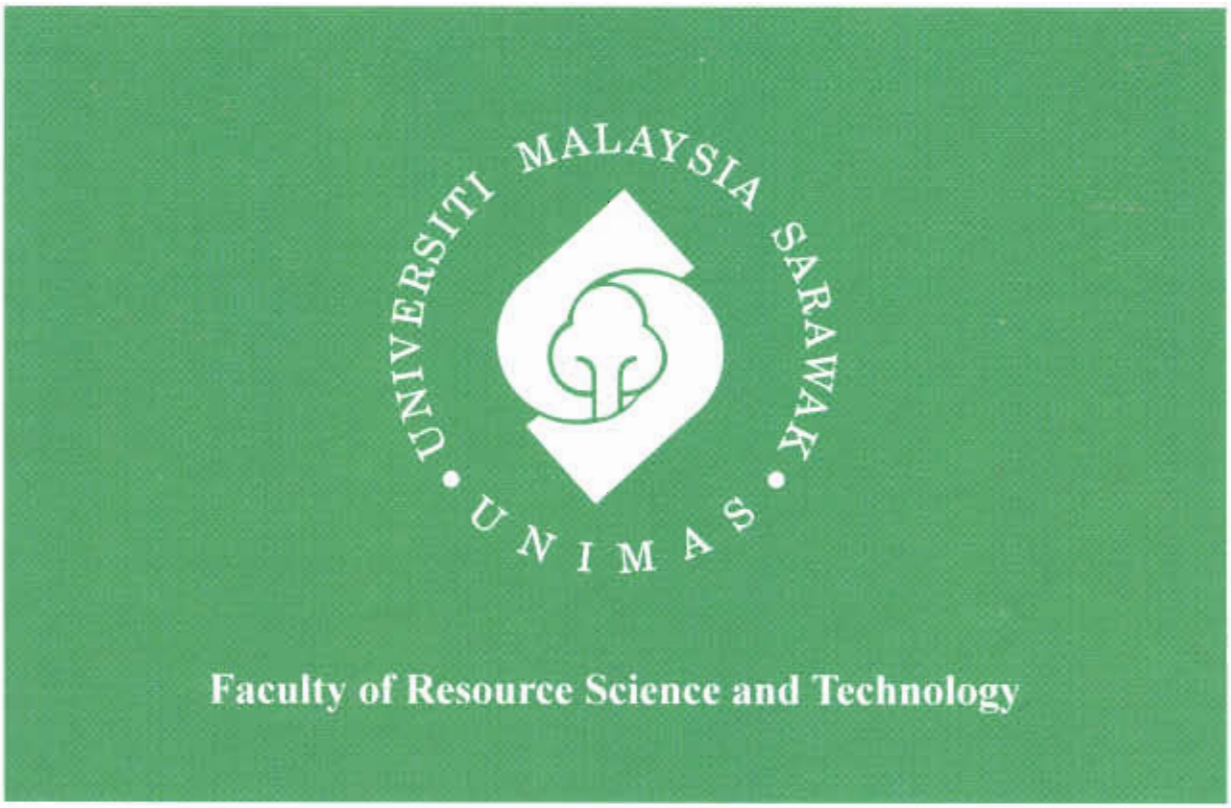

Expression Study of Proline-rich EVH1 Ligand *(Prel)* Family Gene in Zebrafish *(Danio rerio)*

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Master of Science
2017

Expression Study of Proline-rich EVH1 (Prel) Family Gene in Zebrafish (*Danio rerio*)

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A thesis submitted

In fulfillment of the requirements for the degree of Master of Science (Biotechnology)

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2017

DECLARATION

I hereby declare that all the work presented in the thesis entirely my own work. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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Pang Shek Li

Date: 16/10/2017

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ABSTRACT

Proline-rich EVH1 ligand (Prel) family belongs to Mig-10/RIAM/Lamellipodin of adaptor proteins (MRL). They share Pleckstrin homology (PH) and Ras-associating (RA) domain which contains the potential profilin-and Ena/VASP binding sites. Prel1 is involved in integrin activation while Prel2 have significant function in cell signaling, cell migration, promoting axon guidance, affecting adhesion and regulating lamellipodial dynamics. However, only Northern analysis is available of selected adult organs in human and mice and the expression profiles have not been characterized in other animal model. In this project, Prel gene family (Prel1, Prel2a and Prel2b) in zebrafish have been identified and characterized. Prel2 was found to have two copies due to duplication-degeneration-complementation (DDC) effect. RT-PCR analysis showed that Prel family genes commenced after 1K cells stage (3 hour post fertilization (hpf)), where the zygotic transcription starts until 5 day post fertilization (dpf). The expression profiles of Prel genes at 24hpf and 34hpf were established. Expression of Prel2a and Prel2b genes due to the DDC effect, Prel2a and Prel2b complement each other to function as whole Prel2 during the embryogenesis while Prel1 remained widely and diffusely expressed at 24hpf and 34hpf. Prel family members are suggested to be involved in somite development of zebrafish.

Keywords: Prel, MRL, DDC, RT-PCR, clone, expression.

Kajian Ekspresi Keluarga Gen EVH1 Ligan yang kaya Prolina (Prel) dalam Ikan Zebra (Danio rerio)

ABSTRAK

Gen keluarga EVH1 Ligan yang kaya dengan Prolina (Prel) dikategorikan sebagai protein sambungan Mig-10/RIAM/Lamelipodin (MRL). Mereka mempunyai Pleckstrin homologi (PH) dan Ras-asosiat (RA) domain yang berpotensi mengikat profilin-dan Ena/VASP. Prel1 dicadangkan terbabit dalam mengaktifkan integrin, manakala Prel2 berfungsi dalam sel-sel isyarat, penghijrahan sel, mempromosikan panduan axon, menjejaskan pelekatan dan mengawal selia dinamik lamelipodia. Bagaimanapun, hanya Northern analisis terhadap organ dalam manusia dan tikus tersedia dan profil expresi belum dicirikan di modal organisme lain. Dalam kajian ini, keluarga gen Prel (Prel1, Prel2a dan Prel2b) telah dikenalpasti dalam ikan zebra. Prel2 telah didapati dua salinan oleh sebab kesan duplikasi-degenerasi-komplementasi (DDC). RT-PCR analisis menunjukkan gen keluarga Prel bermula 1K sel tempoh sampai 5dpf. Ekspresi profil keluarga gen Prel di 24hpf dan 34hpf telah diwujudkan. Prel2a dan Prel2b telah melengkapkan satu sama lain melalui peringkat perkembangan; manakala Prel1 diekspresi di kawasan somit ikan. Ekspresi tersebut mencadangkan keluarga gen Prel terlibat dalam pembangunan somit dalam embrio ikan zebra.

Kata kunci: *Prel, MRL, DDC, RT-PCR, klon, ekspresi.*

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LIST OF ABBREVIATIONS

RIAM	Rap1-GTP interacting adaptor protein molecule
Prel	Proline-rich Ena/VASP ligand
MRL	Mig-10/RIAM/Lamellipodin
Grb7	Growth Factor Receptor Bound protein 7
RA	Ras-association
PH	pleckstrin-homology
DDC	Duplication-degeneration-complementation
°C	Degree Celsius
cm	centimeter
hpf	Hour post fertilization
ICAM	intercellular adhesion molecule
YSL	yolk syncytial layer
RNA	Ribonucleic aid
DNA	Deoxyribonucleic acid
Pax	Paired box protein
eng	Engrailed
MHB	midbrain-hindbrain boundary
Ena/VASP	Enabled/Vasodilator
EVH	Ena/VASP homology
Arp 2/3	Actin-related protein 2/3
Mena	Mammalian Ena
VASP	vasodilator-stimulated phosphoprotein
VCAM	vascular cell adhesion molecule

pLNs	peripheral lymph nodes
PKC	Protein Kinase C
Evl	Ena/VASP-like
Lpd	Lamellipodin
GM	Grb-Mig
GTP	Guanosine triphosphate
shRNA	Short hairpin RNA
PtdIns(3,4)P_2	phosphatidylinositol(3,4)bisphosphate
WAVE	WASP-family verprolin homologous protein 1
WASP	Wiskott-Aldrich syndrome protein
ADP	Adenosine diphosphate
Cdc	Cell division control protein
PI3K	Phosphoinositide 3-kinase
PTU	1-phenyl-2-thiourea
DEPC	Diethylprocarbonate
mL	mililitres
Tm	Annealing temperature
nmole	Nanomoles
cDNA	Complementary Deoxyribonucleic acid
mM	Millimolar
µM	micromolar
M	Molar
µL	microlitres
pg	Pikograms

mg	Milligrams
ng	Nanograms
DTT	Dithiothreitol
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
$MgCl_2$	Magnesium chloride
$CaCl_2$	Calcium chloride
rpm	rotation per minute
V	Voltage
E. coli	*Escherichia coli*
LB	Luria Broth
OD	Optical density
IPTG	Isopropyl β-D-1-thiogalactopyranoside
X-gal	5-bromo-4-chloro-3-indoly-β-D-galactopyranoside
SOC	Super optimal broth with catabolite repression
NEB	New England Biolabs
NCBI	National center for biotechnology information
BLAST	Basic local alignment search tool
BLASTn	Basic local alignment search tool nucleotide
U	unit
DIG	Digoxigenin
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline with Tween 20
PFA	Paraformaldehyde

SSC	Sodium saline citrate
SSCT	Sodium saline citrate with Tween-20
HM	hybridization mixture
BSA	Albumin bovine serum
MABT	Maleic acid buffer with Tween-20
NBT	Nitro Blue Tetrazolium
BCIP	5-Bromo-4-Chloro-3-indolyl phosphate
MUSCLE	Multiple Sequence Comparison by Log-Expectation
Kb	Kilobases

CHAPTER 1

INTRODUCTION

Activation of cellular integrins is playing key role to most of the physiological events like cell migration, assembly of the extracellular matrix, the immune response, and hemostasis (Lee *et al.*, 2009). Integrins are transmembrane receptors that are bridges for cell adhesion and migration (Takala & Ylänne, 2012). They interact with both signals from intracellular and extracellular and vice versa. The Ras-family of small GTPases are crucial signaling element that control integrin function and mainly thought to play a role in cell proliferation and cell survival (Takala & Ylänne, 2012).

Rap1 is a small GTPase belongs to Ras-family protein which has pronounced role in integrin-dependent cell adhesion and spreading. It has been proved that they regulate the affinity and avidity of integrins in leukocytes and in platelets as well as epithelial cells (Takala & Ylänne, 2012). It is the main GTPase involved in "*inside-out*" signaling pathways (Medraño-Fernandez *et al.*, 2013). In addition the Rap1, the cytoskeleton protein talin is another essential mediator of activation of integrin (Medraño-Fernandez *et al.*, 2013) through binding of talin to the cytoplasmic domain of integrins (Takala & Ylänne, 2012). The activation of this event however is facilitated by Rap1-GTP interacting adaptor protein molecule (RIAM). RIAM also termed as Proline-rich Ena/VASP ligand 1 (Prel1) (noted that in this project RIAM assigned as Prel1 to avoid confusion to the previous nomenclature) which was identified by Lafuente *et al.* (2004).

Prel1 belongs to the Mig-10/RIAM/Lamellipodin (MRL) family of adaptor proteins (Lafuente *et al.*, 2004). Lamellipodin (Lpd also named as Prel2, noted that in this project Lpd assigned as Prel2 to avoid confusion with previous nomenclature) has the highest

homology with Prel1 (Lafuente *et al.*, 2004). The MRL members share similar domain composition, with consecutive Ras-association (RA) and pleckstrin-homology (PH) domain with a more divergent proline-rich C terminus, which contains the potential profilin- and Ena/VASP binding sites (Legg & Machesky, 2004).

The previous studies regarding Prel1 and Prel2 are northern blotting in adult mice organs and knockdown of the gene in mice cell. Northern blot analysis showed that Prel1 is broadly expressed (Lafuente *et al.*, 2004). It is highly expressed in skeletal muscle, heart, liver, bone marrow and expressed moderately in the liver, pancreas, leukocyte and lymph node. Another independent research according to Jenzora *et al.* (2005) showed that western blotting of Prel1 strongly expressed in spleen and thymus, moderately in the brain, heart, lung and weak expression in liver, kidney and stomach. While Prel2 is highly expressed in the brain, heart, ovary and developing embryos (Krause *et al.*, 2004).

Prel1 was proven by Lafuente *et al.* (2004) to act as a link between Rap1-GTP and Profilin either direct binding to Profilin or recruitment of Ena/VASP-Profilin complexes suggested that Prel1 is involved in integrin activation. Besides, Prel1 is also essential for trafficking of lymphocytes from blood into peripheral lymph nodes and bone marrow (Su *et al.*, 2015). The knockdown Prel1 in T cells suggests Prel1 is the principal effector of Rap1 with regard regulating lymphocyte integrins since it is an adaptor molecule that interacts and activates talin (Su *et al.*, 2015). While overexpressed of Prel1 result in induced more cell spread and extension of lamellipodia (Lafuente *et al.*, 2004).

Prel2 negatively regulating adhesion which has the opponent effect compare to Prel1. Instead of binding to Rap1, Prel2 prefer binding to phosphoinositides with its PH domain and lead to localize at the leading edge (Legg & Machesky, 2004). Prel2 was

identified as Ena/VASP binding partner and colocalized exclusively at the tips of filopodia and lamellapodia (Krause *et al.*, 2004). Overexpression produces faster lamellipodia formation however knockdown of Prel2 result in impairment of lamellipodia formation.

According to Postlethwait *et al.* (2004), teleost fish tend to have the partitioning of subfunctions that co-orthologs of human genes due to tetraploidization (whole-genome duplication). Previous studies showed zebrafish often has two orthologs of human genes (Postlethwait *et al.*, 2004). In addition, it is believed that most of the genes in mammals might have the possibility of two homologues in zebrafish that have split the gene's original function between themselves. Due to gene duplication, Prel genes become Duplication-degenerative-complementation model which two genes complement each other by retaining the full set subfunctions present in the original ancestral gene (Force *et al.*, 1999). It brings some disadvantageous to study since two such paralogues have to be isolated and analyzed. However, according to Force *et al.* (1999), the duplicated gene might be preserved by degenerative loss of complementary (DDC) theory. Moreover, the defection or premature of the embryo caused by gene's essential functions might cover the late mammalian gene function (Postlethwait *et al.*, 2000). Thus, the provided benefit that zebrafish might overcome by the splitting of these two functions (Postlethwait *et al.*, 2000). Hence, zebrafish is a model organism to study the evolution of gene duplicates and the role of both genes in the origin of evolutionary innovations.

Zebrafish became popular animal model for scientific studies since it offers few advantages that suit for genetic and development studies (Eisen & Weston, 1993). Zebrafish genome has fully mapped (http://www.sanger.ac.uk/Projects/D_rerio) and it shares significantly similar with human genome including non-coding genome. Approximately 70% of genes associated with diseases in humans have functional

homologs in the zebrafish (Chitramuthu, 2013). Moreover, zebrafish lays 200 to 300 embryos per spawning. The transparency of embryo makes them amenable to follow or observe the organogenesis (Chitramuthu, 2013) and the major organ primordial is formed by 24 hours after fertilization (Kimmel *et al.*, 1995) make *in situ* mRNA hybridization analysis easier to handle. Compare to other animal model, zebrafish is easy and inexpensive to maintain and raise (Chitramuthu, 2013).

Currently the expression data for Prel1 and Prel2 available is only northern blot on adult organs in human and mice according to previous studies. The expression patterns have not been extensively characterized in any embryonic model. In addition, late mammalian gene might be concealed by defects or premature death of embryos caused by gene's essential early function. Hence, zebrafish model with DDC event might overcome this problem by splitting the gene into two (Postlethwait *et al.*, 2000).

1.1 Objectives of This Study

1) To characterize the Prel family gene from different stages of zebrafish embryos.
2) To establish the expression profile of Prel genes spatially and temporally at the embryonic stages.

The presence of two copies of Prel2 genes has helped to prove the occurrence of gene duplication in zebrafish genome. This duplicative event is a key mechanism of evolving novel gene function during evolution. Once the genes are characterized and expression profile is established, the data will be helpful for distinguishing Prel2a and Prel2b functionally.

CHAPTER 2

LITERATURE REVIEW

2.1 Zebrafish (*Danio rerio*)

The zebrafish is a tropical freshwater fish which belongs to the family Cyprinidae of the order teleost (Froese & Pauly, 2015). It is distributed throughout South and Southeast Asia, with the highest species diversity in north-eastern India, Bangladesh and Burma (Barma 1991, cited in Spence *et al.*, 2008). It is native to tributaries of the River Ganges in India and Bangladesh and where the water at temperature between 18°C-24°C. There is approximately 4-6cm of five uniform, pigmented, horizontal, blue strips on an adult fish. The male is torpedo-shaped, with gold stripes between the blue stripes while female has larger, whitish belly and silver stripes instead of gold. It is omnivorous and the food sources are normally zooplankton, phytoplankton, small insects or insect larvae. Zebrafish lifespan in captivity is about 2 to 3 years, although it could be prolonged to 5 years in ideal conditions (Spence *et al.*, 2008).

For the past few decades, wide variety of living organisms such as *Drosophila melanogaster*, *Caenorhabditiselegans*, *Mus musculus*, *Rattus norvegicus* and primates are used for biological studies. However, zebrafish (*Danio rerio*) has come to attention for genetic studies and biomedical research recently as the procedures for genetic and embryological manipulation for zebrafish are much simple and cost for maintenance is cheaper compared to other mammals (Hsu *et al.*, 2007; Spence *et al.*, 2008).

During 1930s, the zebrafish was being used as classical developmental and embryonical model. Early studies drew on the unique combination of the optical clarity of

the embryos and embryological manipulability due to its transparency and hence allowing *in vivo* visualization of cell-biological events (Lieschke & Currie, 2007). The entire embryo cell can remain visible up to early larval stage. Zebrafish genetics techniques in cloning, mutagenesis, transgenesis and mapping approaches were developed through the 1980s.

Generation time of zebrafish is short, it reach adulthood around 3-4 months. It breeds all year round; female can spawn every 2-3 days and a single clutch may contain several hundred eggs. Diameter of its embryo is approximately 0.7mm and is optically transparent, while the yolk is being sequestered into a separate cell. In addition, fertilization is external so live embryos are accessible to manipulation and can be monitored throughout all developmental stages under a dissecting microscope (Kimmel *et al.*, 1995).

Despite of their transparent embryos and rapid organogenesis. zebrafish has been established as a tool for academic developmental biologists during 1970s and 1980s; it is the first vertebrate used for large scale mutagenesis screen which yielding thousands of mutations some of which recapitulated human diseases. There are some reasons why zebrafish is popular for experiment according to Hsu *et al.* (2006). First, the organogenesis of zebrafish is rapid and large numbers of embryos are generated due to high fecundity of zebrafish. Female is able to spawn around 300 eggs per week under ideal conditions. In addition, zebrafish embryo is amenable to gene expression study, examination of specific gene function by transgenic development, antisense gene knockdown, and through large-scale mutagenesis (Hsu *et al.*, 2006).

One of the most important characteristics is the high degree of conservation of genome between human and zebrafish which is approximately 75% of similarity. Therefore, it is considered an ideal organism for studying human diseases. The genome size of human is twice of the zebrafish, however due to gene duplication, it might encode a more number of genes. Gene duplication may diverge but the diverged genes together will fulfill the function of a single mammalian gene; allowing a more detailed dissection of function.

Figure 2.1: Zebrafish (Adapt from http://www.noldus.com/animal-behavior-research/solutions/zebrafish).

2.2 Developmental Stages

As mentioned, one of the advantages of zebrafish embryo is translucent, which allows researchers to observe its development under microscope. In this study, development stages must be understood and observe accordingly. There are seven broad stages of embryogenesis which are zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching periods were defined according to Kimmel *et al.* (1995).

During zygote period, the fertilized egg is approximately 0.7mm in diameter, where the cytoplasm streams towards animal pole to form blastodisc. Based on Kimmel *et al.* (1995), the duration of cleavage period is 0.75 to 2.25 hour post fertilization (hpf).

Transition during this period is from 2-cell stage to 64-cell stage which occurs rapidly and synchronically. The cytoplasmic divisions are meroblastic and horizontal when it reaches the end of the blastodisc to partially cleave the zygote cell from yolk cell.

After cleavage, blastula stage begins with the formation of blastodisc, a sphere of cell sitting atop the yolk at the 18-cell stage until the time of onset gastrulation. During the midblastula transition, the blastodisc which lie against the yolk and remain cytoplasmically connected to it release their cytoplasm and nuclei together into the immediately adjoining cytoplasm of yolk cell forming yolk syncytial layer (YSL). When the formation of YSL started, epiboly begins. The yolk cell is completely surrounded by YSL and blastodisc in the end of gastrulation (Webb & Miller, 2007).

Gastrulation starts onset 50% epiboly until 1-4 somites formation. A thickened marginal region called germ ring appears, serve as the primary germ layers and undergo differential convergence extension movements according to their position along dorso-ventral axis. The formation of germ ring is due to the involution of dorsal marginal cells back upon itself. Thus, there are two germ layers within the germ ring. The upper layer (the epiblast) continues to feed cells into the lower (the hypoblast) throughout gastrulation. The ectoderm correspond to the cells in the epiblast in the end of gastrulation will give rise to such tissues as epidermis, the central nervous system, neural crest, and sensory placodes. While the hypoblast gives rise to both mesoderm and endoderm. Embryonic shield is formed by a marked streaming of cells towards likely dorsal side of the germ ring in both the epiblast and the hypoblast (Webb & Miller, 2007). A narrowing and elongation of the primary embryonic axis occurs as the shield extends toward the animal pole. The aggregation of marginal cells establishes the tail bud, which comprises cells deriving from the dorsal organizer as well as ventral marginal cells in the end of gastrualtion. The dosal

organizer later will form the chordo-neural hinge which differentiated into notochord, hypochord and ventral neural tube (Kimmel *et al.*, 1995).

After epiboly, segmentation period starts with develop of somites and neural tube. The rudiments of the primary organs become visible, the tail bud becomes more prominent and the embryo elongates. Neurulation starts as the neural plate fold into crest and form the three main of parts of the brain which are the forebrain, midbrain and the hindbrain. Somites are developed by the paraxial mesoderm surrounding notochord. The first somite formed anteriorly and posterior one last. The first cells differentiate morphologically and the first body movement occurs (Kimmel *et al.*, 1995). Development of the Kupffer vesicle and the subdivisions of the brain also occur at this stage. Figure 2.2 gives an overview of development of embryo zebrafish from zygote stage to segmentation period.

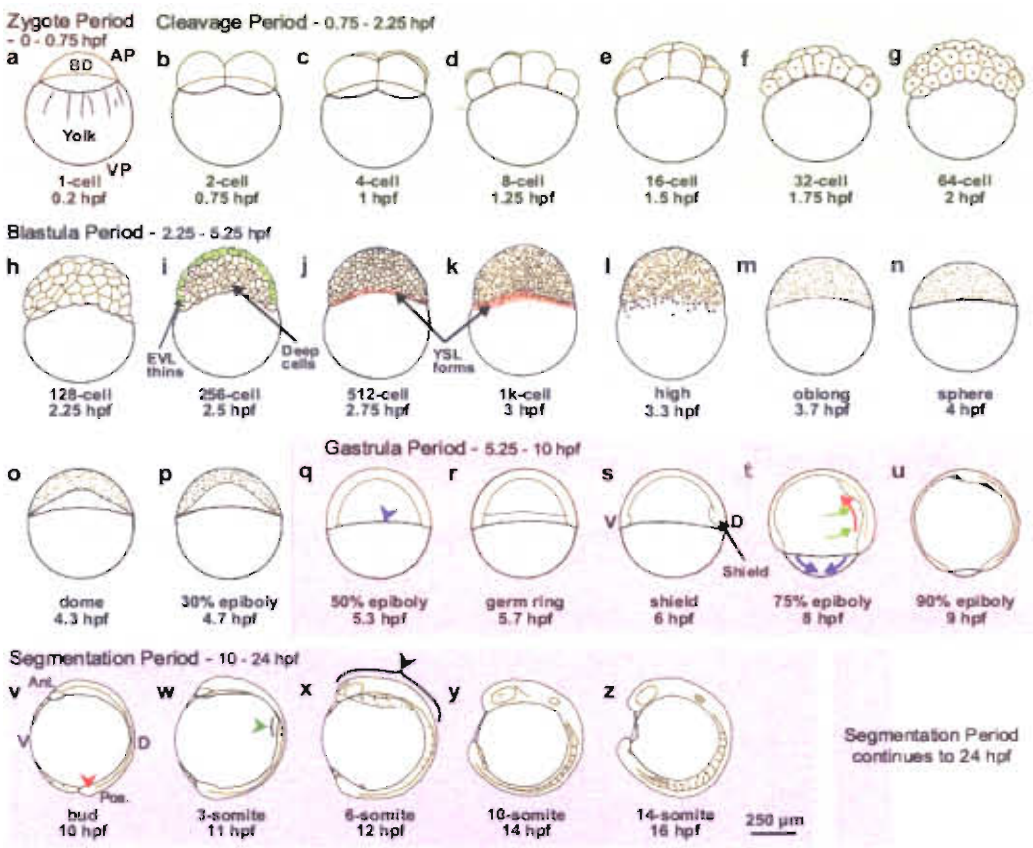

Figure 2.2: First 24 hour post fertilization of embryo zebrafish. Adapted from (http://www.devbio.biology.gatech.edu/?page_id=204)

The transition of pharyngula stage is from Prim-5 (24hpf) to long pec (48hpf). During pharyngula stage, the embryo is now organized bilaterally and a well-developed notochord. New complete set of somites is extended to the end of long post-anal tail. The body axis begins to straighten and the first seven pharyngeal arches develop rapidly. The brain has developed into five distinct lobes. The heart begins to beat at the onset of this stage and circulatory system formed. The movement of the embryo is still uncoordinated because the pectoral fins just begin to develop.

According to Kimmel *et al.* (1995), the time of hatching is not useful as a staging index for embryo zebrafish, because the embryo hatch sporadically during the whole third day of development according to standard temperature (28.5°C). The hatching period transitions from long-pec (48hpf) to protruding mouth (72hpf). During the hatching period, the pectoral fin elongated and each mesenchymal condensation will form the girdle cartilages. Now the circulatory channel appears as a continuous loop at the base of the enlarging fin. Jaws and gilla are also developing along the side of the pectoral fins. In the early stage of the hatching period a small open mouth is developed while a dramatic repositioning of mouth is occurred during the last 12 hours of embryogenesis. In the end of this stage, the mouth is wide open and protrudes anteriorward just beyond the eye. Rapid development of mouth and jaw will continue in early larva. The timeline and stages of embryonic development under microscope is attached in Appendix 1.

2.3 Gene Duplication

A question has been asked for few decades which is how a single-celled bacterium evolved into multi-cellular complex organisms like human (Bergman, 2006; Magadum *et al.*, 2013). The current primary hypothesis to explain this phenomenon is gene duplication. Gene duplication is a major mechanism that generates the new genetic material during the evolution which one gene gives rise two genes cannot be operationally distinguished to each other (Magadum *et al.*, 2013). It is also believed as a primary source of emerging new functions of gene (Ohno, 1970, cited in Lynch & Force, 2000) and play important role in evolution (Magadum *et al.*, 2013).

Gene duplication was first discovered by Bridges (1936) in a mutant fruit fly *Drosophila melanogaster*, which exhibit extreme reduction in eye size when the gene is duplicated from doubling of a chromosomal band (Zhang, 2003). Since then, the importance of gene duplication in supplying raw material to biological evolution has been recognized (Zhang, 2003). During 1970s, Ohno (1970) discussed the fate of duplicated genes in *Evolution by gene duplication* and he concluded that gene duplication is the only way that new functions of gene can arise, and argues that in the past whole genomes were duplicated causing the change from invertebrates to vertebrates, which could occur only if whole genomes were duplicated (Bergman, 2006; cited in Magadum *et al.*, 2013). This idea was considered "outrageous" to scientists at first until the development of molecular biology techniques.

Another research team, Kellis *et al.* (2004) also agreed on that "whole-genome duplication followed by massive gene loss and specialization has long been postulated as a powerful mechanism of evolutionary innovation". One of the most respected Darwinists of

the 20th century, Ernst Mayr, he was agreed on the duplicated gene was paralogous gene. It will have the same function as its sister gene at first. Notwithstanding, the second copy of gene will evolve having its own mutations and it might acquire new functions. The original copy of gene will evolve too and such direct descendants are called orthologues gene (Bergman, 2006).

Gene duplication can occur by unequal crossing over, retroposition or chromosomal duplication (Zhang, 2003) duplicative transposition and including the doubling of the entire chromosome complement i.e. polyploidization (Taylor & Raes, 2005; Magadum *et al.*, 2013,). Unequal crossing over could contain part of the gene, an entire gene, or several genes depending on the region of crossing over. It produces tandem repeated sequences. It is different from retroposition which result in the region lack of introns and regulatory sequences, presence of poly-A sequence and presence of flanking short direct repeats. It event occurred due to when a message RNA (mRNA) is retrotranscribed to complementary DNA (cDNA) and then inserted back into genome (Zhang, 2003; Magadum *et al.*, 2013). Duplicative transposition involves duplication and movement of DNA fragment within the genome which ranging in size from 1 to more than 200 kilobases created by duplicative transposition. Human DNA fragments created by duplicative transposition were identified by using fluorescence *in situ* hybridization (FISH) and bioinformatics tools. Those DNA fragments have been found contain repetitive DNA, portions of genes (intron-exon structures), and complete genes (Taylor & Raes, 2005). Polyploidization is the fourth major mechanism of formation duplicated genes. It is the multiplication two or more genomes are brought together into the same nucleus. The chromosome doubling is occurred after hybridization and it is normally occurred in plant.

2.3.1 Duplication-Degeneration-Complementation (DDC) model

The theory proposed by Ohno (1970) that one of the duplicated genes usually degenerates within a few millions years while another copy of gene will retain the original function (Force *et al.*, 1999; Magadum *et al.*, 2013) made a huge impact on evolutionary research. However, the empirical data shows that the rate of gene duplication is much greater than predicted by the classical model. There are three observations according to Force *et al.* (1999) that the classical theory was contradicted to the actual event. The numerous cases are much higher compared to the predicted one. Second, with the action of purifying selection on both copies of duplicate genes, the nucleotide substitution pattern is consistent. The segregation of null alleles in survived populations is relatively insignificant for loci that have avoided non-functionalization in both duplicate genes (Ferris & Whitt, 1978). In addition, positive selection must be offsetting the null alleles for high degree of duplicate-gene preservation, which supported by the observation of rates of accumulation of expressed mutations in both copies of genes are less than the neutral expectation (Lynch & Force, 1999).

In addition, the previous proposed fate of duplicate gene may be an overly simplistic view of the gene structure. Singular function of gene might fit in the classical theory, but the genes often have several functions, each of them are controlled by different DNA regulatory elements (Piatigorsky & Wistow 1991; Hughes 1994; Kirchhamer *et al.*, 1996; Arnone & Davidson, 1997, cited in Force *et al.*, 1998). The justification was done by showing the spatial and temporal partitioning of expression pattern for gene duplicates in a wide variety of species (Force *et al.*, 1999).

The current theory suggests that there are three outcomes result from gene duplication event. Non-functionalization which is one copy of gene may become silenced by degenerative mutations. Another outcome would be one copy may require a novel, beneficial function and become preserved by natural selection, with another copy retain the original function (neo-functionalization). The third outcome would be both copies become partially compromised by mutation accumulation to the point at which their total capacity is reduced to the level of the single-copy ancestral gene (subfunctionalization) (Lynch & Force, 1999). The model presented below focus on how the subfunctionalization helps with the preservation of duplicate genes in the absence of positive selection, by partitioning the repertoire of gene expression patterns of ancestral alleles.

Duplication-degeneration-complementation (DDC) process is when two chromosomes complementing each other by retaining the full set subfunctions present in the original ancestral gene in order for an organism to remain viable (Force *et al.*, 1999). Gene duplication undergoes two phases under DDC model. Three alternative fates might occur and the first two (non-functionalization and neo-functionalization) of duplication corresponds to classical model's outcome. The third scenario would be subfuntionalization which each duplicate experience reduction or loss of expression for different subfunctions by degenerative mutation. Neofunctionalization and subfunctionalization will undergo random resolution of persisting redundant subfunctions in order to preserve the gene during phase II. The three potential fates of duplicate gene under DDC model is shown in Figure 2.3.

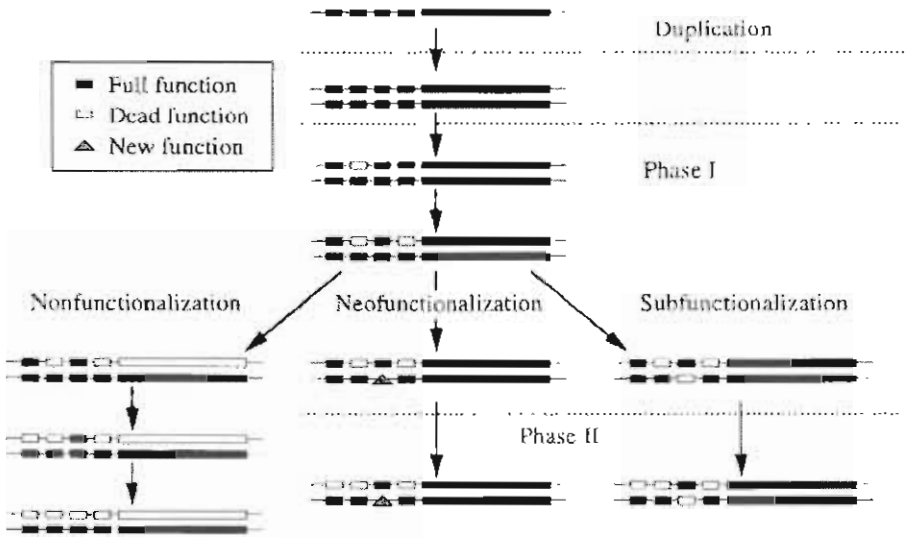

Figure 2.3: The diagram shows three potential fates of duplicate gene pairs with multiple regulatory regions.

According to Detrich *et al.* (2009), zebrafish will often has two genes whieh are both orthologs of a single mammalian gene because during early in zebrafish lineage there was an extra tetraploidizaiton. One of the examples that support the DDC model is *Pax6* locus in mouse and *pax6a* and *pax6b* in zebrafish loci. There is two pax6 were produced by teleost i.e. pax6a and pax6b while there is only Pax6 in mammals (Kleinjan *et al.*, 2008). *Engrailed2a* (*eng2a*) and *eng2b* genes in zebrafish are another example of DDC model. *eng2a* and *eng2b* is a pair of orthologues gene of mammalian *Engrailed* (*En2*). According to Postlethwait *et al.* (2004), *En2* is expressed in somites, anterior hindbrain, jaw muscles, and midbrain-hindbrain boundary (MHB) of a mouse. *Eng2a* and *eng2b* in zebrafish are specifically expressed in the somites and anterior hindbrain, respectively; however, the common expression parts of both organisms are MHB and jaw muscle (Fjose *et al.*, 1992, cited in Kondoh & Kuroiwa, 2014).

The current suggestion is that zebrafish may have two copies of many unique genes in mammals has implications for comparative molecular embryology. The suggestion

concluded that after the isolation and analysis of both zebrafish orthologs of mammalian gene, zebrafish developmental genetics will be more robust when two exist (Postlethwait *et al.*, 2000). In this study, proline-rich EVH1 ligand (*Prel*) family gene is another example of DDC model. Currently there are three prel genes in zebrafish which are *Prel1*, *Prel2a* and *Prel2b*. They are orthologues to mammalian Prel1 and Prel2.

2.4 Proline-rich EVH1 Ligand (Prel) Family Genes

Over more than three decades, the movement of the cells within the tissue is one of the fundamental problems in biology. Eukaryotic cell assemble networks of actin filaments adjacent to the plasma membrane to carry out many fundamental processes such as maintenance morphology; amoeboid locomotion; and cell-cell interaction (Hansen & Mullins, 2015). According to Krause *et al.* (2004), actin cytoskeleton remodeling provides the driving force for cell migration. As far as we know, actin was mean filaments, cables, contraction, motility and so forth. Hence, it is important that to comprehend the signaling pathways that regulate the actin polymerization cycle in living cells at molecular level (Krause *et al.*, 2004).

There are a few actin regulators that control actin polymerization directly such as Enabled/Vasodilator (Ena/VASP) protein family and Actin-related protein 2/3 (Arp 2/3) complex (Legg & Machesky, 2004). The Ena/VASP protein family is associated with adherens-type cell-matrix and cell-cell junctions, microfilaments and highly dynamics membrane regions (Ball *et al.*, 2002). This family protein comprises Mena (mammalian Ena), vasodilator-stimulated phosphoprotein (VASP) and Ena/VASP-like protein (Evl). Ena/VASP interacts with its binding partners via EVH1 motif which is a globular domain

that binds short, proline rich sequences (Hansen & Muslins, 2015). Bacteria effector ActA; the focal adhesion protein Zyxin; an organizer of dorsal stress fibers, paladin; T-cell receptor signaling protein, Fyb/SLAP; axon guidance factors, Robo/Sax-3; and the mig-10/RIAM/Lamellipodin (MRL) protein, which all of them contain tandem repeats of high-affinity EVH1 binding sequence. By recruiting polymerization competent profiling-actin complexes to Ena/VASP proline-rich binding site, the actin filament formation is accelerated (Ball *et al.*, 2000). It is a modulator of cell migration and localize to focal adhesion, stress fibres and the very tips of lamellipodia and filopodia (Jenzora *et al.*, 2004).

Ena/VASP protein family was defined structurally by a proline-rich core domain (Legg & Machesky, 2004). All the Ena/VASP family members shared a conserved domain structure. An N-terminal amino-terminal Ena/VASP homology 1 (EVH1) domain followed by a proline-rich central region and a C-terminal carboxy-terminal Ena/VASP homology 2 (EVH2) domain. According to Krause *et al* (2004), EVH1 domain binds to proteins that containing specific proline-rich consensus site (F/W/Y/L)PPPPX(D/E)(D/E)(D/E)Φ (X = any amino acid, Φ = hydrophobic amino acid) (abbreviated as FPPPP) which is crucial for intracellular signaling or signal transduction in postsynaptic compartments of certain chemical synapses (Ball *et al.*, 2000). While the central proline-rich region providing the binding sites for SH3 and WW domain-containing proteins and for the G-actin binding protein Profilin. The tetramerization of Ena/VASP proteins is mediated by EVH2 domain through a coiled-coil motif and binds to G- actin binding protein Profilin (Krause *et al.*, 2003).

Lamellipodia is regulated by Ena/VASP proteins and Scar/WAVE to activate Arp2/3 complex to nucleate branched actin during lamellipodia formation (Carmona *et al.*, 2016). Lamellipodia is crucial for driving the leading edge of cells and enabling them to

move (Legg & Machesky, 2004). During 2004 in the *issue of Developmental Cell*, Lafuente *et al.* and Krause *et al.* introduce two of the Mig-10/RIAM/Lamellipodin (MRL) protein family that consist membrane-association domains, signaling domains and Ena/VASP-binding sequences and are likely function as adaptors to regulate the actin cytoskeleton (Legg & Machesky, 2004). The two studied protein named Rap1-GTP interacting adaptor molecule (RIAM) and Lamellipodin (Lpd). RIAM is also known as proline-rich EVH1 ligand 1 (Prel1) while Lpd is named as proline-rich EVH1 ligand 2 (Prel2).

Prel1 and Prel2 are two further mammalian orthologues of Mig-10. Database search showed that Prel1 is similar with growth factor receptor bound (Grb) protein family, the Prel2 show the highest homology notwithstanding. Unlike Grb proteins, Prel genes do not direct interaction between small GTPases (Ras or Rho family) (Hansen & Mullins, 2015). Thus, Lafuente and his team concluded that Prel1 is belongs to MRL family after comparing the domain structure between this two protein families (Lafuente *et al.*, 2004). MRL protein family shares some similar structural properties according to Leggs and Machesky. The conserved domains in MRL protein family are pleckstrin-homology (PH) and Ras-association (RA) domain along with more divergent proline-rich C terminus, which contain the potential profilin- and Ena/VASP binding sites (Leggs & Machesky, 2004). In addition, all these three domains contain conserved patch of 27 residues that anticipated forming a coiled-coil region. The figure 2.4 shows the domain composition of MRL family. Human RIAM belongs to MRL family rather than Grb protein family due to proline-rich domain and its function.

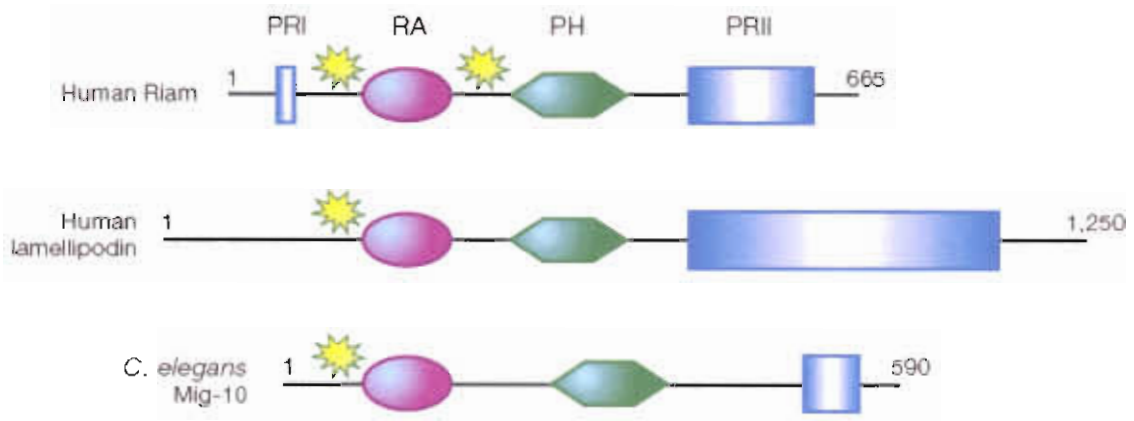

Figure 2.4: Domain composition of MRL (Mig-10/RIAM/Lamellipodin) family protein. The MRL share a conserved domain structure. PR1 and PR2, proline-rich domains: RA, Ras-association domain; PH, pleckstrin-homology domain; yellow star, putative coiled-coil domain.

The RA and PH domains contain approximately 90 and 100 amino acid respectively (Holt and Daly, 2005). The former module was found in variety proteins, some of which are known to be Ras-GTP effectors (Wojcik *et al.*, 1999, cited in Holt & Daly, 2005). PH domain was discovered often bind specifically to phosphoinositides and resulting in localization of the resident protein to a particular membrane compartment of cell (Lemmon, 2004, cited in Holt & Daly, 2005). These two domains embedded in Grb-Mig (GM) region consists approximately 300 amino acid which discovered by Manser *et al.* (1997).

Both C-terminus of Prel1 and Prel2 contain putative binding sites for EVH1 domains, which could bind up to six and four EVH1 molecules respectively (Lafuente *et al.*, 2004; Krause *et al.*, 2004), SH3 domains, and Profilin. Despite that, the C-terminus of Prel2 is 500 amino acids longer than Prel1's while the N terminus of Prel1 can harbor two additional putative EVH1 binding sites and one additional potential coiled-coil domain (Lafuente *et al.*, 2004). Both of the protein was further investigated separately by Krause *et al* (2004) and Lafuente *et al* (2004).

Prel1 was identified by Lafuente and his team through yeast two-hybrid system to search for Rap1-interacting molecules in T cell. With a constitutively active Rap1E63 mutant was used as bait, Rap1 was identified as the potential interacting protein and the protein was named RIAM (Prel1) (Lafuente *et al.*, 2004; Leggs & Machesky, 2004). Prel1 has the functional six binding sites for Profilin and FPPPP motifs respectively. As mentioned earlier, the FPPPP motifs in Prel1 has the potential bind up with Ena/VASP protein, thus it seems to have the potential for cytoskeletal interactions. Later, the research team proceeds to prove that Prel1 could indeed bind to Ena/VASP, both *in vitro* and *in vivo*. The interaction between Prel1 and Ena/VASP and Profilin prompt them to investigate the effect of Prel1 on actin cytoskeleton by overexpression and knockdown experiments.

The overexpressed Prel1 in Jurkat T cells showed Prel1 colocalized with F-actin at the leading edge of cell, consistent with localization of Ena/VASP proteins. There is two effects were observed when Prel1 was overexpressed: the cell spreading of lamellipodia was extended and caused β1 and β2 integrin-mediated adhesion (Lafuente *et al.*, 2004). The further experiment carried out was especially important because authors originally found Prel1 bind to Rap1 based on its ability, which controls by β1 and β2 integrins (Leggs & Machesky, 2004). The small GTPase Rap1 belongs to Ras superfamily which involved in transmitting signals within cells. Rap1 plays a role in cell adhesion and spreading. A study conducted by Ohba *et al.* 2001 showed that fibroblasts deficient for Rap1 display impaired adhesion and migration accelerated. It provides a link between Rap1-GTP and profilin either by direct Prel1 binding to Profilin or by Prel1 recruitment of Ena/VASP-Profilin complexes (Lafuente *et al.*, 2004).

The experiment was proceed further whether Prel1-induced adhesion requires Ena/VASP by disrupting the interaction between Ena/VASP and the proteins have EVH1

binding site and at the same time Prel1 was overexpressed. The results suggested that Prel1 binding Ena/VASP do not require for Prel1-induced adhesion. To further test whether Prel1 is a downstream effector of Rap1, Prel1 was knockdown by using shRNA. The reduction of adhesion showed that Prel1 is required for Rap1-induced adhesion (Lafuente *et al.*, 2004). In a nutshell, the effect on Prel1 on adhesion is likely through Rap1 but not Ena/VASP (Leggs & Machesky, 2004).

Prel2 was identified as an EVH1 ligand through screening the public databases for protein that harboring motifs FPPPP. The study conducted by Krause *et al.* suggests that Prel2 can affect adhesion, however, it is negatively regulating adhesion compare to Prel1 (Leggs & Machesky, 2004). All six EVH1 binding sites of Prel2 were proved that it binds to Ena/VASP *in vitro* and *in vivo* directly by Krause *et al.* (2004).

Not similar to Prel1, RA domain of Prel2 does not seems to bind to Rap1 however it interacted with K-Ras, N-Ras, H-Ras and R-Ras-3 through this region (Leggs & Machesky, 2004). PH domain of Prel2 binds to phosphoinositides. PH domains are phospholipid binding modules that often possess specificity for phosphatidylinositol(3,4)bisphosphate (PtdIns(3,4)P_2) (Krause *et al.*, 2004). Binding of lamellipodin to PtdIns(3,4)P_2 suggest a mechanism by which Ena/VASP proteins localize at leading edge. Prel2 was concentrated and colocalizes with Ena/VASP at the tips of lamellipodia and filopodia, which cone of growth cone filopodia is a structure that plays a key role in guidance signal detection and growth cone motility. The signal seems directly received from phosphoinositides through its PH domain and Ras superfamily proteins through its RA domain to Prel2 (Krause *et al.*, 2004).

The function of Prel2 protein was determined by conducting knockdown and overexpression experiments. Prel2 overexpression formed faster lamellipodial protrusions that turned into ruffles as reported by Krause *et al.* The cells were protruded significantly faster velocity compared to the control sample based on quantification. By neutralized the Ena/VASP function, Krause and his team followed up the dependence of Prel2 to Ena/VASP. Depletion of Ena/VASP, the Ena/VASP protein was artificially recruited to mitochondria (Bear *et al.*, 2002). The velocity of lamellipodia was reduced, which proved that the effect of overexpressed Prel2 could be reversed by blocking Ena/VASP function. The lamellipodia showed a significantly lower velocity of protrusion when Prel2 was knockdown. In addition to the significant lower velocity, the F-actin content in Prel knockdown cells reduced too (Krause *et al.*, 2004).

According to Krause *et al* (2004), overexpression of Prel2 results in increase lamellipodial protrusion velocity but less stable lamellipodia which frequently form nonproductive ruffles. These observations are similar to overexpression of Ena/VASP (Krause *et al.*, 2003). Phenotypes of knockdown Prel2 are slow protrusion of lamellipodial but continue extend for longer times and steadier protrusion. However, Krause *et al.* suggested that Prel2 might regulate other effector of actin cytoskeleton given that the impairment of lamellipodia formation was more severe than phenotype knockdown of Ena/VASP.

Besides overexpression and gene knockdown, northern blot analysis showed that Prel1 is broadly expressed according to Lafuente *et al.* (2004). It is highly expressed in skeletal muscle, heart, liver, bone marrow and expressed moderately in the liver, pancreas, leukocyte and lymph node. Another independent study with western blot data revealed the strong expression of Prel1 in the spleen and thymus, moderate expression in the brain,

heart, lung and weak expression in liver, kidney and stomach (Jenzora *et al.*, 2005). Recently the experiment of gene knockdown Prel1 in mice was conducted by Su *et al.* (2015) and showed that Prel1-deficient mice are viable and fertile; it suggests that there is other gene products could compensate for Prel1 deficiency. Interestingly the study revealed that Prel2 expression was not induced in the spleen or platelets of Prel1-deficient animals (Su *et al.*, 2015).

According to Legg and Machesky, the different functions of Prel1 and Prel2 were due to the binding specificity of RA and PH domains. The RA domain in Prel2 may associated with Ras GTPases while the PH domain preferentially with PtdIns(3,4)P_2 and bind up to four Ena/VASP. Unlike Prel2, RA domain of Prel1 associates with Rap1 rather than Ras, uncharacterized PH domain and several proline-rich motifs could bind up to six Ena/VASP proteins. The following figure shows the differences functions of these two proteins.

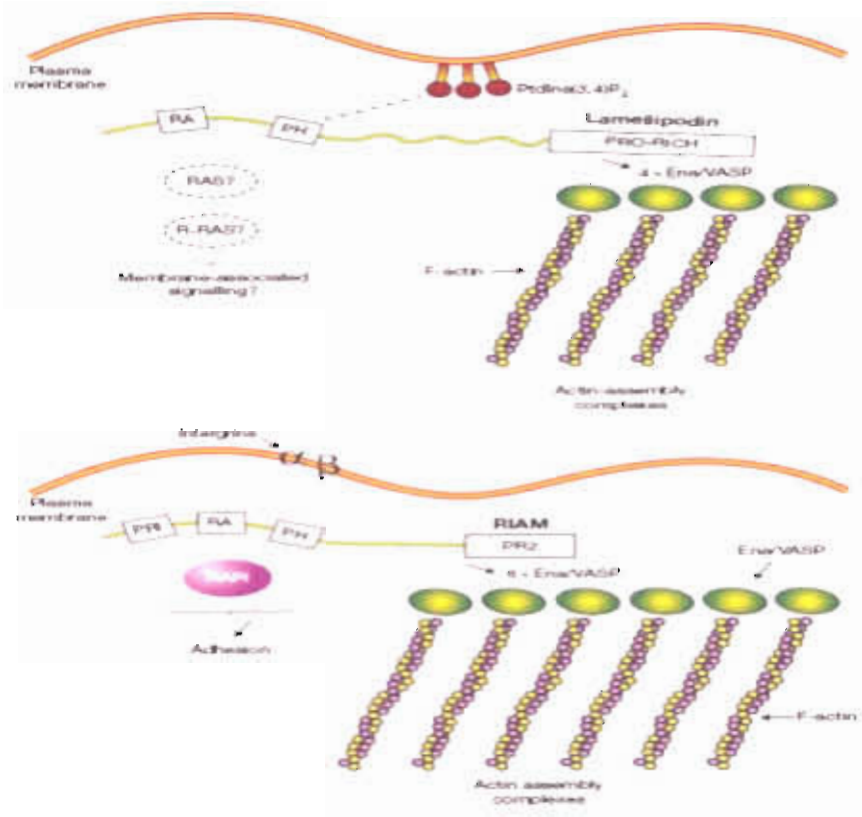

Figure 2.5: Comparison of the domains and putative activities of Prel2 and Prel1 at the plasma membrane. Prel2 seems play key role for Ena/VASP localization in plasma membrane and for the dynamics of lamellipodial actin assembly. However, Prel1 associate with Rap1 mediates β1 and β2 integrin adhesion. Adapted from (http://www.nature.com/ncb/journal/v6/n11/fig_tab/ncb1104-1015_F1.html).

CHAPTER 3

MATERIALS AND METHODS

Figure 3.1 was the procedures have been carried out in this study. The methodology was divided into three parts which including sample preparation, molecular cloning and gene expression.

Sample preparation including maintain the fish stock, breeding, sorting different stages of embryos and fixing the embryo for long term storage. The embryo was prepared for RNA extraction and for whole mount *in situ* hybridization purpose.

Molecular cloning was performed started from RNA extraction from 10 different stages of embryo and then cloned the targeted genes into plasmid in order to multiply the number of copies of genes. The plasmids were cut into the proper orientation for ripoprobe synthesis purpose. Once the probes were synthesized, the expression of the genes spatially and temporally was performed by using whole mount *in situ* hybridization. Before start, the phylogeny analysis was done by constructing a phylogeny tree to find the relatedness between the homology genes from different species.

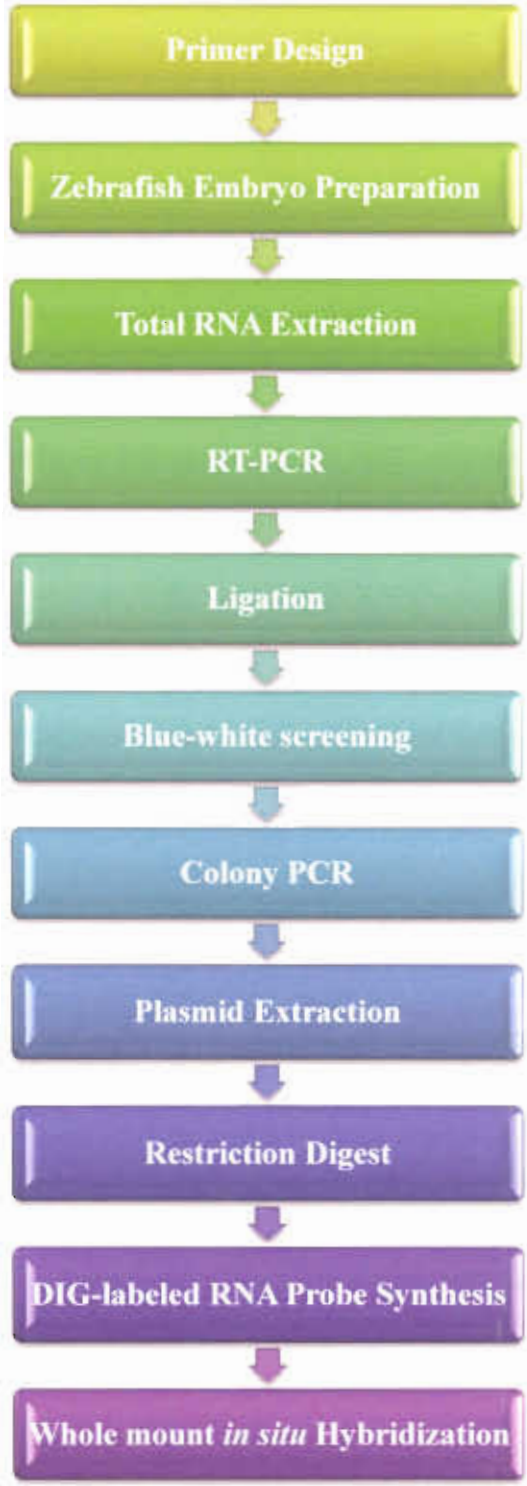

Figure 3.1: Flow chart of methodology

3.1 Phylogenetic Analysis

3.1.1 Retrieving DNA Sequences

To perform phylogenetic analysis, the two main databases used to retrieve the sequences are National Center for Biotechnology Information (NCBI) (hhtp://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org). The peptides of orthologues of interest were employed as starting sequences for extensive BLAST searches in NCBI and Ensembl databases.

3.1.2 Multiple Sequence Alignment and Phylogeny Tree Construction

To understand the relationships of zebrafish Prel family genes to other organism, a phylogenetic tree construction was performed by retrieving sequences from different species of Prel family genes. The sequences retrieved from NCBI and Ensembl search engine were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (http://www.ebi.ac.uk/Tools/msa/muscle/). MUSCLE is a public domain, multiple sequence alignment software for protein and nucleotide sequence. After that, the highest sequence identity to the mammalian orthologues was then further processed and presented with MrBayes 3.2.2 (http://mrbayes.sourceforge.net).

3.2 Sample Preparation

3.2.1 Fish Stock

The zebrafish were obtained from Animal Biotechnology Laboratory, under Molecular Biology Department, Universiti Malaysia Sarawak. The zebrafish were kept in a circulating system that provides continuous aeration to maintain the water quality. The water temperature was maintained between 26°C to 28°C while the photoperiod conditions were 14:10 (light: dark). The fish tanks were cleaned regularly every week to maintain the healthy aquatic environment. The fishes are fed with dry food twice per day. All the maintenance was followed the standard operation protocol in ZFIN (Westerfield, 2000).

3.2.2 Zebrafish Breeding

According to ZFIN (Westerfield, 2000), the zebrafish were photoperiodic in their breeding, the embryos were produced during morning and sunrise. Thus, the photoperiod in Animal Biotechnology Laboratory is mimicking the natural by using an automatic timer (14 hour light: 10 hour dark) to control the day/night time cycle. The breeding tanks were set up at 4pm to 5pm. Several female and male adults were selected and separated. A pair of zebrafish will put in the breeding tank with the ratio 1:1 or 1:2 (male: female). The breeding tank contained a layer of marbles to prevent the parents to consume the embryos. The eggs were collected by using a sieve. The embryos were then washed with distilled water before they were put into embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $MgCl_2$, with 10^{-5}% methylene blue to inhibit fungal growth). The embryos were then examined under a dissecting microscope (Olympus SZ51, Japan) and the unfertilized or unhealthy eggs were removed. The embryo medium was changed every 24 hours and the

embryos older than 24 hour post fertilization (hpf) were treated with 0.0045% 1-phenyl-2-thiourea (PTU) (Sigma Chemical Co. St Louis, MO, USA) to inhibit melanogenesis (Karlsson *et al.*, 2001) for embryo fixation purpose. Figure 3.2 was the breeding tank of zebrafish in Animal Biotechnology Laboratory.

Figure 3.2: Breeding tank of zebrafish with ratio 1:1 (male: female) in Animal Biotechnology Laboratory.

3.2.3 Embryo Sorting

A total number of 10 different stages of embryos were selected for total RNA isolation. The developmental stages selected for this study were 3hpf, 8hpf, 11hpf, 18hpf, 24hpf, 36hpf, 48hpf, 72hpf, 96hpf and 120hpf. The developmental stages of embryos were selected according to Kimmel *et al.* (1995) and they were observed using dissecting microscope (Olympus SZ51, Japan). The embryos were transferred into a Diethylprocarbonate (DEPC) (Amresco) treated 1.5 mL eppendorf tube respectively (all the tips, tubes and water use for total RNA isolation were treated to prevent RNase contamination prior the experiment). The embryos were homogenized with syringe and stored at -20°C in Tri reagent solution (Molecular Research Center, Inc) for total RNA isolation purpose.

3.3 Molecular Work

3.3.1 Primer Design

The primer pairs were designed by using Primer3 (http://waldo.wi.mit.edu/cgi-bin/primer3.cgi/primer3www.cgi). All suitable primer pairs suggested by Primer3 software were than analyzed for hairpin, palindromes, dimmers, GC content and annealing temperature (Tm) with NetPrimer (http://www.premierbiosoft.com/netprimer/netprimer.html). Primer with a rating at least 85 (out of 100) were selected and obtained from IDT (Integrated DNA Technologies, Singapore), 25 nmole of synthesis, standard desalting.

3.3.2 Total RNA Isolation

The total RNA isolated from ten different stages of zebrafish embryos were 3hpf (1K-cell), 8hpf (75% epiboly), 11hpf (5 somites), 18hpf (18 somites), 24hpf (Prim-5), 36hpf (Prim-25), 48hpf (Long-pec), 72hpf (Protruding mouth), 96hpf and 120hpf (adult) by using Tri reagent® (MRC, Inc, Cincinnati).

Approximately 100 embryos were washed twice with PBS for 5 minutes before they were put in a 1.5 mL eppendorf tube. The excessive PBS was removed and 1 mL of Tri reagent was added. The embryos were homogenized using a gauge and syringe. The mixtures were centrifuged at 12,000 rpm for 10 minutes at 4°C. Supernatant was transferred to a new tube after centrifugation. After that, 200 µL of chloroform was added to the samples. The samples were shaken vigorously for 15 seconds prior to centrifugation at 12,000 rpm for 15 minutes at 4°C. The samples were allowed to sit for 5 minutes to

permit the complete dissociation nucleoprotein complexes. The upper aqueous phase was transferred into new tube and precipitated by adding 500 µL of ice cold isopropanol to each sample and allowed incubate at room temperature for 10 minutes. The samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C and the supernatant was discarded. To wash the RNA pellet, 70% ethanol was added and centrifuged again at 7,500 rpm for 5 minutes at 4°C. The RNA pellet was air dried for 5 minutes and then resuspended in 15 µL DEPC treated water and stored in -80°C freezer.

3.3.3 Agarose Gel Electrophoresis and Quantification RNA Samples

To visualize the extracted RNA, 0.5 µL of RNA sample was run on a 1% agarose gel at 65V for approximately 25 minutes. 1 µL of the RNA sample was quantified by using NanoDrop 2000c UV spectrophotometer (Thermo Fisher Scientific Inc).

3.3.4 cDNA Synthesis

cDNA synthesis for each stages embryo of RNA was carried out using SuperScript ™ III First-Strand Synthesis System (Invitrogen, Gronigen, Netherlands) according to manufacturer's instruction. The whole process was done on ice unless incubation part. Total volume of 10µL of first-strand cDNA mixture contains 1µg of RNA, 50ng of random hexamers, 10mM dNTPs mixture and DEPC treated water.

The negative and positive controls were done as the same as standard reaction however different sample of RNA and primer according to the manufacturer's protocol as described as Table 3.1.

Table 3.1: Negative and positive control of first-strand cDNA mixture

Component	Positive RT Control (µL)	Negative RT Control (µL)
Diluted total HeLa RNA (100 pg/µL)	1.0	1.0
Oligo$(dT)_{20}$	1.0	1.0
10 mM dNTP mix	1.0	1.0
DEPC-treated water	7.0	7.0
Total Volume	10.0	10.0

The mixture was incubated at 65°C for 5 minutes using thermal cycler (Bio-rad). The mixture was chilled on ice for 5 minutes after incubation. At the same time, reverse transcription reaction aliquots were prepared as stated in Table 3.2.

Table 3.2: cDNA synthesis mixture

Component	Standard reaction	Positive control	Negative control
10X RT Buffer	2.0 µL	2.0 µL	2.0 µL
25 mM $MgCl_2$	4.0 µL	4.0 µL	4.0 µL
0.1 M DTT	2.0 µL	2.0 µL	2.0 µL
RNaseOUT	1.0 µL	1.0 µL	1.0 µL
SuperScript III RT	1.0 µL	1.0 µL	-
DEPC-treated water	-	-	1.0 µL
Total Volume	10.0 µL	10.0 µL	10.0 µL

Table 3.2 shows cDNA synthesis mixture of standard, positive and negative reaction mixture component. The incubated mixture was mixed with the cDNA synthesis mixture and centrifuges briefly before incubate at 25°C for 10 minutes and followed by 50°C for 50 minutes. While the control reactions were incubated at 50°C for 50 minutes due to different

usage of primer. For termination of reaction, the reactions were incubated at 85°C for 5 minutes. 1 µL of RNase H was added to each reaction and then incubated at 37°C for 20 minutes. The cDNA synthesis reactions were stored at -20°C for PCR purpose.

3.3.5 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To amplify the targeted fragment, PCR was carried out in a total 25 µL per reaction. A total 25 µL of PCR master mix was prepared as 1X Green GoTaq® Flexi Buffer, 1.5 mM $MgCl_2$, 1.25 mM dNTP mixture, 1µM forward and reverse primer set, 2 µg cDNA, 1U Taq Polymerase and nuclease free water.

The positive control was prepared separately with beta-actin primer. Table 3.3 shows that primer sequence and annealing temperature of each primer set for PCR.

Table 3.3: List of primer pairs designed for RT-PCR.

Gene Name	Primer Sequence	Annealing temperature
Prel1	F 5' AAA GCT GGT GGT GAA AGT GG 3' R 5' TTG AGC ATG AAG CAG TGG TC 3'	56 °C
Prel2a	F 5' GCG AAG GCT GAG AAA ATA CG 3' R 5' TTC TGG ATC TGA GGG TGC TT 3'	59 °C
Prel2b	F 5' TGG TGG ATG AAA GAC AGA CG 3' R 5' GCT TCC CAT ACT TGG CAA TG 3'	61 °C
β-actin	F 5' TGC CCA TCT ACG AGG GTT AC 3' R 5' CTC GTG GAT ACC GCA AGA TT 3'	60 °C

The PCR programmed as: initial template denaturation at 95 °C for 2 minutes, followed by 34 PCR cycles consisting of denaturation at 94 °C for 30 seconds, annealing step 45 seconds at certain annealing temperature depend on different primer pairs and final

extension at 72 °C for 5 minutes. To check the existence of targeted genes, the PCR products were run on 1% of agarose gel at 90 V for 45 minutes.

3.3.6 PCR Product Purification

The 10 different stages of PCR products from different primer pairs were purified by using Promega Wizard® SV Gel and PCR Clean-up System kit (Promega, Madison, USA) according manufacturer's instruction. The gel was excised from 0.7% agarose gel after electrophoresis. 10 µL membrane binding solution per 10 mg of gel slice was added. The excised gel was melted at 60 °C until it was completely dissolved. The dissolved gel was transferred to the assembled minicolumn with collection tube. The tube was centrifuged at 12,000 rpm for 1 minute at room temperature. 700 µL of membrane wash solution was added after the supernatant was discarded. The tube was centrifuged again at 12, 000 rpm for 1 minute. After that this step repeated again with 500 µL membrane wash solution was added. The empty collection tube with open lid was centrifuged again to allow evaporation of any residue ethanol. The minicoumn was transferred to a new sterile eppendorf tube and 50 µL of pre-warm nuclease-free water was added. To elude the purified product, the tube was centrifuged at 12,000 rpm for 1 minute after incubate at room temperature for 1 minute. The final product was visualized by using agarose gel electrophoresis at 90 V for 45 minutes. The products were sent for direct sequencing.

3.3.7 Ligation of Purified PCR Product

Purified RT-PCR products were used as DNA fragments for gene cloning and pGEMT-Easy Vector System (Promega, Madison, USA) was use to clone the targeted genes. About 25 ng of purified PCR product was ligated into 50 ng pGEMT-Easy vector. Ligation reactions were set up as stated in Table 3.4. After the mixtures were set up, the reactions were incubated at 4 °C for overnight. The reaction mixtures were transformed into competent cells later on.

Table 3.4: Ligation reaction set up component and amount.

Component	Standard reaction (μL)	Positive control (μL)	Negative control (μL)
2X Rapid Ligation Buffer, T4 DNA Ligase	5	5	5
pGEMT-Easy Vector (50ng/μL)	1	1	1
Purified PCR Product	2 (13 ng/μL)	-	-
Control insert DNA	-	2	-
T4 DNA Ligase (3 Weiss units/uL)	1	1	1
Nuclease-free water	1	1	3
Total	10	10	10

3.3.8 Competent Cell Preparation

The competent cell preparation protocol is adapted from Sambrook *et al.* (1989).

The JM109 *Escherichia coli* (*E. coli*) from glycerol stock was took out and plate on Luria Broth (LB) agar plate and incubated for overnight. A few define colonies from LB agar plate was inoculated in 50 mL LB; the culture was incubated at 37 °C and shake for

overnight. About 2 mL of the culture was transferred into fresh 50 mL LB medium and shake until the OD_{600} became 0.4 - 0.6 and immediately the flask were put on ice for half an hour. To pellet the cells, the culture was centrifuged at 6,000rpm for 15 minutes at 4°C. The supernatant tube was stand inverted for 1 minute to allow the last traces of fluid to drain away. The pellet was resuspended in 2 mL of ice-cold 0.1 M $CaCl_2$ and 25% sterile glycerol. Approximately 200 µL of each suspension of competent cell was transferred into sterile 1.5 mL eppendorf tubes. All the tubes were snap freezed in liquid nitrogen and stored in -80°C freezer.

3.3.9 Transformation of JM109 High-Efficiency Competent Cells

LB/Ampicilin/IPTG/X-Gal (Ampicilin 100µg/ml, 0.2mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), and 20µg/mL 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (X-gal)) plates were prepared for blue-white screening purpose. JM109 *E. coli* competent cells were took out from freezer and thawed on ice for 5 minutes. To start with the transformation, 2 µL of each ligation reaction was aliquot into new sterile tube and put on ice. Each of the aliquot ligation reaction was mixed with 50 µL of the competent cell. The mixture was gently flicked and incubated on ice for 20 minutes. The mixture was heat-shocked for 45 seconds in water bath at 42 °C and immediately put the tubes on ice for 2 minutes. The 50 µL of the transformed cells was transferred carefully into Falcon tube with 950 µL of SOC medium. The culture was incubated in incubator at 37 °C for 2 hours. 100 µL of each transformation culture were plated onto LB/Ampicilin/IPTG/X-Gal plates. The plates were incubated overnight at 37°C and the colonies were checked using colony PCR method.

3.3.10 Colony PCR

A few white colonies were picked up from each of the transformation plates by using sterile tip and inoculated into LB medium. 1 µL of the medium was pipette and put into sterile 0.2 mL PCR tube. Colony PCR was carried out and one reaction consist of 5 µL of 5X GoTaq Flexi PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mixture, 1 µM of forward and reverse primer, 1 unit of Taq polymerase and sterile water (Promega, Madison, USA). The PCR cycle was performed as initial template denaturation at 95 °C for 2 minutes, followed by 34 PCR cycles consisting of denaturation at 94 °C for 30 seconds, annealing step 45 seconds at certain annealing temperature depend on different primer pairs and final extension at 72 °C for 5 minutes. To check the existence of targeted genes, the PCR products were run on 1% of agarose gel at 90 V for 45 minutes. The LB with colony was incubated at 37 °C, shaking at 130 rpm for overnight for plasmic extraction purpose.

3.3.11 Plasmid Extraction using Alkaline Lysis

Plasmid extraction was carried out according to Sambrook *et al.* (1989) protocol. The culture was transferred into 1.5 mL microfuge tube and centrifuged at 12,000 rpm for 30 seconds at 4 °C. The supernatant was discarded and the tube was stand inverted to leave the pellet as dry as possible. The bacterial was resuspended in 100 µL of solution I (resuspend solution) (50 mM glucose, 25 mM Tris-Cl pH8, 10 mM EDTA pH8). Approximately 200 µL freshly made solution II (Lysis buffer consist of 0.2N NaOH and 1% SDS) was added into the concentrated bacteria culture. The mixture was inverted approximately five times gently. The mixture was put on ice for few minutes. After that, 150 µL of ice-cold solution III (Neutralization buffer consists 5 M potassium acetate, 11.5

mL glacial acetic acid and 28.5ml sterile water) was added and inverted about 10 times prior to storage on ice for 5 minutes.

The mixture was centrifuged at 12,000 rpm for 5 minutes at 4 °C and the supernatant was transferred into a fresh tube. To precipitate the DNA, one volume of isopropanol was added and mixed by vortexing. The mixture was allowed to stand for 2 minutes at room temperature. The mixture was centrifuged at 12,000 rpm for 5 minutes at 4 °C. The supernatant was removed and the tube was stand inverted on paper towel to allow all of the fluid drains away. The pellet was washed with ice-cold 70% ethanol and centrifuged at 10,000 rpm for 1 minute at 4°C. The supernatant was discarded as described above. The pellet was air-dried for 10 minutes and resuspended in nuclease-free water and stored at -20 °C. To visualize the extracted plasmid, agaorse gel electrophoresis was performed at 90V, 45 minutes.

3.3.12 Plasmid minipreparation using QIAprep® Spin Miniprep Kit

To get better quality of plasmid, plasmid extraction using QIAprep® Spin Miniprep kit (Qiagen, Netherlands) was performed. To pellet the bacteria, 3 mL of bacterial overnight culture was centrifuged at 12,000 rpm for 3 minutes at room temperature. The bacterial pellet was resuspended in 250 µL buffer P1 (resuspend solution). Buffer P2 (Lysis buffer) was added and mixed thoroughly by inverting 4 to 6 times until the solution become clear. 350 µL buffer N3 (neutralization buffer) was added and mixed immediately by inverting the tube 4 to 6 times. The mixture was centrifuged at 13,000 rpm for 10 minutes at room temperature. The supernatant was transferred to spin column and centrifuged for 1 minute at 12,000rpm. The flow-through was discarded and the spin column was resembled. About

500 µL of PB buffer (recommended if strain JM109 was use) was added and centrifuged for 1 minute at 12,000 rpm. To wash the column, 750 µL buffer PE (washing buffer: absolute ethanol was added prior undergo experiment) was added and centrifuged for 1 minute at 12,000 rpm. To remove the remaining residue, the tube was centrifuged with open-lid for 1 minute at 12,000 rpm. The QIAprep column was then transferred on fresh 1.5 mL microcentrifuge tube. To elute the DNA, 35 µL of pre-warm nuclease-free water was added to the center of the QIAprep spin column, and allowed to stand for 3 minutes at room temperature. Finally, the tube was centrifuged for 1 minute at 13,000 rpm. The final product was stored at -20 °C. To visualize the extracted plasmid, agaorse gel electrophoresis was performed at 90V for 45 minutes.

3.3.13 Restriction Digest

To check for the existence of inserts in the plasmid, restriction digests were carried out. Approximately 3 µL of plasmid DNA was digested with 5 unit of appropriate restriction enzyme. The restriction digest reaction consists of 1 µL of 10X restriction buffer, 3 µL plasmid DNA, 0.5 µL restriction enzyme and topped up with sterile water to a total of 10 µL. The components were mixed and incubated at specific reaction temperature at 37°C for 3 hours. The digested DNA was then separated by agarose gel electrophoresis at 90V at 45 minutes. Most of the enzymes were purchased from NEB (New England Biolabs, Beverly MA, USA) or Promega (Madison, USA). Table 3.5 showed the enzymes and the buffers were used to cut the recombinant plasmid.

Table 3.5: restriction enzyme used to check the presence of targeted inserts in recombinant plasmid.

Gene Name	Enzyme	Buffer
Prel1	*BsaI*	CutSmart®
Prel2a	*Sac I*	Buffer D
Prel2b	*Pst I*	Buffer H

3.3.14 Plasmid Sequencing

Approximately 20 µL of Prel1, Prel2a and Prel2b insert plasmids were sent to 1st BASE Laboratories Sdn Bhd, Selangor for direct sequencing. The sequencing results were analyzed using the NCBI BLAST tool on the World Wide Web (http://www.ncbi.nlm.nih.gov/) to confirm the insert and to identify insert orientation. The recombinant plasmids were further analyzed for linearizing the plasmid using SnapGene Viewer software and NEB cutter v2.0 on internet (http://tools.neb.com/NEBcutter2/index.php).

3.3.15 Linearization Plasmid

After checking the orientation of the insert, approximately 10 µg of plasmid DNA containing the clone of interest was linearized with 50U of an appropriate restriction enzyme (New England Biolabs, Beverly MA, USA). Same as the restriction digest above; the reaction consists 20 µl of 10X restriction buffer, 10 µg plasmid DNA, 50U of an appropriate enzyme and topped up with sterile water to a total 200 µl. The mixture was incubated to appropriate temperature for 7 hours. To terminate the digestion reaction, an

appropriate temperature was applied to the mixture for 15 minutes. To check for the complete digestion, 1 µL of the digestion was loaded on a 1% agarose gel. The remaining sample was purified using QIAquick® Gel Extraction kit (Qiagen, The Netherlands).

3.3.16 Purification of Linearized Plasmid DNA

Absolute ethanol was added into PE buffer prior to the experiment. The DNA was excised from 0.7% agarose gel using a clean scalpel and it was weight. Three volumes of buffer QG was added to the gel (100mg gel equal to 100µL). The tube was incubated at 50°C for 10 minutes until the gel was completely dissolved. The tube was pipetted every 3 minutes to help to dissolve the gel. After the gel was completely dissolved, 1 volume of isopropanol was added and mixed. The mixture was transferred to QIAquick spin column. The sample was centrifuged at 12,000 rpm for 1 minute at room temperature and the flow-through was discarded. Another 500 µL buffer QG was added to the column and centrifuged again at 12,000 rpm for 1 minute. To wash the DNA, 750 µL of PE buffer was added and incubated at room temperature for 1 minute. After that the tube was centrifuged at 13,000 rpm for 1 minute the flow-through was discarded. The QIAquick column was transferred into a clean 1.5 mL microcentrifuge tube and kept at room temperature for 5 minutes. To elute the DNA, 30 µL of pre-warmed nuclease-free water was added and incubated for 3 minutes. Last, the tube was centrifuged at 14,000 rpm for 5 minutes. The purity of the DNA was checked by loading 1 µL of the product on 1% agarose gel. The concentration of the purified products were estimated by compare them to lambda Hind III ladder (New England Biolabs, Beverly MA, USA).

3.3.17 Digoxigenin-labeled RNA Probes Synthesis

The protocol following is adapted from DIG-RNA labeling kit (Roche, Germany). In a total volume of 20 µL reaction, 1 µg of the linearized plasmid was combined with 2µL 20X DIG-NTP labeling mixture, 2 µL 10X transcription buffer, 1 µL RNase inhibitor and 2 µL RNA polymerase and DEPC treated water was topped up to a total volume of 20 µL (Digoxegenin RNA Labeling Kit, Roche, Germany). An appropriate polymerase was used to synthesis antisense RNA probe (Table 3.6). After incubation at 37°C for 2 hours, 1 µL aliquot was stored at -20°C. To eliminate the DNA, 1 µL RNase-free DNase was added to the remaining reaction. To clean the RNA, 5 µL of 4M LiCl and 140 µL of absolute ethanol were added and incubated overnight at -20°C. The next day, the samples were taken out and centrifuged at 14,000 rpm for 15 minutes at 4°C. To wash the RNA pellet, 70% ethanol was added and pelleted by centrifugation at 14,000 rpm for 1 minute at 4°C. The pellet was air-dried for 5 minutes followed by resuspended in 15 µL DEPC treated water; the product was stored at -80°C. 1 µL of the product was taken and compared to the RNA product which aliquot previously without DNase treatment. Table 3.6 shows the chosen enzymes to synthesize DIG-labeled probes. For each gene, the orientation of probe produced is given alongside the appropriate restriction enzyme to linearize the plasmid and corresponding polymerase used for RNA synthesis.

Table 3.6: DIG-labeled probes used for *in situ* hybridization of Prel family gene with a positive control MyoD gene.

Probe Name	Orientation	Restriction Enzyme	RNA Polymerase	Vector
Prel1	Sense	*Nco*I	SP6	pGEMT Easy
	Antisense	*Nde*I	T7	pGEMT Easy
Prel2a	Sense	*Nco*I	SP6	pGEMT Easy
	Antisense	*Sal*I	T7	pGEMT Easy
Prel2b	Sense	*Nco*I	SP6	pGEMT Easy
	Antisense	*Sal*I	T7	pGEMT Easy
MyoD (positive control)	Antisense	*Bam*HI	T7	pBlueScript II SK(+)

3.4 Gene Expression: *In situ* Hybridization on Whole Mount Zebrafish Embryos

Whole mount *in situ* hybridization (WISH) was carried out according to Thisse *et al.* (2008) with some modifications.

3.4.1 Fixation Zebrafish Embryo

After the fishes laid eggs, the eggs were collected and cleaned by embryo medium. The embryos were allowed to grow until the targeted stages were reached. For the embryo older than 24hpf, the embryo medium was changed to Danieau medium (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, and 5 mM HEPES) with 0.0045% of 1-phenyl-2-thiourea (Sigma) in order to prevent melanin pigmentation. Embryos were dechorionated by using sharpened needles before fixing them in 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) (2.7 mM KCl, 137 mM NaCl, 0.01 M Phosphate

buffer (Na_2HPO_4 and KH_2PO_4) (pH7.2)) overnight at 4°C. The next day, the fixed embryos were dehydrated in the order of the following methanol concentration for 5 minutes each: 25% MeOH/75% PBS, 50% MeOH/50% PBS, 75% MeOH/25% PBS, and finally 100% methanol for 15 minutes at room temperature and then stored at -20°C prior proceed with *in situ* hybridization.

3.4.2 Day 1: Pre Hybridization and Hybridization of embryos

The embryos stored at -20°C were rehydrated by washing them in the order of following solutions for 5 minutes each: 75% MeOH/25% PBS, 50% MeOH/50% PBS, 25% MeOH/75% PBS and finally 4X 5 minutes in PBST (0.1% Tween-20 in PBS). To permeablise the 24hpf embryos, Proteinase K (10 µg/mL) was added in PBST and incubated for 10 minutes. Embryos between 30 and 48hpf were digested with same concentration of Proteinase K for 20 minutes and 30 minutes respectively. The embryos older than 48hpf were digested with 10 µg/mL of Proteinase K for 30 minutes. After the digestion, embryos were washed in 2X 5 minutes in PBST. To stop the digestion reaction, the embryos were refixed in 4% PFA in PBS for 20 minutes and washed again 4X 5 minutes in PBST to remove the residue of PFA. The embryos were then transferred into new DEPC treated microcentrifuge tube.

The embryos were pre-hybridized in hybridization mixture (HM) (50% formamide, 5X sodium saline citrate (SSC), 1 M citric acid and 0.1% Tween-20 (pH6.0)) for 4 hours at 68°C. 1 µL of DIG-labeled RNA probe was diluted in 100 µL of hybridization mixture (50 mL hybridization mixture contains 25 mL formamide, 12.5 mL 20X SSC, 0.5 mL 50 mg/mL tRNA, 0.5 mL of 5 mg/mL heparin, 0.46 mL 1 M citric acid and 0.25 mL 20%

Tween-20 and 10.7 mL of DEPC-treated water (pH6.0)). Pre-hybridization mixture was replaced with hybridization mixture with DIG-labeled RNA probes respectively. Then, the embryos were incubated at 68°C for overnight. The HM with probes were saved and re used until the producing weak signals.

3.4.3 Day 2: Washing and Incubation of Embryo with Anti-DIG Antibody Alkaline phosphatase

On day 2, non specific bound probe was eliminated by stringent washing with one quick pre-warmed HM at 68°C followed by 75% HM/ 25% 2X SSCT (SSC with 0.1% Tween-20) at 68°C for 10 minutes, 50% HM/ 50% 2X SSCT at 68°C for 10 minutes, 25% HM/ 75% SSCT at 68°C for 10 minutes and 2X SSCT at 68°C for 10 minutes. After that, the embryos were washed with two highly stringent washes by using 0.2X SSCT for 30 minutes twice at 68°C, followed by 75% 0.2X SSCT/ 25% PBST for 10 min at room temperature, 50% 0.2X SSCT/ 50% PBST for 10 minutes at room temperature, 25% 0.2X SSCT/ 75% PBST for 10 minutes at room temperature and lastly PBST for 10 minutes at room temperature. The embryos were pre-incubated and blocked for 4 hours at room temperature in blocking buffer in MABT containing 2% sheep serum and 2mg/ml albumin bovine serum (BSA) (Sigma, USA). Lastly, the pre-incubated embryos were then incubated with anti-DIG antibody (diluted 1: 5000 in blocking solution) (ABCAM, USA) overnight at 4°C with agitation.

3.4.4 Day 3: Washing and Labeling Reaction

To remove unbound antibodies, the embryos were washed with 7X 15 minutes MABT at room temperature under gentle agitation. Right after that, embryos were washed with 3X 5 minutes at room temperature in alkaline tris buffer which was freshly made (50 mL of alkaline tris buffer consists 5 mL 1 M Tris HCl pH9.5, 2.5 mL 1 M $MgCl_2$, 1 mL 5 M NaCl, 0.5 mL 20% Tween-20, and DEPC-treated water to a total 50 mL). To stain the embryos, approximately 700 µL of labeling mixture (50 mL of labeling mixture contain 225 µL Nitro Blue Tetrazolium (NBT), 175 µL 5-Bromo-4-Chloro-3-indolyl phosphate (BCIP) 49.6 mL alkaline tris buffer) was added in the tube. The reaction was allowed to occur in the dark. The staining reaction was monitored regularly under a dissecting microscope with light on at most 30 minutes for the first 5 hours. The embryos were allowed to stain up to 48 hours. The incubation time depends on the signal of probes. The reaction was stopped by washing the embryos in stop solution (PBST, 1 mM EDTA, pH 5.5) for several times. To wash away the excessive Dig-antibody and non specific product, the embryos were washed with cleaning solution (125 mM Glycine, 0.1% Tween-20) and then absolute ethanol for 5 minutes each case. To improve the optical clarity of stained embryos they were transferred to 50% glycerol/50% PBS for few hours and then 100% glycerol prior inspection under SZ51 dissecting microscope (Olympus, Japan) and DM750 (Leica, Germany). Pictures were captured using camera mounted to the Leica DM750 microscope.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Phylogenetic Analysis

Figure 4.1 shows the phylogenetic tree shows the Prel family members in different species. Numbers at branches given a score based on 250,000 runs. The peptide sequences are retrieved from NCBI genbank (https://www.ncbi.nlm.nih.gov/) and Ensembl (http://asia.ensembl.org/index.html). Gene bank accession number and Enseml Gene ID: Prel1 *Danio rerio* (zebrafish) (NP_956928.1), Prel1 *Xenopus tropicalis* (frog) (ENSXETP00000064069), Prel1 *Tetraodon nigroviridis* (green spotted puffer) (ENSTNIP00000002887), Prel1 *Takifugu rubripes* (fugu) (ENSTRUP00000013765), Prel1 *Rattus norvegicus* (rat) (NP_001094047.1), Prel1 *Pan troglodytes* (chimpanzee) (ENSPTRP00000004018), Prel1 *Oryzias latipes* (medaka) (ENSORLP00000016235), Prel1 *Mus musculus* (mouse) (NP_062329.2), Prel1 *Homo sapiens* (human) (NP_061916.3), Prel1 *Gasterosteus aculeatus* (stickleback) (ENSGACP00000021670) and Prel1 *Bos Taurus* (cow) (NP_001029765.1); Prel2 *Homo sapiens* (human) (AAS82582.1), Prel2 *Macaca mulatta* (monkey) (XP_014966237.1), Prel2 *Pan troglodytes* (chimpanzee) (ENSPTRP00000048840), Prel2 *Rattus norvegicus* (rat) (ENSRNOP00000033491), Mig-10 *Caenorhabditis elegans* (roundworm) (sp|P34400.3), Prel2a *Danio rerio* (zebrafish) (ENSDARP00000082723), Prel2b *Danio rerio* (zebrafish) (ENSDARP00000061155), Prel2a *Gasterosteus aculeatus* (stickleback) (ENSGACP00000019984), Prel2b *Gasterosteus aculeatus* (stickleback) (ENSGACP00000011325), Prel2a *Oryzias latipes* (medaka) (ENSORLP00000006622), Prel2a *Takifugu rubripes* (fugu) (ENSTRUP00000020782), Prel2b *Takifugu rubripes* (fugu) (ENSTRUP00000040327),

Prel2a *Tetraodon nigroviridis* (green spotted puffer) (ENSTNIP00000017549) and Prel2b *Tetraodon nigroviridis* (green spotted puffer) (ENSTNIP00000014487).

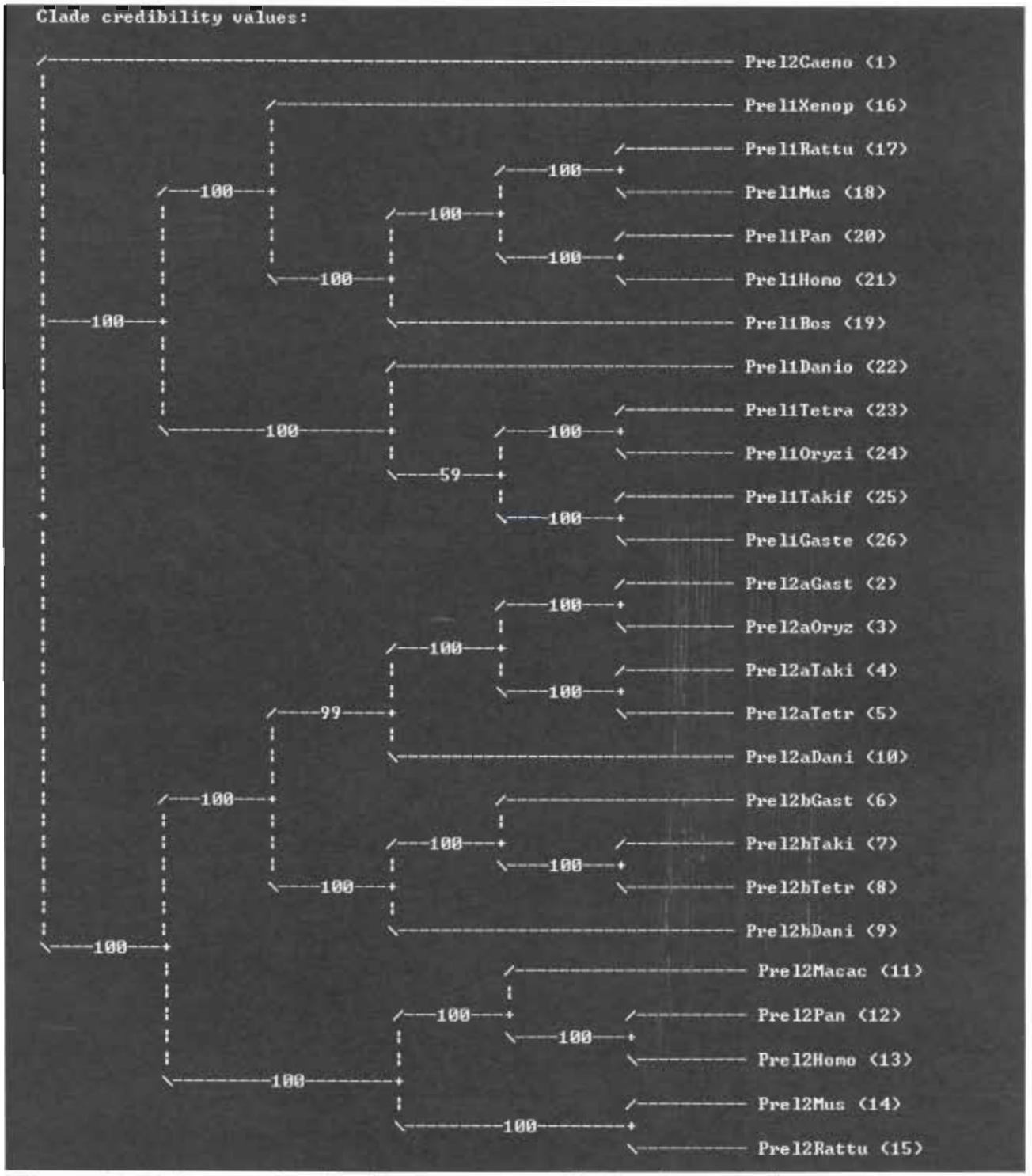

Figure 4.1: Phylogeny of Prel family genes generated by Bayesian analysis.

Figure 4.1 shows the Bayesian inference of the majority rule phylogeny tree based on 26 peptides sequences of Prel family with Bayesian posterior probabilities are accordingly indicated besides the branch nodes. Mig10 gene of *C. elegans* is outgroup between Prel1 and Prel2 genes. Bayesian support value below 95PP is considered as weak and below 90PP considered not supported. As shown in figure 4.1, the tree was divided into three groups including outgroup. The first group is Prel1 sequences and it divided into two branches, one is composed of 6 species of mammals, and another one was 5 species of fishes. While the second group is Prel2 sequences and it divided into two branches too. The first branch consists of 9 species of fishes; another one consists of 5 species of mammals. All clades show high Bayesian support value excluding the branches of *Tetraodon nigroviridis* (green spotted puffer) and *Takifgu rubripes* (fugu). The relatedness of Prel1 and Prel2 were shown in the phylogenetic tree; the results implied that the phylogenetic tree based on the Prel family genes were reliable and generally revealed their evolutionary distance and it shows they are homolog with the other Prel family gene in different species.

4.2 Total RNA Extraction from 10 Different Development Stages of Zebrafish Embryo

Different stages of embryo were sorted for total RNA extraction. The result of RNA extraction shows in Figure 4.2.

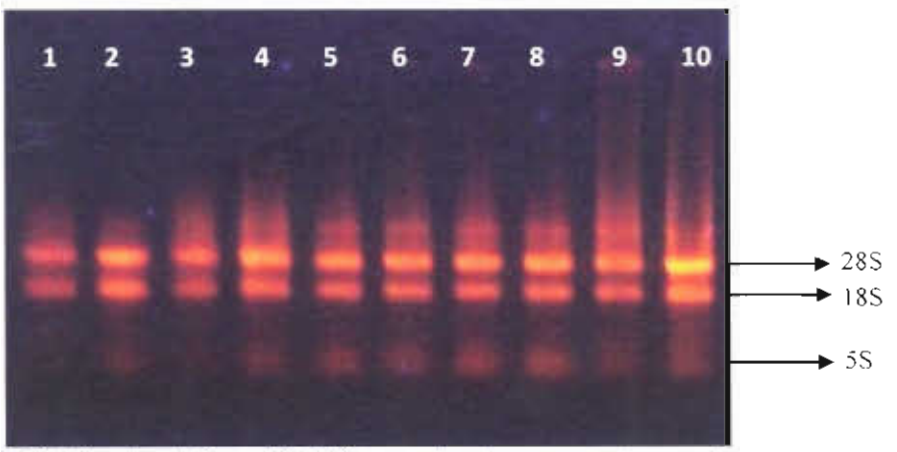

Figure 4.2: Total RNA isolated from 10 different development stages of zebrafish embryo. Lane 1 (3hpf), Lane 2 (8hpf), Lane 3 (11hpf), Lane 4 (18hpf), Lane 5 (24hpf), Lane 6 (36hpf), Lane 7 (48hpf), Lane 8 (72hpf), Lane 9 (96hpf) and Lane 10 (120hpf).

Figure 4.2 shows the RNA product from 10 different development stages of zebrafish embryo. Three distinct bands which represent 28S, 18S and 5S were seen clearly on gel.

4.3 Quantitative RNA

Prior complementary DNA (cDNA) synthesis, the purity and quantity of RNA was checked and the results show in Table 4.1.

Table 4.1: UV Spectrophotometry of RNA samples.

RNA from Different Stages	Concentration (ng/μL)	$A_{260/280}$	$A_{260/230}$
3hpf	1223	1.87	1.92
8hpf	2045	2.04	1.76
11hpf	1992	2.09	1.71
18hpf	2587	2.05	1.89
24hpf	2060	2.17	2.09
36hpf	2489	2.12	2.09
48hpf	2152	2.14	1.73
72hpf	2784	2.14	1.61
96hpf	2205	1.92	2.05
120hpf	2740	2.10	2.17

The RNA purity, concentration and integrity were checked for downstream application which depends upon having high quality and intact RNA. The concentrations of RNA were ranging about 1μg to 3μg per μL (1223ng to 2740ng). According to NanoDrop (Thermo Fisher Scientific, USA), the ratio absorbance at 260nm and 280nm is used to assess the purity of nucleic acid. The extracted RNA was ranging from 1.87 to 2.17. A ratio of A260/280 approximately 2.0 for RNA is considered as "pure" (Thermo Fisher Scientific, USA). There are two of the RNA were lower than 2.0 might indicate the presence of protein, phenol or other substance that absorb strongly at 280nm. As for A260/230 measure secondary purity of nucleic acid. The ratio normally is higher than A260/20 which in the range of 2.0-2.2 is considered pure. Some of the RNA were not reach 2.0 might indicate the presence of contaminants which absorb at 230nm (Thermo Fisher Scientific, USA).

4.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The extracted RNA was converted to cDNA via reverse transcription in order to study it with same molecular approaches used for DNA. Figures 4.3 until 4.10 are the optimized PCR product of Prel genes.

4.4.1 RT-PCR Prel 1

Figure 4.3 to 4.6 shows optimized the RT-PCR product of Prel1, negative and positive controls from 10 different stages of zebrafish embryo.

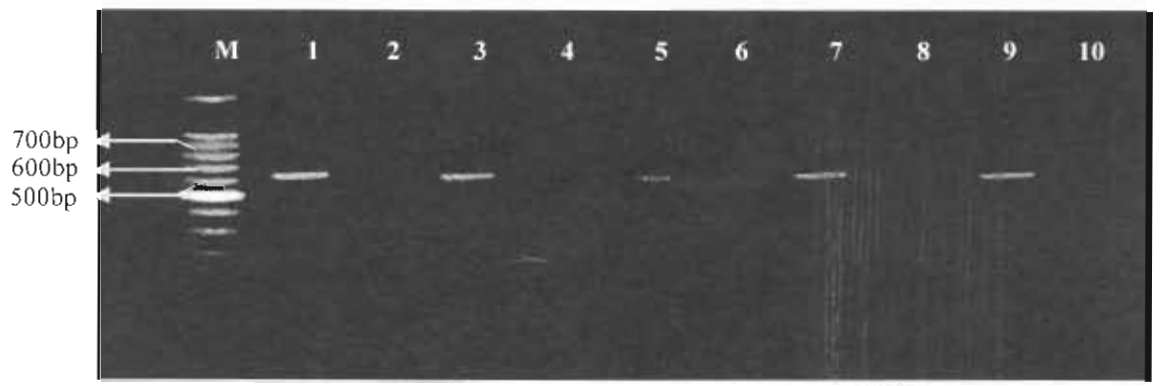

Figure 4.3: RT-PCR Prel1 product from 5 different stages of RNA. Lane M (100bp ladder); Lane 1 (3hpf); Lane 3 (8hpf); Lane 5 (11hpf); Lane 7 (18hpf); Lane 9 (24hpf); Lane 2,4,6,8 and 10 are negative control.

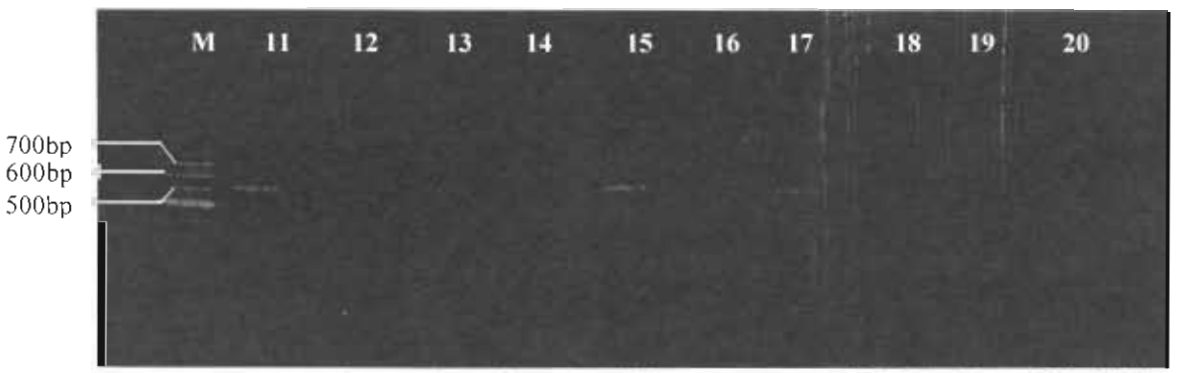

Figure 4.4: RT-PCR Prel1 product from 5 different stages of RNA. Lane M (100bp ladder); Lane 11 (36hpf); Lane 13 (48hpf); Lane 15 (72hpf); Lane 17 (96hpf); Lane 19 (120hpf); Lane 12, 14, 16, 18 and 20 are negative control.

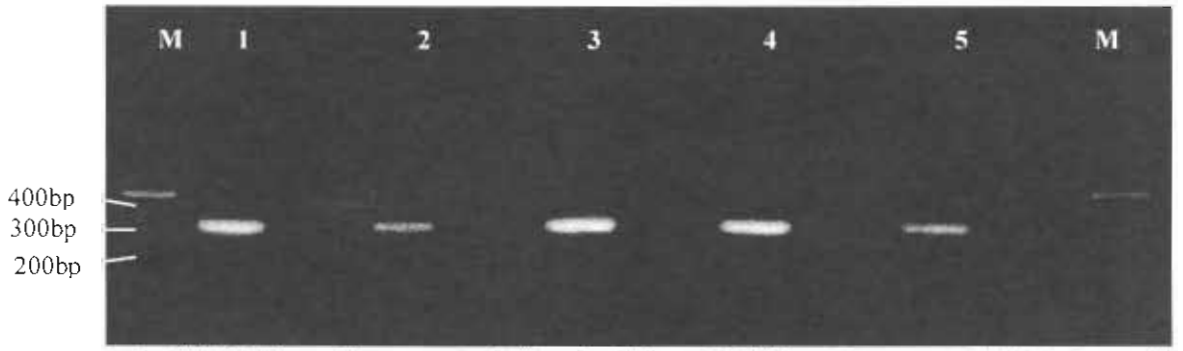

Figure 4.5: RT-PCR positive control (β-actin) from 5 different stages of RNA. Lane M (100bp ladder); Lane 1 to 5 (3hpf, 8hpf, 11hpf, 18hpf and 24hpf).

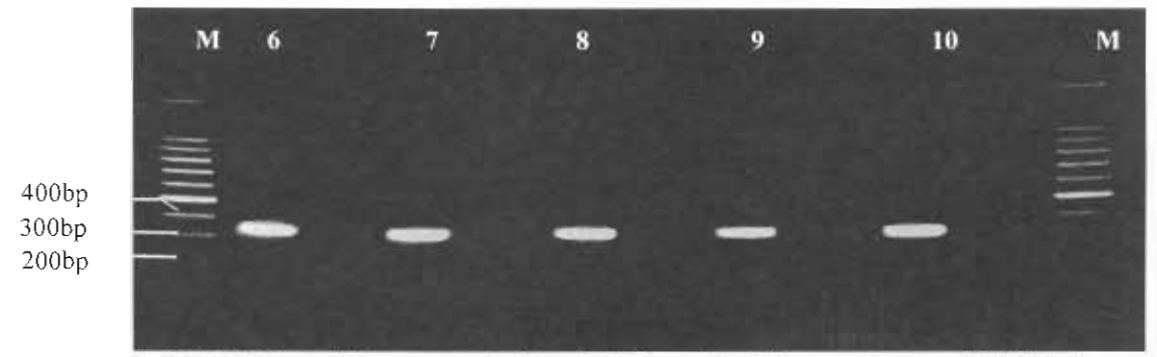

Figure 4.6: RT-PCR positive control (β-actin) from 5 different stages of RNA. Lane M (100bp ladder); Lane 6 to 10 (36hpf, 48hpf, 72hpf, 96hpf and 120hpf).

Figure 4.3, Figure 4.4, Figure 4.5, and Figure 4.6 shows the RT-PCR products of Prel1 and positive control from 10 different stages of zebrafish embryo. To validate the analysis, the negative controls and positive controls of each respective gene correspond to the 10 different development were done. The β-actin gene (positive control) was showed on the Figure 4.5 Figure and 4.6 with expected size which is approximately 300bp. Prel1 gene is present start from 3hpf until 120hpf of the embryo stage (blastula stage, segmentation stage, pharyngula stage, hatching stage and larval stage). It suggests that Prel1 play role along the early development stage. Cell adhesion formation in crucial in orchestrates morphogenesis throughout the embryonic development (Ridley *et al.*, 2003). Large groups of cell migrate collectively as sheet to form the resulting three-layer embryo during gastrulation stage and then those cells migrate from various epithelial layers to target location; they differentiate

to form the specialized tissue (Ridley *et al.*, 2003). Thus, the data from RT-PCR is match with the theory that cell adhesion is important for early development; Prel1 plays an important role in direction finding during cell migration *in vivo* and *in vitro* (Lagarrigue *et al.*, 2015).

4.4.2 RT-PCR Prel 2a

Figure 4.7 and Figure 4.8 shows optimized the RT-PCR product of Prel2a, negative and positive controls from 10 different stages of zebrafish embryo.

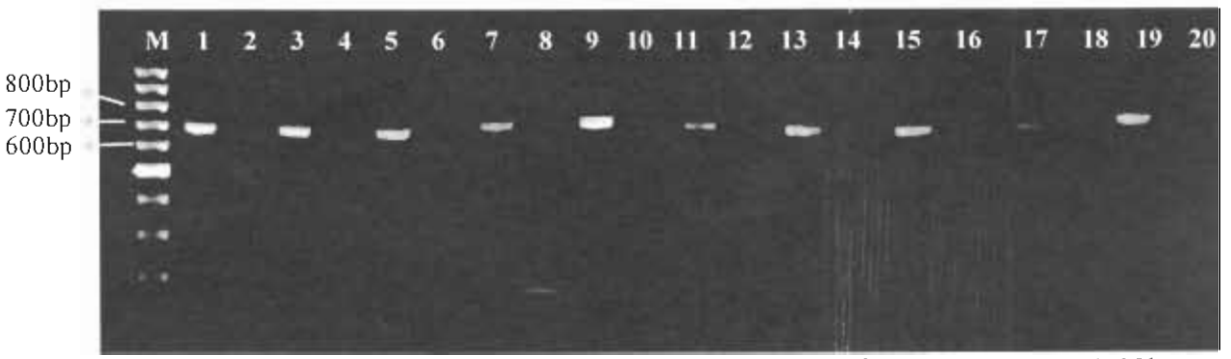

Figure 4.7: RT-PCR Prel2a product from 10 different stages of RNA. Lane M (100bp ladder); Lane 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19 are 3hpf, 8hpf, 11hpf, 18hpf, 24hpf, 36hpf, 48hpf, 72hpf, 96hpf and 120hpf respectively. Lane 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 are negative controls.

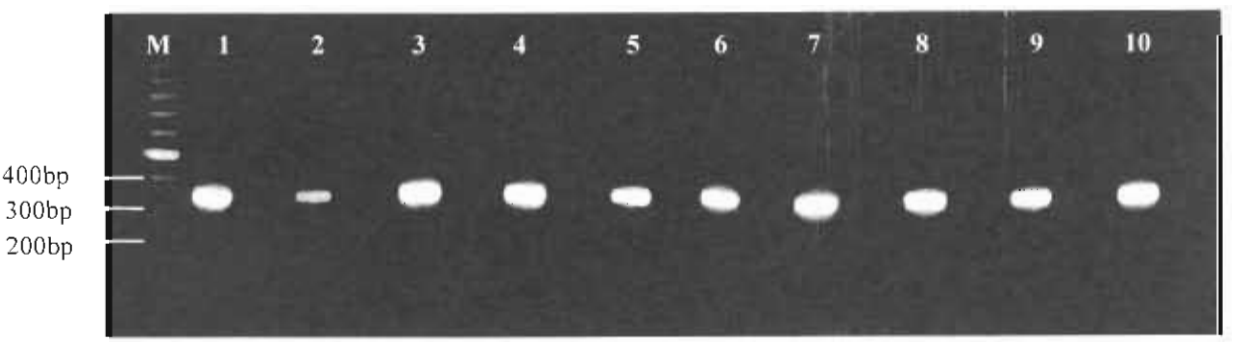

Figure 4.8: RT-PCR product positive control of 10 different stages of RNA. Lane M (100 bp ladder); Lane 1 to 10 (3hpf, 8hpf, 11hpf, 18hpf, 24hpf, 36hpf, 48hpf, 72hpf, 96hpf and 120hpf).

Figure 4.7 shows the RT-PCR product of Prel2a from 10 different stages of embryo zebrafish while Figure 4.8 shows the positive control i.e. β-actin gene from 10 different stages of embryo. The product size of Prel2 is approximately 700bp which is expected size as the primer set was designed. Cell migration is crucial for many processes for example embryonic development, tissue repair, axon extension or path finding and even including metastasis (Lagarrigue *et al.*, 2015). According to Kurosaka and Kashina (2008), cell migration over short and long distances plays a critical role at all stages of development, from gastrulation to the formation of an adult organism which match with the result Figure 4.7, Prel2a appeared along the 10 different stages (from blastula until larval stage).

4.4.3 RT-PCR Prel 2b

Figure 4.9 and Figure 4.10 shows optimized the RT-PCR product of Prel2b, negative and positive controls from 10 different stages of zebrafish embryo.

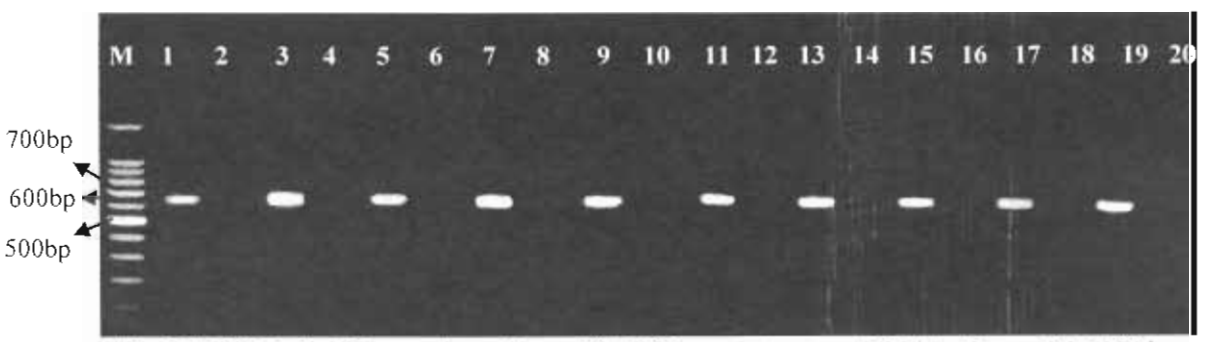

Figure 4.9: RT-PCR Prel2b product from 10 different stages of RNA. Lane M (100bp ladder); Lane 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19 are 3hpf, 8hpf, 11hpf, 18hpf, 24hpf, 36hpf, 48hpf, 72hpf, 96hpf and 120hpf respectively. Lane 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 are negative control.

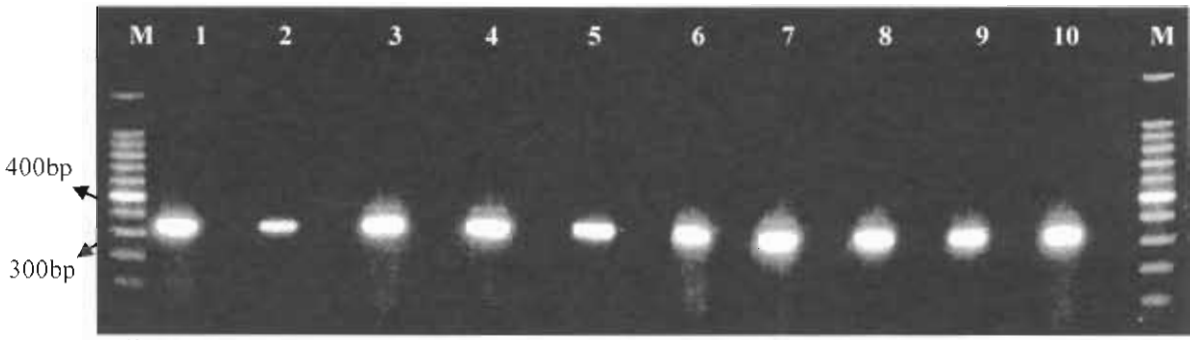

Figure 4.10: RT-PCR positive control of 10 different stages of RNA. Lane M (100 bp ladder); Lane 1 to 10 (3hpf, 8hpf, 11hpf, 18hpf, 24hpf, 36hpf, 48hpf, 72hpf, 96hpf and 120hpf.

Figure 4.9 and Figure 4.10 shows RT-PCR product of Prel2b and positive control gene from 10 different stages of embryo zebrafish respectively. The product size of Prel2b is approximately 650bp as expected. Prel2b and Prel2a are subunits for entire Prel2 which responsible for cell protrusion during development of an organism.

4.5 PCR Purification

The RT-PCR products were purified. Figure 4.11 shows the result of purification.

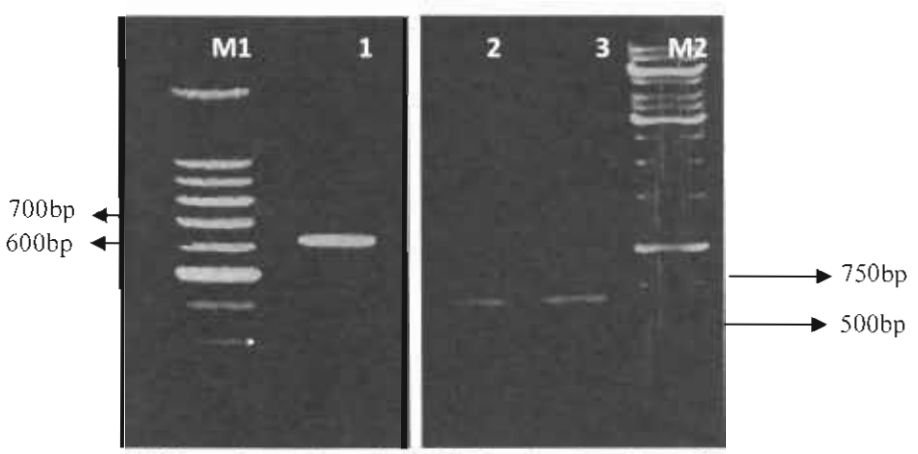

Figure 4.11: Purified PCR product. Lane M1: 100bp ladder; Lane M2: 1Kb ladder; Lane 1: Purified PCR Prel1 product; Lane 2: Purified PCR Prel2b product; Lane 3: Purified PCR Prel2a product.

Figure 4.11 displays the purified product of Prel1, Prel2a and Prel2b. PCR products are purified to remove the remaining dNTPs, primers, Taq polymerase and salt for the downstream purpose. Three genes were successfully purified as distinct purified products are clearly seen on gel picture. Purification step was done to avoid incorporation of primer-dimers or other extraneous reaction products and increase the rate of number of white colonies containing the relevant insert (Promega Corporation, USA).

4.6 Direct Sequencing

The nucleotide sequence search was carried out using BLASTn through the NCBI server and Table 4.2 shows the results.

Table 4.2: the description of the BLASTn result.

Gene	Accession	Description	Query coverage	E-value	Max Identity
Prel 1	NM_200634.1	Danio rerio amyloid (A4) precursor protein-binding, family B, member 1 interacting protein (apbb1ip), mRNA	99%	0.0	99%
Prel 2a	XM_005159957.3	PREDICTED: *Danio rerio* Ras association (RalGDS/AF-6) and pleckstrin homology domains 1b (raph1b), transcript variant X6, mRNA	99%	0.0	99%
Prel 2b	XM_005167508.3	PREDICTED: *Danio rerio* Ras association (RalGDS/AF-6) and pleckstrin homology domains 1a (raph1a), transcript variant X6, mRNA	100%	0.0	99%

BLASTn result shows show 99% of its identity; about 99% to 100% of the query coverage and 0.0 of E-value (expect value). The description of the blastn result also match with the desire product from RT-PCR with 0.0 random background noise. Hence, those products were used for ligation reaction. The blastn match alignment result is shown in Appendix 2 for further information.

4.7 Blue-white Screening

The purified PCR products were cloned into pGEMT Easy vector and plasmid was heatshocked into JM109 *E. coli*. Figure 4.12A-D and Figure 4.13E-J shows the blue-white screening plates of Prel family genes.

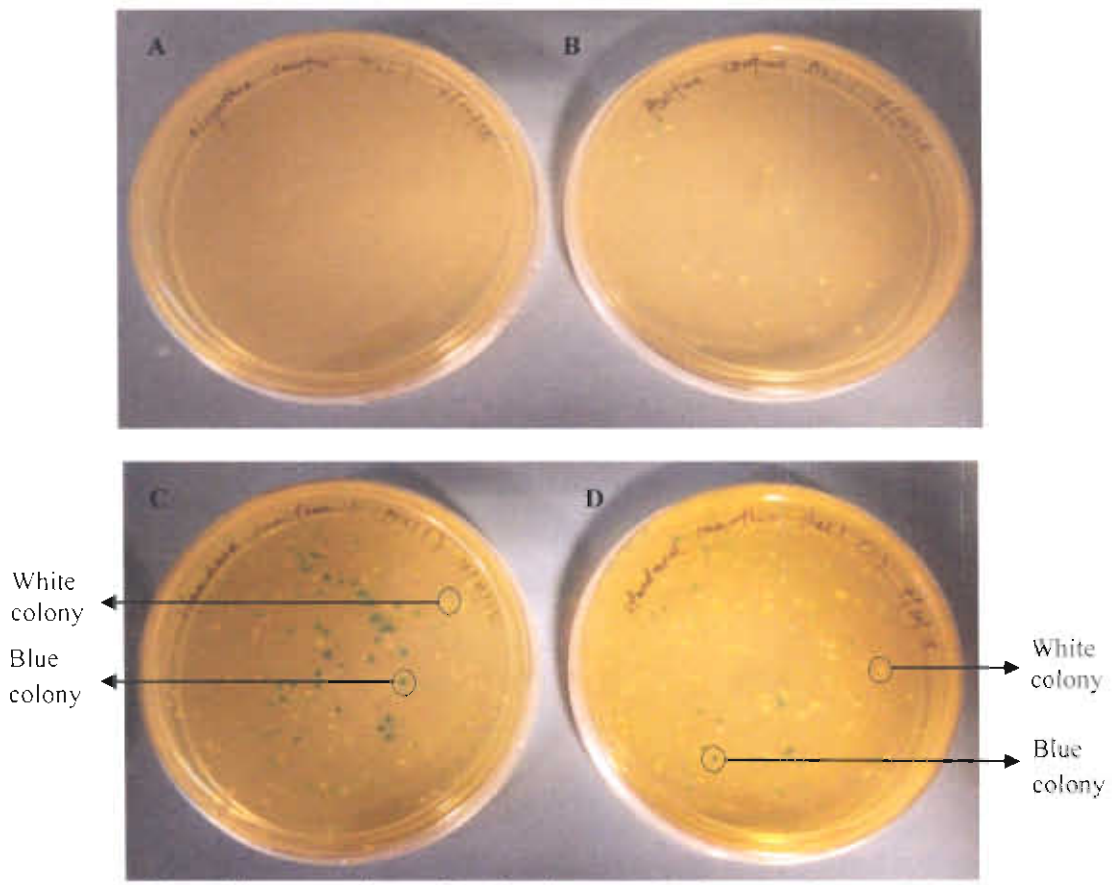

Figure 4.12: Blue-white screening of Prel1 plates A: Background control; B: Positive control; C and D: replicate reaction of Prel1.

Figure 4.12 panel A to D shows the blue white screening plates of Prel1, negative and positive controls. White colonies represent the plasmid ligated with gene of interest while blue is non-recombinant plasmids. Blue color in cells mean the cells contain functional β-galactosidase (which lacZα was not interrupt) while white colonies means the X-gal is not hydrolyzed indicate the lacZα was disrupted. Several white colonies were picked for colony PCR reaction (Sambrook & Russell, 2001).

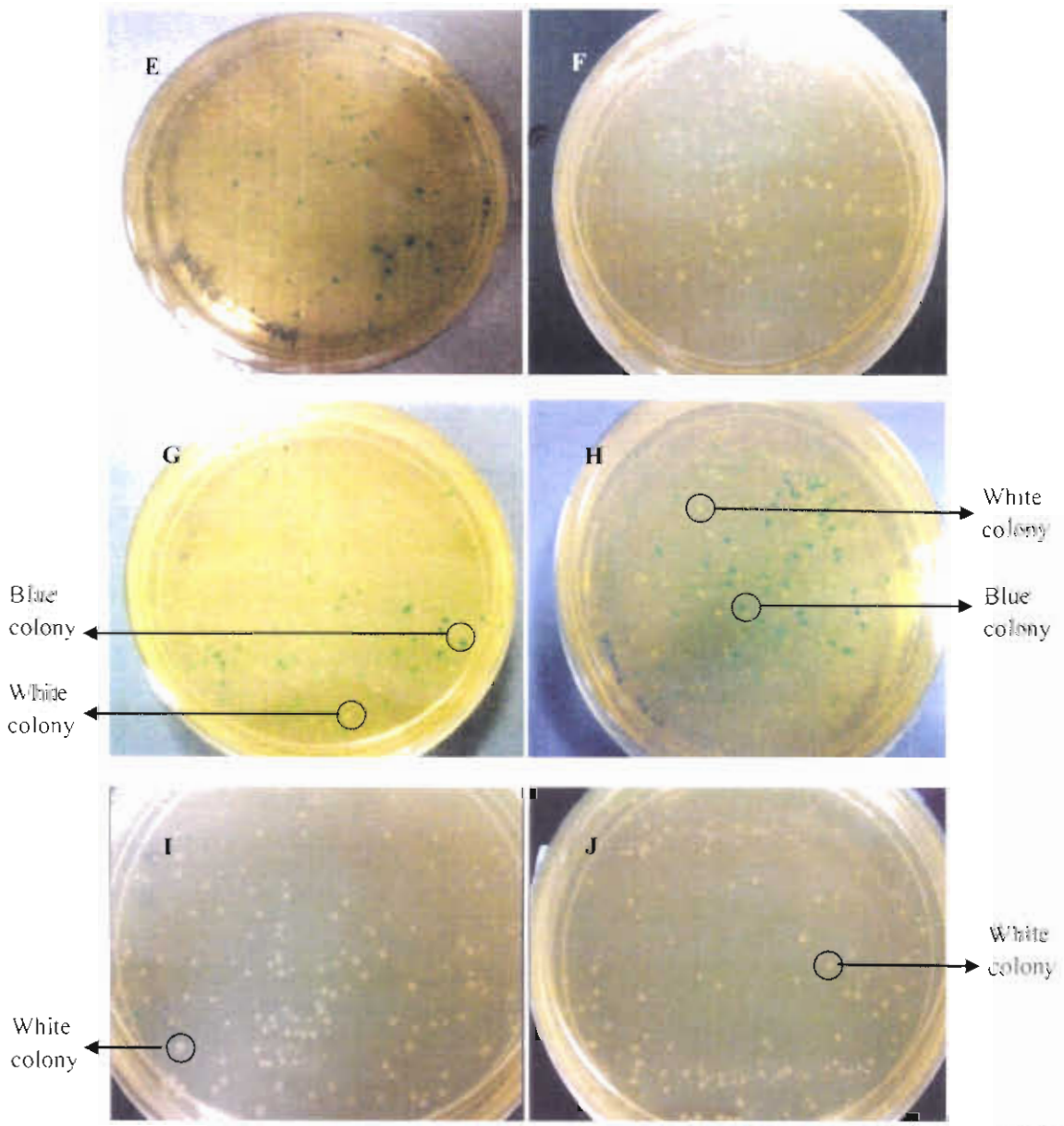

Figure 4.13 Blue white screening of Prel2a and Prel2b. E: Background control; F: Positive control G and H: Replicate reaction of Prel 2a gene; I and J: Replicate reaction of Prel 2b gene.

Figure 4.13 panels E to J displays the results blue white screening of Prel2a and Prel2b. Several white colonies were picked for colony PCR to check to presence of targeted genes.

4.8 Colony PCR

Several white colonies were picked up for colony PCR to check the presence of Prel genes and Figure 4.14 shows the result of colony PCR.

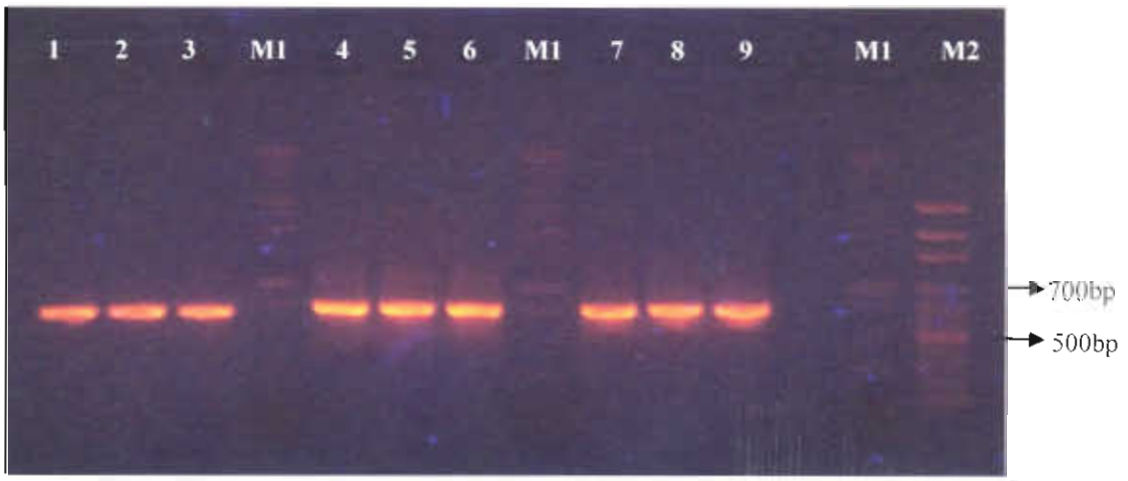

Figure 4.14: Colony PCR product from cloning plates. Lane M1: 1Kb ladder; Lane M2: 100bp ladder; Lane 1 to 3: PCR product Prel 1; Lane 4 to 6: PCR product Prel 2a; Lane 7 to 9: PCR product Prel 2b.

Figure 4.14 show the colony PCR product of Prel1. 2a and 2b. At least three distinct colonies from each plate were picked to screen the presence of targeted genes. The gel picture shows sharp bands from each colony and lies within the expected size which indicates the colonies contain recombinant plasmids. The remaining bacteria were poured into 5mL of LB contain ampicilin and shake for overnight until the reading of OD_{600} become 0.4-0.6 and proceed for plasmid extraction.

4.9 Plasmid Extraction

The colonies which contain the targeted genes were subculture for plasmid extraction and Figure 4.15 shows the extraction result.

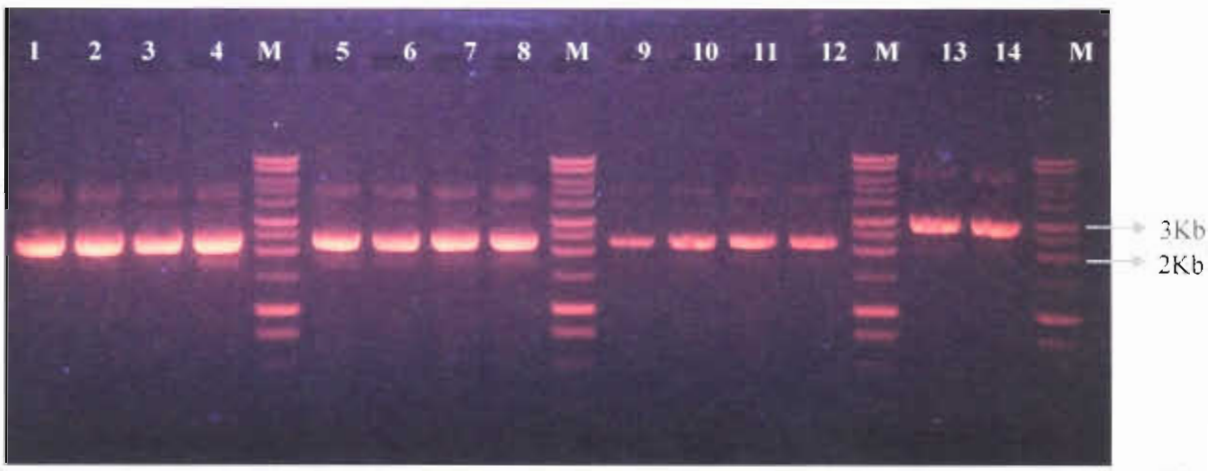

Figure 4.15: Extracted recombinant plasmid. Lane M: 1Kb ladder; Lane 1 to 4: Plasmid with Prel 1 gene; Lane 5 to 8: Plasmid with Prel 2a gene; Lane 9 to 12: Plasmid with Prel 2b gene; Lane 13 and 14: Plasmid with MyoD gene (positive control).

Figure 4.15 shows the recombinant plasmid with Prel1, Prel2a, Prel2b and MyoD. Based on the gel picture, all the plasmids were extracted successfully. There is more than one band shown in a single lane due to there are few types of plasmid such as nicked, linear, supercoiled and circular, single-stranded of plasmids. In the picture, supercoiled DNA with the inserted gene was the brightest. The band size for Prel genes are about 2 Kilobase pair to 3 Kilobase pair, however the predicted size should range approximately 3,500 base pair. This is due to the ladder used was not plasmid ladder which result the size slightly different from the expected. Recombinant plasmid with MyoD gene was included in plasmid extraction in order to synthesis the positive control probe for *in situ* hybridization purpose.

4.10 Quantitative Plasmid DNA

The extracted plasmids were quantified to check the purity and concentration. Table 4.3 shows the result of spectrophotometry.

Table 4.3: UV Spectrophotometry of recombinant plasmids.

Plasmid	Concentration (ng/µl)	$A_{260/280}$
Prel1	246.5	2.01
Prel2a	298.3	1.90
Prel2b	217.1	1.95
MyoD	235.8	1.89

Ratio of absorbance at 260 nm and 280 nm about 1.8 is generally accepted as pure for DNA (Thermo Fisher Scientific, USA). $A_{260/280}$ of all plasmid was ranging 1.89 to 2.01 which indicates the plasmid DNA is quite pure. The concentration of the plasmid is ranging 0.2µg to 0.3µg per µL. The concentration of plasmid is important for getting sufficient amount of digested plasmid to synthesize DIG-labeled RNA probes.

4.11 Plasmid Sequencing

The plasmid sequences search was carried out using BLASTn through the NCBI server and Table 4.4 shows the results.

Table 4.4: BLASTn output for plasmid Prel family gene sequences.

Gene	Accession	Description	Query coverage	E-value	Max Identity
Prel1	NM_200634.1	*Danio rerio* amyloid (A4) precursor protein-binding, family B, member 1 interacting protein (apbb1ip), mRNA	99%	0.0	99%
Prel2a	XM_005159957.3	PREDICTED: *Danio rerio* Ras association (RalGDS/AF-6) and pleckstrin homology domains 1b (raph1b), transcript variant X6, mRNA	99%	0.0	99%
Prel2b	XM_005167508.3	PREDICTED: *Danio rerio* Ras association (RalGDS/AF-6) and pleckstrin homology domains 1a (raph1a), transcript variant X6, mRNA	100%	0.0	99%

BLASTn result shows show 99% of its identity; about 99% to 100% of the query coverage and 0.0 of E-value. The description of the blastn result is match with the desire gene. Apbb1ip is another nomenclature of Prel1, while Raph1 is Prel2. The purpose of blast the sequences is to verified the orientation of inserted gene. The pGEMT Easy vector carry two universal RNA polymerase promoters: SP6 and T7 promoters. When the orientations

of the plasmid were identified, the DIG-labeled RNA probes were *in vitro* synthesis by using the correct polymerase.

4.12 Restriction Digest

Prior convert the plasmids DNA into DIG-labeled RNA probes, the plasmids were digested using appropriate enzyme. The enzymes were selected using NEBcutter (http://tools.neb.com/NEBcutter2/). The digested plasmid is shown in Figure 4.16.

Figure 4.16: Restriction digest of recombinant plasmids. Lane M: 1Kb ladder; Lane 1 and 2: digested plasmid Prel 1 (Nde I/Nco I); Lane 3: undigested plasmid Prel 1; Lane 4 and 5 digested plasmid Prel 2a (Nde I/Nco I); Lane 6: undigested plasmid Prel 2a; Lane 7 and 8: digested plasmid Prel 2b (Nde I/Nco I); Lane 9 undigested plasmid Prel 2b; Lane 10 digested plasmid MyoD (BamHI); Lane 11 undigested plasmid MyoD.

Figure 4.16 shows the result of plasmid digestion. Digested recombinant plasmid of Prel1, Prel2a and Prel2b product size are range approximately 3.5 Kilobases while recombinant plasmid of MyoD is about 4 Kilobase. The plasmids were digested using two types of enzymes in order to synthesis sense and antisense probes. The antisense and sense probes were generated according the orientation of the plasmid because the ligation of PCR is in random form. The appropriate enzymes were choosing using NEBcutter V2.0 and Appendix 3 is attached to provide illustration of it. The antisense RNA probe is reverse

complement and it will bind to the targeted sense mRNA in the embryo while sense RNA probe is the negative control of *in situ* hybridization. The plasmids were digested using different enzymes according to the orientation, thus, different promoter polymerases (either T7 polymerase or SP6 polymerase in this case) were used later on. The linearized plasmids were purified prior synthesized to DIG-labeled RNA probes. Recombinant plasmid with MyoD was came with known orientation, thus the plasmid was digest according to recommended enzyme.

4.13 DIG-labeled RNA Probe Synthesis

The purified plasmid DNA was converted into DIG-labeled RNA probe. Figure 4.17 shows the result of synthesized probes.

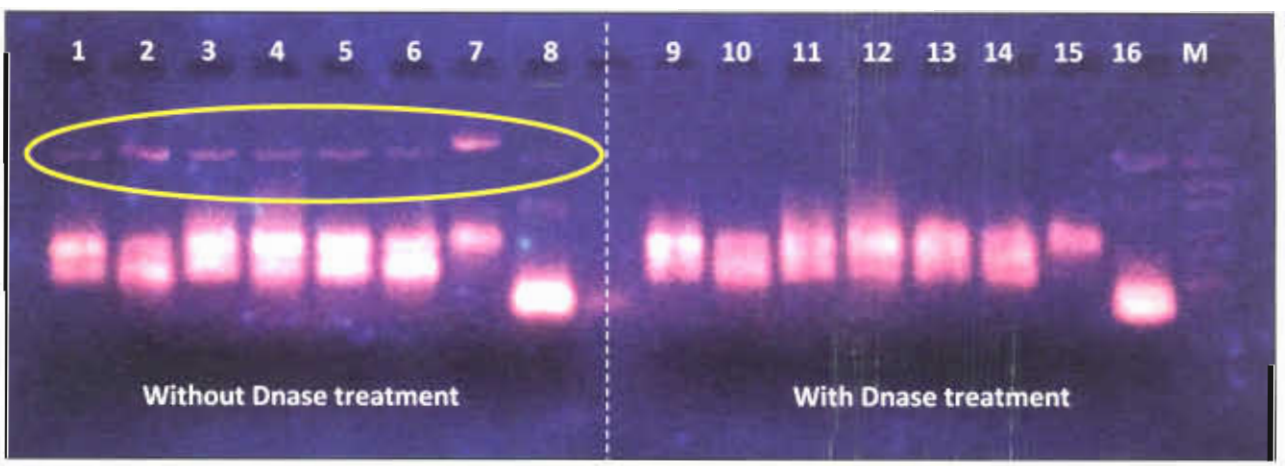

Figure 4.17 DIG-labeled sense and antisense riboprobes. Lane M: 100bp ladder; Lane 1 to 8 are Riboprobes without Dnase treatment; Lane 9 to 16 are Riboprobes with Dnase treatment.

Figure 4.17 shows the *in vitro* transcripted DIG-labeled RNA probes. Prior convert into RNA, the plasmid was purified to making it more efficient transcription by eliminate salts, proteins, RNAse and etc. Hence, it is important for adding DNase to get rid of remaining

DNA in the reaction. Figure 4.17 compare the riboprobes with and without Dnase treatment. At the left side of the picture, the reactions were not treated with Dnase, there is still some remaining DNA (yellow circle). Whereas the right side of the figure 4.16 the riboprobes are clean, no DNA remaining. The DIG labeled, single stranded RNA probes of defined length by *in vitro* transcription of template DNA in the presence of digoxigenin-UTP, using SP6 or T7 RNA polymerase were generated. Agarose gel was used for running electrophoresis and it causes the RNA bands were smearing; denaturing gel should be used for checking the integrity of RNA (Thermo Fisher Scientific, USA).

4.14 Quantitative DIG-labeled RNA Probe

The quality and quantity of the riboprobes were checked through UV spectrophotometry, the results shows in Table 4.5.

Table 4.5: UV Spectrophotometry of DIG-labeled riboprobes.

Probe	$A_{260/280}$	$A_{260/230}$	Concentration (ng/μL)
Prel1 sense	1.98	2.10	1098
Prel1 antisense	2.12	2.15	1221
Prel2a sense	2.20	1.94	1147
Prel2a antisense	2.02	1.91	1007
Prel2b sense	1.90	1.86	985
Prel2b antisense	1.94	1.88	993
MyoD	2.13	2.05	1094

The quality and the quantity of the riboprobes are very important for the downstream purpose. The RNA should be "pure" without other substance to avoid degradation and

affect the procedure of downstream; while the concentration should be approximately 20-100ng of the DIG-labeled RNA probe per mL of hybridization mix (Roche, Germany). Excess probe might lead to high background problem, thus the amount of probe being used must be known. According to Table 4.5, the absorbance of 260 nm and 280 nm and absorbance of 260 nm and 230 nm reading show the riboprobes are considered "pure" (NanoDrop, USA).

4.15 Whole Mount *In situ* Hybridization

The DIG-labeled probes were used for 3 days procedure whole mount *in situ* hybridization.

The result shows in Figure 4.18A to G and Figure 4.19H to N.

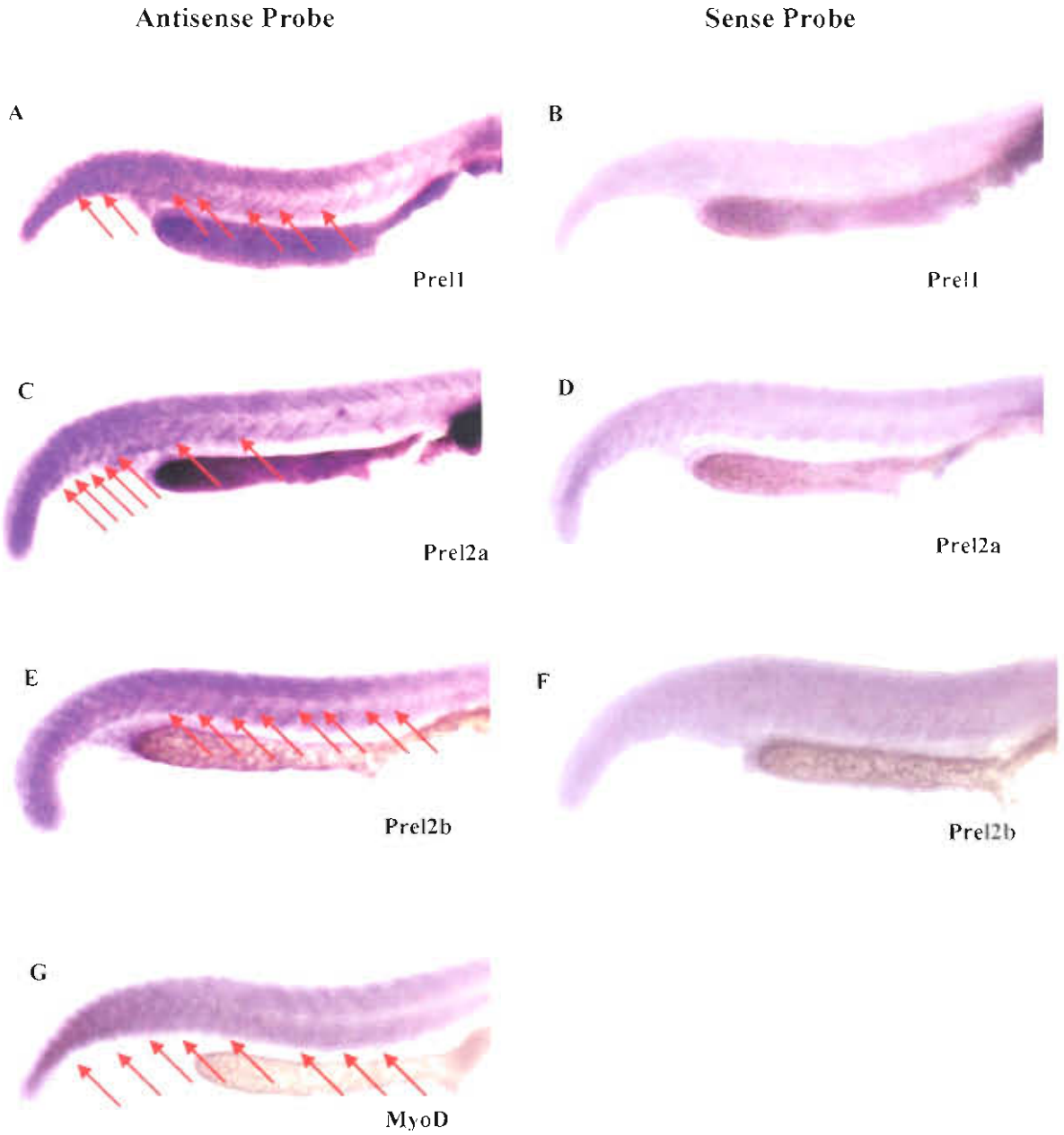

Figure 4.18: Expression pattern of Prel1, Prel2a and Prel2b at 24hpf embryo by *in situ* hybridization. Figure 4.18A: Prel1 antisense probe; 4.18B: Prel1 sense probe; 4.18C: Prel2a antisense probe; 4.18D: Prel2a sense probe; 4.18E: Prel2b antisense probe; 4.18F: Prel2b sense probe; 4.18G: positive control, MyoD.

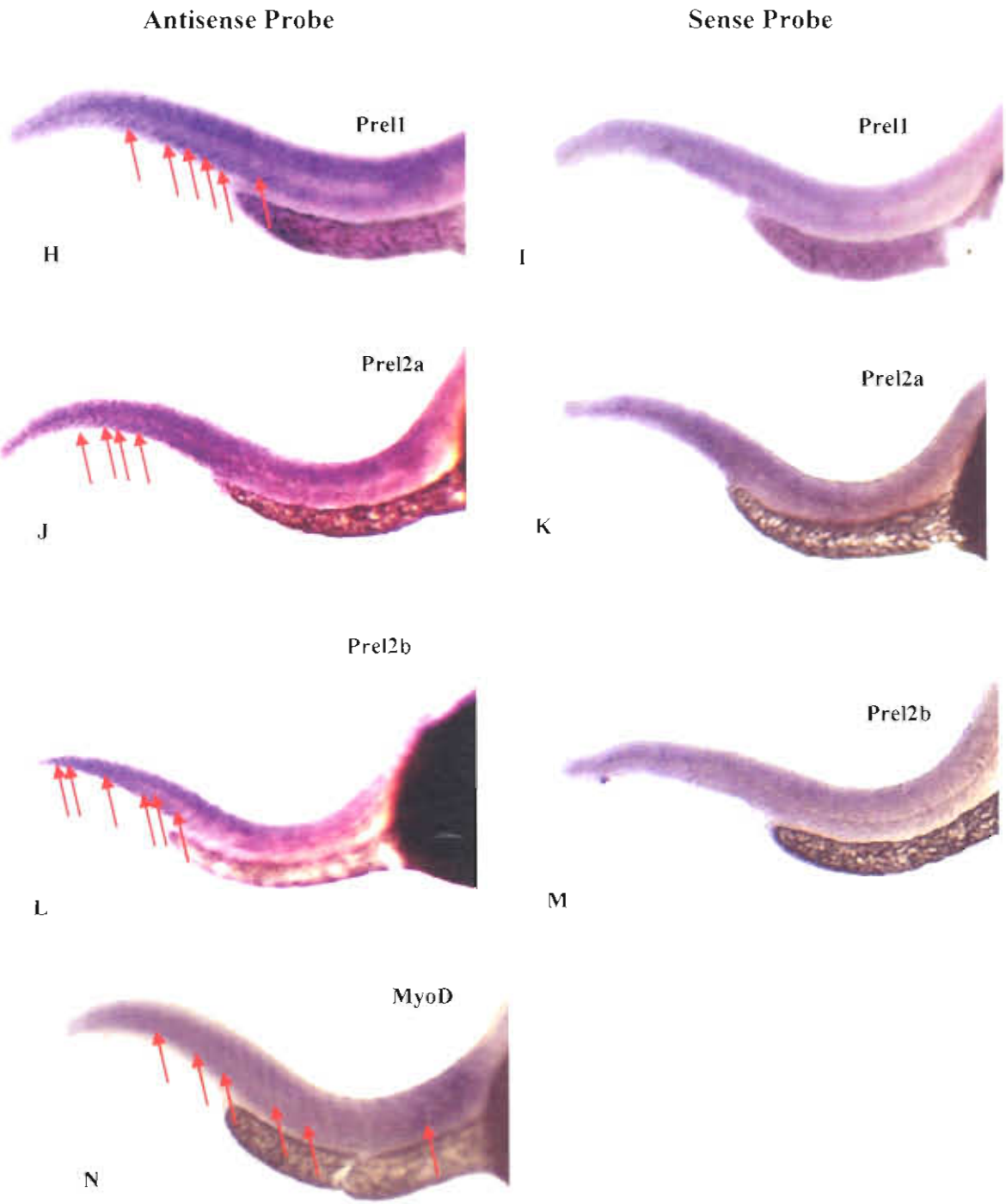

Figure 4.19: Expression pattern of Prel1, Prel2a and Prel2b at 34hpf embryo by *in situ* hybridization. Figure 4.19H: Prel1 antisense probe; 4.19I: Prel1 sense probe; 4.19J: Prel2a antisense probe; 4.19K: Prel2a sense probe; 4.19L: Prel2b antisense probe; 4.19M: Prel2b sense probe; 4.19N: positive control, MyoD.

Figure 4.18A to Figure 4.18G are the expression pattern of Prel1, Prel2a, Prel2b and MyoD in 24hpf embryo. Blue staining indicates localization of the mRNA for each gene. Red arrows point to the position of some somite boundaries. The expression in the somite at 24hpf is stronger than compared to expression at 34hpf. The first row, Figure 4.18A and Figure 4.18B are the expression of Prel1 gene. The antisense probe expressed evenly along the trunk region while there is no expression for the sense probe. Figure 4.18C and Figure 4.18D are the expression of antisense and sense probes of Prel2a respectively. Prel2a gene is expressed along the trunk but it express stronger at the tail region. While the Figure 4.18E and Figure 4.18F are the expression of Prel2b gene. Antisense Prel2b probe was bind along the trunk too; however, the expression shows thicker at the middle of the trunk which is different from Prel2a. The sense probe of Prel2a and Prel2b got no expression in the embryo. As for the positive control, MyoD gene (Figure 4.18G), it is strongly expressed along the somites. The expression of Prel family gene is consistent with the RT-PCR analysis.

Figure 4.19H to Figure 4.19N show the expression pattern of Prel family genes at 34hpf zebrafish embryo. Figure 4.19H and Figure 4.19I show the expression of Prel1 and the negative control. Prel1 antisense probe express along the somites while there is no expression for the negative control. Figure 4.19J and Figure 4.19K are the expression pattern of Prel2a which bind along the trunk, however this time, it express stronger at the middle of the trunk which is different from Prel2b (Figure 4.19L) which express at the somite of tail region.

Cell migration is very crucial mechanism for developing embryo to define their correct shape and positioning during organogenesis and in the tissues and in the organs of the adult body (Kurosaka & Kashina, 2008). Fail to coordinate cell migration might lead to

multiple human syndrome such as neurological disorders, congenital heart diseases, physical and mental retardation (Kurosaka & Kashina, 2008). There are four steps of migration: polarization, protrusion, adhesion and retraction. In this discussion we focus on lamellipodia protrusion and adhesion since Prel1 and Prel2 have pronounced function in cell protrusion, adhesion (Lafuente *et al.*, 2004; Krause *et al.*, 2004) and endocytosis (Bodo *et al.*, 2017) which means Prel family genes are very vital for early development of embryo.

Cell protrusion and adhesion formation is caused by actin polymerization and adhesion formation which regulated by Ena/VASP, profilin, ADP/Cofilin, capping proteins, cross linkers, Arp2/3 complex, WAVE/WASP and Rac/Cdc42, talin, PKC, Rap1 and PI3K (Krause & Gautreau, 2014; Ridley *et al.*, 2003). Prel1 and Prel2a has proven that they interact with Ena/VASP, profiling, Rap1, talin and activate Arp2/3 according to previous studies (Krause *et al.*, 2004; Lafuente *et al.*, 2004; Jenzora *et al.*. 2004), hence, Prel family genes are involving in actin polymerization and integrins activation (Hansen & Mullins, 2015). Cell migration requires membrane protrusion at the cell front which the plasma membrane is provided by actin polymerization (Krause & Gautreau, 2014) followed by the extension of ruffles or a leading pseudopod (Friedl *et al.*, 2004). Once the protrusion cell extension touches extracellular matrix ligands, adhesion receptor of integrins families become engage, cluster, attach to the substrate and recruit cytoplamic adaptor and signaling proteins and then connecting to actin filaments. The resulting local interaction regions are multifocal defined as focal adhesion (Friedl *et al.*, 2004).

Cell migration over short and long distance plays a key role at all stages of development, onset of gastrulation until the formation of an adult organism (Gilbert, 2000). Mesenchymal cells are responsible for most migratory event that takes place during the development (Kurosaka & Kashina, 2008). In vertebrate segmentation, a specialized

mesodermal structure called the somite (Pourquiè, 2003) and they govern the metamerism of all somite-derived tissues including axial skeleton, the dermis of the back and all striated muscle of the adult body (Sawada *et al.*, 2000). During segmentation of zebrafish, the first pair of somite is formed approximately at 10.5hpf (Kimmel *et al.*, 1995). Adhesive properties of the cells in the anterior region presomitic mesoderm and undergo mesenchymal to epithelial transitions around the loosely organized mesenchymal cells (Stickney *et al.*, 2000) to form muscle cells (somite). According to Lagarrigue *et al.* (2015), Prel1 or Prel2 can drive the formation of an integrin-talin complex containing activated integrins and they enriched at the protruding leading edge of migrating cells and associated with growing actin filaments that form "sticky fingers" to sense extracellular matrix and guide cell migration. Thus, Prel1 and Prel2 are extremely crucial for developing embryo.

There are numerous data on mouse knockouts of cell migration components, however, according to Su *et al.* (2015), Prel1-deficient mice are viable and fertile suggested other gene product can compensate for Prel1 deficiency. Nevertheless, the study found out Prel1-deficient mice got peripheral lymph nodes (pLNs) were depleted of both B and T cells and recirculating B cells were disminished in the bone marrow, the spleen hypercellular, albeit with a relative deficiency of marginal zone B cells. This the finding shows Prel1 is essential for efficient adhesion to intercellular adhesion molecule (ICAM) 1 and vascular cell adhesion molecule (VCAM) 1 and for the proper trafficking of lymphocytes to secondary lymphoid organs (Su *et al.*, 2015). Depletion of Prel2 in pyramidal neurons arising from the ventricular during corticogenesis in the mouse embryo elicited an abnormal cortical phenotype. In addition, knockdown Prel2 also reduced the number of primary processes in multipolar neurons accumulating in the mulitpolar cell accumulation zone before they initiate their radial migration in the developing cortex

(Bodo *et al.*, 2017). Thus, in the mammalian central nervous system, Prel2 contributes to neuronal morphogenesis, neuronal migration during development (Bodo *et al.*, 2017).

The positive control (Figure 4.18G and 4.19N) of the whole mount *in situ* hybridization is MyoD gene. The function of MyoD in development is to commit mesoderm cells to a skeletal myoblast lineage and then regulate that continued state. Thus, it is strongly expressed along the somites. In zebrafish, there are 5 family proteins were identified to be involved in the segmentation and boundary formation including *her*, *notch*, *delta*, *mesp* and *ephrin* (Stickney *et al.*, 2000). According Durbin *et al.* (1998), *her1* is expressed dynamically in pre-segmental somatic plate of zebrafish (Muller *et al.*, 1996; Palmeirim *et al.*, 1997). It suggests that *her1* responsible for the establishment of a segmental pattern within this region of the embryo (Durbin *et al.*, 1998). Expression of *mespa* and *mespb* occurred during segmentation in 1 to 3 stripes in the anterior parts of somites primordial which is very similar to *her1* and *her4* (Sawada *et al.*, 2000). Among *notch* family members, *notch6* and *notch1b* were highly expressed at the posterior region of presomitic mesoderm whereas *notch5* and *notch1b* were expressed at the anterior part of mature somite (Sawada *et al.*, 2000). The *Eph* family in believed involved in intercellular signaling and neural tube patterning (Durbin *et al.*, 1998). Several *ephrins* such as *Eph-A-L1*, *ephrin-B2* and *EphA4* are expressed in somites and also restricted anterior region of presomitic mesoderm of presumptive somites (Durbin *et al.*, 1998).There is four members delta in zebrafish which *deltaC* and *deltaD* have the similar expression pattern with the *eph* family (Haddon *et al.*, 1998).

Prel1 was detected and expressed along the somite at 24hpf and 34hpf. One of the noticeable results of Prel2 gene expression was the complementary, temporal and spatial expression in developing somites. At 24hpf, Prel2a was expressed in the posterior tail and

may regulate the patterning of subdivision or presomitic mesoderm, while Prel2b shows strong expression in anterior region, where the morphologically distinct somites has formed, suggests Prel2b might regulate proper somite formation after the subdivision of the presomitic mesoderm. The expression of Prel2a and Prel2b remain 34hpf even though the segmentation complete at 24hpf. However, the weak expression can be observed at 34hpf. It might suggest that they are complementary to each other as DDC model. Prel2a was completely down regulated and showed weak expression at the posterior trunk at 34hpf; Prel2b was strongly expressed in the posterior tail region. Prel family members were strongly suggests playing a role during segmentation. The complementary expression is shown in Figure 4.20.

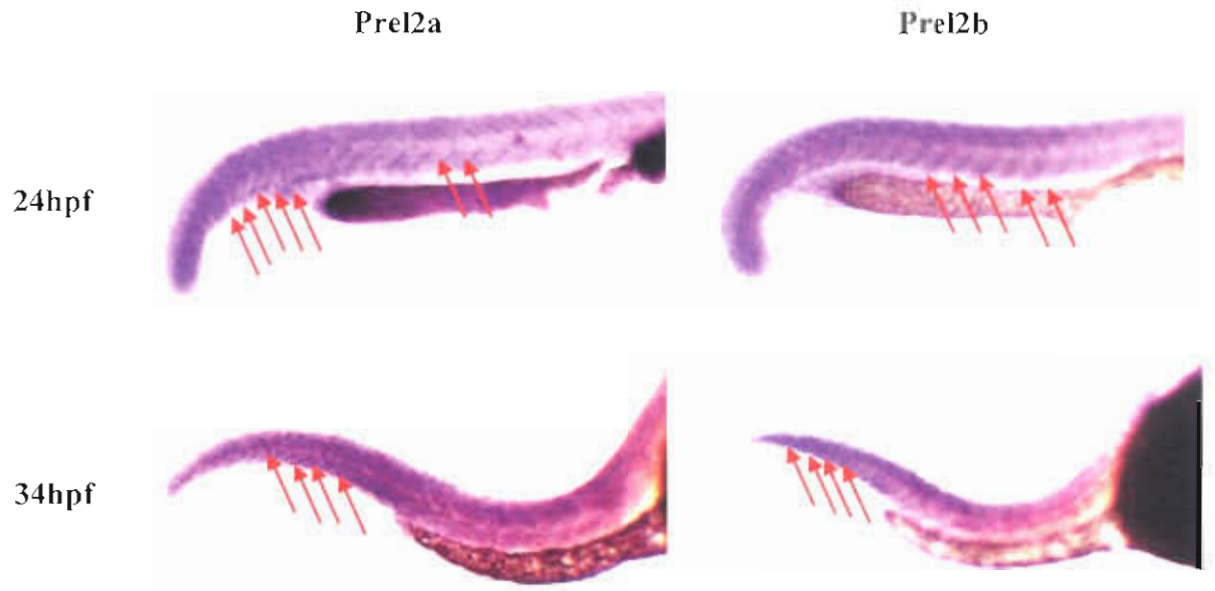

Figure 4.20: Comparison expression pattern of Prel2a and Prel2b at 24hpf and 34hpf embryo respectively.

Figure 4.20 shows Prel2a and Prel2b gene were complementary each other during early development of embryo zebrafish which is matched with the DDC model: subfunctionalization. Subfunctionalization is duplicated gene pairs with two or more copies of the ancestral gene and they complement each other to retain the full function of the original gene (Force *et al.*, 1999).

CHAPTER 5

CONCLUSION AND FUTURE WORKS

5.1 Summary of the Findings

The Prel family members are adaptor proteins that have been shown to play a role in cell migration. However, the expression data is only available from Northern blot of adult organs in mice and human. Moreover, the expression patterns have not been extensively characterized in any animal model. Throughout this project, a total number of three Prel family genes have been characterized. Expression profile of Prel family genes was been established to give insight into the future work. Multiple sequence alignment and phylogenctic tree were performed and the identity of these orthologues has been confirmed. The most interesting findings were the expression of Prel2a and Prel2b genes due to the DDC effect, Prel2a and Prel2b complement each other to function as whole Prel2 during the embryogenesis while Prel1 remained widely and diffusely expressed at 24hpf and 34hpf.

5.2 Future Works

To enhance the findings of complementation of Prel2a and Prel2b. double *in situ* hybridization has to be carried out. Although there are numerous of studies have done the knockdown on mice, however, the function between Prel2a and Prel2b still remain unknown due to there is only duplication-degeneration-complementation (DDC) occurred in zebrafish. Since the late mammalian gene might be concealed by defects or premature

death of the embryos caused by gene's essential early function, the procedure of knockdown and overexpression of Prel2a and Prel2b should be carried out in the future.

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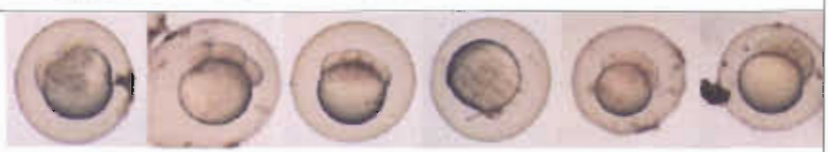
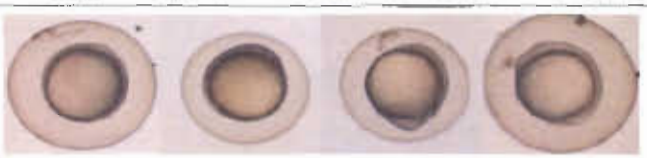
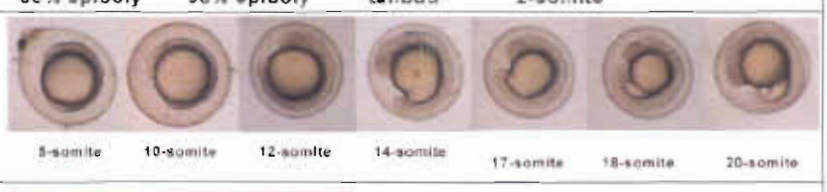
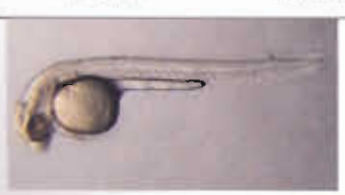
APPENDICES

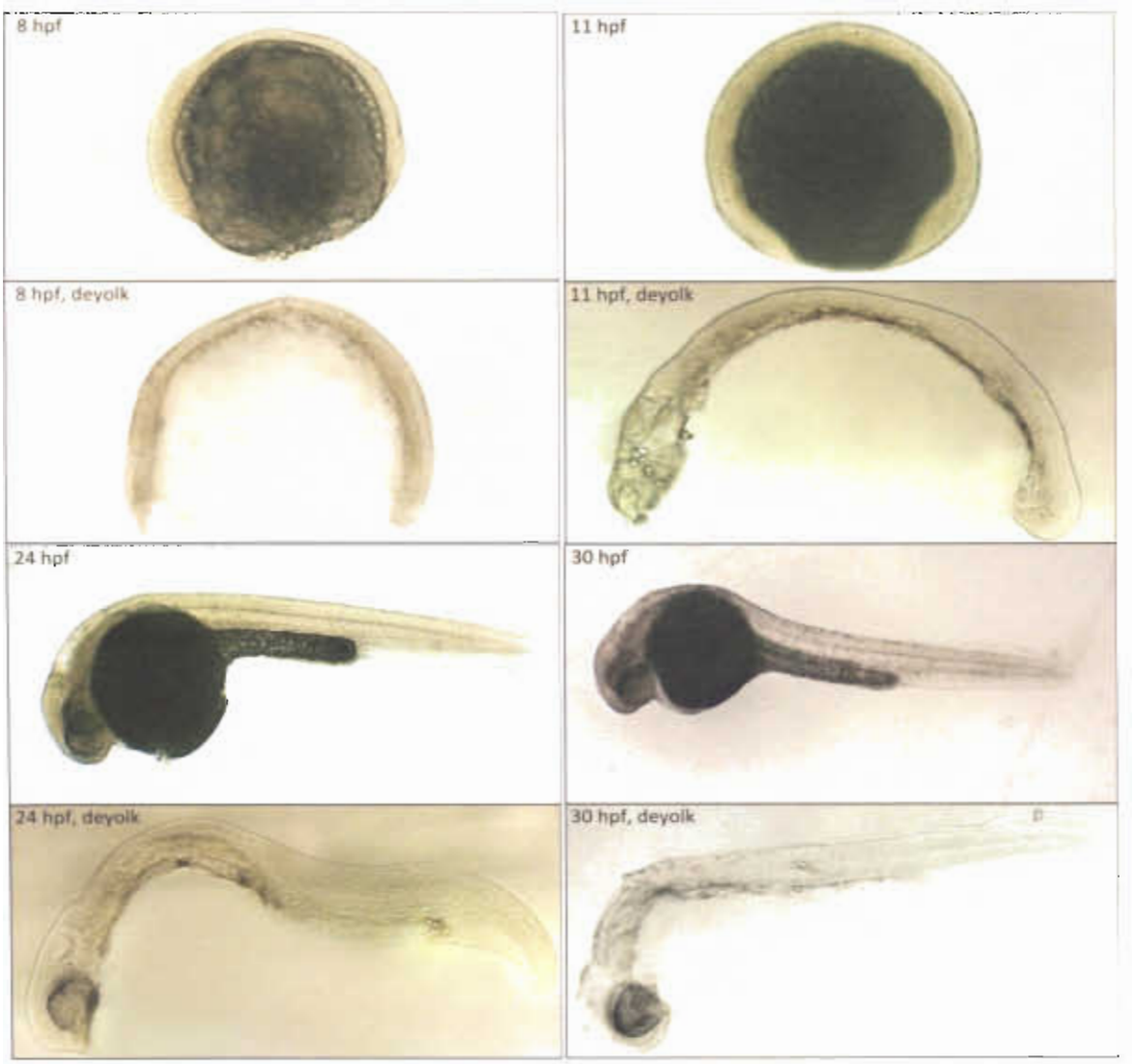
Appendix 1

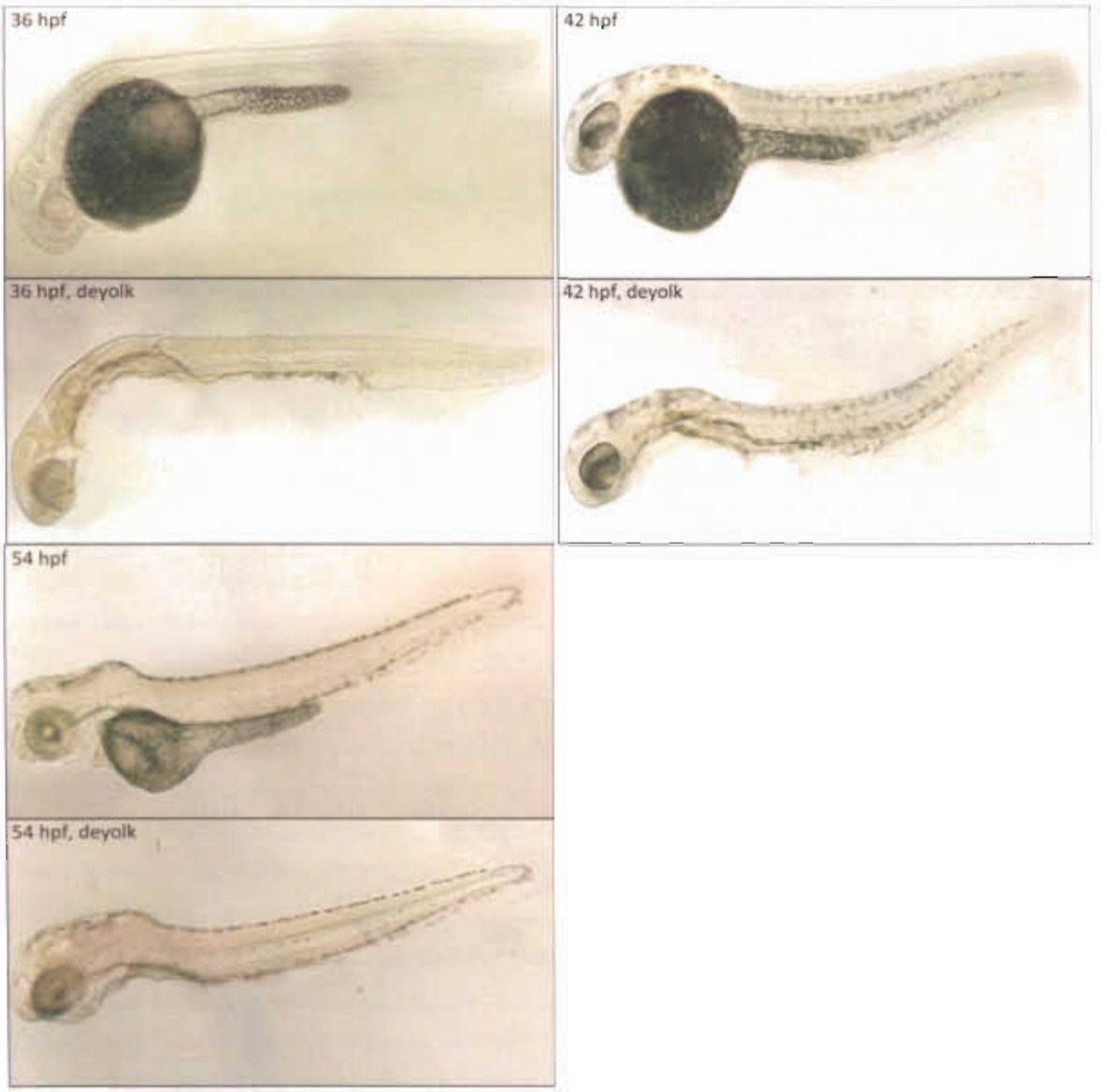
Zebrafish embryo at different stages.

Duration	Stages	Image
0 – 0.75 hpf	Zygote	
0.75-2.25 hpf	Cleavage	
2.25-5.25 hpf	Blastula	
5.25-10.33 hpf	Gastrula	
10.33-24 hpf	Segmentation	
24-48 hpf	Pharyngula	
48-72 hpf	Hatching	
72hpf – 30 days	Larval	

Appendix 2

7 stages of embryo after deyolk: 8hpf, 11hpf, 24hpf, 30hpf, 36hpf, 42hpf and 48hpf. The yolk is removed for better visualization purpose.

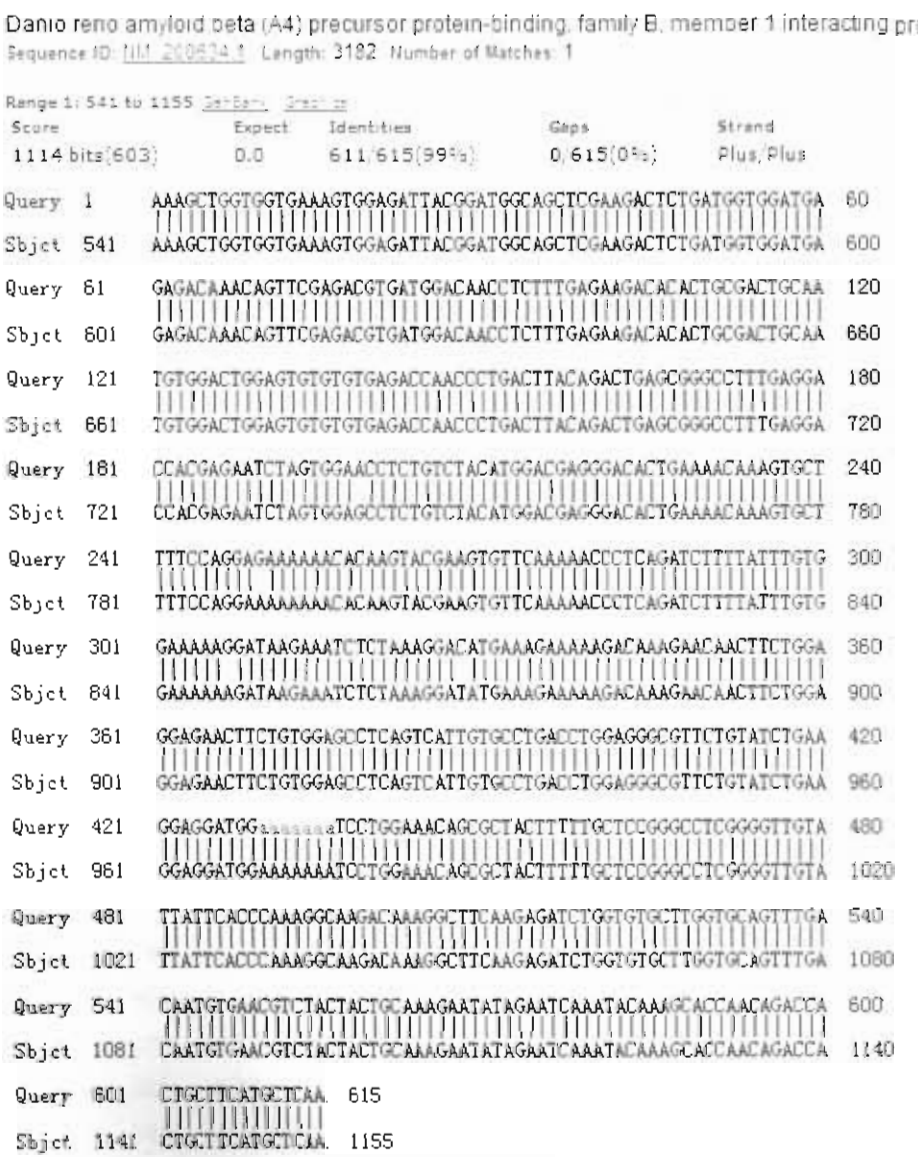



36 hpf
42 hpf
36 hpf, deyolk
42 hpf, deyolk
54 hpf
54 hpf, deyolk

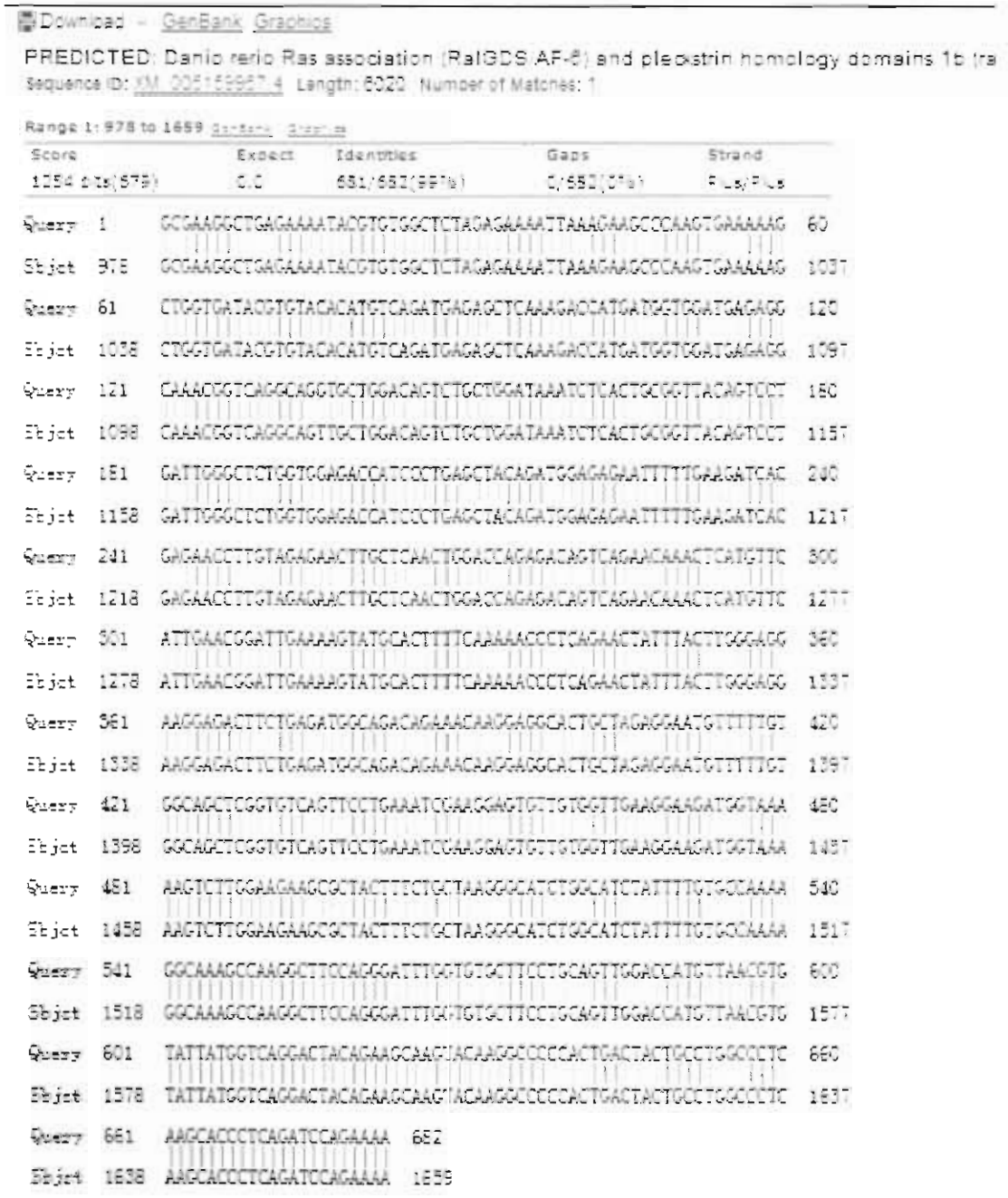
Appendix 3

Nucleotide sequence search using BLASTn through NCBI server.

Blastn Result: Prel1 Sequence



Blastn result: Prel2a



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PREDICTED: Danio rerio Ras association (RalGDS/AF-6) and pleckstrin homology domains 1b (ra

Sequence ID: XM_005165927.4 Length: 6020 Number of Matches: 1

Range 1: 978 to 1659 Graphics

Score	Expect	Identities	Gaps	Strand
1254 bits(679)	0.0	681/682(99%)	0/682(0%)	Plus/Plus

```
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Sbjct  978   GCGAAGGCTGAGAAAATACGTGTGGCTCTAGAGAAAATTAAAGAAGCCCAAGTGAAAAAG  1037
Query  61    CTGGTGATACGTGTACACATGTCAGATGAGAGCTCAAAGACCATGATGGTGGATGAGAGG  120
Sbjct  1038  CTGGTGATACGTGTACACATGTCAGATGAGAGCTCAAAGACCATGATGGTGGATGAGAGG  1097
Query  121   CAAACGGTCAGGCAGGTGCTGGACAGTCTGCTGGATAAATCTCACTGCGGTTACAGTCCT  180
Sbjct  1098  CAAACGGTCAGGCAGTTGCTGGACAGTCTGCTGGATAAATCTCACTGCGGTTACAGTCCT  1157
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Sbjct  1158  GATTGGGCTCTGGTGGAGACCATCCCTGAGCTACAGATGGAGAGAATTTTTGAAGATCAC  1217
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Sbjct  1218  GAGAACCTTGTAGAGAACTTGCTCAACTGGACCAGAGACAGTCAGAACAAACTCATGTTC  1277
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Sbjct  1278  ATTGAACGGATTGAAAAGTATGCACTTTTCAAAAACCCTCAGAACTATTTACTTGGGAGG  1337
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Sbjct  1338  AAGGAGACTTCTGAGATGGCAGACAGAAACAAGGAGGCACTGCTAGAGGAATGTTTTTGT  1397
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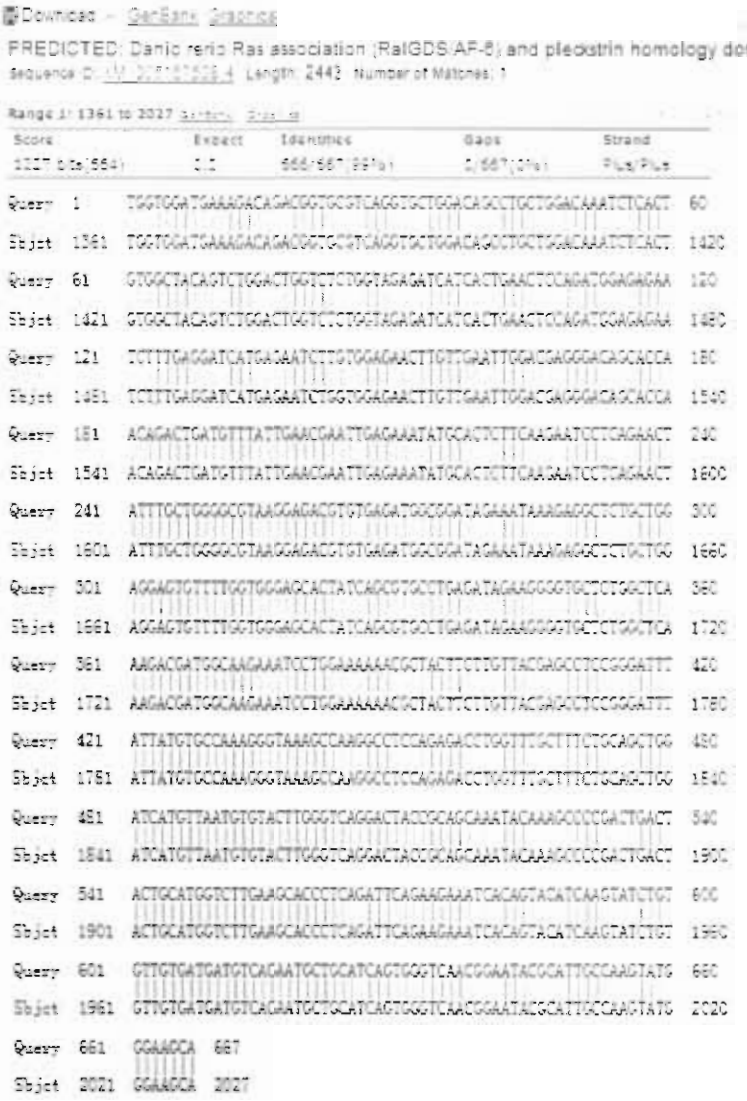
Sbjct  1638  AAGCACCCTCAGATCCAGAAAA  1659
```

Blastn result: Prel2b



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PREDICTED: Danio rerio Ras association (RalGDS/AF-6) and pleckstrin homology domains 1a (raph1a), transcript variant X6, mRNA

Sequence ID: XM_021184838.1 Length: 2443 Number of Matches: 1

Range 1: 1361 to 2027 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
1227 bits(664)	0.0	666/667 (99%)	1/667 (0%)	Plus/Plus

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Sbjct  1421  GTGGCTACAGTCTGGACTGGTCTCTGGTAGAGATCATCACTGAACTCCAGATGGAGAGAA  1480
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Sbjct  1481  TCTTTGAGGATCATGAGAATCTGGTGGAGAACTTGTTGAATTGGACGAGGGACAGCACCA  1540
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Sbjct  1721  AAGACGATGGCAAGAAATCCTGGAAAAAACGCTACTTCTTGTTACGAGCCTCCGGGATTT  1780
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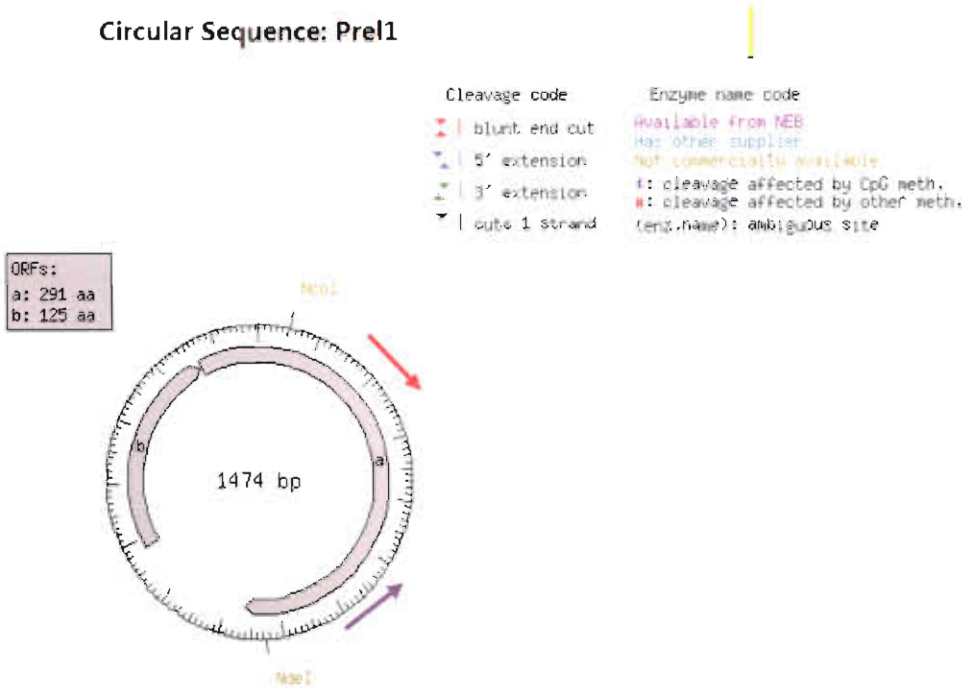
Query  481   ATCATGTTAATGTGTACTTGGGTCAGGACTACCGCAGCAAATACAAAGCCCCGACTGACT  540
Sbjct  1841  ATCATGTTAATGTGTACTTGGGTCAGGACTACCGCAGCAAATACAAAGCCCCGACTGACT  1900
Query  541   ACTGCATGGTCTTGAAGCACCCTCAGATTCAGAAGAAATCACAGTACATCAAGTATCTGT  600
Sbjct  1901  ACTGCATGGTCTTGAAGCACCCTCAGATTCAGAAGAAATCACAGTACATCAAGTATCTGT  1960
Query  601   GTTGTGATGATGTCAGAATGCTGCATCAGTGGGTCAACGGAATACGCATTGCCAAGTATG  660
Sbjct  1961  GTTGTGATGATGTCAGAATGCTGCATCAGTGGGTCAACGGAATACGCATTGCCAAGTATG  2020
Query  661   GGAAGCA  667
Sbjct  2021  GGAAGCA  2027
```

Appendix 4

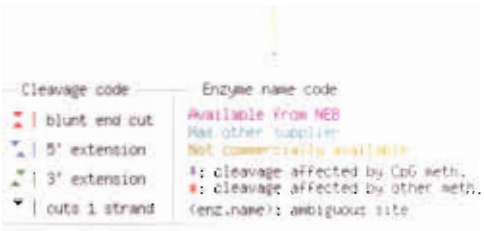
Suitable enzymes were identified to digest the plamid by using NEBcutter V2.0.

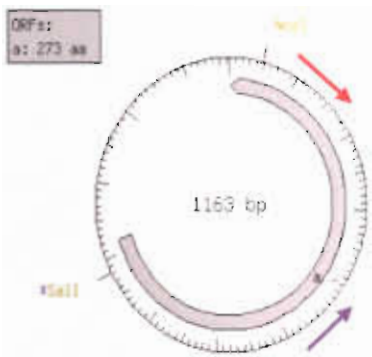
NEBcutter V2.0: Prel1



NEBcutter V2.0: Prel2a

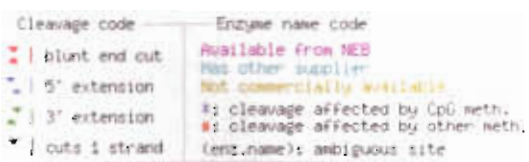
Circular Sequence: Prel2a

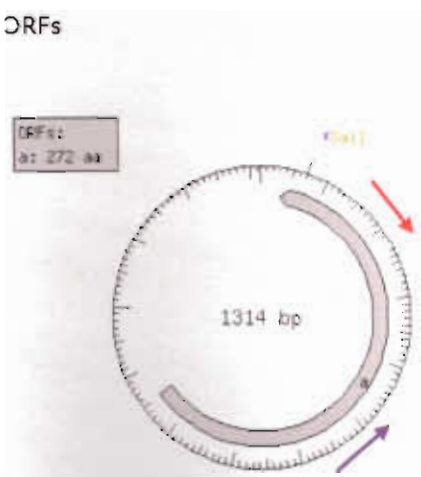




NEBcutter V2.0: Prel2b

Circular Sequence: Prel2b





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Title of Thesis	Expression Study of Proline-rich (Prel) Family Gene in Zebrafish (Danio rerio)				
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