binding to the pyrimidine tract thus inhibiting the binding of U2AF [89]. This results in selection of a 3' splice site from an alternative downstream exon. Another example of a splicing repressor is the PTB splicing factor which inhibits the binding of U2AF65 to the pyrimidine tract [90-91]. However, repression of splicing is generally a co-operative effort of more than one repressor binding to multiple silencer sites. There are two models for such repression. The first one states that the multiple silencers and the repressors bound to them create a 'zone of silencing' which antagonises splicing factor binding. The other model states that the region bound between the repressors is looped out which prevents binding of splicing factors [92-93].

There are numerous examples of combinatorial control in splicing. Positive and negative regulators of splicing act in concert with each other and it is the interplay between these elements that modulates and fine-tunes the process of splicing. One example is the splicing of mammalian *Src*. In non-neuronal cells, exon N1 in *Src* is excluded due to binding of PTB which prevents binding of U1 snRNP. However, in neuronal cells, exon N1 is included in the mature transcript. In this case, there is activation of splicing by hnRNPs that bind to a downstream ISE. In addition, nPTB, a neuronal-specific paralogue of PTB, binds to the same region as PTB but is less repressive than PTB. Hence, binding of nPTB cannot negate the binding of

activating effect of hnRNPs and there is an overall activating effect on splicing [94-96].

1.3.4.5 Other Methods of Splicing Regulation

Alternative splicing may also be regulated by histone modification. Studies have shown that exons generally have different histone modifications compared to introns [97]. Constitutive exons show a higher nucleosomal occupancy compared to alternative exons [98-99]. There are exons that show evolutionary conservation of histone modifications [100]. It is possible that these histone marks could play a part in alternative splicing regulation [101]. Apart from histone modification, other modifications such as DNA methylation are also believed to be involved in splicing regulation [102].

The secondary structure of a pre-mRNA can also regulate the recognition of the splice site. Exons of genes like *tau* and *fibronectin* are regulated by their secondary structure [103-104].

Phosphorylation of splicing factors is also an important step in the regulation of alternative splicing. These phosphorylation events are in turn regulated by various signal transduction pathways [105-107]. Phosphorylation of the RS domain of the SR proteins is a key step in its binding to the pre-mRNA. For example, phosphorylation of SRp38 reduces its ability to bind to U1 snRNP and increases its binding to Tra2-alpha, another splicing factor [108].

1.3.4.6 Alternative Splicing and Diseases

Alternative splicing plays such a crucial and widespread role in the development and maintenance of life that its deregulation leads to several different human diseases. In many cases, diseases are caused by point mutations in the splice sites and formation of abnormal mRNAs due to aberrant splicing reaction. For example, thalassemia is caused by mutations in the 5' splice site of genes which affect its binding to the U1 snRNP [109-110]. There are also many cases where the splicing factors are mutated leading to abnormal splicing. For example, Spinal Muscular Atrophy (SMA) is a collection of diseases caused by loss of the *SMN1* gene. The SMN protein encoded by this gene regulates many of the snRNP binding to the pre-mRNA [111].

Aberrant alternative splicing is a hallmark of cancer [112-117]. Many of the proteins that play an important role in cancer development and progression are alternatively spliced. Changes in alternative splicing influence many aspects of cancer such as cell proliferation, cell invasiveness, angiogenesis, methylation defects and chemotherapy resistance. An example of a proto-oncogene affected by the SF2/ASF splicing factor is *Ron* which plays an important role in epithelial-mesenchymal transition [118]. There are many investigations being done on the relationship between splicing regulation and cancer progression. Genes such as *Cyr61* show a switch in their isoforms which promotes metastasis [119]. Many splicing factors whose expression is controlled by c-myc are up-regulated in cancer [120]. An

illustration of direct involvement of aberrant splicing in cancer would be the pyruvate kinase gene. Tumours generally undergo anaerobic glycolysis and hence have lower requirements for oxygen and different gene expression profiles compared to normal cells which have low rates of glycolysis and obtain their energy from oxidation of pyruvate in mitochondria. The switch to anaerobic glycolysis in cancer cells is aided by the up-regulation of one of the isoforms of pyruvate kinase produced as the result of aberrant splicing [121].

1.3.4.7 Requirement for Alternative Splicing

Alternative splicing plays a diverse and essential role in life. This process is controlled by splicing programs involving around 50-300 regulatory proteins. These splicing programs control the vast majority of gene splicing bringing about drastic physiological effects [122-123]. Alternative splicing can have a very prominent effect on the molecular properties of the protein. It can change its structure and function. It can include or exclude exons that can affect the coiling, stacking, polarity, and crystal structure of a protein. Alternative splicing can also affect the binding of a transcription factor to DNA as well as protein-protein interaction. It can modify the structure of the transactivation domain of a protein which can either enhance or reduce its binding to the promoter region. This can either render the isoform inactive or act as a dominant negative or even modulate its activity [124]. For example, the Kruppel family of transcription factors have both DNA binding and protein-protein interaction domains. However, as the result of alternative splicing,

different isoforms are produced, some of which retain both the DNA binding and protein-protein interaction domains, while others only retain the DNA binding domain [125].

Alternative splicing can also modulate the intracellular localization signal of a protein. It can introduce or remove an exon which codes for nuclear localization or cytosolic localization signals. Alternative splicing can redistribute a protein isoform among the different cellular compartments. The variants can have dramatically different functions in different subcellular compartments [126]. For example, the *lipin* gene encodes products that have a nuclear or a cytosolic localization depending on the alternative splicing of an exon. The nuclear form functions as a transcription factor whereas the cytosolic form has phosphatase activity [127-128]. Alternative splicing can also affect the untranslated region of the RNA. For example, alternative splicing involving the 3' UTR affects transcript localization, stability and translation efficiency [129-130].

Alternative splicing can affect protein association with the membrane, and can produce isoforms of proteins that are either soluble or membrane-bound by inclusion or exclusion of exons encoding the transmembrane region. An example is Tumour Necrosis Factor Receptor (TNFR) which has both soluble and membrane bound isoforms [131]. Alternative splicing can alter the enzymatic activity of a protein. Kinase activity can be switched on or off by inclusion or exclusion of exons in the active centre. Alternative splicing

can also affect the specificity and efficiency of enzyme binding to its substrate [132].

In addition to all these functions, alternative splicing plays a role in evolution by creating diversity among species. Exons that play important roles are conserved in evolution and the non-functional exons are lost and eliminated over time. Thus, alternative splicing can be considered as one of the driving forces of evolution. Alternative splicing is also a part of the diversity among different species. Comparison of human and mouse have shown that about 11% of the exons are alternatively used in one species but not in the other [133-134].

1.4 ALTERNATIVE SPLICING IN NEURONAL DEVELOPMENT

The nervous system is a complex and dynamic system that comprises hundreds of different cell types that form a network of connections with each other, all of which need to be precisely regulated. Approximately 76% of the genes expressed during brain development show high levels of alternative splicing [135]. Thus, alternative splicing plays a key role in generating and maintaining the high level of molecular and cellular diversity that is characteristic of the nervous system. The different types of neuronal cells form synapses with each other that are extremely dynamic in nature. They disunite, reconstruct, rejoin and reform, a process known as synaptic plasticity. Synaptic plasticity governs our ability to sense, memorise, learn, interpret and respond to stimuli. Hence, it is extremely crucial to maintain and

control this dynamic phenomenon with precision. Synaptic plasticity has been shown to be heavily modulated by alternative splicing of several genes as indicated below. Apart from synaptic plasticity, alternative splicing is involved in many other crucial processes such as neurogenesis, migration and axon guidance. There are many examples of neurological disorders that are caused by disruption of splicing in the brain [136-138].

The first neuronally-spliced variant described in the literature is *calcitonin* mRNA which was previously known to play a role in the thyroid. *Calcitonin* is alternatively spliced in neurons resulting in the production of neurotransmitter CGRP which is very different from the protein produced in thyroid [139-140]. Alternative splicing has since been found to regulate several major neuronal activities, and is prominent in the genes encoding neurotransmitters, receptors and channels. For example, N-methyl-D-aspartate (NMDA) R1 is a subunit of the NMDA receptors, which are glutamate channel receptors that regulate neuronal synaptic plasticity. The localization of NMDA R1 at the synapse is very important for determining its function. There are seven or more isoforms of NMDA R1 with each playing a distinct role in fine-tuning its position at the synapse [141]. Similarly, N type calcium channels are voltage gated channels at the synapse that play an important role in regulating neurotransmitter release in the nervous system. Alternative splicing can produce different isoforms of the channels with fine modulation in their structure resulting in different activation kinetics [142].

Alternative splicing also plays an important role in synaptic connectivity and nervous system development. For example, neurexin is a neuronal cell surface receptor that plays an important role in synapse formation and reformation. There about 100 different isoforms of neurexin, many of which perform distinct functions in synapse formation and neuronal cell recognition [143-144]. Alternative splicing can also modulate axon guidance. Dscam is an axon guidance receptor in *Drosophila* with approximately 38000 different splice variants [145].

1.4.1 Neuronal Splicing Factors

There are several neuronal splicing factors that play crucial roles in orchestrating neuronal development and function. These factors may be tissue-specific or general splicing factors that play a specific role in neurons. The general splicing factors may be ubiquitously expressed but their expression in the neurons may be controlled by post-translational modification such as phosphorylation [146-147].

nSR100 (neural specific SR-related protein of 100 kDa) is one of the most important neuronal splicing factors identified to date. nSR100 is known to regulate neuronal differentiation and neurite extension [148]. It generally promotes exon inclusion in the target gene. Introns flanking exons regulated by nSR100 are filled with pyrimidine rich motifs [148]. These polypyrimidine tracts are in turn recognised by the PTB1 and PTB2 splicing factors. It has

often been observed that PTB splicing factors and nSR100 work in concert with each other.

PTB (polypyrimidine tract binding proteins) are general splicing factors that are differentially expressed in neurons. They are mainly involved in alternative splicing of genes that regulate synaptic maturation, dendrite spine formation and neuronal differentiation [149]. PTB1 and 2 have non-overlapping expression patterns in the developing brain. PTB splicing factors are mainly involved in exon skipping [150]. PTB is involved in the splicing of neuronal receptors such as GABA and calcitonin-CGRP [151].

CUGBP (CUG binding proteins) are ubiquitously expressed RNA-binding proteins which play an important role in alternative splicing of genes involved in neurogenesis and neurotransmission. CUGBP is involved in both exon inclusion and exclusion depending on the position of the binding region in the intron.

Rbfox1 and Rbfox2 are tissue-specific splicing factors that show very high expression in the neuromuscular system and regulate alternative splicing by binding to a conserved UGCAUG element [152]. These splicing factors can also act as activators or repressors of alternative splicing depending on the position of the binding domain in the pre-mRNA [153]. Rbfox proteins play a very important role in mature circuitry and neuronal development [154].

1.5 ALTERNATIVE SPLICING OF DAB1

The *Dab1* gene spans >1 Mb; however, the open reading frame of the main Dab1 isoform is only 1668 bp [155]. There have been reports of many alternatively spliced forms of *Dab1* which have been documented in mouse, human, zebrafish, lizard and chick [48, 56, 155-163]. There are several alternative splicing regulatory mechanisms in play in *Dab1* including alternative promoter utilization, alternative cassette exon, and mutually exclusive alternative exons [155, 160, 162, 164].

All the major Dab1 isoforms have the PI/PTB domain; however there are variations in the central region (that contains all the tyrosine phosphorylation sites) and the C-terminal domain [164]. Alternative splicing in *Dab1* primarily involves exons 6 to 10 which encode the central tyrosine phosphorylation domain. Inclusion or exclusion of exons within this region results in the presence of different tyrosine phosphorylation sites in the protein [54]. As a result, many of the isoforms are differentially phosphorylated and hence may have different downstream targets and effects.

1.5.1 Dab1 Alternative Splicing in Chicken

The different isoforms of Dab1 are differentially expressed at different stages of brain development. In chicken, there are two well-characterized isoforms of Dab1 that are expressed in the developing brain and retina [160]. Dab1-Early (*Dab1-E*) is missing exons 7 and 8 and is expressed at early

stages of development, mostly in undifferentiated and proliferating cells in the retina and brain. The exclusion of exons 7 and 8 leads to loss of 105 bp, which results in loss of a 35 aa region that contains the Y198 and Y220 phosphorylation sites. However, the Y185 is converted from a SFK recognition site (YQXI) into an Abl recognition site (YQVP) resulting in a Dab1 protein that has no SFK phosphorylation sites but retains two Abl phosphorylation sites and hence shows reduced tyrosine phosphorylation [55, 160]. The other major isoform of Dab1 in chicken is Dab1-Late (*Dab1-L*). *Dab1-L*, expressed at later stages of development than *Dab1-E*, includes both exons 7 and 8 but excludes exon 9b (57 bp). Dab1-L retains all four tyrosine phosphorylation sites and therefore shows high levels phosphorylation (Figure 1.6). There are no known motifs in the amino acids encoded by exon 9b [160].

Studies have shown that both the Dab1-E and Dab1-L isoforms have distinct functions during development. Transfection of retinal cells with a GFP-tagged Dab1-L expression construct leads to strong induction of tyrosine phosphorylation and Src phosphorylation, concomitant with widespread formation of thin and elongated processes, a characteristic of neuronal differentiation [160]. However, transfection of retinal cells with a GFP-tagged Dab1-E expression constructs has no effect on tyrosine phosphorylation, Src phosphorylation or the morphology of retinal cells. The expression of GAP-43, a neuronal protein associated with axonal outgrowth during development, regeneration and sprouting, is up-regulated by approximately two-fold in Dab1-L transfected cells when compared to Dab1-E transfected cells [160].

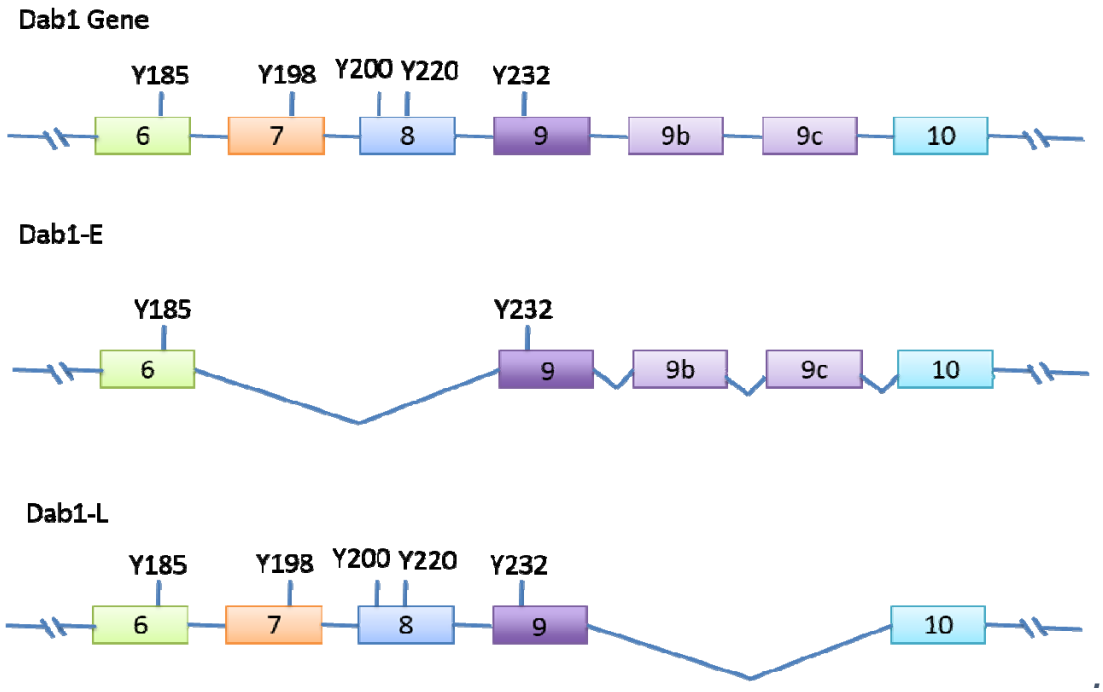

Figure 1.6 Dab1-E and Dab1-L isoforms.

1.5.2 Dab1 Alternative Splicing in Mouse

The mouse *Dab1* gene also undergoes alternative splicing resulting in the production of at least 11 different isoforms in the developing brain. All the isoforms have a common PI/PTB domain and C-terminal region, but have different combinations of exons in their central tyrosine phosphorylation domain. The four Dab1 tyrosine phosphorylation sites are located in four adjacent exons: exon 6 (Y185), exon 7 (Y198), exon 8 (Y220) and exon 9 (Y232). There is either inclusion or exclusion of exons 7 and/or 8, inclusion or exclusion of exons 9b and 9c, or alternate 5' splice site usage in exon 9, or different combinations of these events, in mouse *Dab1* variants [56]. These splicing events lead to either removal or rearrangement of the tyrosine phosphorylation sites. For example, removal of exon 7 removes the Y198 phosphorylation site but results in the joining of exon 6 (containing Y185Q) to exon 8 (containing Y200I) leading to the formation of $Y^{185}QY^{200}I$ motif which is identical to $Y^{198}QY^{200}I$. However, removal of exon 8 results in the removal of $Y^{200}I$ and joins $Y^{198}Q$ to $V^{222}P^{223}$ leading to formation of an $Y^{198}QVP$ tyrosine phosphorylation site [56].

The expression pattern of Dab1 in the mouse is much more complex than that in the chicken as there are many more Dab1 isoforms in mouse. However, the general trend that is observed in both mouse and chicken is exclusion of exons 9b and 9c when exons 7 and 8 are included and vice versa. Exons 7 and 8 are predominantly excluded in the early stages of

mouse brain and retina development but included in the later stages, whereas exons 9b and 9c are included in the early stages but excluded in the later stages of mouse brain and retina development [56, 160].

Previous work in the lab has shown that the different isoforms of mouse Dab1 are differentially tyrosine phosphorylated and recruit distinct sets of SH2 domain proteins [56, 165]. Variants 1 and 5, which retain all four tyrosine phosphorylation sites, show highest levels of tyrosine phosphorylation and recruitment of Src, PI3K and Socs2 SH2 domains [56]. Variants 2 and 3, which lack exon 7 and exon 8, respectively, with each containing a single YQXI tyrosine phosphorylation site, are unable to recruit Src, PI3K or SOCS. However, binding of Dab1 variants 2 and 3 to Crk/CrkL and Nckβ, SH2 domains is not affected. As variants 2 and 3 each retain two YXVP tyrosine phosphorylation sites, these results are in line with the literature indicating that Crk/CrkL and Nckβ both bind to YXVP [54, 166]. Variants 4 and 6 lack both exons 7 and 8 and do not retain any YQXI sites. These variants didn't interact with any of the tested SH2 domains [56].

These combined results lead to formulation of a model whereby the different Dab1 isoforms co-ordinately regulate and modulate the Reelin signaling pathway at different stages of development. During early stages of development, alternative splicing excludes exons 7 and/or 8. These early Dab1 isoforms are mainly expressed in proliferating precursor cells or early born neurons [56, 165]. The absence of SFK tyrosine phophorylation sites and absence of overall tyrosine phosphorylation in early Dab1 isoforms

suggests that these isoforms do not induce the Reelin signaling cascade which underlies neuronal polarization and early neuronal migration. As the brain matures, splicing results in inclusion of exons 7 and 8 in the late born neurons. These Dab1 isoforms are capable of fully activating Reelin downstream effectors thereby contributing to neuronal migration and the inside-out lamination in the cerebral cortex. *Dab1* splicing therefore appears to play a key role in the regulation and fine tuning of the Reelin signaling pathway. Nova-2 is a neuronal splicing factor that is involved in the splicing of *Dab1* and a key focus of my project.

1.6 NOVA SPLICING FACTORS

Nova (Neuro-oncological ventral antigen) is a family of mammalian splicing factors that regulate alternative splicing in the CNS. These splicing factors were first discovered as autoantigens in paraneoplastic opsoclonus myoclonus ataxia (POMA), a disorder associated with breast cancer and motor dysfunction [167]. The Nova family comprises two members: Nova-1 and Nova-2. Both these proteins are restricted to the CNS and, together, are involved in approximately 700 alternative splicing events most of which affect genes involved in axon guidance and synaptic plasticity [168]. Nova binds to a cluster of YCAY (pyrimidine-C-A-pyrimidine) motifs on the pre-mRNA transcript [169-170]. Nova can either enhance or inhibit the inclusion or exclusion of an exon depending on the position of its binding site in the intron. Splicing of many important genes is controlled by Nova splicing factors,

including GlyRα2 (Glycine Receptor Alpha2) and GABA (Gamma-AminoButyric Acid), these are inhibitory receptors which are the best studied examples of substrates of Nova [169].

Nova-1 and Nova-2 share a high degree of similarity, with 75% similar amino acids [167, 171]. Both Nova-1 and Nova-2 have three highly-conserved KH (hnRNPK Homology) domains with short connecting sequences (50 amino acids for Nova-1 and 26 amino acids for Nova-2) between the first and second KH domains, and long connecting sequences (179 amino acids for Nova-1 and 204 amino acids for Nova-2) between the second and third KH domains. KH domains are protein domains that recognise and bind to RNA, they can also bind to single stranded DNA. KH domains are approximately 70 amino acids long with characteristic patterns of hydrophobic residues [172-173]. A family of about 50 proteins containing KH domains are involved in various functions related to RNA such as translation, alternative splicing, RNA processing and mRNA localization. [167].

Nova-1 and Nova-2 have very similar RNA binding motifs, both binding to sequences rich in YCAY; however, their expression profiles differ. Nova-1 is mainly expressed in the subcortical CNS structure during brain development whereas Nova-2 is expressed throughout the CNS, particularly in cortex, olfactory bulb, thalamus, inferior colliculus, inferior olive, and the internal and external granule cell layers of the cerebellum [174]. *Nova-1* and *Nova-2* double knockout mice die 2 to 3 weeks after birth [139]. *Nova 1-/-* mice show weakness and tremulousness due to motor neuron atrophy [139].

Darnell *et al*. performed protein-RNA crosslinking and immunoprecipitation (CLIP) experiments using Nova-1 or Nova-2, which resulted in the identification of 500 mRNAs that are bound by both the Nova proteins such as JNK2, neogenin, and gephyrin pre-mRNAs [175].

It is noteworthy that Nova-1 has two isoforms, one including exon 4 (isoform 1) and the other excluding exon 4 (isoform 2) [176]. The exon 4 encodes threonine phosphorylation sites which make isoform 1 more highly phosphorylated than isoform 2. However, no differences in splicing efficiency have been reported for the two isoforms to date. Studies have shown that *Nova-1* exon 4 has 5 repeats of YCAY and that Nova-1 binding to exon 4 can lead to exclusion of exon 4 [176], suggesting a regulatory loop between isoform 1 and isoform 2.

As mentioned earlier, Nova splicing factors can act as both silencers and enhancers of splicing depending on the position of the YCAY clusters in the pre-mRNA. Genome wide studies have identified five major locations of YCAY clusters in the pre-mRNA [170]. Two of these locations span splicing enhancers called NISE2 and NISE3 (Nova intronic splicing enhancers). These YCAY clusters are associated with increased Nova-dependent exon inclusion. The remaining two locations of YCAY clusters span splicing silencers known as NESS1 and NESS2 (Nova exonic splicing silencers) which increase Nova-dependent exon skipping (Figure 1.7) [170].

Nova promotes silencing of splicing and exon skipping by inhibiting the formation of complex E. Complex E is formed when the U1 snRNP and

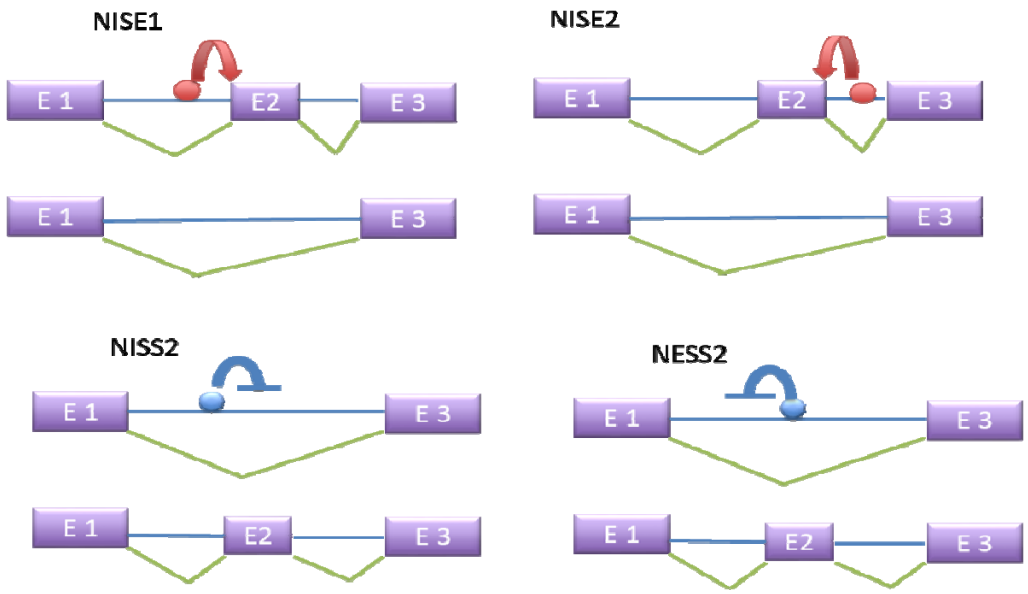

Figure 1.7 Nova dependant splicing enhancer and silencer. The red circles are splicing enhancers and the blue circles are splicing silencers. NISE stands for Nova Intronic Splicing Enhancer, NISS stands for Nova Intronic Splicing Silencer and NESS stands for Nova Exonic Splicing Silencer.

accessory factors bind to the pre-mRNA and commit it to splicing. Nova-1 inhibits the access of U1 snRNP to the 5' splice site. This happens when there is an intact cluster of YCAY at the 5' splice site. Over-expression of Nova has been shown to reduce the formation of U1-RNA complex by 2.5-fold [170]. Nova can also interact with the pre-mRNA before snRNP binding, thus modifying the pre-spliceosomal complex [155, 170]. Studies have shown that Nova may enhance splicing by binding to the 3' splice site and promoting the spliceosomal assembly [170]. As well, Nova may bind to the substrate and affect RNA folding [170].

1.6.1 Nova and Neuronal Development

There have been many genome wide studies and gene expression microarray analyses that suggest that Nova is involved in almost 7% of alternative splicing events that take place in the brain [177]. In addition to alternative splicing that affects the brain in general, Nova is also associated with differential splicing in different regions of the brain. For example, genes like *NMDA NR1* show Nova-dependent alternative splicing in the cortex and spinal cord [139, 178].

Nova is at the top of the hierarchical network of regulating proteins in the brain and is involved in the splicing of transcripts that encode proteins that regulate both structure and function of the synapse. In particular, Nova is involved in synapse biogenesis, synaptic transmission, cell-cell signaling, and regulation of synaptic plasticity, cell shape, axonogenesis and neurite

morphogenesis. Nova is also involved in the regulation of neurotransmitter receptor and neurotransmitter release, as well as the regulation of long term potentiation of inhibitory postsynaptic currents [139].

Nova proteins have been reported to shuttle between the nucleus and cytoplasm [179]. This indicates that Nova has other functions apart from splicing. Nova was found to be involved in the localization of *GIRK2* (G protein-activated inward rectifier potassium channel 2) mRNA by binding to its 3' UTR region [179]. Nova can also bind to the 3' UTR region of *GlyR α2* mRNA in the brain. In the ventral horn motor neurons, Nova is transported along with *GlyR α2* pre-mRNA up to the dendrite where Nova regulates *GlyR* splicing [180].

Another study has shown that Nova co-localizes with gephyrin in the dendrite [179]. Gephyrin is a postsynaptic marker for inhibitory synapses [181], suggesting that Nova may also be involved in the inhibitory synapses. These combined data indicate that Nova plays a dynamic and crucial role in normal brain development.

1.6.2 Regulation of Dab1 Alternative Splicing by Nova-2

As indicated previously, alternative splicing of *Dab1* may play a crucial role in fine tuning the effects of Reelin signaling during different stages of mouse brain development. However, little is known about what regulates alternative splicing of *Dab1*. Yano *et al*. reported that Nova-2, a neuronal splicing factor, is responsible for exclusion of exons 9b and 9c in *Dab1* at later

stages of development in brain [171]. Knockout of Nova-2 in mouse brain results in severe migration defects in the cortical and Purkinje cells and defects in laminar structure formation. However, no change in the progenitor cell population was observed in these mice, with the defects primarily associated with post-mitotic cells.

Nova-2 is expressed in the post-mitotic migratory cells of the cortical region from embryonic day E14.5 to postnatal day P0 in wild-type mice. *Dab1* transcripts containing exons 9b and 9c are found at high levels in *Nova-2* knockout mouse brain. In contrast, exons 9b and 9c are excluded in wild-type mice brain at this developmental stage. *In utero* electroporation of wild-type *Dab1* (excluding exons 9b and 9c) in *Nova-2* knockout mice results in the recovery of the brain defects associated with *Nova-2* knockout mice [171]. Interestingly, by P0, both exons 9b and 9c are excluded in *Nova-2* knock-out mice, indicating that that there are other factors involved in the exclusion of exons 9b and 9c in mouse brain.

1.7 REGULATION OF PHOSPHORYLATION BY SHP-2

As discussed earlier, protein tyrosine phosphorylation and dephosphorylation play an important role in regulating protein-protein interaction and cell signaling. Shp-2 is an important example of a cytosolic SH2-domain containing tyrosine phosphatase involved in numerous signal transduction processes [182-183].

Shp-2 is ubiquitously expressed in all cell types and tissues. It has two tandem SH2 domains that bind to pTyr thereby activating Shp-2 phosphatase activity. Shp-2 also has a proline-rich domain that binds to SH3 domains of other proteins such as SFK [184]. Shp-2 is involved in a wide range of pathways and signaling transduction processes and interacts with a plethora of proteins such as Ras, Raf, MAP kinase, Jak-Stat and PI3K. Shp-2 can function at multiple sites of signal transduction and help in signal relay. It can interact with initiator growth factors such as PDGF and EGF, as well as with proteins that play intermediate roles in signal relay such as Grb-2, Jak2, and p85 subunit of PI3K. In most cases, the ultimate effect of Shp-2 interaction is enhancing signal transduction [185].

1.7.1 Tandem SH2 Domains

Shp-2 has two tandem SH2 domains at the N-terminus and one catalytic phosphatase (PTB domain) domain at the C-terminus [185]. The SH2 domains in the N-terminus are structurally packed against the PTB domain, blocking its access to the substrate and rendering it inactive and unable to bind to pTyr. However, in the presence of a dually phosphorylated substrate, the SH2 domain in the C-terminus binds to the pTyr thereby stimulating phosphatase activity by about 10 fold [186]. The next step that occurs when substrate is present is the dissociation of the N-terminal SH2 domains from the PTB domain. This removes the steric blockage of the catalytic site increasing phosphatase activity by 100 fold [187]. Thus, the N-

terminal SH2 domains act as an allosteric switch for the catalytic domain. Structural analysis of the two tandem SH2 domains has shown that they are set wide apart (50 A) and are perpendicular to each other. This renders the two SH2 domains more flexible in the active state [188].

1.7.2 Binding Specificity of Shp-2

Previous studies have established that the pTyr and the 3 residues C-terminal to it are important for SH2 domain recognition and binding. A vital interaction which is common among all SH2 domains is the insertion of the pTyr side chain into a deep pocket in the SH2 domain where the pTyr of the target forms a bidentate interaction with an arginine residue in the SH2 domain. The residues adjacent to the pTyr, specifically the three residues immediately C-terminal to pTyr, interact with the adjacent residues in the target protein and enable high affinity interaction between them. Thus, this interaction with the three residues located adjacent to pTyr also contributes to the selectivity of the SH2 domain [189]. However, for some SH2 domain proteins including Shp-2, residues both N- and C-terminal to pTyr contribute to the binding specificity of the protein. For example, aliphatic amino acids with hydrophobic R groups (e.g., leucine, isoleucine, valine, and threonine) located at the -2 position of pTyr enhance binding to Shp-2 [189]. There have been a number of studies that show that the residues beyond +3 relative to pTyr are also important in determining the binding specificity of Shp-2. By screening peptide libraries, it has been shown that the Shp-2 SH2 domain

prefers an aromatic residue in the +5 position [189]. In addition to the SH2 domain, the phosphatase domain also plays an important role in determining the specificity of the substrate. There have been suggestions of a combinatorial model whereby the SH2 domain guides Shp-2 to the correct cellular location and the phosphatase domain selects the appropriate substrates [184, 190].

It has previously been reported that tyrosine phosphorylation of Shp-2 leads to increased activity. Phosphorylation at Y542 and Y580 stimulate the N-SH2 and C-SH2 domains, respectively [190]. Serine and threonine phosphorylation of Shp-2 has also been reported, although the exact sites have not been identified [191]. Moreover, Shp-2 is also regulated by reactive oxygen species (ROS) and there is evidence that hydrogen peroxide and other ROS generated by growth factors and cytokine stimulation can act as secondary messengers [192].

1.7.3 Biological Functions of Shp-2

Shp-2 is a ubiquitously expressed protein which plays a range of important roles. The importance of Shp-2 in mammalian development is demonstrated by the fact that *Shp2*$^{-/-}$ mice are embryonic lethal [193]. Mutation and deletion analysis of Shp-2 have shown that Shp-2 is involved in a host of functions such as limb development, gastrulation, mesodermal differentiation and hematopoietic differentiation [193].

There is considerable evidence pointing to a role for Shp-2 in neuronal fate specification. In the brain, deletion of Shp-2 leads to impairment of cortical differentiation manifested by a reduction in the number of neurons and oligodendrocytes and an increase in astrocytes. There is also a reduction in the number of proliferative cells and increased apoptosis of neural progenitor cells [194]. The latter cells also show a reduced activation of Erk, but an enhanced activation of STAT3, which may result in abnormal neurogenesis and gliogenesis.

Shp-2 binds to tyrosine phosphorylated proteins through its SH2 domains and dephosphorylates them. However, there have been a few reports where Shp-2 gets tyrosine phosphorylated and binds to other SH2 domains and acts as an adaptor protein to promote the association of other proteins. For example, Shp-2 promotes the binding of c-kit and Grb2 protein leading to the activation of the Ras signaling pathway in hematopoietic cells [195]. Gbr2 can bind to EGF directly but not to PDGF. In this case, Shp-2 serves as an adaptor protein linking Grb2 and PDGF [196-197]. Intriguingly, there have been reports indicating that Shp-2 can also promote phosphorylation of a protein. For example, Shp-2 has been reported to be involved in hormone angiotensin II (ANG II) induction of Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. Shp-2 facilitates JAK2 association with ANG II receptor AT1 by acting as an adaptor between these two proteins. This association promotes JAK2 phosphorylation thereby activating the JAK/STAT cascade leading to cell proliferation [198].

1.7.4 Shp-2 and Cancer

Many crucial signaling pathways are regulated by tyrosine phosphorylation which is controlled by the balanced action of PTKs such as Src, Abl and PTPs such as PTEN and Shp-2. As discussed earlier, Shp-2 has both inhibitory and stimulatory effects on many signaling pathways that are closely associated with cancer and deregulation of Shp-2 expression and function has been associated with various types of human cancer [199]. Somatic mutation of the *Shp-2* gene has been reported in ~33% of juvenile myelomonocytic leukemias [200], 4-5% of acute myeloid leukemias [201] and 6% of acute lymphoblastic lymphoma [202]. Low frequency mutations in the *Shp-2* gene have also been detected in solid tumours such as lung and colon cancer. Many of the oncogenic mutations in *Shp-2* are concentrated in the PTP domain or the N-terminal SH2 domain. This can stop the N-SH2 from binding to the catalytic domain and hence increase the activity of Shp-2 [200].

The principle transforming effect of mutated Shp-2 is the activation of the Ras-ERK pathway. A large proportion of Shp-2 substrates are proteins that negatively regulate the Ras-ERK pathway. These include receptor tyrosine kinases or tyrosine phosphorylated adaptor proteins that recruit Ras-GTPase, a negative regulator of Ras. Oncogenic mutations in Shp-2 leads to an increase in the half-life of Ras-GTP by interfering with the activity of Ras-GAP (Ras-GTPase activating protein) that catalyses the inactivation of Ras-GTP and attenuates the Ras signaling pathway. Hence, *Shp-2* mutation

results in increased activity of Ras which leads to cell transformation and tumour formation [203-204]. Shp-2 can also activate pathways involving AKT and STAT5. Mast cells derived from transgenic mice injected with mutated *Shp-2* show hypersensitivity to interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation resulting in increased proliferation, and activation of ERK, AKT and STAT5 [205].

1.7.5 Dab1 and Shp-2

As mentioned earlier, Dab1 is an adaptor protein that undergoes tyrosine phosphorylation and recruits multiple SH2 domain proteins that transduce the Reelin signal. *Dab1* undergoes developmentally regulated alternative splicing which affects its phosphorylation and activity. However, there may be other mechanisms that control the phosphorylation and activity of Dab1. It has been shown previously that Shp-2 interacts with Dab1 based on phosphopeptide affinity purification; however, Dab1 did not co-immunoprecipitate with Shp-2 and thus the association between Dab1 and Shp-2 could not be confirmed [61]. Two of the tyrosine phosphorylation sites in Dab1, Y185 (A-V-pY185-Q-T-I)] and Y198 (P-V-pY198-Q-Y-I), resemble the consensus Shp-2 binding motif, [I/V/L]-x-(p) Y-x-x-[I/V/L] [206].

As discussed earlier, Dab1 phosphorylation plays a key role in neuronal migration during brain development through the Reelin signaling pathway [207-208]. Tyrosine-phosphorylated Dab1 helps to recruit SH2 binding domain proteins that further transmit and regulate downstream

signals. Alternative splicing of *Dab1* modulates its tyrosine phosphorylation thereby fine-tuning the Reelin signaling pathway and modulating the complex mechanism underlying neuronal migration [56]. However, the mechanism regulating *Dab1* alternative splicing remains poorly understood.

1.8 RESEARCH OBJECTIVES

Nova-2 is involved in the splicing of *Dab1* exons 9b and 9c in embryonic brain. However, at later stages of development, splicing of exons 9b and 9c occurs independent of Nova-2. Here, we examine other splicing factors that may be involved in *Dab1* splicing. The Nova family of proteins has a second member called Nova-1. Nova-1 has two isoforms, one which includes exon 4 (isoform 1) and the other which excludes exon 4 (isoform 2) [176]. Previous data from our lab showed that the expression of Nova-1 isoform 2 correlates with Dab1 splicing [176]. Here, I study the impact of Nova-1 on the splicing of *Dab1* using overexpression and knockdown assays.

In addition, I have examined whether Dab1 phosphorylation is affected by Shp-2. My work demonstrates that Shp-2 binds to Dab1 and that it can regulate the phosphorylation of Dab1. I have used overexpression of Src (the kinase that phosphorylates Dab1) and Shp-2 to study the interplay between these two SH2 domain enzymes by examining their effect on Dab1 tyrosine phosphorylation.

Chapter 2:

MATERIALS AND METHODS

2.1 P19 Cell Culture

P19 cells are mouse teratocarcinoma cell line [209]. P19 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 7.5% bovine serum and 2.5% fetal calf serum. P19 cells can be differentiated into cells of neuronal lineage in the presence of retinoic acid [210]. For neuronal differentiation, P19 cells were cultured in petri dishes (to allow formation of embryoid bodies) at a density of 10^5 cells/ml in the presence of 1 μM all-trans-retinoic acid (RA) (Sigma R2625) on Day 0 [211]. On Day 2, medium containing cells that had started forming embryoid bodies were collected in a tube and embryoid bodies allowed to settle to the bottom of the tube. The medium was removed and replaced with fresh DMEM supplemented with serum and 1 μM RA. On Day 4, the embryoid bodies were trypsinizsed and broken down into single cells. These single cells were cultured in DMEM plus 10% fetal calf serum on tissue culture plates coated with 100 ng/ml poly-D-lysine. Poly-D-lysine was used to allow cells to adhere to the plates. On Day 6, the cells were treated with 5 μg/ml Ara-C (Sigma C1768) in order to kill the proliferating cells. The cells completed neuronal differentiation by Day 8. Cells were harvested at different stages (Day 0, Day 2, Day 4, Day 6 and Day 8) and used for either RNA or protein analysis.

2.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Primer Preparation

Total RNA was prepared using the TRIzol reagent. Briefly, cell pellets obtained from a 10 cm tissue culture plate were resuspended in 1 ml of TRIzol reagent. Two hundred µl chloroform were added to the solution and the mixture was centrifuged at 12000 g for 15 minutes at 4ºC. During this extraction step, the chloroform goes to the upper aqueous layer whereas the phenol goes to the bottom (organic) layer. The RNA is located in the upper aqueous layer and is separated from the proteins which are located in the organic layer. The upper aqueous layer was then transferred to a fresh tube and 500 µl of isopropanol was added to the solution followed by mixing and centrifugation to precipitate the RNA. The pellet was then washed with 1 ml of 75% ethanol, dried and dissolved in water. The cDNA was prepared using Superscript II reverse transcriptase (Invitrogen), five µg of template RNA and oligo d(T) as the primer using the conditions specified by Invitrogen (10 mM dNTPS, 0.1 M DTT, 20 units of RNase inhibitor, and 5X first strand buffer that contains 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$).

Analysis of Dab1 alternative splicing was carried out using: (i) primers flanking exons 7 and 8 of Dab1: P1, 5'-CGGCTGAACCTGTTATCCTG-3'; P2, 5'-CTTCCTTC TTTTGGCTGGTG-3' and (ii) primers flanking exons 9b and 9c of Dab1: P3, 5'-TGTGCCAAAAAGTCAACCTG-3'; P4, 5'-CAGCAGTGCCGAAAGACATA-3' [160]. The PCR conditions were 35 cycles of: 94ºC - 30 seconds, 58ºC - 30 seconds, 72ºC - 30 seconds. The primers used for Nova1 RT-PCR analysis spanned exons 3 and 5, and consisted of forward primer, 5'-CCACCGTTAATCCTGATCG-3' and reverse primer, 5'-

CGGTTTTGTTCAGGTTCTCC 3'. The primers used for Nova2 RT-PCR analysis spanned exons 1 to 3, and consisted of forward primer, 5'-CTTCCTGAAGGTGCTCATCC-3' and reverse primer, 5'-GCTCCTCCCTTACCGATGAT-3. The conditions for PCR amplification using these primers were 33 cycles of: 94ºC - 30 seconds, 58ºC - 30 seconds, 72ºC - 30 seconds. The other splicing factors were examined using the following primers: nPTB, forward primer, 5'-CTGGCAACAGAGGAAGCAG-3', reverse primer, 5'-AATGGCTGGGTCTAACGCA-3'; CUGBP1, forward primer, 5'-ACACCCGTAAAGCTGCATTA-3', reverse primer, 5-CACTTG GTGTGTTCTGAGCT-3'; CUGBP5, forward primer, 5'-CTCAGACAGCCTTGCACGA-3, reverse primer, 5'-CACTGTTGTCTGTTGCTGC -3'; Fox1, forward primer, 5'- CGCCACACA GACAGATGATG-3', reverse, 5'- TTGATACACTACTCCGCCATA-3'; Fox2, forward, 5'- CC AGACCAGTGAACATAACC-3', reverse 5'-TGCCGTAACCGTCGCTGTAA-3'; Fox3 forward primer, 5'-AGACTCATCCTGAGCAGCCA-3', reverse primer, 5'- GATCAGCAGCG GCATAGACT-3'; Tra2β, forward primer, 5'- ATCAAAGTCCAGGTCCAGGT-3', reverse primer- 5'- TCGTACCCTCTGTCATAATAG-3'; NSSR, forward primer, 5'- ACGACACCAG GTCTGAAGAT-3', reverse primer, 5'-ACTGTTTCTAGGACTGTAAGA-3'. The ELAV primers used were: ELAV1, forward primer, 5'- ATGTCTAATGGTTATGAAGA -3', reverse primer, 5'-AATCTCTGCGCCTGGTGGT -3'; ELAV2, forward primer, 5'-ACACAACTGTCTAATGGGCC -3', reverse primer 5'-

CCATTTAGGCCTTTGATAGCT -3'; ELAV3, forward primer 5'-GAGATGGAGCAGCTCTTCT -3', reverse primer 3'-GTCATGGTGACGAAGCCGA -4'; ELAV4, forward primer, 5'-CCATGGAGCCTCAGGTGTC-3', reverse primer, 5'-TTGTTGGCAAACTTCACAGTAA -3'.

2.3 Generation of Nova1 Isoform Expression Constructs

Total RNA was extracted from P19 cells on Day 8. cDNA was prepared by reverse transcription using five µg of total RNA as described above. RT-PCR of Nova1 was carried out using Nova1 forward primer containing a *Cla* 1 cut site (underlined) (5'-AATATCGATAATGATGGCGGCAGCTCCC-3') and Nova1 reverse primer containing a *Xba*1 cut site (underlined): 5'-GAGTCTAGATCAACCCACTTTCTGAGGATT-3'. Reactions were electrophoresed in 1% agarose in Tris-acetate-EDTA buffer. Two cDNA fragments of 1525 bp and 1454 bp were resolved, cut out of the gel, and eluted using a gel purification kit (Fermentas). The two purified cDNA fragments were inserted into p3X-FLAG-CMV vector at the *Cla*1 and *Xba*1 sites. The two fragments were confirmed to be the two isoforms of Nova1 by DNA sequencing.

2.4 Cell Culture and DNA Transfection

Neuro2A, a mouse neuroblastoma cell line, and HEK 293T, a human embryonic kidney cell line, were cultured in DMEM supplemented with 10%

fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Primary retinal cultures were prepared from embryonic day (ED) 5 or ED10 chick retinal tissue dissociated with trypsin [212]. Transfection of cultured cell lines was performed 24 hours after plating the cells, whereas transfection of primary cultures was carried out 48 to 96 hours after plating depending on cell density. The DNA (5 – 10 µg/100 mm plate) was introduced into cell lines using PEI (Polyethylenimine) at a ratio of 1:4 (DNA:PEI). The medium was replaced 16-18 hours after transfection. Cells were harvested and lysed in RIPA buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM PMSF, 1 mM Na3VO4 and 1X Complete protease inhibitor cocktail (Roche)] and stored at -80ºC for immunoprecipitation or western blot analysis. For transfection of primary retinal cultures, we used the calcium phosphate precipitation method as previous described [160].

2.5 Western Blot Analysis and Co-Immunoprecipitations

For western blotting, cell lysates obtained by harvesting cells with RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% deoxycholate, 1% TritonX-100, 1 mM Na3VO4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1X Roche Complete protease inhibitor) were separated in a SDS-PAGE gel. The proteins were transferred to nitrocellulose membrane by electroelution in transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 10% methanol). Membranes were immunostained with the following antibodies: rabbit anti-

Dab1 (100-4101-225 Rockland: 1:5000); mouse anti-actin (A5441 Sigma, 1:100,000); mouse anti-GFP (G6795 Sigma, 1:2000); mouse anti-phospho-tyrosine antibody pY 4G10 (Millipore, 1:5000); mouse anti-Src p60 (a kind gift from J. Brugge, 1:2500); mouse anti-Shp-2 (610622 BD Transduction Laboratories, 1:5000); rabbit anti-Nova1 (07-637 Upstate Biotechnology, 1:2500), mouse anti-Crk (610036, BD Transduction Laboratories, 1:2500), and rabbit anti-Nckβ (07-100, Upstate, 1: 2000).

For co-immunoprecipitations, cell lysates were prepared using a modified RIPA buffer. As the normal detergent concentration in RIPA buffer would be very harsh for a co-IP, the detergents deoxycholate and Triton X-100 were used at 0.1% instead of 1%. The lysates were precleared with Protein G Sepharose beads (GE Healthcare) for 45 minutes at 4˚C, followed by incubation with primary antibodies for 3 hours at 4˚C. Immunoprecipitates were collected with Protein G Sepharose beads for 90 minutes at 4˚C. The beads were then washed using wash buffer (RIPA buffer without the protease inhibitor) followed by addition of loading dye (0.5 M Tris-HCl pH 6.8, 20% glycerol, 1 M DTT, 10% Bromophenol Blue, 10% SDS). Immunoprecipitates were separated by SDS-PAGE and immunoblotted with the appropriate antibodies.

When the same membrane was to be used for a different antibody, the membrane was stripped. Stripping of a blot was done by adding 300 µl β-mercaptoethanol to 50 ml of stripping buffer (62.5 mM Tris-HCl pH 6.7, 2% SDS). The blot was immersed in this solution at 60ºC for 30 minutes, shaking

every ten minutes. The blot was then washed with Tris-buffered saline and Tween 20 (0.1%) for ten minutes, blocked with 10% milk and immunostained with a different antibody.

2.6 Immunostaining

P19 cells were cultured as previously described with the exception that on Day 4, the cells were plated on cover slips coated with poly-D-lysine in 24 well plates. On Day 8, the medium was removed and the cells were washed with PBS. The cells were then fixed by adding 4% paraformaldehyde to each well for ten minutes. To prepare 4% paraformaldehyde, powder was added to PBS and the mixture heated sufficiently for the paraformaldehyde to go into solution without boiling. Cells were fixed for ten minutes. The paraformaldehyde was removed, and the cells were washed with PBS twice. The cells were then permeabilized by adding 0.2% Triton X-100 for five minutes and then again washed with PBS twice. Sodium tetraborohydrate (0.5 mg/ml) was added to the cells as a blocking agent for ten minutes followed by two washes in PBS. Primary antibody, in this case TUJ1 diluted at 1:4000, was added to the cells and incubated overnight in a humid chamber at 4ºC. The cells were then incubated with mouse secondary antibody conjugated with Alexa 488 (green fluorescent dye) for 90 minutes at room temperature. This was followed by DAPI nuclear staining and finally the cover slips were mounted on slides for viewing using a fluorescence microscope.

2.7 Generation of Ultracompetent Cells

A single DH5α bacterial colony was picked from a plate that had been incubated for 16 - 20 hours at 37ºC. The colony was transferred to 25 ml of LB broth, incubated for 6 - 8 hours at 37ºC with vigorous shaking. Three flasks with 250 ml LB broth were inoculated using this starter culture with the first flask inoculated with 10 ml starter culture, the second flask inoculated with 4 ml starter culture and the third flask inoculated with 2 ml starter culture. The flasks were incubated overnight at 18-22ºC with vigorous shaking. When the OD_{600} of one of the cultures reached 0.55, that culture was incubated in an ice water bath for 10 minutes to stop bacterial growth. The cells were then harvested by centrifugation at 2500g for 10 minutes at 4ºC. The pellet obtained was resuspended in 80 ml of Inoue transformation buffer (0.5 M PIPES pH 6.7, 55 mM $MnCl_2$ 15 mM $CaCl_2$, 250 mM KCl) by swirling. The cells were again centrifuged at 2500g for 10 minutes at 4ºC and resuspended in 20 ml ice-cold Inoue buffer along with 1.5 ml DMSO and incubated on ice for 10 minutes. Aliquots of the suspension were prepared and snap-frozen by immersion in a liquid nitrogen bath and stored at -80ºC. The efficiency of these ultracompetent cells was approximately $1X10^9$ colony forming units per ml.

2.8 Design and Generation of Lentiviral ShRNA

The lentivirus system plentilox 3.7 was used to deliver Nova1 shRNA into P19 cells. The complete virus is produced when the cells are transfected

with the plentilox construct along with other packaging vectors. The packaging vectors are pRSV-REV (rev gene), pRRE (gag/pol gene) and pVSVG (env gene). The DNA sequences corresponding to the shRNA were inserted at the *Hpa*I and *Xho*I restriction enzyme sites located in the multiple cloning region of plentilox 3.7. The shRNA was designed based on the criteria established by Tom Tuschl [213]. Briefly, an RNA sequence of 18-20 bases (CCTACAACCTCAGACCACCGTTAAT) was selected from the target mRNA. A 5'G was added to the sequence to make it compatible with the U6 promoter. A 5'T was added in front of the G as *Hpa*I digestion creates a blunt end upstream of the -1 position of the U6 promoter. The 5'T allows recovery of the -1 position required for U6 promoter activity. A loop sequence TTCAAGAGA [214] was inserted immediately downstream of the 5' T-G-(CCTACAACCTCAGACCACCGTTAAT). A reverse complementary sequence of T-G-CCTACAACCTCAGACCACCGTTAAT was then inserted downstream of the loop, followed by a polyT (six Ts) tail to terminate the sequence. Finally, a C was added at the 3' end. The antisense strand is complementary to the original strand, but contains an AGCT overhang. The C at the 3'end of the sense (top) strand along with the AGCT at the 3' end of the antisense strand constitute an *Xho*I restriction enzyme site that can be used for cloning into the plentivirus 3.7 vector. Thus the final sequence for the Nova1 shRNA lentivirus construct is:

5'–TGCCTACAACCTCAGACCACCGTTAATTTCAAGAGAATTAACGGTGGT CTGAGG TTGTAGGCATTTTTTC -3' (top strand) and

3’-ACGGATGTTGGAGTCTGGTGGCAATTAAAGTTCTCTTAATTGCCACCA

GACTCCAACATCCGTAAAAAAGAGCT – 5’ (bottom strand).

The two strands were annealed, electrophoresed in a 15% polyacrylamide gel, cut out of the gel, purified by phenol/chloroform extraction and ligated into plentilox 3.7 digested with *Hpa*I and *Xho*I. The construct was then transformed into ultracompetent DH5α cells.

2.9 Lentiviral Infection

The plentilox 3.7 containing the sequences coding for the shRNA along with other packaging vectors were transfected into 293T cells using PEI. The medium was replaced 16-18 hours after transfection. After an additional 24 hours, the medium was collected and stored at -80ºC. The supernatant contains the lentiviral particles. The collected medium was added to Day 5 P19 cells. The cells were exposed to the viral particles overnight along with 8 µg/ml polybrene to increase infection efficiency. The P19 cells were harvested on Day 8 for RNA or protein extraction.

Chapter 3:

RESULTS

3.1 P19 Neuronal Differentiation

The P19 cells were cultured as depicted in Figure 3.1 in order to induce neuronal differentiation. Cells at Day 0, Day 2, Day 4 were in the form of embryoid bodies. These cells represent neuronal progenitor cells. By Day 8, long processes characteristic of mature neuronal cells were observed. P19 cells at Day 8 were immunostained with an antibody to neuronal tubulin or TUJ1. P19 cells showed strong immunoreactivity to anti-TUJ1 antibody, with staining observed in both cell bodies and elongated processes (Figure 3.2). TUJ1 was excluded from the nucleus. Based on these data, P19 cells are a good model for studying neuronal differentiation.

3.2 Dab1 Expression in P19 Cells

P19 cells undergo neuronal differentiation in culture. To examine *Dab1* splicing in differentiating P19 cells, cells were harvested at Day 0, Day 2, Day 4, Day 6 and Day 8. RNA was extracted and RT-PCR carried out using primers flanking exons 9b and 9c to detect *Dab1-E*-like spice variants. As previously observed in developing mouse brain and chick retina, both these exons were included at the earlier stages of P19 growth (Day 0 and Day 2) (Figure 3.3). These results indicate that *Dab1-E*-like splice forms are expressed by P19 cells prior to neuronal differentiation. On Day 4, when the cells start the process of neuronal differentiation, a switch in Dab1 forms was

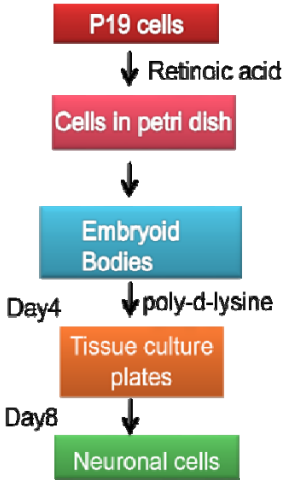

Figure 3.1.The different stages of P19 neuronal development.

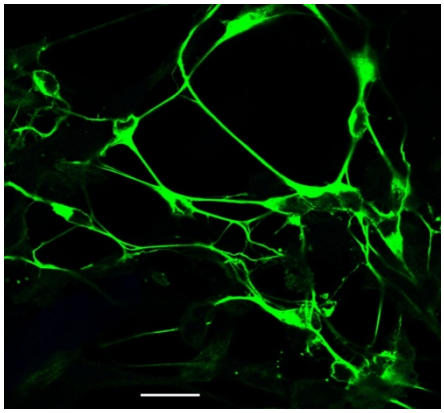

Figure 3.2. P19 neuronal differentiation. P19 cells undergo neuronal differentiation in the presence of 1 μM retinoic acid (RA). P19 cells are cultured on petri dishes for four days in the presence of RA to promote the formation of embryoid bodies. Embryoid bodies are precursor neuronal cells. Embryoid bodies are then broken down with trypsin and plated on tissue culture plates until Day 8 (shown here) at which time the cells have undergone complete neuronal differentiation, forming elongated processes and expressing neuronal tubulin TUJ1 (green).

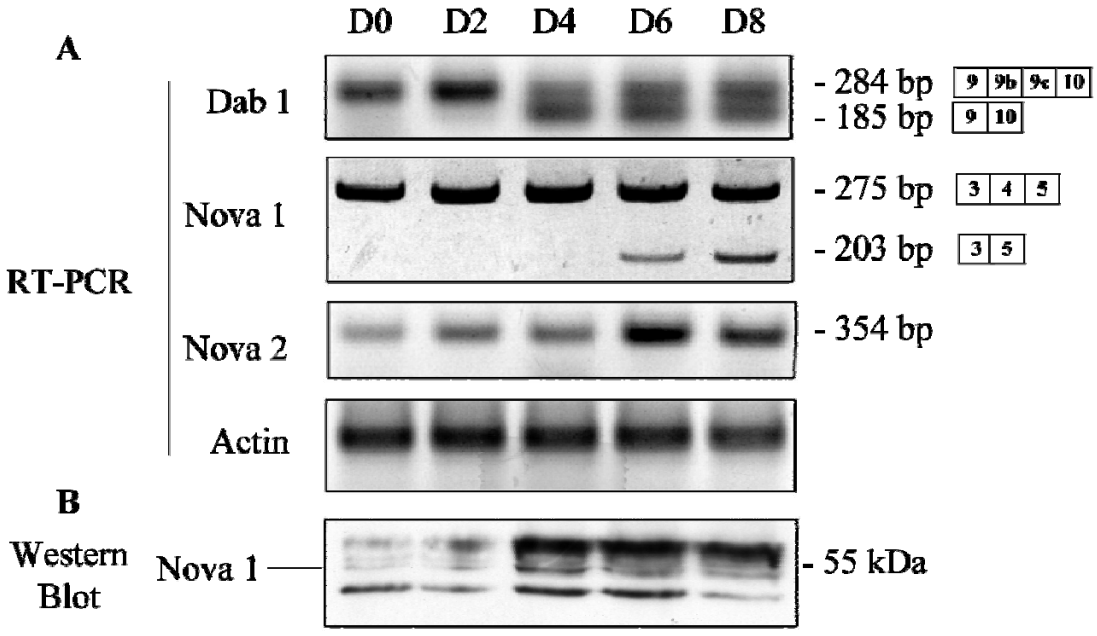
D0
D2
D4
D6
D8
A
Dab 1
- 284 bp
9 9b 9c 10
- 185 bp
9 10
Nova 1
- 275 bp
3 4 5
- 203 bp
3 5
RT-PCR
Nova 2
- 354 bp
Actin
B
Western Blot
Nova 1
- 55 kDa

Figure 3.3. Expression of Dab1 and Nova1 in P19 cells. (A) The cDNAs obtained from the P19 cells at different stages (Day 0 or D0, D2, D4, D6 and D8) were used for analysis of Dab1 splicing. RT-PCR analysis was carried out using primers spanning Dab1 exons 9 to 10. RT-PCR reactions were electrophoresed in a 2% agarose gel in Tris-acetate-EDTA buffer. The top band represents Dab1 splice forms that include exons 9b and 9c while the lower band represents Dab1 splice forms that exclude exons 9b and 9c. The same samples were used to conduct RT-PCR using primers spanning Nova1 exons 3 to 5. The RT-PCR reactions were electrophoresed in a 1.5% agarose gel in Tris-acetate-EDTA buffer. The higher band represents the larger splice form of *Nova1* which includes exon 4 whereas the lower band excludes exon 4. The induction of the smaller *Nova1* splice product is observed at Day 6 whereas *Dab1* splicing of exons 9b and 9c is observed at Day 4. The samples were also used for RT-PCR analysis using Nova2. The expression of Nova2 increases with progression of development peaking at Day 6 and going down at Day 8. Actin was used as a loading control. (B) Western blotting was carried out using protein lysates from P19 cells at D0, D2, D4, D6 and D8. The samples were electrophoresed in an 8% SDS-PAGE gel and proteins transferred to a nitrocellulose membrane. The membrane was immunostained with rabbit anti-Nova1 antibody. Nova1 expression increases with development, again correlating with *Dab1* splicing.

observed, with exons 9b and 9c being excluded in approximately half of the *Dab1* transcripts based on relative band intensities (Figure 3.3). These results indicate that P19 neuronal differentiation is accompanied by a switch from *Dab1-E*-like (including exons 9b and 9c) splice forms to *Dab1-L*-like (excluding exons 9b and 9c) splice forms, as previously observed *in vivo*. Hence, the P19 cells can replicate the process of *Dab1* alternative splicing when the cells undergo neuronal differentiation.

3.3 Expression of Nova and Other Factors in P19 Cells

Based on semi-quantitative RT-PCR analysis, *Nova2* transcript levels increase as P19 cells undergo neuronal differentiation. Highest levels of Nova2 were observed at Day 6, with an approximately two-fold reduction in Nova2 levels observed at Day 8. Little to no change in the levels of *Nova1* mRNA containing exon 4 was observed in differentiating P19 cells (Figure 3.3). However, the *Nova1* splice form that excludes exon 4 was clearly induced at Day 6 with a very weak signal observed at Day 4 and no signal observed at Day 0 and Day 2 (Figure 3.3). Western blot analysis of Nova1 shows a prominent increase in Nova1 expression from Day 2 to Day 4 (Figure 3.3). Our data indicate a general correlation between *Dab1-E* to *Dab1-L* transition and expression of Nova2, with delayed expression of Nova1 lacking exon 4 in relation to the appearance of a Dab1-L-like splice form that excludes exons 9b and 9c.

In an attempt to identify other splicing factors that may play a role in the *Dab1-E* to *Dab1-L* transition, splicing factors previously shown to be expressed in neurons and/or to play a role in the splicing of neuronally-expressed proteins were analysed. The splicing factors selected for analysis were Tra2β, NSSR, Fox 1, Fox 2, Fox 3, CUGBP1 and CUGBP5 (Table 3.1). RT-PCR data for these 7 splicing factors in P19 cells are shown in Figure 3.4. Fox 1 and CUGBP5, and to a lesser extent Fox 3, showed increases in RNA levels that coincided with neuronal differentiation. These factors may potentially play a role in *Dab1* alternative splicing as the observed increase in expression correlates with the splicing out of exons 9b and 9c upon neuronal differentiation.

Apart from splicing factors, RNA binding proteins can also influence Dab1 splicing. ELAV binding proteins that are implicated in increased expression of Nova1 by binding to its 3' UTR region were also studied. *ELAV* 3 and 4 showed increases in RNA levels in differentiating P19 cells (Figure 3.5). *ELAV1* RNA peaked at Day 6 whereas correctly-sized ELAV2 products were not detected.

3.4 Overexpression of Nova1 isoforms in Neuro2A cells

Neuro2A is a mouse neuronal-like neuroblastoma cell line that expresses key players (Crk, Src, Nckβ and Shp-2) involved in Reelin-Dab1 signaling (Figure 3.6). Moreover, Neuro2A endogenously expresses the *Dab1-E*-like splice form; i.e., the splice form that includes exons 9b and 9c

	Splicing Factor	Binding Site	Tissue	Reference
1	PTB	CUCUCU, UCUUC	Widespread	[215]
2	nPTB	CUCUCU	Neurons, myoblasts, testis	[216]
3	HuB	AU-rich elements (ARE)	Neurons	[217]
4	NAPOR	UG-rich sequences	Brain, heart, skeletal muscle	[218]
5	Fox ½	UGCAUG	Neurons, muscle, heart	[219]
6	hnRNP H	GGGA	Widespread	[220]
7	hnRNP A1	UAGGGA/U	Widespread	[221]
8	Tra2β	GAA repeats GHVVGANR*	Widespread	[222]

***H= A, C or U, V=A, C or G,R= A or G**

Table 3.1. Neuronal splicing factors and their binding sites.

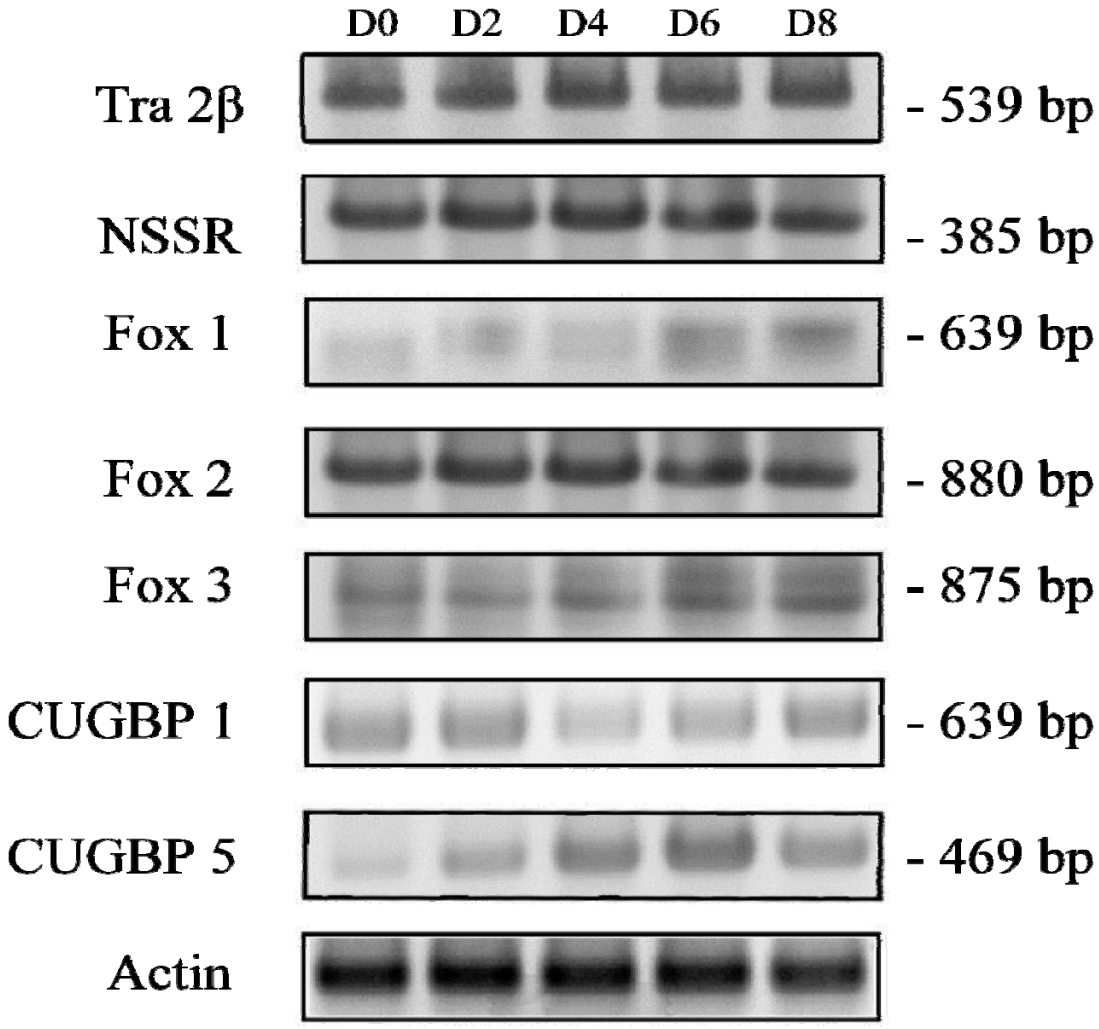

Figure 3.4. Expression of other splicing factors in P19 cells. RNAs extracted from P19 cells at the indicated time points were used for RT-PCR analysis using primers specific for the indicated neuronal and ubiquitous splicing factors. The products were electrophoresed in a 1% agarose gel in Tris-acetate-EDTA buffer. Fox 1, Fox 3 and CUGBP5 show correlation with Dab1 alternative splicing. Actin was used as the loading control.

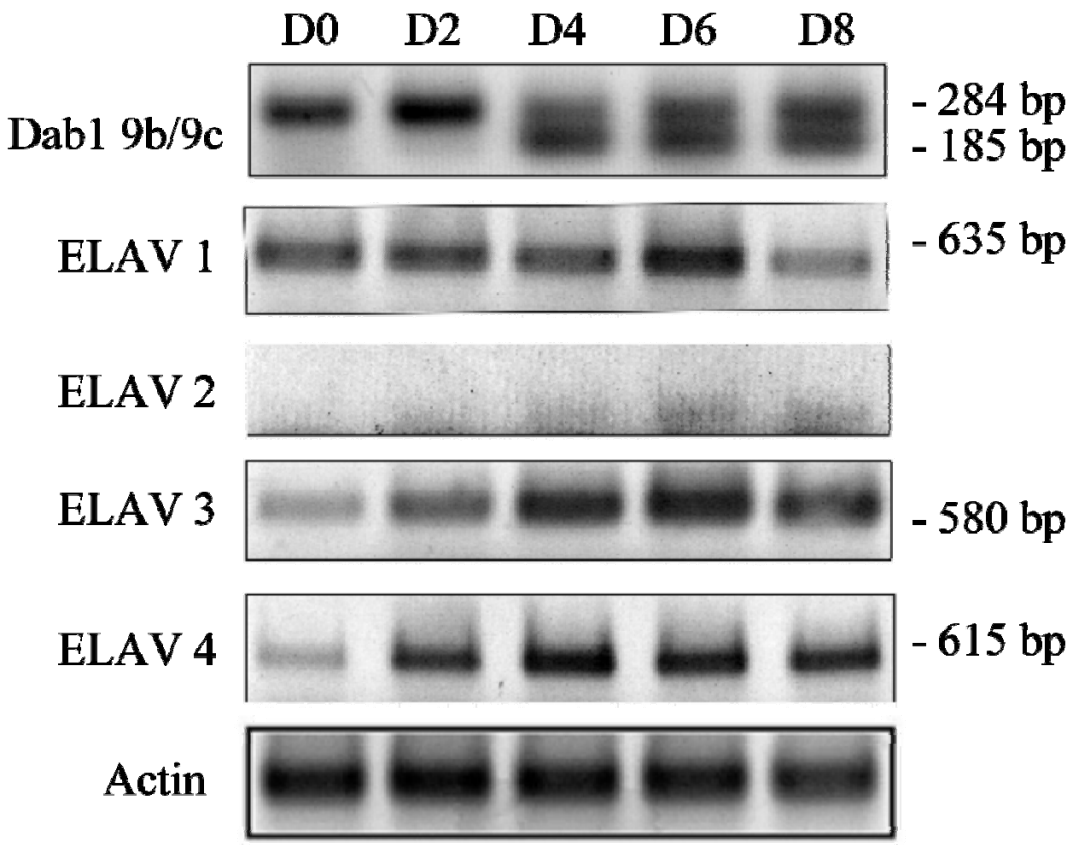

Figure 3.5 Expression of ELAV RNA binding proteins in P19 cells. cDNAs from P19 cells at the indicated time points were used for RT-PCR using primers specific for the four different members of the ELAV family. Actin was used as the loading control. The samples were electrophoresed in a 1% agarose gel.

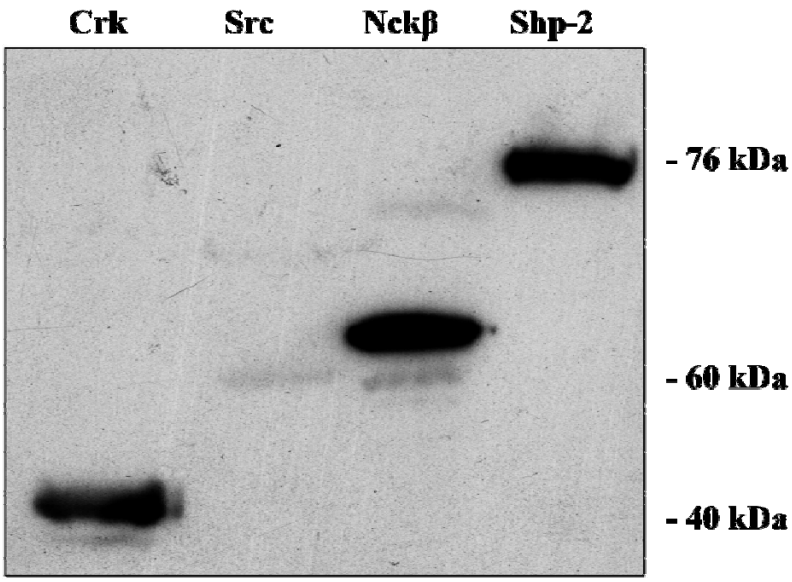

Figure 3.6 Expression of SH2 domain proteins in Neuro2A. Cell lysates were obtained from Neuro2A cells and 50 µg of cell lysates were loaded in four different lanes of a 10% SDS-polyacrylamide gel. The gel was electrophoresed and transferred to a nitrocellulose membrane. Each lane was cut out separately and immunostained with anti-Src, anti-Crk, anti-Nckβ and anti-Shp-2 antibodies. After immunostaining and signal detection, the membrane was put together and exposed to film. Key components of the Reelin-Dab1 signaling pathway (Crk, Src, Nckβ and Shp-2) are expressed in Neuro2A cells.

(see control lane in Figure 3.7). As Neuro2A cells are much easier to transfect than P19 cells, we used these cells to examine the effect of overexpressing Nova1 on *Dab1* splicing. Expression constructs of both the long (including exon 4) and short (excluding exon 4) forms of *Nova1*, along with empty vector, were transfected into Neuro2A cells. Cells were harvested 48 hours after transfection. RT-PCR analysis of the cDNA obtained from transfected Nuero2A was carried out using primers spanning *Dab1* exons 9 to 10 (Figure 3.7). The control lane shows absence of exons 9b and 9c splicing with the observed band including both exons 9b and 9c. Similar results were obtained upon transfecting *Nova1* isoform 1. However, overexpression of *Nova1* isoform 2 resulted in induction of Dab1 splicing and exclusion of exons 9b and 9c.

3.5 Nova1 Knockdown in P19 Cells

As previously discussed, P19 cells express a Dab1-E-like splice form that includes exons 9b and 9c prior to their differentiation into neuronal cells. Neuronal cell differentiation results in the expression of a Dab1 splice form that excludes exons 9b and 9c. Nova1 expression in P19 cells increases with neuronal cell differentiation. To further address the possibility that Nova1 plays a role in *Dab1* exons 9b and 9c splicing, we used siRNAs to knockdown Nova1 in proliferating P19 cells (prior to neuronal cell differentiation). These experiments were not successful due to low transfection efficiency. We

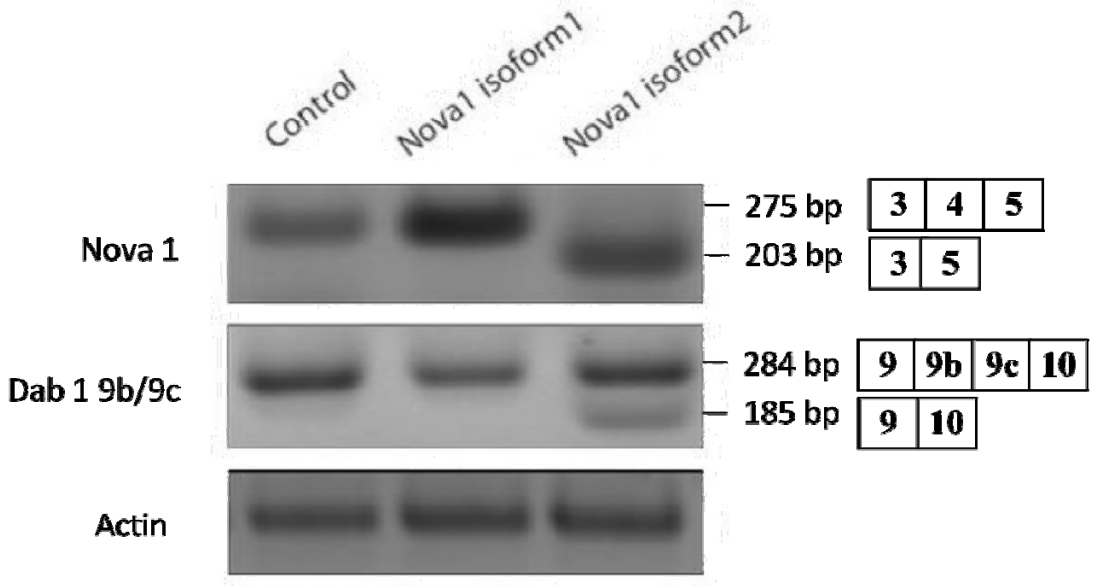

Figure 3.7 Overexpression of Nova1 isoforms in Neuro2A. The Neuro2A cells were transfected with control (empty) vector, Nova1 isoform 1 (with exon 4) expression vector, and Nova1 isoform 2 (without exon 4) expression vectors. RT-PCR was carried out using primers spanning exons 3 to 5 in Nova1 and exons 9 to 10 in Dab1. Overexpression of Nova1 isoform 1 had no effect on Dab1 splicing; however, overexpression of Nova1 isoform 2 resulted in induction of Dab1 exons 9b and 9c exclusion.

therefore attempted to knockdown *Nova1* in P19 cells using ShRNA inserted in lentiviral vector plentilox 3.7.

Nova1 shRNA targeting both forms of *Nova1* were inserted into plentilox 3.7 and introduced into P19 cells at Day 5, the day after the P19 embryoid bodies were dissociated and plated on poly-D-lysine tissue culture plates. At Day 5, P19 cells are no longer dividing and hence, lentiviral infection is the most efficient method to introduce shRNA into the cells. Infection efficiency was monitored by fluorescence microscopy using GFP expression as a surrogate marker for the presence of lentivirus in the cells. Reduced levels of both *Nova1* variants were observed in P19 cells infected with the lentivirus-Nova1 shRNA construct compared to control cells (Figure 3.8). RT-PCR analysis of *Dab1* exons 9b and 9c in P19 infected cells showed an increase in the Dab1 variant that includes exons 9b and 9c upon *Nova1* knockdown (Figure 3.8). While an increase in the intensity of the band that excludes exons 9b and 9c was also evident, a similar increase in actin levels was observed which indicates a higher concentration of cDNA in the second sample. However, the increase in the higher band, the band that includes exons 9b and 9c, is proportionately much higher when compared to actin. These combined results suggest a role for Nova1 in the exclusion of *Dab1* exons 9b and 9c.

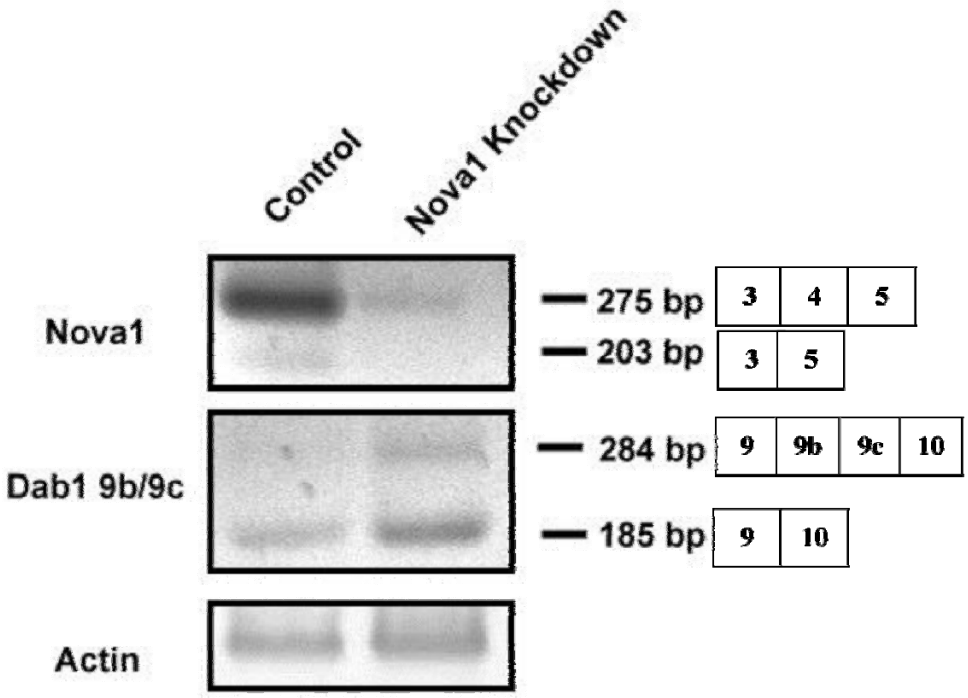
Control
Nova1 Knockdown
Nova1
275 bp
3
4
5
203 bp
3
5
Dab1 9b/9c
284 bp
9
9b
9c
10
185 bp
9
10
Actin

Figure 3.8 Effect of Nova1 knockdown on Dab1 splicing. *Nova1* shRNA was introduced into the lentiviral construct plentilox 3.7 and infected into P19 cells on Day 5. Control used was plentilox 3.7 containing scrambled shRNA. The cells were harvested on Day 8 and cDNA was prepared from the total RNA obtained using TRIzol. RT-PCR for *Nova1* was carried out using primers flanking exons 3 and 5. The control lane shows the presence of both the isoforms of Nova1, whereas the knockdown lane shows the reduction in both isoforms. RT-PCR for *Dab1* was carried out using primers flanking *Dab1* exons 9b and 9c (in exons 8 and 10). The control shows the presence of the isoform that excludes exons 9b and 9c with a barely detectable higher molecular band which likely includes exons 9b and 9c. Upon Nova1 knockdown, the higher molecular weight band that includes exons 9b and 9c is much more abundant when compared to control.

3.6 Expression of SH2 Domain Proteins in P19 Cells

Dab1 shows a switch from non-tyrosine-phosphorylated to highly tyrosine phosphorylated as neuronal cell differentiation progresses. This switch enables certain SH2 domain-containing proteins to bind to Dab1 and propagate the Reelin-Dab1 signal. These SH2 domain-containing proteins play a very important role in determining the effect of Dab1 on neuronal cell migration and hence it is very important to understand and characterize the roles of these SH2 domain proteins in differentiating neurons. The SH2 domain proteins Src, Crk, Nckβ and Shp-2 have previously been shown to interact with Dab1, with level of interaction varying depending on the tyrosine phosphorylated state of Dab1. In an attempt to better understand the downstream signaling pathways induced or repressed by the different Dab1 isoforms, Gao et al., studied the interaction between the different Dab1 isoforms and specific SH2 domains [56]. I contributed to this work by examining the expression of Src, Crk, NCKβ and Shp-2 in differentiating P19 cells. The goal of this experiment was to determine whether there was a correlation between the expression of SH2 domain proteins and Dab1-E/Dab1-L-like isoforms. All four SH2 domain proteins were expressed in P19 cells (Figure 3.9). However, there was no clear correlation between the expression of Src, Crk, NCKβ and Shp-2, and the expression of Dab1-E versus Dab1-L isoforms (compare Figures 3.3 and 3.9).

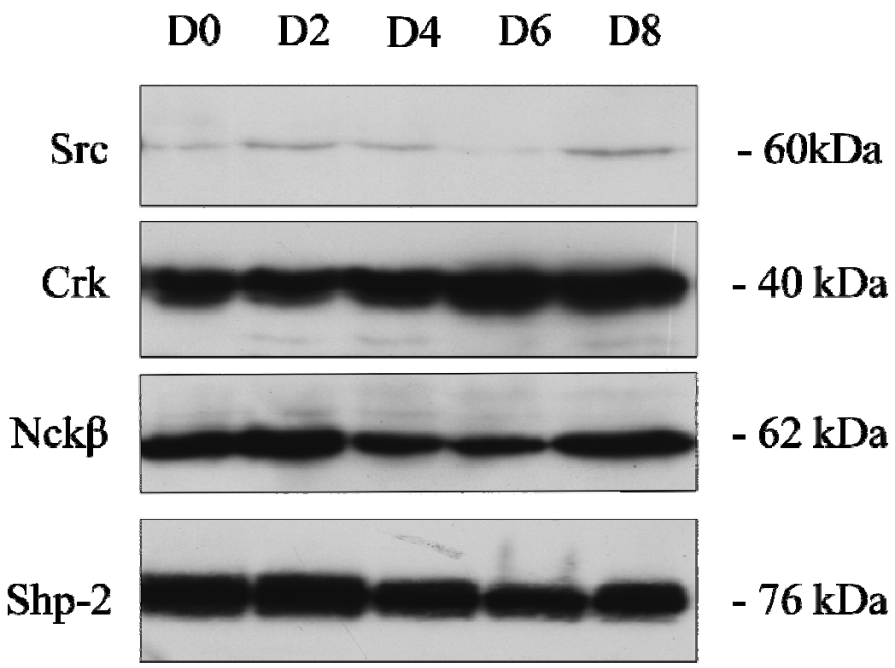

Figure 3.9. Expression patterns of SH2 domain proteins in P19 cells. P19 cell lysates were prepared from P19 cells at Day 0, Day 2, Day 4, Day 6 and Day 8. Fifty μg of cell lysates prepared from each sample were loaded in duplicate and electrophoresed in two separate 8% SDS-polyacrylamide gels. The proteins were transferred to a PVDF membrane and immunostained with anti-Src, anti-Crk, anti-Nckβ and anti-Shp-2 antibodies.

3.7 Interaction of Shp-2 with Dab1

As mentioned in the Introduction (Section 1.7), Shp-2 is a tyrosine phosphatase that recognizes two of the four phosphor-tyrosine sites in Dab1: Y185 (A-V-pY185-Q-T-I)] and Y198 (P-V-pY198-Q-Y-I). To determine whether Shp-2 might be involved in the regulation of Dab1 phosphorylation, we examined Neuro2A cells which endogenously express both Dab1-E and Shp-2. To specifically address whether Shp-2 interacts with Dab1-L but not Dab1-E, the Dab1-L-like isoform (including exons 7 and 8 and excluding exons 9b and 9c) and Dab1-E-like isoform (including exons 9b and 9c and excluding exons 7 and 8) were overexpressed in Neuro2A cells using GFP-tagged Dab1 constructs. Cells were harvested 48 hours after transfection and protein lysates prepared as described in Materials and Methods. Co-immunoprecipitations were carried out using anti-Shp-2 antibody. Western blot analysis was used to demonstrate that anti-Shp-2 antibody can efficiently immunoprecipitate Shp-2 (Figure 3.10). Next, interaction between Shp-2 and the Dab1 isoforms was examined using an anti-Dab1 antibody that recognises both the Dab1-E-like and Dab1-L-like isoforms. Both these Dab1 isoforms showed a similar level of interaction with Shp-2 (Figure 3.10). A high molecular weight band was seen in Dab-L lane which represents phosphorylated Dab1-L.

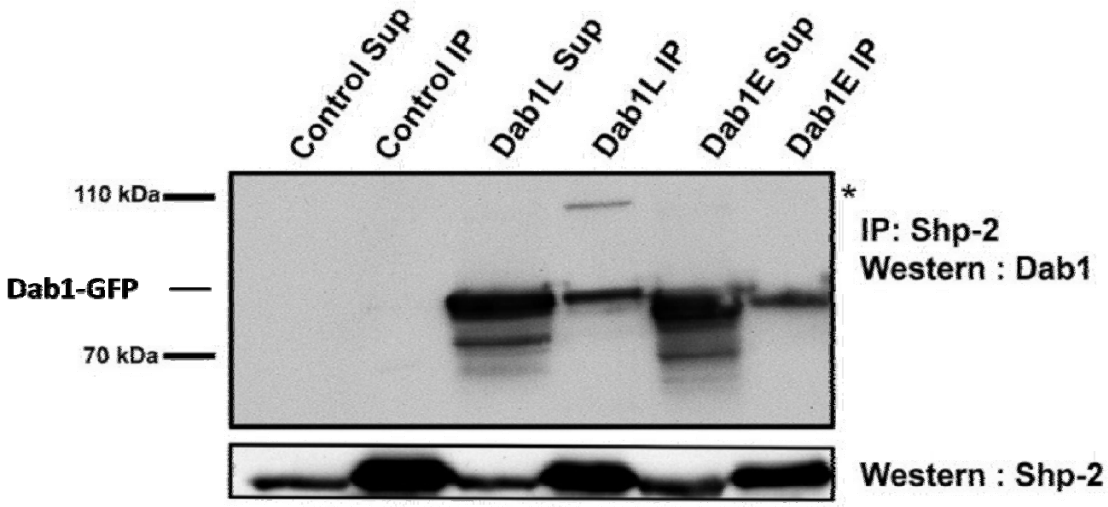

Figure 3.10. Co-Immunoprecipitation of Dab1 and Shp-2 in Neuro2A cells. Lysates obtained from Neuro2A cells transfected with Dab1-L, Dab1-E and control (GFP- empty vector) were used to immunoprecipitate Shp-2 using mouse anti-Shp-2 antibody (BD Transduction Laboratories). Immunoprecipitated samples were then electrophoresed in an 8% SDS-PAGE gel and transferred to a nitrocellulose membrane and immunostained with anti-Dab1 antibody (Rockland). Both Dab1 isoforms show similar levels of binding to Shp-2. An extra band (indicated by the asterisk) is observed in the Dab1-L IP lane, likely representing phosphorylated Dab1-L. The blot was stripped and immunostained with anti-Shp-2 antibody to confirm immunoprecipitation. The experiment was repeated twice.

3.8 Interaction between Dab1, Src and Shp-2 in Neuronal and Non-neuronal Systems

Regulation of Dab1 phosphorylation plays a very important role in its downstream effects. Our results indicate that Shp-2 interacts with Dab1 and thus may be involved in the regulation of Dab1 phosphorylation. As Src is known to phosphorylate Dab1-L, it is possible that Src and Shp-2 SH2 domains play competing roles in Dab1-L phosphorylation. To address this possibility, we overexpressed Dab1-L, Src and Shp2 in Neuro2a cells. Five different plates of Neuro2A cells were transfected. The first plate was transfected with a control vector with GFP tag, the second plate with a GFP-tagged Dab1-L expression construct, the third plate with a GFP-tagged Dab1-L expression construct and Src expression construct, the fourth plate with a GFP-tagged Dab1-L expression construct and Shp-2 expression construct, and the fifth plate with a GFP-tagged Dab1-L expression construct along with both the Src and Shp-2 expression constructs. Cells were harvested 48 hours after transfection and lysates prepared. Co-immunoprecipitations were carried out using anti-GFP antibody. Western blot analysis using anti-GFP antibody showed very efficient immunoprecipitation of GFP-Dab1-L (Figure 3.11). The immunoprecipitated samples were immunostained with anti- phosphotyrosine antibody to see the effect of Src and Shp-2 overexpression on Dab1 phosphorylation. As expected, strong induction of GFP-Dab1-L phosphorylation was observed upon Src overexpression. Unexpectedly, Dab1 phosphorylation was similarly induced with Shp-2 overexpression. However,

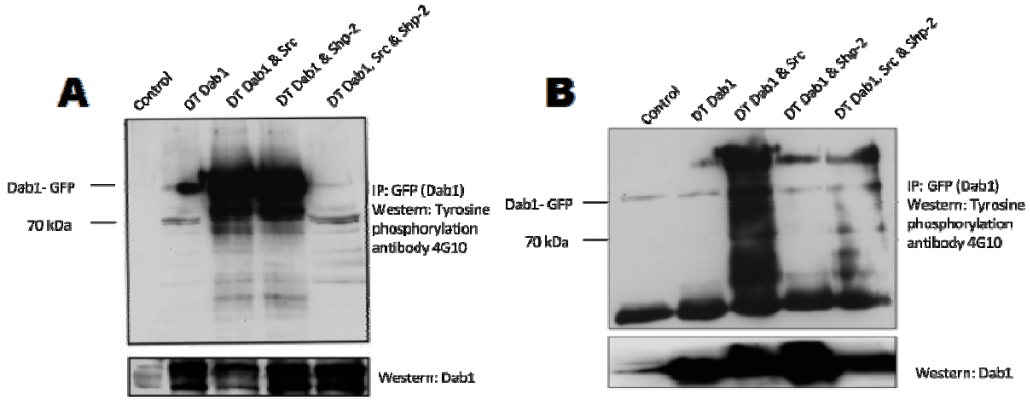
A
Control
DT Dab1
DT Dab1 & Src
DT Dab1 & Shp-2
DT Dab1, Src & Shp-2
Dab1- GFP
70 kDa
IP: GFP (Dab1)
Western: Tyrosine phosphorylation antibody 4G10
Western: Dab1
B
Control
DT Dab1
DT Dab1 & Src
DT Dab1 & Shp-2
DT Dab1, Src & Shp-2
Dab1- GFP
70 kDa
IP: GFP (Dab1)
Western: Tyrosine phosphorylation antibody 4G10
Western: Dab1

Figure 3.11. Effect of Shp-2 on Dab1 phosphorylation. (**A)** Cell lysates prepared from Neuro2A cells transfected with GFP empty vector; GFP-Dab1-L; GFP-Dab1-L and Src; GFP-Dab-L and Shp-2; and GFP-Dab1-L, Src and Shp-2, were used in immunoprecipitation of GFP-Dab1-L fusion protein with mouse anti-GFP antibody (Sigma). Immunoprecipitates were electrophoresed in a 8% SDS-PAGE gel, transferred to a nitrocellulose membrane and immunostained with the mouse anti-phospho-tyrosine antibody pY 4G10 (Millipore). The blot was stripped and immunostained with anti-Dab1 antibody to confirm immunoprecipitation. Overexpression of either Src or Shp-2 increased Dab1 tyrosine phosphorylation. Overexpression of both Src and Shp-2 prevented Dab1 tyrosine phosphorylation. This experiment was repeated five times. (**B)** The same experiment was conducted using 293T cells instead of Neuro2A cells. Src overexpression resulted in increased Dab1 tyrosine phosphorylation. In contrast to Neuro2A, Shp-2 overexpression in 293T cells prevented Dab1 tyrosine phosphorylation. Co-expression of both Shp2 and Src also prevented Dab1 tyrosine phoshorylation. Although this experiment was only done once by me, similar results in 293T cells were obtained by a former graduate student in the lab, Tina Gao.

expression of both Src and Shp-2 had no effect on Dab1-L phosphorylation when compared to control transfectants (Figure 3.11A).

To determine whether induction of Dab1 tyrosine phosphorylation by Shp-2 was specific to neuronal cells, we conducted the same experiment in 293T, an embryonic human kidney cell line. As shown in Figure 3.12B, different results were observed with Shp-2. In contrast to the elevated levels of Dab1-L tyrosine phosphorylation observed in Neuro2A upon overexpression of GFP-Dab1-L and Shp-2, only background signal was observed under these conditions in 293T cells (Figure 3.11B).

3.9 Identification of Shp-2 Binding Sites in Dab1

Shp-2 shows interaction with both the Dab1-E and Dab1-L isoforms (Figure 3.10). Shp-2 is known to bind to its targets using its SH2 domain. There are four tyrosine phosphorylation sites (Y^{185}, Y^{198}, Y^{220}, Y^{232}) in Dab1-L that represent potential binding sites for the Shp-2 SH2 domain. Dab1-L with mutations in all four putative binding sites, singly or in combination, were overexpressed in Neuro2A cells using expression constructs previously generated in the lab [55]. Cell lysates from these transfected cells were immunoprecipitated with anti-mouse Shp-2 antibody and immunostained with an anti-rabbit Dab1 antibody which recognizes all Dab1 isoforms (Rockland). A strong interaction was observed between Shp-2 and wild-type Dab1-L (Figure 3.12). No interaction was detected between Shp-2 and the Dab1 Y^{185} and Y^{220} mutants, demonstrating the importance of these phosphorylation

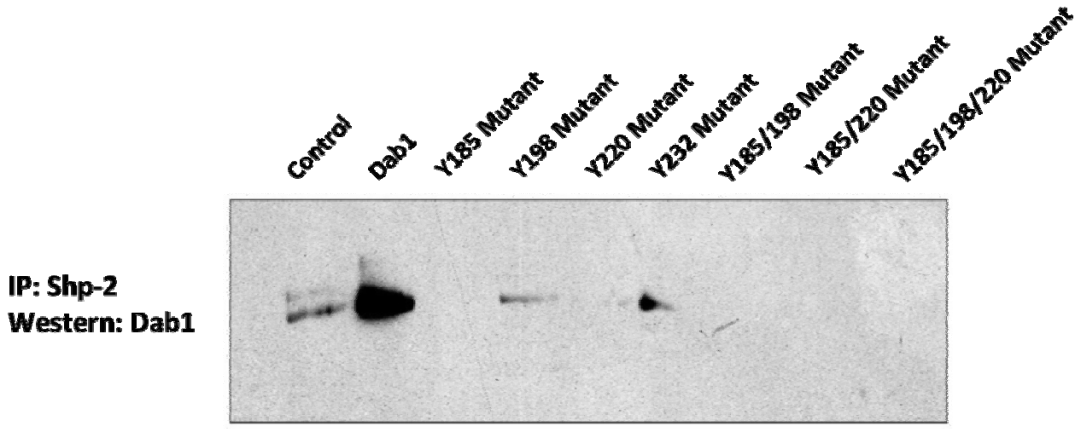

Figure 3.12 Shp-2 binding to Dab1 mutants. The Neuro2A cells were transfected with control (GFP empty vector), wild type and mutant Dab1-L. Dab1-L was mutated at Y^{185}, Y^{198}, Y^{220}, Y^{232}, Y^{185}/Y^{198}, Y^{185}/Y^{220}, and $Y^{185}/Y^{198}/Y^{220}$. Cell lysates were immunoprecipitated with anti-Shp-2 antibody and the blot immunostained with rabbit anti-Dab1 antibody. Binding of Shp-2 to wild-type Dab1-L, and to a lesser extent Y^{198} and Y^{232} Dab1-L mutants, is observed. This experiment was repeated four times, with the best data shown here.

sites for Shp-2 binding. Reduced interaction between Shp-2 and Dab1 Y^{198} and Y^{232} was observed. As expected, the double mutants and the triple mutant showed no interaction with Shp-2.

Chapter 4:

DISCUSSION

AND

FUTURE DIRECTIONS

4.1 DISCUSSION

4.1.1 Neuronal models for studying Dab1 splicing and regulation of Dab1 phosphorylation

There are many model systems for studying neuron-related events. I used two *in vitro* models: Neuro2A and P19 cells. Neuro2A cells are mouse neuroblastoma cells that are at an early stage of neuronal differentiation. These cells mimic one particular stage of neuronal development, the early transitory stage. As shown in this thesis, Neuro2A cells produce key players involved in the Reelin-Dab1 downstream signaling. In addition to expressing the appropriate proteins for our study, these cells are easy to culture and transfect. The transfection efficiency of Neuro2A using PEI (1:4 ratio of PEI:DNA) is close to 90%. Thus, these cells serve as an appropriate model system for studying Dab1-related splicing and expression events at the early transitory stage of neuronal differentiation.

P19 is a mouse embryonic teratocarcinoma cell line that can be induced to undergo neuronal differentiation with retinoic acid. These cells normally exist in an undifferentiated state; however, when cultured in the presence of retinoic acid, these cells undergo neuronal differentiation. Upon RA treatment, P19 cells first form embryoid bodies. When embryoid bodies are trypsinized at Day 4 and placed on a tissue culture plate where they can adhere, the cells stop dividing and undergo late stage differentiation forming neuronal cells with long processes that express neuron-specific markers such as TUJ1. Thus, these cells undergo the full spectrum of neuronal

differentiation. As demonstrated in this thesis, P19 cells mimic the neuronally-induced Dab1 splicing events previously observed in mouse brain and chick retina models. Thus, P19 represent an important tool for studying the expression pattern of Dab1 spicing factors and other proteins associated with Dab1 splicing. P19 cells, however, are very difficult to transfect and hence, cannot easily be used for manipulation of gene expression. These cells are best suited for studying endogenous gene expression patterns through the spectrum of neuronal differentiation. Thus, P19 and Neuro2A have pros and cons that make them useful for investigating different aspects of Dab1 splicing and phosphorylation.

4.1.2 Role of *Dab1* Splicing in Differentiating Neuronal Cells

The Reelin signaling pathway plays a central role in the migration of neurons during brain development. However, there are reports indicating that the effect of Reelin varies depending on neuronal cell type and the differentiation state of the neuron. Reelin can affect the migration of neurons by regulating their migratory mode, speed, and polarity [223]. In spite of extensive study, the mechanism underlying such a finely-tuned response to Reelin, which leads to concerted and coordinated migration of different neuronal cells in the brain, is not clear. One of the most intriguing possibilities is regulation of the Reelin signal by the different isoforms of Dab-1. There is evidence suggesting that the different Dab1 isoforms are expressed at different stages of development and in distinct populations of cells. As

different Dab1 isoforms likely recruit different downstream interacting proteins due to the presence of different combinations of tyrosine phosphorylation sites [53-56, 155, 160, 164-165, 212], this allows a high level of variability in how individual neurons respond to the Reelin signal. As seen in chicken retina and brain, the early isoform Dab1-E is expressed at the earlier stages of development whereas the late isoform Dab-L is expressed at the later stages of development [160]. The same trend is observed in mouse brain [56]. In both these species, the inclusion and exclusion of exons 7, 8, 9b and 9c are developmentally co-ordinated, with exons 7 and/or 8 excluded, and exons 9b and 9c included, at early developmental stages, and exons 7 and/or 8 included, and exons 9b and 9c excluded, at late stages of development.

Not only are the Dab1 isoforms expressed at distinct stages of development, they are also expressed in distinct population of cells, further underlining their importance in modulating Dab1 function. In mouse brain, the Dab1-E like isoforms (including exons 9b and 9c) are mainly found in the proliferating progenitor cells in the ventricular zone. In contrast, the Dab1-L like isoforms (including exons 7 and 8) are found in the more mature cells of the cortical zone [56]. In chick retina, Dab1-E is found in progenitor cells whereas Dab1-L is expressed in differentiating migratory cells [160]. These results indicate that: (i) Dab1 plays a dynamic role in neuronal differentiation and migration during central nervous system development and (ii) alternative splicing of *Dab1* plays an integral role in fine-tuning the effect of Dab1 in different sets of cells in the developing central nervous system.

In P19 cells, Day 0 and Day 2 are characterized by the presence of precursor cells that are undergoing rapid cell division. At these developmental stages, Dab1 E-like isoform are expressed. At later stages, from Day 4 to Day 8, when cells are undergoing neuronal differentiation, Dab1-L like isoforms are produced. Dab1-E like isoforms lack tyrosine phosphorylation sites due to splicing of exons 7 and/or 8 [55, 59]. This prevents the phosphorylation and activation of Dab1-E or only partial phosphorylation and activation of Dab1-E-like isoforms. As a consequence, the expression of Dab1-E-like isoforms results in minimal propagation of the Reelin signaling pathway and hence, little or no neuronal cell migration [56].

The switch from Dab1-E to Dab1-L during neuronal cell differentiation may therefore play a key role in determining when neuronal can migrate. In fact, Dab1-E has been found to maintain the progenitor cell pool in chick retina *in ovo* electroporated with a *Dab1* shRNA construct [55]. As development progresses, the progenitor cells exit the cell cycle and form neuronal precursor cells that are ready for migration [224-226]. At this stage, there is a switch from non-tyrosine phosphorylated Dab1-E to tyrosine phosphorylated Dab1-L-like isoforms which leads to activation of the Reelin-signaling pathway [55]. This results in neuronal migration and formation of the inside out laminar structure observed in the cerebral cortex [55].

4.1.3 Nova Family of Splicing Factors

Nova is the first group of splicing factors found to be tissue-specific, with abundant expression in developing brain. The Nova family of RNA splicing factors plays a very crucial role in neuronal development. It comprises Nova1 isoforms 1 and 2 and Nova2. Nova1 isoform 2 is missing the penultimate exon, exon 4 [227]. This exon has putative threonine and serine phosphorylation sites. Accordingly, isoform 1 shows ten-fold higher phosphorylation than Nova1 isoform 2 [176]. However, exclusion of exon 4 has not been shown to affect its splicing factor properties thus far [176]. Knockout of *Nova1* in mice results in death one week after birth. Nova1-deficient neurons undergo widespread apoptosis, leading to death [139]. *Nova2* knockout mice show extensive migration defects, similar to those observed in Reelin or *Dab1* mutant mice [228].

Studies of *Nova2* knockout mice indicate that Nova2 is involved in *Dab1* splicing of exons 9b and 9c during a particular window of time (E14.5 to E16.5) in development [162]. After E16.5, exclusion of exons 9b and 9c in *Dab1* occurs even in *Nova2* knockout mouse brain [162]. This indicates the presence of another factor that is involved in *Dab1* splicing and that acts in concert with Nova2 to maintain the balance between the spliced isoforms of Dab1 at different stages of development.

Nova1 may also be involved in the splicing of *Dab1* exons 9b and 9c. In this thesis, we report that the expression patterns of Nova1 and Nova2 in P19 generally correlate with *Dab1* splicing events that result in exclusion of

exons 9b and 9c. Furthermore, we show that manipulation of Nova levels, particularly Nova1 isoform 2, in Neuro2A results in a switch in the *Dab1* splicing pattern. Nova1 shares the same binding site as Nova2, thus the binding sites identified for Nova2 in the intronic region of exon 9b and 9c may also be the binding site for Nova1. It is possible that both Nova1 and Nova2 are involved in *Dab1* splicing. However, we found that Nova1 isoform 2, the splice variant of *Nova1* that lacks exon 4, is first expressed in P19 cells at Day 6, two days after the appearance of the Dab1-L-like isoform which excludes exons 9b and 9c. This inconsistency may be explained by Nova2 already playing a role in Dab1 splicing on Day 4, with Nova1 isoform 2 taking over this role by Day 6. *Nova2* RNA peaks at Day 6 with a reduction in signal intensity observed at Day 8. Thus, Nova1 and Nova2 may be acting in concert to orchestrate *Dab1* splicing at different stages of development. This possibility could be further addressed by examining Nova2 at the protein level and by examining *Dab1* splicing and Nova expression at additional stages in P19 (particularly Day 5).

4.1.4 Other Factors Possibly Involved in Dab1 Splicing

The expression of other neuronal and ubiquitous splicing factors was examined during development to identify additional factors that might be involved in *Dab1* splicing. Different expression patterns were observed for these factors in P19 at different stages of differentiation. Three factors in particular, namely Fox 1, Fox 3 and CUGBP5, showed increased expression

at the point when Dab1 exons 9b and 9c exclusion is first observed in P19 cells at Day 4. Fox proteins are expressed in both muscle tissue and neurons where they play important roles in splicing. Fox 1 has been shown to act as splicing enhancer in neurons by binding to UGCAUG splice enhancer elements [229]. Similarly, Fox 3 binds to UGCAUG enhancer elements downstream of an intron thereby enhancing its splicing [230]. CUGBP5, similar to all the CUGBP proteins, promotes exon inclusion and plays an important role in brain development [231].

There are proteins like ELAV RNA binding proteins which are known to bind to the *Nova1* 5' UTR region and either make them more stable or increase their turnover [232]. The ELAV family of proteins are neuron-specific RNA binding proteins that are involved in multiple RNA processing functions [217]. As shown in this thesis, ELAV3 and ELAV4 RNA levels increase with differentiation in P19 cells, and hence may act upstream of Nova in *Dab1* splicing mechanism. ELAV proteins are in turn activated by protein kinase C alpha (PKCα)–mediated threonine phosphorylation [233]. This phosphorylation also has been shown to affect the *Nova1* binding capacity of ELAV4. It is thus possible that a cascade of proteins including PKCα/ELAV/Nova regulate Dab1 splicing (Figure 4.1).

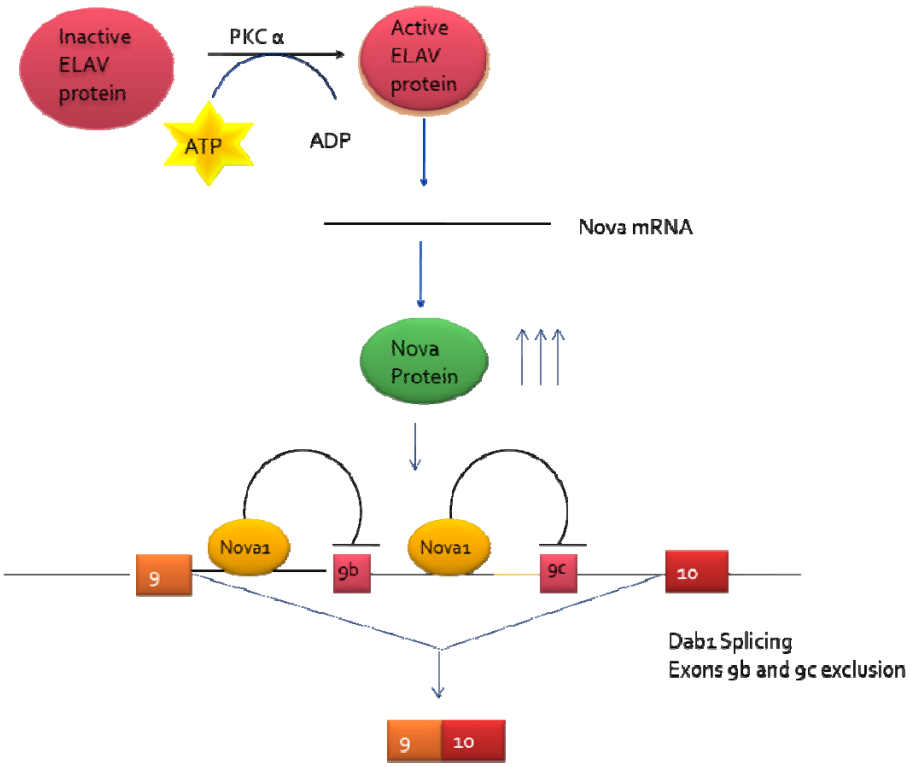

Figure 4.1. ELAV and Nova regulation of Dab1 splicing. This model shows that at the later stages of neuronal differentiation, PKCα phosphorylates and activates ELAV. Once activated, ELAV binds to *Nova1* mRNA 3' UTR region and stabilizes it, thus increasing its expression levels. In turn, Nova1 binds to the *Dab1* intron region upstream of exons 9b and 9c, and promotes splicing of exons 9b and 9c.

4.1.5 Regulation of Dab1 Phosphorylation by Shp-2

Shp-2 is a SH2 binding phosphatase which recognises and binds substrates via its SH2 domain. Binding to a substrate activates Shp-2's enzymatic phosphatase domain. However, there have been a few reports indicating that Shp-2 can also act as an adaptor protein [196-197]. For example, Shp-2 can bind to a substrate using its SH2 domain and recruit other proteins using its second SH2 domain. Importantly, Shp-2 has been shown to induce phosphorylation by recruiting other kinases [198]. Thus, Shp-2 is a highly dynamic protein with multiple functions. Our results suggest that Shp-2 interacts with Dab1. However, the effect of Shp-2 on Dab1 tyrosine phosphorylation is complex. In the non-neuronal cells 293T cells, Shp-2 functions as a phosphatase, with Dab1 dephosphorylation observed when Shp-2 is co-transfected with Dab1-L. In the presence of both Src and Shp-2, Dab1-L remains unphosphorylated. However, in Neuro2A cells which express properties of early neurons, Dab1 is phosphorylated in the presence of either Src or Shp-2. However, when both Src and Shp-2 are over-expressed, Dab1-L remains unphosphorylated. These results suggest that Shp-2 may be acting as regulator of Dab1 phosphorylation. Thus, when Dab1 is hyperphosphorylated by Src, Shp-2 acts as a phosphatase and dephosphorylates it. However, when Dab1 is hypophosphorylated, Shp-2 acts as an adaptor protein that recruits other kinases to phosphorylate it. The difference between the neuronal and non-neuronal system suggests that Shp-

2 may be recruiting neuronal tissue-specific kinases for phosphorylation of Dab1, that are absent in 293T cells (Figure 4.2).

4.2 FUTURE DIRECTIONS

4.2.1 Investigation of the Interaction between Nova1 and Dab1

Putative Nova1 binding sites have already been identified in the *Dab1* gene. However, the interaction between Nova1 splicing factor and *Dab1* mRNA needs to be confirmed. Experiments such as RNA-Immunoprecipitation (RNA-IP) could be used to validate Nova1 binding to the putative Nova1 binding sites in *Dab1*. The other important aspect of Nova1 studies is to study its expression pattern. Dab1-L is primarily expressed in the outermost cortical layer of the brain during cortical migration at E14.5 to E16.5 [56]. There is no expression of Dab1-E in these migrating cells [56]. Therefore, it will be worthwhile to look at the correlation between expression of the two different isoforms of Nova1 and Dab1-E and Dab1-L in the developing central nervous system. This experiment could be carried out by *in situ* hybridization using isoform-specific probes. The difference between Nova1 isoforms 1 and 2 is a 71 bp exon (exon 4). The primers used to prepare the probe have to be designed in such a way that the primers for the larger isoform span just the 71 bp exon of *Nova1* whereas the primers for the smaller isoform should cover a region of around 600-700 bp, with the reverse primer binding to the junction of exons 3 and 5.

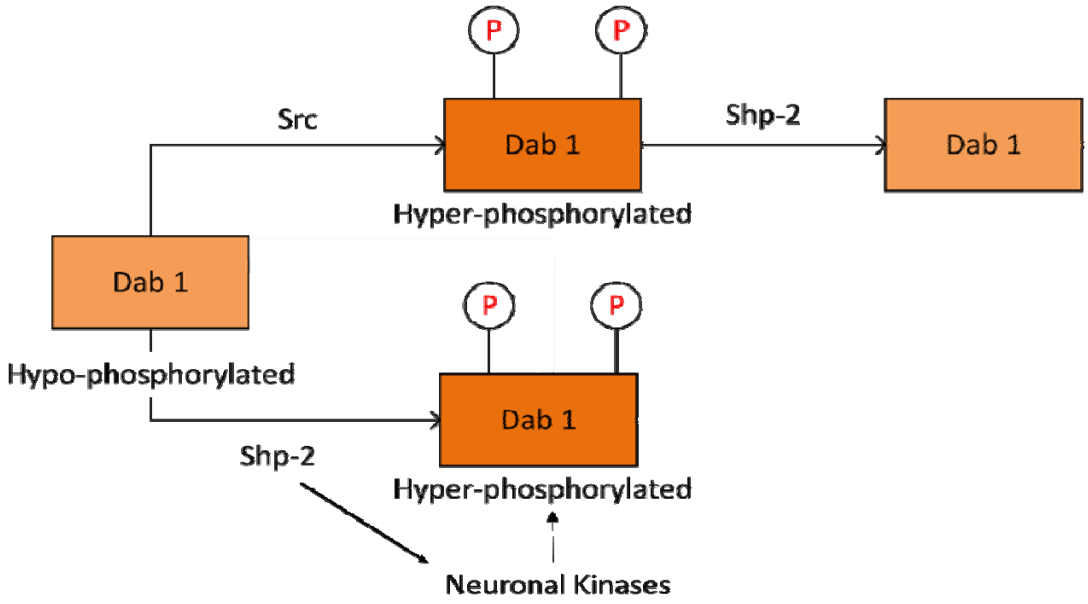

Figure 4.2. Regulation of Dab1 phosphorylation by Shp-2. In a neuronal system, when Dab1 is hypo- or un-phosphorylated, it can be phosphorylated by both Src kinase and Shp-2. In this context, Shp-2 acts as an adaptor protein for other kinases. However, when Dab1 is hyper-phosphorylated (e.g. by Src), Shp-2 then turns into a phosphatase and reduces the phosphorylation of Dab1. This model demonstrates how Shp-2 may play a role in maintaining the fine balance of Dab1 phosphorylation.

4.2.2 Identification of Other Splicing Factors Involved in Dab1 Splicing

The expression of splicing factors such as Fox 1, Fox 3 and CUGBP5 coincides with *Dab1* splicing during brain development. Scanning *Dab1* exons 9b, 9c, and the intronic regions around these exons will help identify any binding sites for the above mentioned splicing factors. Minigene cassettes can be designed to study the effect of these splicing factors on *Dab1* splicing using cell cultures. Minigene cassettes should contain the important splicing elements that surround exons 9b and 9c, along with exons 9b and 9c. These cassettes can be transfected into neuronal cells such as Neuro2A along with splicing factor(s) of interest. The effect on splicing can be monitored by PCR. Mutation of the putative binding sites will help identify the actually binding sites of the splicing factors. Mutation and deletion of the intronic regions will help narrow down the splicing elements that promote exclusion of exons 9b and 9c. The inclusion and exclusion of exons 7 and 8 occurs simultaneously with the exclusion and inclusion of exons 9b and 9c, respectively. Therefore, similar studies can be carried out to identify splicing factors involved in exons 7 and 8 splicing.

4.2.3 Shp-2 Regulation of Dab1 Phosphorylation

In order to confirm the hypothesis that Shp-2 maintains the fine balance of Dab1 phosphorylation with the help of kinases that are specifically expressed in neurons, another neuronal system will be studied. Retroviral constructs for *Dab1, Src* and *Shp-2* will be produced that can be transfected

into primary cultures of chick retina. We have already demonstrated that alternative splicing of *Dab1* occurs in primary retinal cultures as a function of time in culture (Sachin Katyal, unpublished work). Protein will be harvested from these transfected cells and western blot analysis carried out to determine the effect of Shp-2 on Dab1 phosphorylation in the presence and absence of exogenous Src and Shp-2. Depending on the results, it may also be necessary to use retroviral constructs to reduce endogenous Src and or Shp-2 levels in these primary retinal cultures. If the effect is found to be similar to that observed in Neuro2a and not 293T cells, this will confirm that Shp-2 regulation of Dab1 phosphorylation is dependent on other neuronal factors and proteins. The goal will then be to identify factors that interact with Shp-2 to regulate Dab1 phosphorylation. This could be carried out by Shp-2 co-immunoprecipitation followed by mass spectrometry.

4.2.4 Effect of ELAV Proteins on Dab1 Splicing

ELAV4 has already been shown to increase the expression level of Nova1 by binding to the 3' UTR region of *Nova1* mRNA [232]. ELAV4 is an important neuronal RNA binding protein which is actively involved in neuronal differentiation and development [234]. ELAV may be one of the factors that regulates *Dab1* splicing by regulating the expression levels of Nova1. The expression patterns of the four ELAVs in the brain at different stages of development will be compared to that of Dab1-E, Dab1-L, Nova1 isoform 1

and Nova1 isoform 2. Over-expression and knockdown of *ELAV*s will also help elucidate their effect on Dab1 splicing.

4.2.5 Dab1 and Notch Signaling

As discussed, Dab1-L and Dab1-E have diverse downstream effects. However, the finely-tuned differences in these downstream effects remain poorly understood. Another downstream effector pathway for Dab1 is the Notch signaling pathway. There are different Reelin-dependent and Reelin-independent effects of Dab1 on the Notch signaling pathway [235-237].

Reelin activates Notch-1 by increasing levels of the Notch-1 intracellular domain (NICD) [235]. Binding of Notch to Dab1 is an essential step in Notch-1-mediated activation by Reelin. Keilani *et al*. have shown that NICD co-immunoprecipitates with Dab1 and that the levels of co-immunoprecipitated NICD and Dab1 increase upon treatment with Reelin [235]. These authors also showed that tyrosine phosphorylation of Dab1 is crucial for this interaction [235]. We know that the Reelin-dependent tyrosine phosphorylated form of Dab1 is Dab1-L. However, there is some indication that Notch may interact with Dab1-E as well [235].

Notch regulates progenitor cell potency by preventing premature cell cycle exit and differentiation of the progenitor pool [238-239]. It is interesting to note that the inhibition of Notch signaling in chick retina mimics the Dab1-E knockdown phenotype. Specifically, the level of ganglion cell differentiation is increased by knocking down both Dab1-E and Notch [240]. Hence, it is

possible that Dab1-L and Dab1-E bind to Notch and activate different downstream pathways. For example, Notch binding to Dab1-L may result in radial glial differentiation and axonal growth, whereas binding of Notch to Dab1-E may result in maintenance of the progenitor pool.

It would be interesting to study the interaction of Dab1-L and Dab1-E with the Notch ICD, and to see what effect overexpression or knock-down of Dab1-E or Dab1-L has on Notch ICD levels. Identification of Dab1-E/Notch-specific and Dab1-L/Notch specific interactors could be identified by co-immunoprecipitation followed by mass spectrometry.

4.3 CONCLUSIONS

The *Dab1* gene shows highly coordinated alternative splicing at different stages of neuronal development. Dab1-E like isoforms, expressed at the earlier stages of development, exclude exons 7 and 8 but include exons 9b and 9c. Dab1-L like isoforms, expressed at later stages of development, include exons 7 and 8 but exclude exons 9b and 9c. I have shown that the embryonal carcinoma cell line P19 mimics developmentally-regulated Dab1 splicing as the cells transition from precursors to differentiated neurons. At Day 0 and Day 2 prior to neuronal differentiation, the Dab1 expressed in P19 cells includes exons 9b and 9c. Later on, when P19 cells differentiate into neurons, Dab1 excludes exons 9b and 9c. Therefore, P19 is a good *in vitro* model for studying the splicing of Dab1 and the factors involved with Dab1 splicing.

Nova2 has already been shown to affect Dab1 splicing. Using P19 as well as Neuro2a, a neuroblastoma cell line that expresses properties of early neuronal cells, I show that Nova1, particularly an isoform of Nova1 that excludes exon 4, likely plays a role in Dab1 splicing in concert with Nova2. I also identify additional splicing factors (e.g Fox 1, Fox3) that may also play a role in Dab1 splicing.

Splicing of Dab1 plays an important role in determining its phosphorylation state as exons that are included and excluded contain tyrosine phosphorylation sites that determine the activity of Dab1 and its downstream effects. In addition to splicing, factors such as Src family kinases and Shp-2 phosphatases also play important roles in Dab1 phosphorylation. Shp-2, while known to function as a phosphatase, can also act as an adaptor protein that promotes phosphorylation by recruiting different kinases. In this thesis, I report that Shp-2 can promote phosphorylation of Dab1-L in the context of a neuronal cell environment; however, in non-neuronal cells such as HEK 293T cells, Shp-2 does not phosphorylate Dab1. These results suggest that Shp-2 may recruit neuron cell-specific kinases that play a role in Dab1-L phosphorylation and activity. When both Src and Shp-2 are expressed at elevated levels, Dab1-L remains in an unphosphoryted state in both P19 and HEK 293T cells. This is a key finding as it demonstrates the importance of contextual phosphorylation and dephosphorylation events in the regulation of Dab1 activity.

Dab1 is a crucial component of the Reelin-signaling pathway that plays a key role in determining the different downstream effect and pathways activated by Reelin-signaling at different stages of development. Different phosphorylation states of Dab1 induce different downstream effects which in turn affect the migration of neuronal cells during brain development. This thesis provides further evidence for the importance of maintaining a fine balance in the phosphorylation-dephosphorylation state of Dab1 through different kinases and phosphatases, as well as different splicing factors involved in the splicing of Dab1.

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