Studies on the interaction of DEAD box helicase p68 with mrhl long non coding RNA

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MASTER OF SCIENCE

as part of Integrated PhD program

(Biological Sciences)

By

Bhavana Kayyar

Molecular Biology and Genetics Unit

Jawaharlal Nehru Centre for Advanced Scientific Research

Jakkur, Bangalore-560064

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DECLARATION

I hereby declare that the work described in this thesis entitled **'Studies on the interaction between DEAD box helicase p68 and long non coding RNA mrhl'** is the result of investigations carried out by myself under the guidance of **Prof. MRS Rao** at Chromatin Biology Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore-560064, India. This work has not been submitted elsewhere for the award of any other degree.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described has been based on findings of other investigators. Any omission, which might have occurred by oversight or misjudgement, is regretted.

December 13, 2016 Bhavana Kayyar

Jawaharlal Nehru Center for Advanced Scientific Research
Jakkur Campus, Jakkur Post
Bengaluru 560 064, INDIA
Office Tel: +91 (80) 2208 2754
Office Fax: +91 (80) 2208 2758
+91 (80) 2208 2468
Email:
mrsrao@jncasr.ac.in

Prof. MRS Rao
Honorary Professor and SERB Distinguished Fellow
Molecular Biology and Genetics Unit

CERTIFICATE

This is to certify that the work described in this thesis entitled **'Studies on the interaction between DEAD box helicase p68 and long non coding RNA mrhl'** is the result of investigations carried out by Ms. Bhavana Kayyar at Chromatin Biology laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, under my supervision.

December 13, 2016 Prof. MRS Rao

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1 Introduction:

1.1 RNA and its classification

The central dogma of molecular biology states that the flow of genetic information in a cell is from DNA through RNA to proteins, making Ribonucleic acid (RNA) one of the three central biomolecules essential for life.

In human beings, around 1%-2% of the genome is protein coding. A vast majority of the genome is non protein coding sequence that was initially considered to be 'junk DNA' that was transcriptionally silent (1). However, the ENCODE project (Encyclopedia Of DNA Elements) has discovered that more than 85% of the genome undergoes transcription to produce non-protein coding RNA (ncRNA) in a process called as pervasive transcription. RNA is classified based on the function, size and sub cellular localization (2). Fig 1.1 shows the present classification of RNA. Based on protein coding potential, RNA is classified into Coding and Non coding RNA. Coding RNA refers to messenger RNA transcripts. Noncoding RNA is further divided based on function into house keeping ncRNA, which includes ribosomal RNA, transfer RNA, snRNA and snoRNA, and regulatory RNA. This is further classified according to the size of the transcripts into short and long non coding RNAs. Short non coding RNAs include microRNA, short interfering RNA and Piwi associated RNAs.

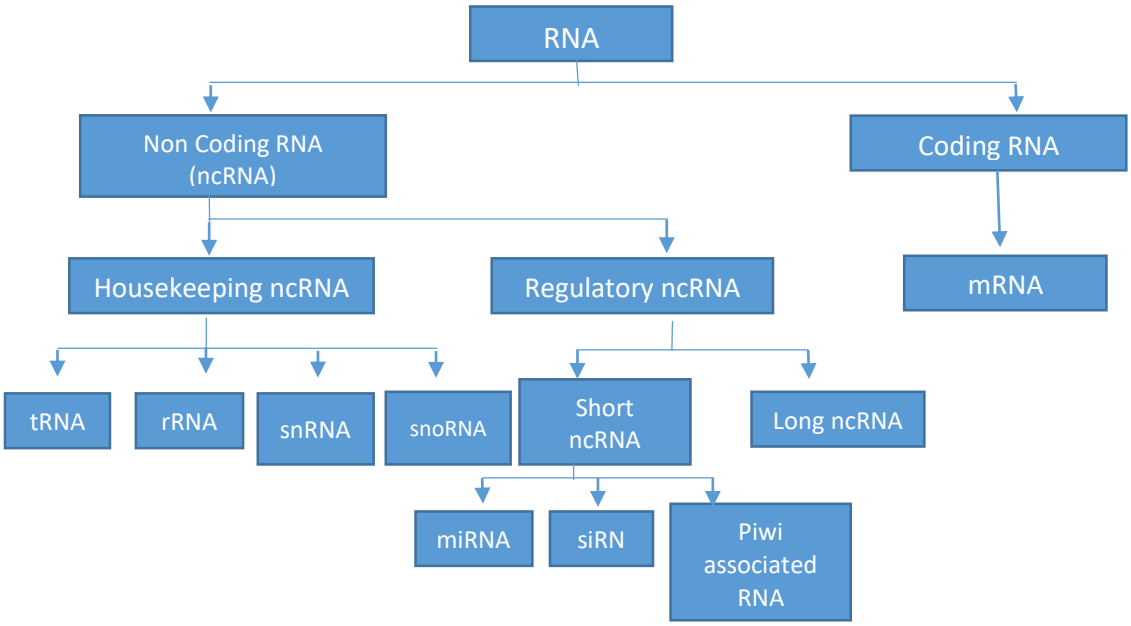

Fig. 1.1 Classification of RNA

NcRNA are expressed abundantly in cells in a developmentally regulated manner. In addition, they also are localized to specific sub cellular compartments indicating that have varied functions within cells (3,4). The housekeeping ncRNA are core players in translation and splicing. Small nucleolar RNAs are involved in guiding chemical modifications such as methylation and peudouridylation in ribosomal RNA and tRNAs by complementary base pairing (5). The snoRNA HBII-52 lacks complementarity to any rRNA or tRNA but exhibits complementarity and promotes inclusion of exon VB of the serotonin receptor 5-HT_{2C}R in humans. This is required for the functioning of this receptor (6). Housekeeping ncRNA can also play a role in regulatory processes as in the case of conserved small nuclear RNA 7SK. This snRNA, along with the protein partners HEXIM 1/HEXIM 2, negatively regulates transcription by reducing levels of RNA polymerase II phosphorylation (7).

Early examples of non coding RNA in regulatory roles include the long non coding RNAs Xist and Tsix which are required for dosage compensation by X chromosome inactivation in mammals(8) and the oncogenic miR 20a(9).

1.2 Long Non Coding RNA (lncRNA):

LncRNAs are currently defined as transcripts larger than 200 base pairs that are transcribed by RNA polymerase II. They are often spliced and polyadenylated but lack an extended open reading frame (ORF) within the sequence. LncRNA can be classified based on their location relative to near by protein coding genes as shown in figure 1.2.

1. **Antisense lncRNAs** or Natural Antisense Transcripts (NATs) are transcribed from the antisense strand of a protein coding gene. Eg : Airn
2. **Intronic lncRNAs** are located within introns of protein coding genes. Eg : *COLDAIR*, mrhl
3. **Divergent** or bidirectional promoter lncRNAs are transcribed within 1kb of promoter in the opposite direction to a protein coding gene. Eg: FMR4
4. **Intergenic lncRNA** are located between two protein coding genes. Eg: *HOTAIR*

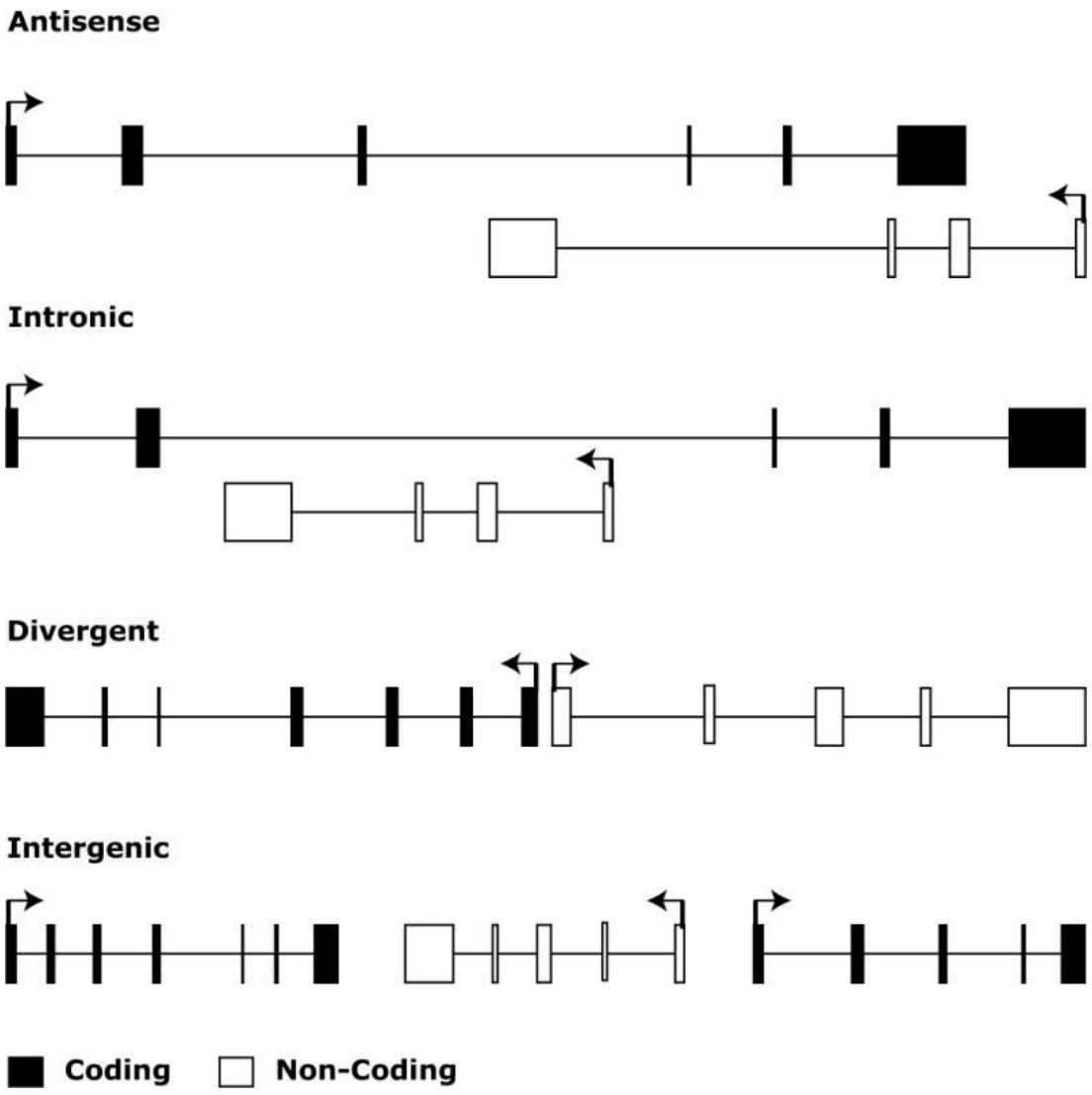

Fig. 1.2 – Genomic locations of Long non-coding RNA. Adapted from Rinn,JL., and Howard Y. Chang. 'Genome Regulation By Long Noncoding Rnas'. *Annu. Rev. Biochem.* (10)

Long non coding RNAs play roles in a myriad of cellular processes and the mechanisms of action can be broadly categorized as follows.

1. **Signal**

LncRNA can act as molecular signals to mark time, space, developmental stage or gene (11).Dosage compensation in mammals by X chromosome inactivation requires the lncRNA Xist which gets transcribed from and coats the same X chromosome, marking it for inactivation. The polycomb repressive complex (PRC2) is recruited and H3K27 trimethylation of the promoter of the Xist gene occurs leading to a repressive chromatin

mark. The heterochromatic mark then spreads to all regions of chromosome where Xist in present (8,12).

Another lncRNA that acts as a signal is the transcript PANDA. Located 5kb upstream of the CDKN1A gene, PANDA expression is activated in a p53 dependent manner during DNA damage. PANDA then interacts with transcription factor NF-YA to limit the expression of proapoptotic genes and induce cell cycle arrest (13).

2. **Decoy**

This archetype of RNA act as a molecular sink for RNA binding proteins which could be transcription factors, chromatin modifiers or other regulatory proteins or miRNA.

The lncRNA MALAT1 (metastasis-associated lung adenocarcinoma transcript 1 is a nuclear RNA that localizes to nuclear speckles. MALAT1 sequesters Serine/Arginine (SR) splicing factors to nuclear speckles. Depletion of MALAT1 results in alternative splicing of a set of pre mRNA (14).

Telomeric repeat RNA (TERRA) that is transcribed from the telomeres, physically interacts with telomerase by complementary base pairing with the template sequence of telomerase RNA. In addition, it also interacts with the telomerase reverse transcriptase (TERT) protein subunit independently of the telomerase template RNA. Telomere bound TERRA appears to bind and sequester telomerase maintaining it in close proximity to telomeric 3' end. The levels of TERRA change in a cell cycle dependent manner – accumulating in G1 phase, continuously decreasing in S phase and reaching its lowest point in S to G2 phase transition. This down regulation may release telomerase allowing the extension of telomeric strand in a cell-cycle dependent manner (15,16).

3. **Guide**

Some of the lncRNAs bind to proteins and direct them to specific target loci. They can be targeted to loci in *cis* or in *trans*. HOTAIR (HOX transcript antisense intergenic RNA) is transcribed from the HOX C locus and silences the HOX D locus located 40kb away. HOTAIR forms a complex with PRC2, histone lysine demethylase (LSD1), co-repressor for elements-1-silencing transcription factor(Co REST), repressor for elements-1- silencing transcription factor (REST) and guide the complex to alter the epigenetic state of the target locus in *trans* (17).

4. Scaffold

LncRNAs can also act as platforms for the assembly of molecular complexes. RNA transcripts bind to different components through different domains simultaneously and bring the effector molecules together in space and time.

The telomerase plays a key role in maintenance of chromosomes by adding back telmoeric repeat DNA that is lost from the ends during replication. The RNA subunit called TERC (Telomerase RNA) provides the template for telomere repeat synthesis. The catalytic protein subunit is the TERT (Telomerase Reverse Transciptase) along with other species specific accessory proteins. The primary role of TERC seems to be that of a scaffold as domains have been identified on TERC that affect TERT binding, accessory proteins binding as well as template usage(18).

Though the mechanisms of action can be classified into broad subtypes, the final effect exerted by two lncRNA acting through the same mechanism can be very different from each other. Fig1.3 illustrates some other functions performed by lncRNAs.

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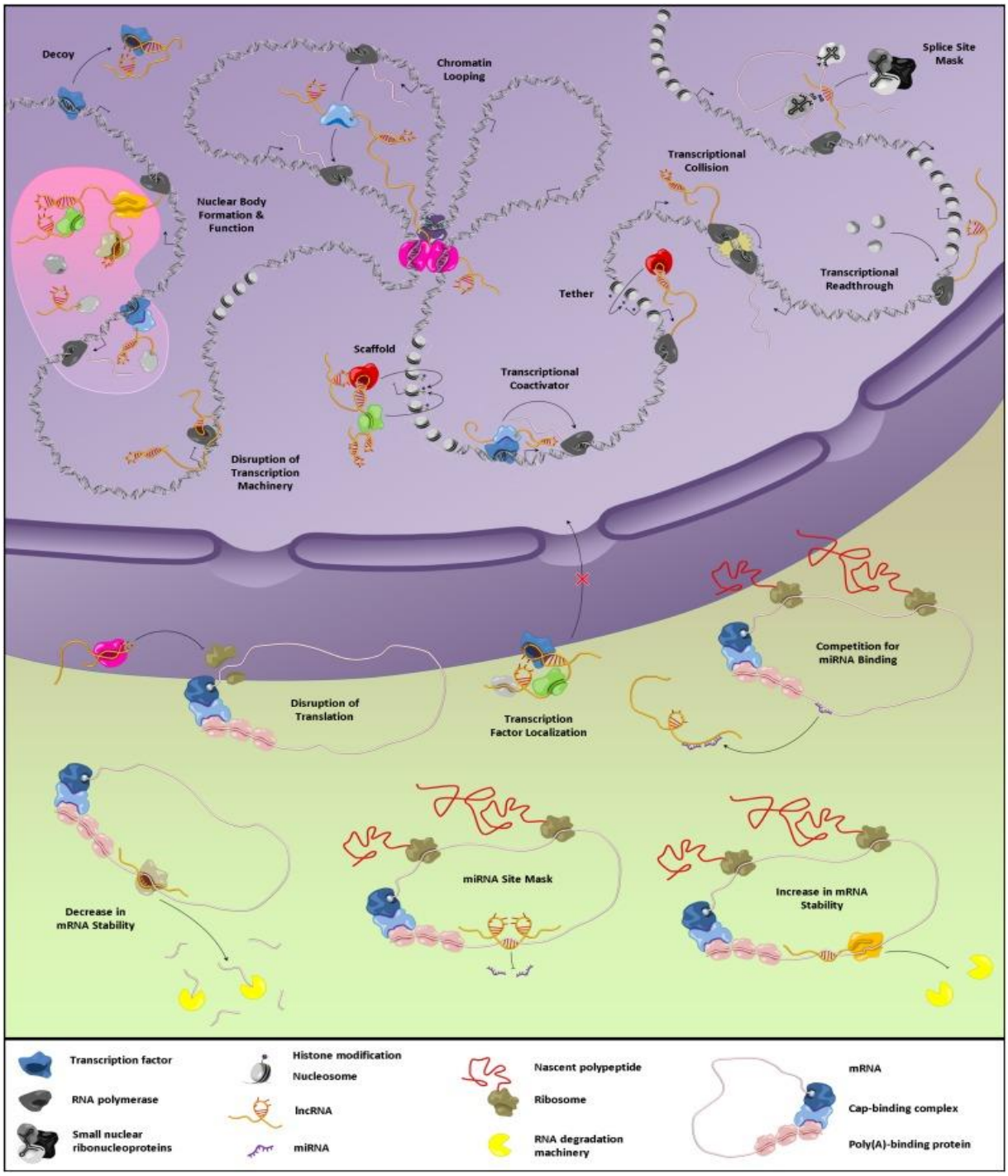


Fig 1.3 – Possible mechanisms of action of lncRNAs - Kung JTY, Colognori D, Lee JT. Long Noncoding RNAs: Past, Present, and Future. *Genetics*. 2013;193(3):651-669. doi:10.1534/genetics.112.146704. (19)

1.3 Novel lncRNA - mrhl

Meiotic Recombination Hotspot Locus (mrhl) RNA is a 2.4-kb long non-coding RNA that is encoded for in chromosome 8 of mouse. It is an intronic lncRNA that is transcribed independently from the 15th intron of *phkb* gene by RNA polymerase II. The transcript is unspliced and polyadenylated and is expressed in liver, spleen, kidney and testis where it localizes to the nucleus (20). It gets processed to an 80-nucleotide intermediate RNA by the Drosha machinery which is also nuclear localized. Although it gets further processed into a 22nt fragment *in vitro*, this miRNA fragment is not detected *in vivo* in mouse spermatogonial cells (21). It is also known that mrhl physically associates with the chromatin. Experiments have shown that mrhl associates with the chromatin at 1370 distinct loci. In addition, microarray expression profiling carried out in mouse spermatogonial cells revealed that around 652 genes were differentially expressed upon mrhl silencing. A comparison of genes associated with mrhl and the genes that are perturbed upon mrhl silencing revealed that around 37 genes are regulated by the physical association of mrhl and are termed GRPAM loci (Genes Regulated by Physical Association of Mrhl). Studies carried out in mouse male germ cells indicate that mrhl RNA plays a functional role as a negative regulator of Wnt signaling along with a protein partner p68, a DEAD box helicase. Activation of Wnt signaling in these cells leads to a down regulation of mrhl transcript levels. Upon down regulation of mrhl, the nuclear localized p68 translocates to the cytoplasm where it forms a dimer with beta catenin. The p68-beta catenin dimer translocates back to the nucleus where beta catenin associates with the transcription factor TCF4 and regulates the expression of Wnt target genes (22,23).

1.4 DEAD box Protein – p68

P68 or DDX5 is one of the founding members of the DEAD box helicase family of proteins. With almost 37 members in humans, the DEAD box family of helicases is one of the largest subfamilies of RNA helicases (24).

The DEAD box helicases are involved in many aspects of RNA metabolism as shown in Fig 1.4.

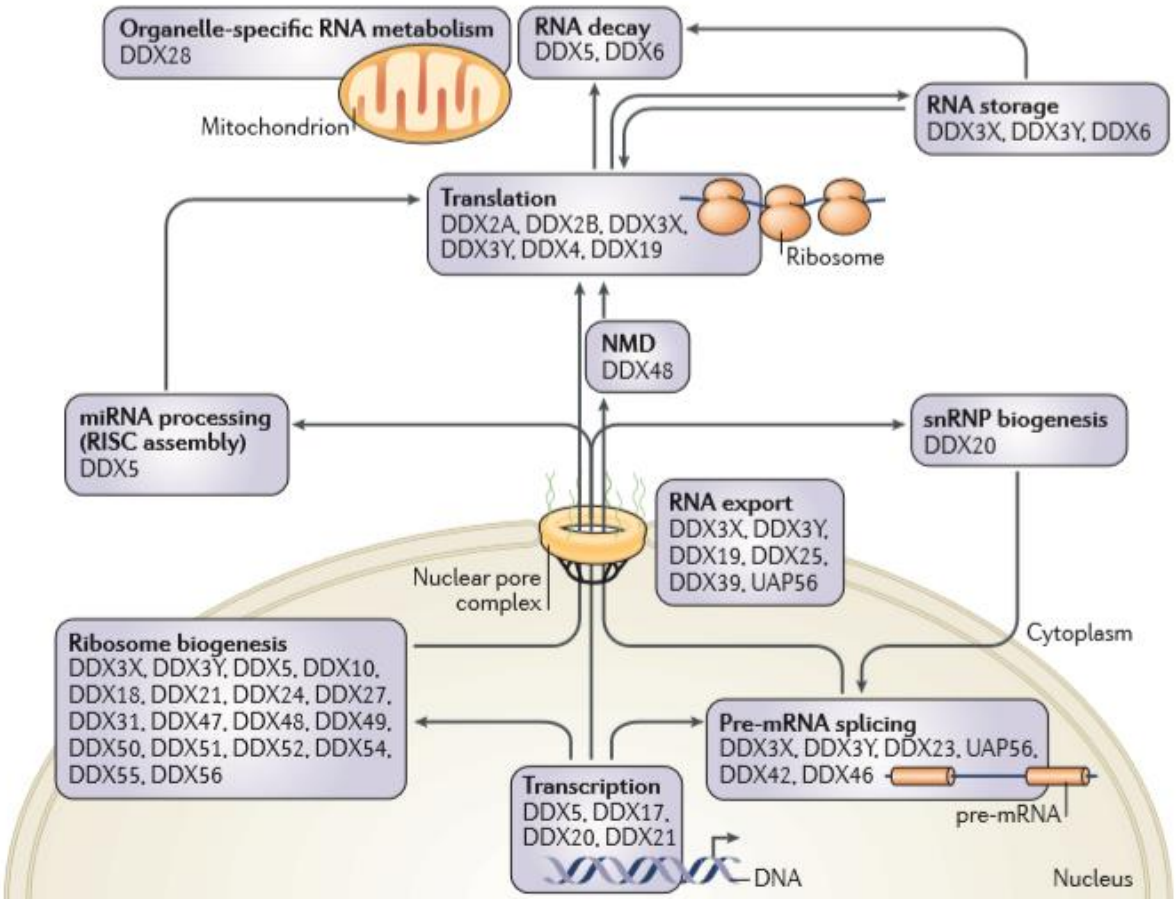


Fig 1.4 – Cellular Processes involving DEAD box helicases. Linder P **,** Jankowsky E**.** From unwinding to clamping- the DEAD box RNA helicase family. Nat Rev Mol Cell Biol. 2011 Jul 22;12(8):505-16 (25)

Some of the functions include pre-mRNA splicing, rRNA processing, development and differentiation, role in transcriptional regulation (coactivator for Estrogen Receptor α) and transcription initiation (25) DEAD box helicases can mediate these functions either by binding to RNA and recruiting other proteins to form a complex, for which helicase activity may not required, or by unwinding duplex RNA or mediating annealing of RNA-both functions requiring helicase activity.

Structurally, the DEAD box helicases have a highly conserved helicase core region and variable N and C terminal regions. The conserved core consists of two identical domains that resemble the bacterial protein Recombinase A (RecA). The helicase core contains atleast 12 characteristic motifs at conserved positions as depicted in Fig 1.5. Motif II has

within it, the Asp-Glu-Ala-Asp (D-E-A-D) conserved sequence after which there proteins are named (25).

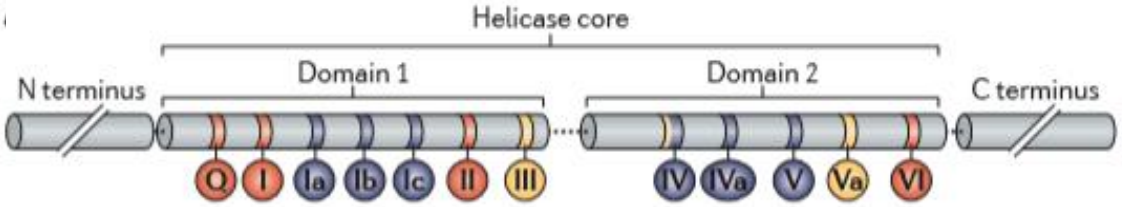
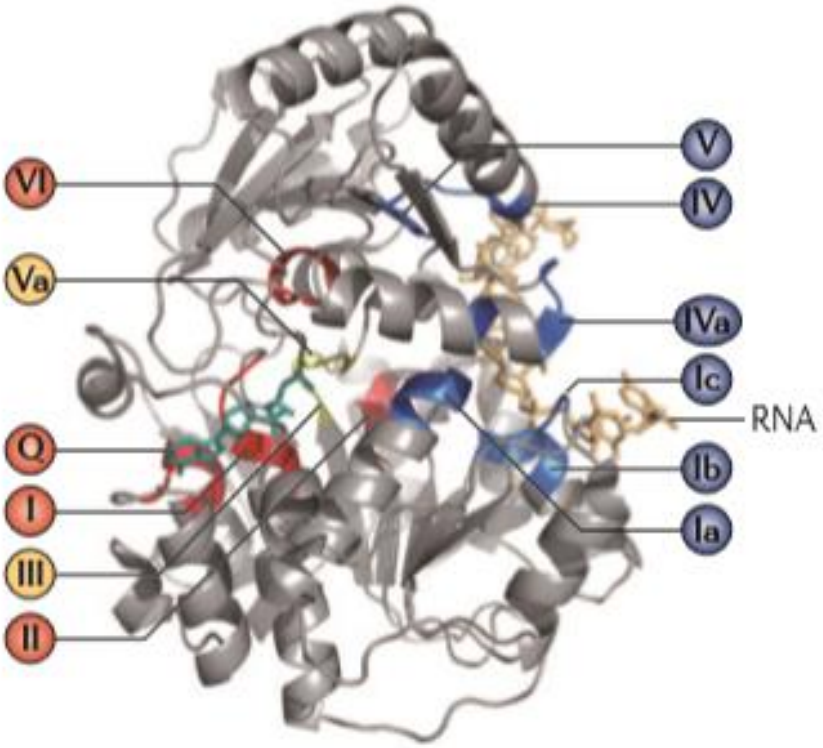


Fig 1.5 –Conserved domains and Motifs of DEAD box helicases. Linder P **,** Jankowsky E**.** From unwinding to clamping- the DEAD box RNA helicase family. Nat Rev Mol Cell Biol. 2011 Jul 22;12(8):505-16 (25)

The two helicase domains form a cleft that harbours the ATP- binding site as can be seen in Fig 1.6. Many studies report that the RNA that associates with the helicase binds to the region opposite the ATP binding site across both the Rec A domains as shown in Fig 1.5. The motifs in blue are the RNA binding motifs and motifs in orange indicate ATP binding motifs.



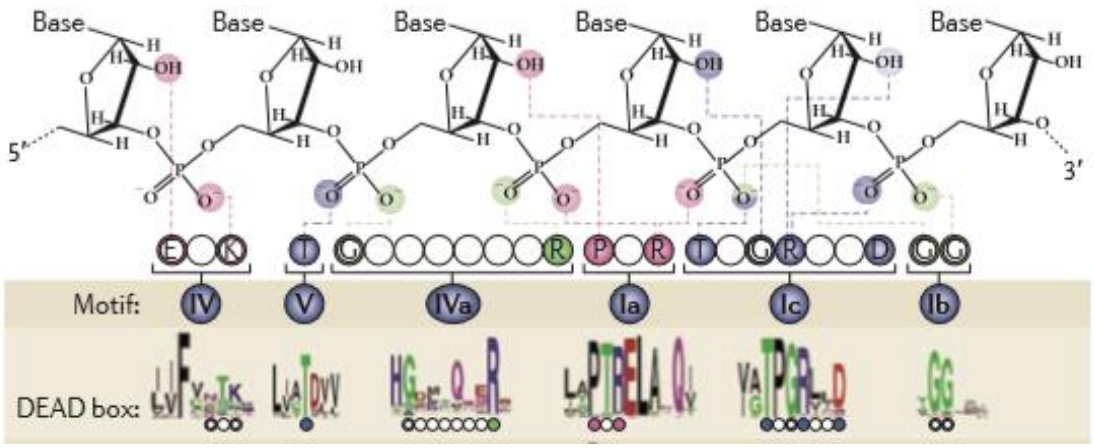


Fig 1.6 – Motifs involved in RNA binding in DEAD box helicases. Linder P **,** Jankowsky E**.** From unwinding to clamping- the DEAD box RNA helicase family. Nat Rev Mol Cell Biol. 2011 Jul 22;12(8):505-16 (25)

However, there are also reports that suggest that DEAD box helicases can interact with RNA substrates through the C terminal tail (26) or can be mediated by RGG motif present within the terminal sequences similar to hnRNP U, also an RNA binding protein (27)

The motifs within the helicase domain implicated in RNA binding are well conserved in p68. In addition to the conserved residues, p68 also has RGG motifs. RGG motif is some proteins is known to mediate nucleic acid binding. In the case of hnRNPU, the RGG motif mediates RNA binding (27).

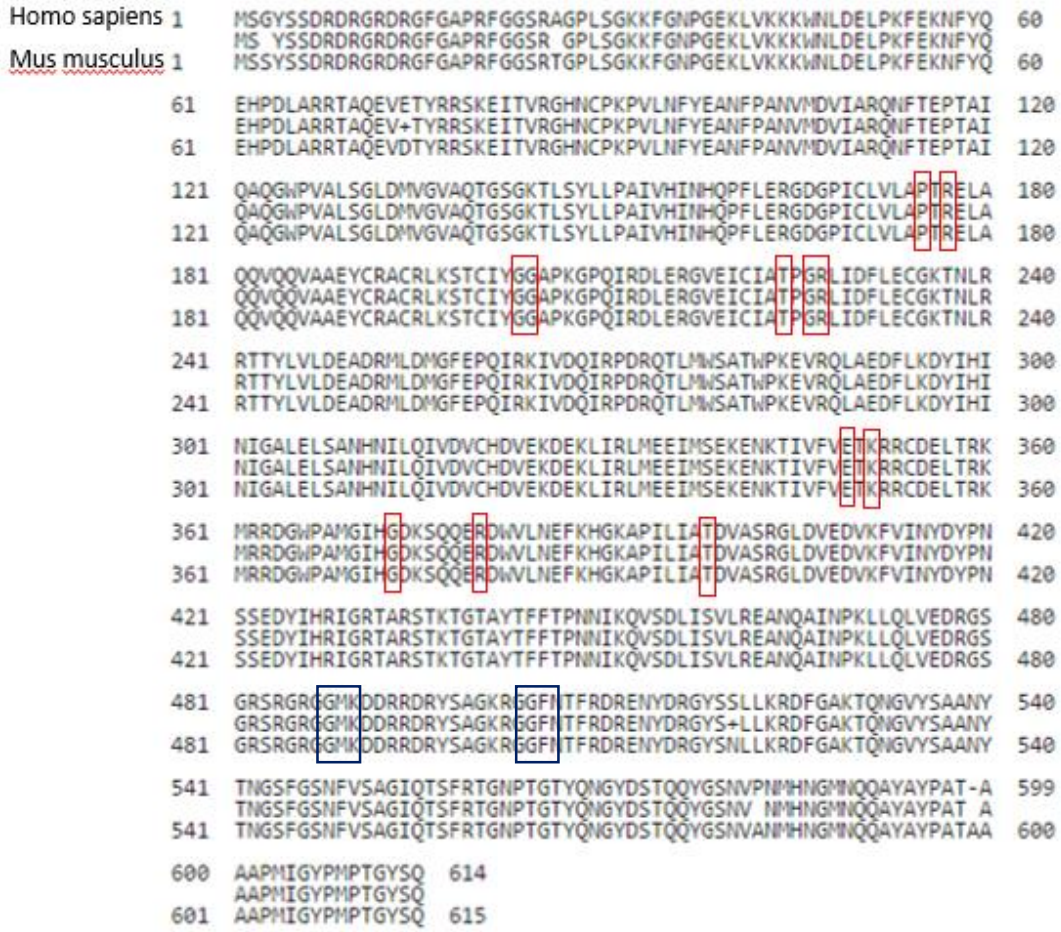


Fig 1.7 – Conserved residues implicated in RNA binding present in human and mouse DDX5 are highlighted in red and RGG motifs in black.

Functionally, p68 is known to play a role as a transcriptional coactivator for Estrogen Receptor α. p68 is required for gene regulation upon mrhl down regulation in mouse spermatogonial cells (20). Mrhl is involved in the nuclear retention of p68.Upon the down regulation of mrhl RNA, the p68 that is localized to the nucleus translocates to the cytoplasm and dimerizes with cytoplasmic beta catenin. This dimer then translocates into the nucleus. The nuclear translocation of beta catenin activates TCF4, a key transcription factor of the Wnt pathway. TCF4- beta catenin complex further binds to the WRE (Wnt Responsive Elements) present in target gene promoters and regulate gene expression. The nuclear translocation of beta catenin does not occur in the absence of p68. In addition, 27 out of the 37 GRPAM loci did not show mrhl occupancy upon p68 silencing. Also, the

expression of these 27 genes were perturbed under the same condition indicating that p68 is necessary for mrhl mediated regulation of these genes (22).

1.5 Wnt Signaling

Beta catenin is the core effector molecule of the canonical Wnt signaling pathway. Signaling by Wnt pathway is a fundamental mechanism which drives cell proliferation, differentiation, organogenesis and embryonic development. The Wnt pathway involves the Wnt ligands (glycoproteins) binding to the Frizzled and LRP family of receptors on the cell surface which set off a cascade of reactions within the cell. This cascade results in Beta catenin translocating to the nucleus where it functions as a transcriptional co activator and this is classified as the canonical Wnt signaling pathway. In the absence of Wnt ligands, beta catenin is targeted for destruction by the multimeric destruction complex, whose components include axin, adenomatous polyposis coli (APC), and GSK3β. Alternatively, Wnt ligand binding to its receptor can activate down stream effectors such as small GTPases of Rho family or heterotrimeric G proteins which result in the release of calcium inside the cell and are classified as the non-canonical Wnt/JNK pathway and non-canonical Wnt/calcium pathway respectively (28). The canonical and non- canonical Wnt signaling pathways are illustrated in Fig 1.8.

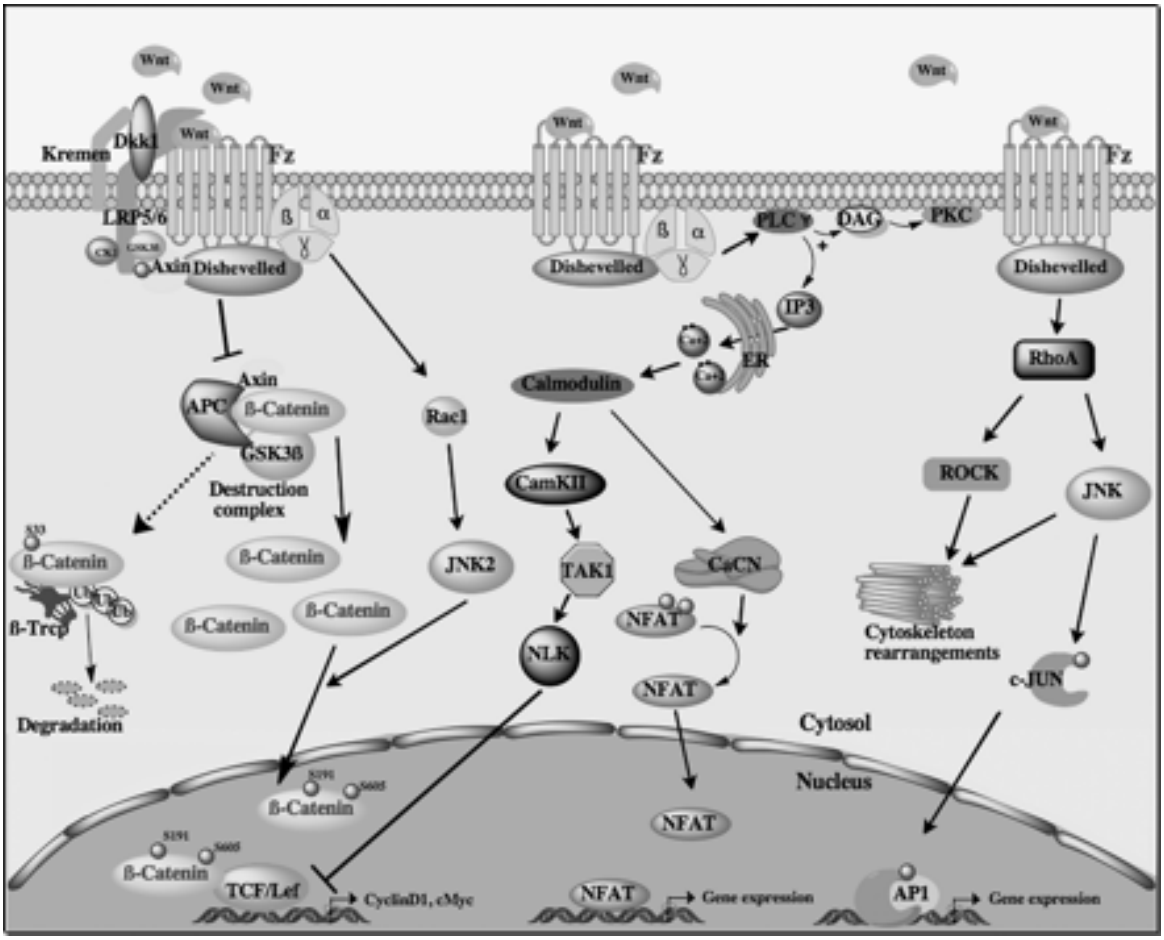


Fig.1.8 Canonical (left) and non-canonical (right) Wnt signaling pathways. Adapted from Rao, T. P., and M. Kuhl. 'An Updated Overview On Wnt Signaling Pathways: A Prelude For More'.2013. *Circulation Research* (29)

Wnt signaling is indispensable for spermatogenesis. The dynamic activation and silencing of Wnt signaling pathway during the various stages of spermatogenesis is required for the maintenance and proliferation of spermatogonial stem cells as well as Sertoli cells and also for the subsequent differentiation of the germ cells (30,31)

The role of mrhl long non coding RNA has been well characterized in mouse Gc1 spg cells (derived from murine B type spermatogonial cells). Through a series of experiments, it has been demonstrated that mrhl is a negative regulator of the Wnt signaling pathway. Several

Wnt signaling pathway genes showed perturbation upon mrhl silencing and TCF4 which is a key tran scription factor of the Wnt target genes was found to be up regulated (21).

The levels of mrhl RNA drop by almost 70% when spermatogonial cells are subjected to exogenous supply of Wnt3A ligand (21). It has been demonstrated that mrhl transcription is regulated by beta catenin and TCF4 that bind to its promoter upon Wnt activation along with the co repressor Ctbp1 (23). Wnt3A mediated Wnt activation and mrhl down regulation results in the upregulation of several pre meiotic and meiotic differentiation markers in Gc1 spg cells as shown in Fig 1.9.

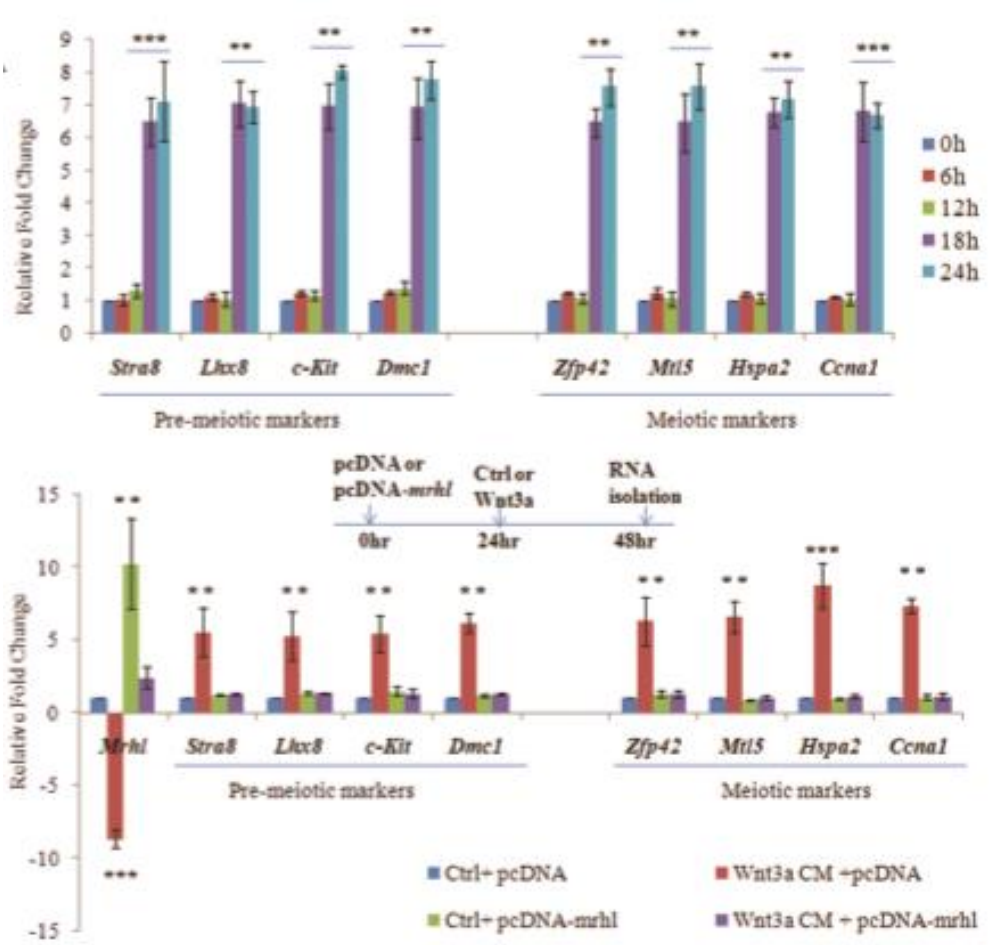


Fig 1.9 Upregulation of differentiation markers on Wnt3A mediated Wnt activation in Gc1 spg cells. Akhade VS *et al*,. Mechanism of Wnt signaling induced down regulation of *mrhl* long non-coding RNA in mouse spermatogonial cells, Nucleic Acids Research, 2016, Vol. 44 (32)

1.6 Aim and Scope of the investigation

As mentioned earlier, p68 is an interacting partner of mrhl and that p68 is essential for mrhl to physically interact with 27 of the 37 target genes. However, little is known about the domains involved in their interaction, both within the protein and RNA. We were interested in analyzing the regions involved in the interaction, both within the protein and RNA

The objectives of this work are as follows.

1. To understand the region of mrhl RNA that interacts with p68.
2. To understand the region of p68 that is involved in the interaction.
3. To understand if p68 interacts with mrhl directly.

2 Materials and Methods

2.1 Materials

All fine chemicals were purchased from Sigma-Aldrich, USA or Thermo Fischer Scientific Inc. All enzymes were purchased from New England Biolabs Inc, USA. Nitrocellulose membranes were obtained from Amersham International, England. Western ECL pico and femto kits were obtained from Thermoscientific, USA. Nylon membrane was obtained from Amersham International, England. Taq DNA polymerase was obtained from TaKaRa Bio, USA. Bio -14-CTP was obtained from Thermo Fisher Scientific.

2.1.1 Plasmids

pGEM3zf(+) plasmid was used for cloning full length as well as truncated mrhl gene fragments for *in vitro* transcription. pGEM3zf(+) possesses ampicillin resistance marker gene. Clones for *in vivo* expression of full length and truncations of mrhl with 3' S1 apatmer fusion and were created in the vector pCDNA3.1 which also possesses ampicillin resistance marker.

pET 22b (+) vector with p68 ORF sequence cloned into it was purchased from Origene.

P3XFLAG-CMV-10 vector was used for cloning of the p68 full length protein gene and internal deletion ORF to include N-terminal FLAG tag.

2.1.2 Bacterial Strains

XL1-Blue cells (Stratagene) were used for transformation of pGEM3zf(+) plasmids. The genotype of XL1-Blue is *end*A1 *gyr*A96(nal^{R}) thi-1 *rec*A1 *rel*A1 *lac gln*V44 F'[::Tn10 *pro*AB^{+} *lac*I ^{q} Δ*(lacZ)*M15] *hsd*R17(r_K^- m_K +)

BL21 cells were used for the transformation of pET22b(+) plasmid and protein expression.

2.1.3 Cell Lines

Cell lines used were Gc1- Spg mouse spermatogonia (B type) and L Wnt -3A cells which were obtained from American Type Culture Collection (ATCC). Cells were maintained in

Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum containing antibiotics (Penicillin and Streptomycin to a final concentration of 50 U/ml)

2.1.3 Antibodies

Rabbit Anti-p68 antibody was raised at the Centre. Rabbit- anti FLAG antibody was purchased from Sigma – Aldrich, USA. Goat Anti-Rabbit Antibody was obtained from Merck GeNei. HRP conjugated Streptavidin was obtained from Abcam, USA.

2.1.4 Primers

All primers were obtained from Sigma-Aldrich, USA. The list of primers is as follows.

Sr. No.	Name of Primer	Sequence of Primer (5' to 3')	Primer Target
1	Clone 1 F	CGCGGATCCTGACTTGCTCTTCATTAGATCATTTC	Mrhl gene
2	Sal1R	GAACCGGTCGACTGTGACCTGAGAGATGCCATC	Mrhl gene
3	Clone 2 F	CGCGGATCCCAGGCGACAAATTCACAACTC	Mrhl gene
4	Sal2R	GAACCGGTCGACATGAACATGATAAATCAGCAGTCAAC	Mrhl gene
5	Clone 3 F	CGCGGATCCATCTATTCCAGAAGGAATTTTAGGTA	Mrhl gene
6	Sal3R	GAACCGGTCGACGACATCTGTAAATGAGAGATGAGTG	Mrhl gene
7	Clone 4 F	CGCGGATCCTCCTGAGGTCGGTCCTTCC	Mrhl gene
8	Sal4R	GAACCGGTCGACAGTTAGGCAGGTAGAAATAACCCAAA	Mrhl gene
9	Clone 5 F	CGCGGATCCGTATTCTTGAGCCAGTTTTAGTCTTTC	Mrhl gene
10	Sal5R	GAACCGGTCGACAGGGTAGTGAATTCACAATGCC	Mrhl gene
11	Clone 6 F	CGCGGATCCGGTATCAAAACTGGCCTTGGAC	Mrhl gene
12	Sal6R	GAACCGGTCGACAGGAGGAATGAAGTATCCACCC	Mrhl gene
13	S1 1R	ATGCCTCGAGCCCGGCCCGCGACTATCTTAC GCACTTGCATGATTCTGGTCGGTCGGTTTGTG ACCTGAGAGATGCCATC	Mrhl gene
14	S1 2R	ATGCCTCGAGCCCGGCCCGCGACTATCTTAC GCACTTGCATGATTCTGGTCGGTCGGTTTATG AACATGATAAATCAGCAGTCAAC	Mrhl gene

15	S1 4R	ATGCCTCGAGCCCGGCCCGCGACTATCTTAC GCACTTGCATGATTCTGGTCGGTCGGTTAG TTAGGCAGGTAGAAATAACCCAAAG	Mrhl gene
16	S1 6R	ATGCCTCGAGCCCGGCCCGCGACTATCTTAC GCACTTGCATGATTCTGGTCGGTCGGTTGCG GCCGCAGGAGGAATGAAGTATCCACCC	Mrhl gene
17	P68 NotI F	GGCGCGGCCGCATGTCGAGTTATTCTAGTGACCGAG	P68
18	P68 del 161-235 R	TTGTTCTTCTCAGATTGGTTTTTGGCTGGTGGTTTATGTGTACAATG	P68
19	P68 del 161-235 F	ACATAAACCACCAGCCAAAAACCAATCTGAGAAGAACAACTTACCTTG	P68
20	P68 del 312-444 R	CTTGCTTTATGTTATTAGGTGTAAAATGGTTTGCACTCAGTTCCAGTG	P68
21	P68 del 312-444 F	GAACTGAGTGCAAACCATTTTACACCTAATAACATAAAGCAAGTGAGCG	P68
22	P68 R	GGCGGATCCATTTACATATACTTCTAAAGTCTTATTGAGAATACCCTG	P68
23	261 FP	TGAGGACCATGGCTGGACTCT	Mrhl
24	261 RP	AGATGCAGTTTCCAATGTCCAAAT	Mrhl
25	Beta actin FP	AGGTCATCATATTGGCAACG	Beta-actin
26	Beta actin RP	TACTCCTGCTTGCTGATCCAC	Beta-actin

Table 2. List of primers

2.2 Methods

2.2.1 Preparation of Bacterial Culture Medium

Bacterial strains were grown in Luria broth (LB) composed of 10g bactotryptone, 5g yeast extract and 10g NaCl in 1L of water. The medium was autoclaved at 121ºC for 20 minutes before use. For LB agar preparation, 15g of agar was added for 1 litre of broth. The medium and plates were supplemented with 100µg/ml ampicillin .

2.2.2 Preparation of Competent Cells and Transformation

Buffer TSS (10ml):

2X LB - 5ml

25% Polyethyleneglycol - 4ml

1M $MgCl_2$ solution - 500µl

Dimethyl Sulfoxide - 500 µl

Buffer was prepared fresh and sterilized by filtration through 0.20µm cellulose nitrate membrane filter.

Competent cells were prepared by modifying the Transformation and Storage Solution (TSS) method.The bacterial strain was inoculated in LB and grown overnight at 37°C. Using 1% of the overnight primary culture as inoculum, a secondary culture was grown at 37°C until it reached an optical density of 0.3 to 0.4 at 600nm. The culture was chilled on ice and the bacterial cell pellet was obtained by centrifugation at 1300xg for 10 minutes at 4ºC. The cell pellet was resuspended in 1/10th of the original volume of TSS. Aliquots of 150µl of competent cells were stored at -80ºC.

For transformation, competent cells were mixed with the desired DNA (approximately 100ng of DNA) and incubated on ice for 30 minutes. The cells were subjected to heat shock at 42ºC for 90 seconds and immediately chilled on ice for 5 minutes. 800µl of recovery LB medium was added and the cells were incubated at 37°C for 1 hour. The cells were then pelleted and resuspended in 100µl of LB and plated on LB agar containing appropriate antibiotic. LB plates were incubated at 37°C.

2.2.3 Plasmid DNA isolation by STET method

STET buffer (100ml):

Sucrose – 8gm

0.5M Ethylenediaminetetraacetic acid (EDTA) – 10ml

1M Tris HCl (pH 8.0) – 1ml

Triton X 100 – 0.5ml

Colonies of Bacteria were cultured in LB with 100µg/ml ampicillin at 37 ºC for 12 to 16 hours. The cells were pelleted and resuspended in 350µl of STET buffer. 25µl of lysozyme (10mg/ml) was added and the vials were placed in a boiling water bath for 40 seconds. The vials were then centrifuged at 15,000Xg for 10 minutes and the pellets were removed. RNase (10 µg/ µl) was added to the samples and incubated at 37 ºC for 60 minutes. To this sample, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and the tubes were centrifuged at 15.000Xg for 10 minutes. The aqueous supernatant layer was transferred to a fresh tube and to this, an equal volume of isopropanol was added and incubated for 15 minutes at room temperature. The samples were centrifuged at 15,000Xg, 4 ºC for 15 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol. The pellets were air dried and resuspened in milliQ water.

2.2.4 Plasmid DNA preparation by Macherey-Nagel NucleoSpin Plasmid Kit

For isolation of plasmid, the required colonies were inoculated in 10ml LB broth containing the appropriate antibiotic. They were grown at 37ºC overnight in a shaker orbital incubator. Plasmid DNAwas isolated from this culture using Macherey-Nagel (USA) Plasmid Mini Kit. Plasmid was eluted in a total volume 50µl. Amount of plasmid DNA isolated was estimated using Nanodrop (ThermoScientific, USA).

2.2.5 Plasmid DNA Preparation by Alkaline lysis and purification by PEG precipitation

Alkaline Lysis Solution I: 10mM EDTA (pH8.0)

50mM Glucose

25mM Tris- Cl (pH 8.0)

Alkaline Lysis Solution II:

0.2N NaOH

1% SDS

PEG – $MgCl_2$ solution:

40% polyethylene glycol (PEG 8000)

30mM $MgCl_2$

Alkaline Lysis Solution III

5mM Potassium acetate – 60ml

Glacial Acetic Acid – 11.5ml

Sterile Water – 28.5ml

Colonies were inoculated in 20ml LB broth containing appropriate antibiotic and grown overnight at 37 ºC at 180rpm. The cells were pelleted at 3,100Xg for 10 minutes and media was aspirated to leave the pellet dry. Pellet was resuspended in 200 µl of ice cold alkaline lysis Solution I. To this, 400 µl of freshly prepared alkaline lysis solution II was added and tubes were inverted to mix. 300 µl of ice cold alkaline lysis buffer III was added and mixed by inverting tubes. Lysate was centrifuged at 15,000Xg for 5 minutes at 4 ºC. RNase was added and the solution was incubated for 1 hour at 37 ºC .Plasmid DNA was recovered by isopropanol precipitation after extraction with phenol: chloroform. The dy plasmid pellet was dissolved in 1ml of sterile water and 0.5ml of PEG-$MgCl_2$ solution was added. Solution was stored at room temperature for 10 minutes and plasmid was collected by centrifugation at 15,000Xg for 20 minutes at room temperature. Traces of PEG was washed with 70% ethanol and the plasmid pellet was air dried and resuspended in sterile water.

2.2.6 Polymerase Chain Reaction

Polymerase Chain reactions were either set up in 50 µl or 10 µl reaction volumes. PCR for amplification of fragments for cloning was performed with Phusion polymerase or XT 20 polymerase for overlap extension PCR and all confirmatory PCRs were set up with Taq polymerase. A typical reaction contained 50-100 ng of template DNA, buffer to a final concentration of 1X, forward and reverse primers to a final concentration of 1mM, mixture of all four dNTPs to a final concentration of 1mM and 1U of polymerase. Denaturation was set up as per manufacturer's instruction for individual polymerase. Annealing was set up at

3 ºC below the Tm of primers for Taq polymerase and XT 20 polymerase at 3 ºC above the Tm of primer for Phusion polymerase as per manufacturer's instructions.

2.2.7 Restriction Digestion

All restriction enzymes were purchased from New England Biolabs, USA. Restriction enzymes were used according to the data sheet. Approximately 1µg of template DNA was used for a reaction volume of 50µl. Reaction was kept for either 3 hours or overnight.

2.2.8 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using 0.8-1.0% agarose gel prepared in 1x TAE (40mM Tris acetate and 1mM EDTA pH 8.0) containing 10µg/100ml of ethidium bromide. Electrophoresis was carried out at 5V/cm of gel.

2.2.9 Gel Elution and PCR clean up

Gel elution and PCR clean up for cloning of mrhl gene sequences were done as instructed in the data sheet using NucleoSpin Gel and PCR Clean-up kit provided by Macherey-Nagel, USA.

2.2.10 Ligation of restriction digested products

T4 DNA ligase was purchased from New England Biolabs, USA and used according to data sheet. Ligation reaction was set up in the ratio of 1:3 or 1:5 of vector to insert ratio in a reaction volume of 20µl. Reaction was incubated at room temperature for 3 hours.

2.2.11 Cloning

Clones of mrhl full length gene and truncations were made in the pGEM3zf(+) vector between the BamHI and SalI sites. Clones were screened for by α complementation

Clones of mrhl full length and truncation were made in the pCDNA3.1 vector between the HindIII and BamHI sites.

Clones of p68 full length and internal deletion ORF were made in p3XFLAG-CMV vector between the NotI and BamHI sites.

Clones were screened for by colony PCR and confirmd by DNA sequencing.

2.2.12 Screening for Transformed clones by α- complementation

As pGEM3zf(+) plasmid contains lacZα sequence, blue – white screening was performed to screen for recombinant plasmid containing colonies. Transformed cells were plated on LB agar supplemented with ampicillin to a final concentration of 100µg/ml, Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1mM and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to a final concentration of 40 ug/ml. Plates were incubated at 37^{o}C for 16 hours. White colonies were picked for further screening.

2.2.13 Expression and Purification of p68

Lysis Buffer:

50mM Tris-Cl buffer (pH 7.5)

150mM NaCl

0.5mM EDTA

10% Glycerol

20mM imidazole

1mM Dithiothreitol (DTT)

Lysozyme to final concentration of 1mg/ml

1mM Phenylmethanesulfonylfluoride (PMSF)

Single bacterial colony was inoculated to 5ml LB supplemented with ampicillin and grown overnight at 37^{o}C in a shaker incubator. From the overnight primary culture, secondary culture was inoculated and grown at 37^{o}C till OD_{600nm} was around 0.6. To this culture, IPTG was added to a final concentration of 1mM to induce protein expression. The culture was further incubated either for 4 hours at 37^{o}C or overnight at 18^{o}C. The cells were pelleted and resuspended in 5ml of lysis buffer and incubated at 30^{o}C for 40 minutes. The cells were then sonicated at 20% amplitude for 10 minutes at 5 seconds on - 5 seconds off cycle. The cell lysate was transferred to ookridge tube and centrifuged at 17,700Xg for 15 minutes at 4^{o}C. The supernatant and the pellet fraction was separated and the pellet was resuspended

in an equal volume of lysis buffer. The pellet was boiled in the presence of loading dye and resolved by SDS-PAGE.

2.2.14 Sodium Dodecyl Sulphate Polyacrylamide electrophoresis (SDS-PAGE)

Composition of 12% separating gel

	10ml	20ml
Water	3.3ml	6.6ml
30% Acrylamide solution	4ml	8ml
1.5 M Tris- Cl pH 8.8	2.5ml	5ml
10% SDS	0.1ml	0.2ml
10% Ammonium persulphate	0.1ml	0.2ml
TEMED	0.006ml	0.012ml

Composition of stacking gel:

	5ml
Water	3.4ml
30% Acrylamide solution	0.83ml
1M Tris Cl pH 6.8	0.63ml
10% SDS	0.05ml
10% APS	0.05ml
TEMED	0.005ml

Electrophoresis buffer

25mM Tris

250mM glycine pH 8.3,

0.1% SDS

6X SDS loading dye

375mM Tris-Cl pH 6.8

10% beta mercaptoethanol

10%SDS

0.03% bromophenol blue

50% glycerol

Proteins were resolved on a 12% polyacrylamide gel containing 0.1% sodium dodecyl sulphate. The gel was electrophoresed at 100V for 3 hours.

2.2.15 Western Blotting

Transfer buffer

7.5g glycine

1.65g Tris,

1% SDS,

20% of methanol

The proteins resolved by 12% SDS-PAGE were transferred on to activated nitrocellulose membrane using Amersham Biosciences semidry transfer unit with the transfer buffer. The transfer was set up at 2.5mA/ cm^2 of blot area. After transfer, the membrane was stained with 0.1% Ponceau (0.1g of Ponceau, 5ml of acetic acid made upto 100ml using water) to check the success of transfer of proteins. The membrane was blocked using 5% skimmed milk in 1x PBS or 3% BSA in 1XTBS for 1 hour at room temperature. The membrane was then incubated with the primary antibody of appropriate titre in 1% skimmed milk in 0.05% PBST or 1%BSA in 1XTBS overnight at 4ºC. The membrane was washed with 0.05% PBST or 0.3% TBST thrice for 10 minutes each. Subsequently the membrane was incubated with the secondary antibody in 1% skimmed milk or 1% BSA in 1XTBS at a dilution of 1:4000 for 1 hour at room temperature. This was followed by three washes of 10 minutes each using 0.05% PBST or 0.3% TBST. Detection was carried out using the Amersham ECL-analysis system.

2.2.16 Purification of IgG from Serum by sequential precipitation with Caprylic Acid and Ammonium Sulphate

IgG from Rabbit serum was precipitated by addition of 4 times serum volume of 60mM sodium acetate (pH 4.0).The pH of solution was adjusted to 4.5 with 1M Tris. 25µl of caprylic acid per ml of serum was added drop wise with vigorous mixing and was kept for stirring for 30 minutes at room temperature. The solution was centrifuged at 10,000X g for 30 minutes at room temperature. The supernatant was filtered through 0.8µm filter and pH was adjusted to 7.4 with 1M Tris. The solution was cooled to 4^oC and 0.277g of ammonium

sulphate per ml of solution was added slowly. The solution was stirred at 4^{o}C till precipitate was formed. The precipitate was pelleted by centrifugation at 10,000Xg for 15 minutes at 4^{o}C. The pellet was resuspended in 1X PBS.

2.2.17 Affinity Purification of Antibody by antigen immobilization on membrane

The pellet fraction of cell lysate was resolved by SDS PAGE and transferred on to nitrocellulose membrane by semi dry transfer. Membrane was stained with Ponceau S staining solution (0.1%(w/v) ponceau, 1%(v/v) acetic acid), protein band was cut out and additional membrane was discarded. Membrane was washed extensively to remove Ponceau stain. The membrane was blocked with 5% skim milk in 0.05% PBS Tween 20 at room temperature for 1 hour. The membrane was washed extensively with PBS T. IgG that was purified by sequential precipitation was bound to membrane overnight at 4^{o}C. The IgG bound membrane strip was then washed with PBS and the antibody was eluted with 0.1M glycine pH 2.5. The eluate was immediately neutralized by adding 1/10th volume of Tris- HCl pH 10.

2.2.18 Culturing of mammalian cells

Mammalian cells were maintained in DMEM supplemented with 10% FBS and 10 U/ml of penicillin and 0.1mg/ml of streptomycin (Complete medium). The cells were grown at 37^{o}C at 5% CO_2 until 95% confluent. For passaging, cells were detached from culture dishes by trypsinisation (0.25% trypsin and 0.02% EDTA) after washing with PBS. Trypsin was neutralized in the presence of complete medium and cells were pelleted at 1500Xg for 4 minutes. Cell pellet was resuspended in complete medium and replated in required number of culture dishes.

2.2.19 Freezing of mammalian cells.

Cells grown to confulency were washed with PBS and trypsinised. The cells were pelleted by centrifugation at 1,500Xg for 4 minutes. The pellet was resuspended in ice cold freezing medium (95% complete medium +5% DMSO) and immediately transferred to chilled cryovials. The vials were stored at -80^{o}C for two days before being transferred to liquid nitrogen.

2.2.20 Thawing of mammalian cells

Frozen stocks were thawed in a circulating water bath at 37^{o}C for 2-5 minutes. The thawed stocks were transferred immediately into 5ml of complete medium to dilute the DMSO. Cells were pelleted by centrifugation at 1,500Xg for 4 minutes. The pellet was gently resuspended in complete medium and seeded in culture dishes.

2.2.21 Preparation of nuclear lysate from cells

Cell Lysis Buffer	**Nuclear Lysis Buffer**
15mM Tris Cl (pH7.4)	20mM Tris Cl (pH 7.4)
2mM $MgCl_2$	2mM $MgCl_2$
15mM KCl	10mM KCl
	150mM Nacl
	0.2% NP-40
	10% Glycerol

Cell pellets were resuspended in 5 times the pellet volume of Cell lysis buffer and stored on ice for 10 minutes with intermittent pipetting. The nuclei were pelleted from the suspension by centrifugation at 13,000Xg for 10 minutes at 4^{o}C. The pelleted nuclei were then resuspended in 5 times pellet volume of nuclear lysis buffer and stored on ice for 30 minutes with intermittent pipetting. The debris was pelleted by centrifugation at 15,000Xg for 10 minutes at 4^{o}C. The protein from the supernatant fraction, which is the nuclear lysate, was quantified by Bradfor's method with Bradford reagent (Bio-Rad).

2.2.22 *In vitro* Transcription

Clones of mrhl made in the pGEM3zf(+) plasmid were linearized with either HindIII enzyme to prepare template for the sense transcript from T7 promoter or with BamHI to prepare the template for the anti-sense transcript from SP6 promoter. The digested plasmids were purified from gel. 1µg of linearized plasmid was incubated along with 1mM each of ATP, CTP, GTP and UTP with either T7 polymerase or SP6 polymerase (Thermo Fisher Scientific) in 1X transcription buffer. For biotin labeling of transcripts, bio-14-CTP and CTP were mixed in a ratio of 1:3 and used to a total final concentration of 1mM. The reaction was incubated

at 37^{o}C for 2 hours 30 minutes. After this, the template DNA was digested by the addition of 2.5Units of Rnase free DNase (Promega) in the presence of DNAse buffer to a final concentration of 1X. The reaction was incubated at 37 ^{o}C for 1 hour. The RNA was extracted from the mix by one phenol: chloroform: isoamylalcohol (25:24:1) extraction and one chloroform extraction. The RNA was precipitated by the addition of isopropanol in the presence of 30µg of glycogen as a carrier at -20^{o}C for 1 hour. The precipitated RNA was collected by centrifugation at 12,000Xg for 10 minutes at 4^{o}C. The pellet was washed once with 70% ethanol and air dried. The dried pellet was resuspended in nuclease free water and stored at -20^{o}C.

2.2.23 RNA pulldown for *In vitro* detection of RNA-associated proteins

Buffer A

150m M KCl

25mM Tris pH 7.4

5mM EDTA

0.5mM DTT

0.5% NP 40

1mM PMSF

100U/ml RNase inhibitor

In vitro transcribed, biotinylated RNA was quantified by spectrophotometry with Nanodrop-1000 (Thermo scientific). 20µg of RNA was heated to 65^{o}C and slowly cooled to allow proper folding of RNA. Nuclear extract containing 1mg of protein was pre-cleared by incubating with 60µl of streptavidin beads (Pierce) at 4^{o}C for 1 hour on a tube rotator. The supernatant was collected after centrifugation at 400Xg for 30 seconds and the volume was duplicated with buffer A supplemented with fresh protease inhibitors, RNAsin and yeast tRNA (0.1µg/µl).To this, 20µg of RNA was added and incubated overnight on tube rotator at 4^{o}C. To isolate RNA bound to proteins, 80 µl of streptavidin agarose beads slurry that was washed with Buffer A was added and incubated on rotator at 4^{o}C for 2 hours. The beads were washed three times with 500µl of buffer for 1 minute on tube rotator. Beads were collected by centrifugation at 400Xg for 30 seconds. The beads were then boiled in 50 µl of

6X SDS loading dye for 10 minutes. The beads were centrifuged at 15,000Xg for 30 seconds and the supernatant dye was resolved on a 12% SDS-PAGE for western blotting to detect p68 protein binding.

2.2.24 Electrophoretic Mobility Shift Assay

10X TBE (pH 7.5 and 8.3)

1M Tris base

1M Boric acid

0.02M EDTA

pH is adjusted with HCl

10X EMSA buffer

300mM Tris-Cl (pH7.5)

1M NaCl

20mM $MgCl_2$

10mM DTT

6X EMSA loading dye

0.6M KCl

10mM Tris-Cl(pH7.5)

5% Glycerol

0.25% w/v Bromophenol Blue

0.25% w/v xylene cyanol

Binding assays were set up in 20µl volume for RNA and protein using 1.32fmol of RNA in the presence of 1X EMSA buffer, presence or absence of 1mM ATP and varying molar ratios of protein: RNA (4:1, 2:1, 1:1 and 0.5:1). The reaction was incubated at 37^{o}C for 30 minutes. The reaction was brought to room temperature and EMSA loading buffer was added to a final concentration of 1X. The entire volume of reaction was loaded and resolved on pre chilled 1% agarose in 0.5X TBE buffer pH 7.5 with the same as running buffer for 1 hour. The electrophoretic set up was maintained at 4^{o}C throughout. The RNA was then transferred onto positively charged nylon membrane with a semi dry transfer apparatus using ice cold 1X TBE buffer pH8.3 as the transfer buffer. Transfer conditions were maintained at 4mA/ cm2 of membrane area for 40 minutes at 4^{o}C. The membrane was immediately subjected to UV crosslinking using the UV stratalinker 1800 at auto crosslink setting (120,000 µJ/cm2). The membrane was then blocked for 1 hour in 3% BSA solution prepared in 1X TBS buffer prepared with DEPC treated water. After blocking, the membrane was incubated with 1:100 dilution of streptavidin – HRP conjugate in 1% BSA in 1X TBS. The

blot was washed thrice with 0.3% TBST and then developed using ECL, similar to Western blot.

2.2.25 Transfection of mammalian cells.

Gc1 spg cells were grown to 60% confluency in complete medium prior to transfection. Cells were washed twice with 1X PBS and antibiotic free DMEM containing 2% FBS was added and incubated at 37^{o}C at 5% CO2 till addition of DNA-lipid comlex. Lipofectamine 2000 transfection reagent (Thermofisher Scientific) was used for transfection experiments. Plasmid DNA was used at concentrations as given below

Size of culture dish	Volume of culture medium	Amount of plasmid DNA	Volume of lipofectamine 2000
90 mm	7.5ml	11.25µg	22.5µl
60 mm (6 well dish)	1.5ml	2.25 µg	4.5 µl

Plasmid DNA and lipofectamine 2000 were diluted in 250 µl each of antibiotic free DMEM containing 2% FBS. The diluted plasmid was added to diluted lipofectamine 2000 and incubated for 20 minutes. The DNA-lipid complex was then added to the cells. 8 hours post transfection, medium containing DNA-lipid complex was aspirated out and cells were washed twice with 1X PBS. Fresh DMEM containing 10% FBS and antibiotics was introduced into the tissue culture dishes and cells were grown upto 48 hours post transfection.

2.2.26 *In vivo* RNA pulldown to detect interacting protein partners

S1 Lysis buffer

10mM Tris-Cl (pH7.4)

150mM NaCl

5mM $MgCl_2$

0.1mM EDTA

0.8% TritonX-100

5% Glycerol

0.5mM PMSF

50U/ml RNase inhibitor

1mM DTT

1X mammalian protease inhibitor cocktail (mPIC)

Clones for mammalian expression of mrhl RNA attached to S1 aptamer were transfected into Gc1 spg cells in 90 mm culture dishes. 48 hours post transfection, cells were harvested by trypsinization after washing twice with 1X PBS. Cells were resuspended in 5 times the pellet volume of S1 lysis buffer and incubated on ice for 15 minutes. The tubes were then centrifuged at 2,500 X g at 4^{o}C for 5 minutes. The protein in the supernatant was quantified by Bradford's assay and 30µl of Streptavidin agarose beads slurry was used per 500µg or protein. The lysate was incubated with beads equilibrate with S1 lysis buffer overnight at 4^{o}C on a tube rotator. The beads were then washed three times with S1 lysis buffer for 1 minute on tube rotator and collected by centrifugation at 400X g for 1 minute at room temperature. The beads were boiled with 50 µl of 6X SDS loading buffer on resolved on 12% SDS-PAGE for detection of p68 by western blotting.

2.2.27 Isolation of total RNA from cells by TRIzol extraction

Cells were homogenized in 1 ml of TRIzol and incubated at room temperature for 5 minutes. 200 µl of chloroform was added and tubes were mixed vigorously for 15 seconds. The tubes were incubated at room temperature for 2 minute 30 seconds. The tubes were centrifuged for phase separation at 12,000X g for 15 minutes at 4^{o}C. The upper aqueous layer was removed and 500 µl of isopropanol per ml or TRIzol was added to the aqueous phase. The tubes were incubated at room temperature at 10 minutes or at -20^{o}C for atleast 30 minutes in the presence of 30µg of Glycogen as carrier. The tubes were centrifuged at 12,000X g for 10 minutes at 4^{o}C to collect the precipitated RNA. The supernatant was discarded and the RNA pellet was washed with 1ml of 70% ethanol and centrifuged at 7,500Xg for 5 minutes. The pellet was dried and resuspended in DEPC water.

2.2.28 Synthesis of First strand of cDNA

RNA was first treated with DNase to remove genomic DNA contamination. RNA was resuspended in 1X DNase buffer RNase free DNase I was used at 1u/ µg of RNA. The reaction was incubated at 37^{o}C for 45 minutes and heat inactivated at 65^{o}C for 10 minutes. Around 2 µg of RNA was used for synthesis of first strand. In 0.5ml PCR tube, RNA, 5µM of Oligo $(dT)_{18}$ primer and nuclease free water to make the volume upto 12µl was added and the reaction was incubated at 65^{o}C for 5 minutes. The reaction was snapped cooled on ice and

1X Revertaid buffer, 1mM dNTP, 20 U of Ribolock RNase inhibitor and 200 U of revert aid Reverse transcriptase was added and reaction was incubated at 42^{o}C for 60 minutes. Reaction was terminated by heating to 70^{o}C for 10 minutes.

2.2.29 RNA immunoprecipitation

IP Lysis Buffer	**RIP Buffer**
50mM HEPES (pH 7.5)	50mM HEPES (pH7.5)
0.4M NaCl	0.1M NaCl
1mM EDTA	5mM EDTA
1mM DTT	10mM DTT
0.5% TritonX-100	0.5% TritonX-100
10% Glycerol	10% Glycerol
	1% SDS

FLAG – tagged constructs of p68 protein were made in mammalian expression vector for RNA immunoprecipitation experiments. The cloned plasmids were transfected into Gc1 spg cells grown in 90 mm tissue culture dishes and used 48 hours post transfection for experiment. Cells were harvested by trypsinization and resuspended in 5 ml of ice cold 1XPBS. Crosslinking was carried out by adding 37% formaldehyde drop by drop into the cell resuspension to a final concentration of 1%. Tubes were incubated on gel rocker for 10 minutes at room temperature. 2M glycine was added to the tubes to a final concentration of 0.25M to quench crosslinking. Tubes were incubated on gel rocker at room temperature for 5 minutes. Cells were pelleted at 100X g at room temperature for 2 minutes. Cells were washed twice in ice cold 1XPBS was collected by centrifugation at 100Xg for 2 minutes at 4^{o}C. Cells were then resuspended in 1 ml of IP lysis buffer and lysed by sonication at 40 % amplitude at 5 second on/5 second off cycle for 2 minutes. Then debris was then pelleted by centrifugation at 13,000Xg for 3 minutes at room temperature. The lysate was then incubated with either 80 µl of pre blocked anti- FLAG M2 agarose beads or 60 µl of agarose beads pre incubated with pre immune serum, overnight at 4^{o}C on tube rotator. Beads were washed three times with 500µl of IP lysis buffer for 1 minute on tube rotator at room temperature and resuspended

in 100µl of RIP buffer. The beads along with the buffer were incubated at 70^{o}C for 1 hour to reverse crosslinking. The tubes were centrifuged at 400X g for 1 minute at room temperature to sediment beads and supernatant was collected. RNA was isolated by TRIzol extraction from the supernatant. cDNA synthesis was set up in 20 µl reaction with around 500ng of RNA as template after DNase treatment. PCR was set up to amplify gene of interest with 1 µl of cDNA as template.

Results and Discussion

3. One on one interaction of p68 and mrhl

3.1 Introduction:

Earlier studies from our laboratory have established that p68 is an interacting partner of mrhl by means of *in vitro* and *in vivo* pulldown assays and RNA immunoprecipitation studies.

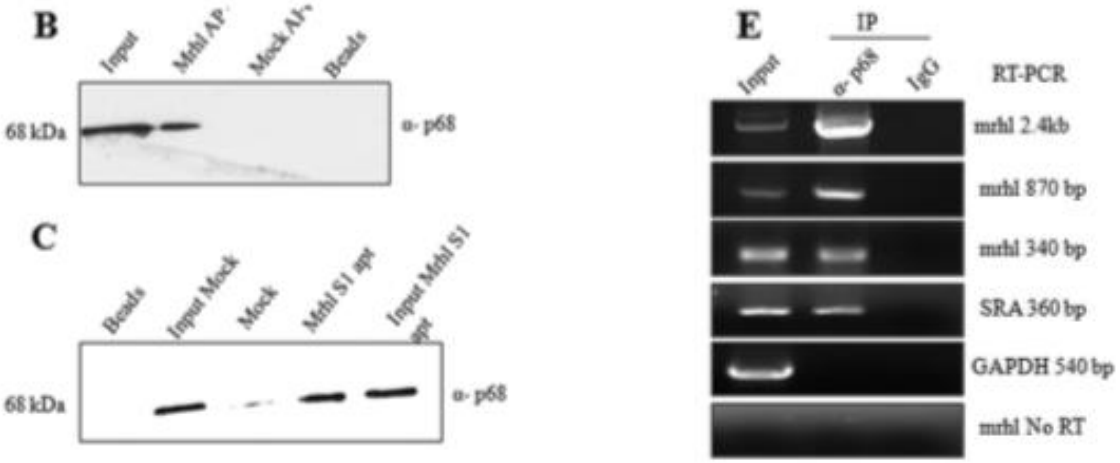


Fig 3.1 – p68 is an interacting partner of mrhl. Demonstrated by *in vitro* pulldown (B), *in vivo* pulldown (C) and RNA immunoprecipitation studies (E). Arun *et. Al.,* mrhl RNA, a Long Noncoding RNA, Negatively Regulates Wnt Signaling through Its Protein Partner Ddx5/p68 in Mouse Spermatogonial Cells,Mol. Cel. Biol. 2012 Aug, (19)

However, it was not known if this protein was binding directly to the RNA or the interaction was mediated through other bridging molecules. P68 possesses the ATP binding domain. Literature reports indicate the requirment of ATP binding and/or ATPase activity for the activity of many DEAD box helicases, it is not common to all members of the family. So, It was of interest to analyze the components of this interaction.

3.2 Cloning of mrhl full length gene in pGEM3zf(+)

2.4kb gene of mrhl was amplified from mouse genomic DNA for cloning into pGEM3zf(+) using primers which contained restriction sites for the enzymes BamHI and SalI. The PCR amplified, digested fragment was ligated into pGEM3zf(+) vector also linearized with BamHI and SalI enzymes. Recombinant clones that were grown in the presence of ampicillin were screened for by PCR and DNA sequencing of the insert.

The plasmid was used for *in vitro* transcription.

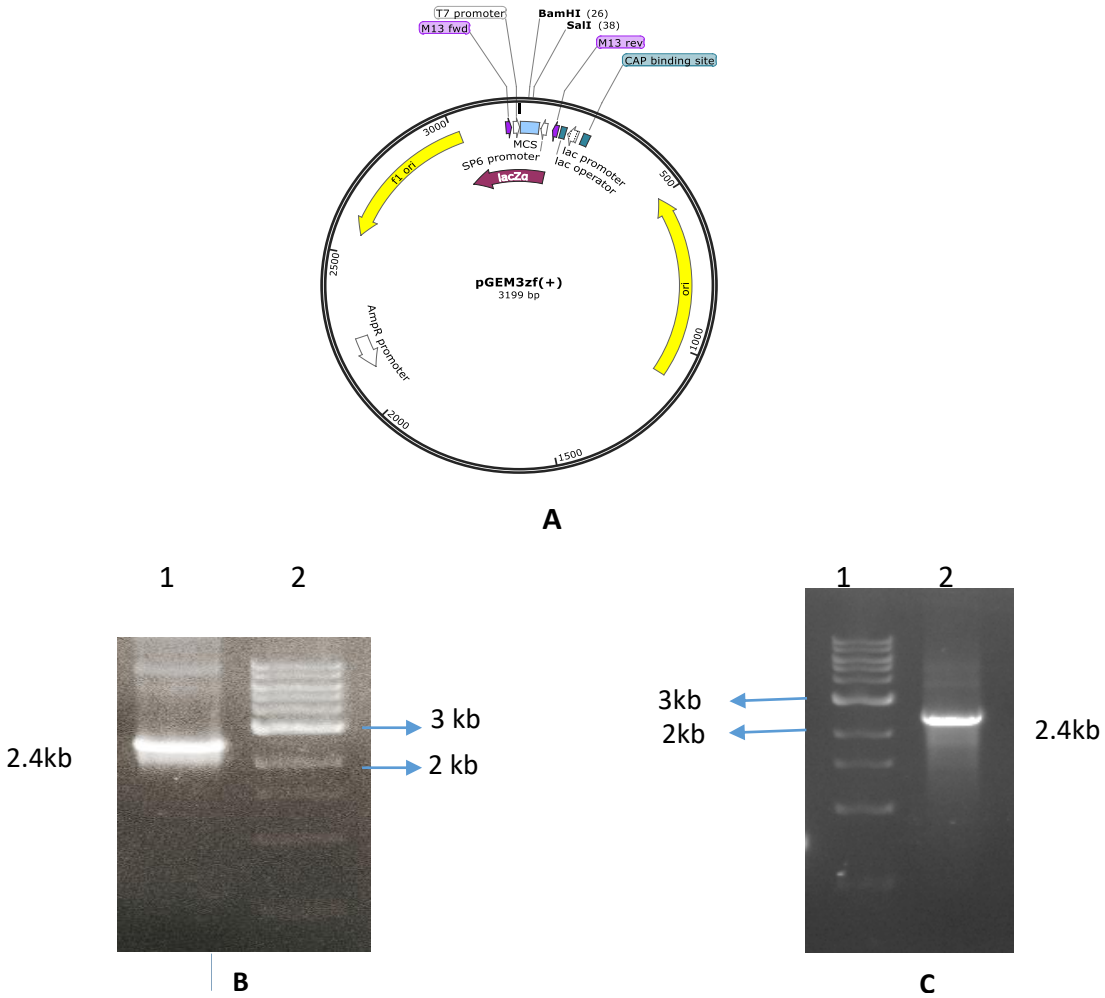


Fig 3.2 – Cloning of mrhl gene in pGEM3zf(+) vector. **A** – Vector map of pGEM3zf(+), **B** – PCR amplification of mrhl gene for cloning, Lane 1 –PCR amplified product, Lane 2 – NEB 1kb ladder, **C** – PCR confirmation of the clone, Lane 1 – NEB 1kb ladder, Lane 2 – PCR product

3.3 *In vitro* Transcription of mrhl and Electrophoretic Mobility Shift Assay

Mrhl gene was cloned down stream of the T7 promoter in the pGEM vector. The vector was linerized down stream of the gene with HindIII enzyme and 1 µg of plasmid was used as template for *in vitro* transcription with T7 RNA polymerase.

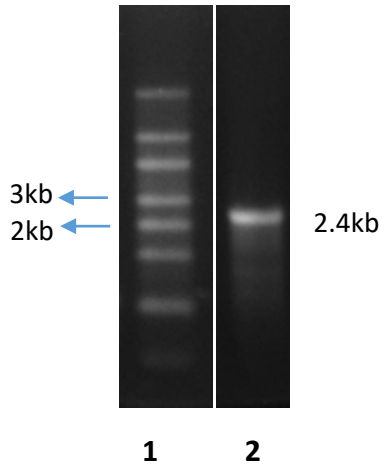


Fig 3.3 – RNA agarose electrophoresis for *In vitro* transcribed mrhl gene. Lane 1 – RNA marker, Lane 2- IVT product

P68 protein that was commercially procured was confirmed by Western blotting

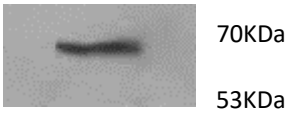


Fig 3.4 - Western blot confirmation of commercial p68 protein.

Electrophoretic mobility shift assay was set up with mrhl *in vitro* transcribed RNA and p68 protein in the presence and absence of ATP. It was observed that there was shift observed in the RNA in the presence of p68 without ATP. However, this shift was more pronounced in the presence of ATP.

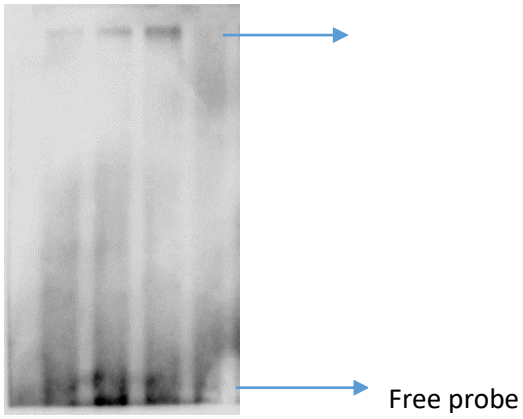


Fig 3.5 – Electrophoretic mobility shift assay for mrhl RNA. Lane 1 – RNA in buffer containing DTT, Lane 2 – RNA and p68 in buffer containing DTT, Lane 3 – RNA and p68 in buffer containing DTT and 1mM ATP, Lane 4 – RNA only

Next, binding reactions were set up with varying molar ratios of p68 to protein. Ratios of 0.5:1, 1:1, 2:1 and 4:1 of p68: mrhl were set up in the presence of 1mM ATP. Gel retardation assay showed increasing concentration of shifted aggregate with increasing amount of protein. Since the signal for retardation is observed very close to the wells, it is possible that no RNA:protein complex is being formed but the signal is due to aggregate formation. The experiment will have to be repeated in the presence of Heparin to preclude non specific RNA:protein interactions

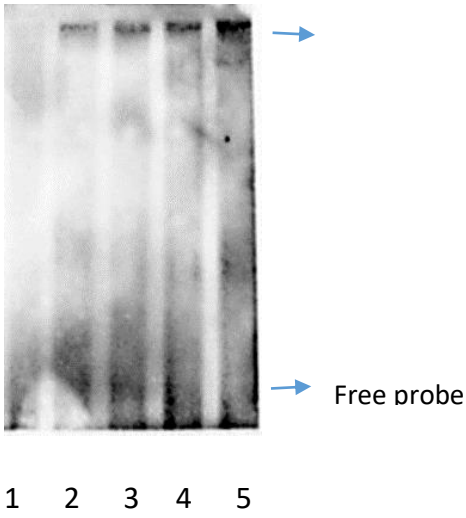


Fig 3.6 – Electrophoretic mobility shift assay with varying p68: mrhl ratios. Lane 1 – RNA only, Lane 2 –0.5:1 molar ratio, Lane 2 – 1:1 molar ratio, Lane 3 – 2:1 molar ratio, Lane 4 – 4:1 molar ratio of p68 : mrhl in the presence of Buffer containing DTT and 1mM ATP.

4. Mapping the region of mrhl that binds to p68

4.1 Bioinformatics analysis of protein binding region of mrhl

The software catRAPID was used to predict the regions on mrhl that might bind to p68 (*Manjira Ghosh, unpublished data*). catRAPID uses an algorithm to predict propensity of interaction between a protein –RNA pair based on secondary structure, hydrogen bonding and Van der waal's forces.

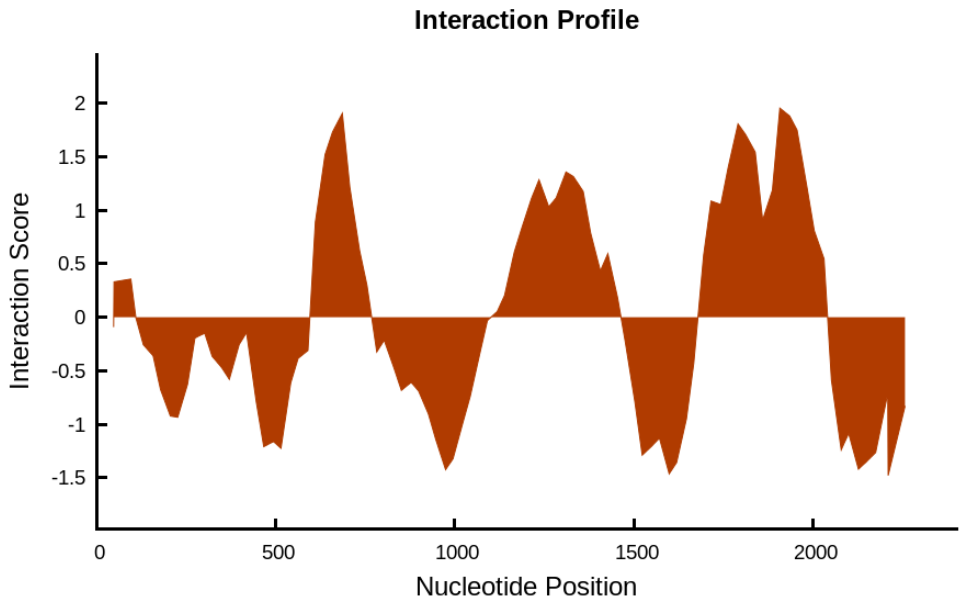


Fig 4.1 – catRAPID fragments prediction for propensity of interaction between mrhl and p68. A – The interaction score (Y-axis) along the RNA sequence (X-axis) shows that three regions within mrhl RNA have high interaction scores making them candidate regions of interaction.

In addition to the regions predicted by catRAPID, another region suspected to interact with p68 A homolog of mrhl within the 15th intron of human *phkb* gene has been recently identified in the laboratory (*Roshan Fatima, unpublished data).* RNA IP followed by high throughput sequencing revealed that hmrhl is an interacting RNA of p68 in human cancer cells (28).

Preliminary BLAST search revealed around 65% homology between mrhl and human mrhl (hmrhl) mapping to the region 1095-2198 nucleotides of mrhl. In addition to the three regions predicted by catRAPID, this conserved region could have the p68 binding domain within it.

Fig 4.2 – BLAST analysis for mrhl and hmrhl shows 65% homology mapping to region 1095-2198 of mrhl.

4.2 Cloning of truncated regions of mrhl

Based on catRAPID software prediction and BLAST results, 5 truncations of mrhl were made as shown in Fig. 4.3. The RNA gene was divided into four regions such that three segments contained regions of predicted propensity for interaction with p68 and one spanned a region that had no predicted propensity for interaction based on catRAPID predictions. One clone spanned the region of homology between mrhl and hmrhl. Clone 1 contained +1 to +420, clone 2 contained +420 to +840, clone 34 contained region +840 to +1690 and clone 56 contained

region +1690 to +2401 of mrhl RNA gene. Clone 36 spanned the region +840 to +2401 to encompass region showing homology to hmrhl

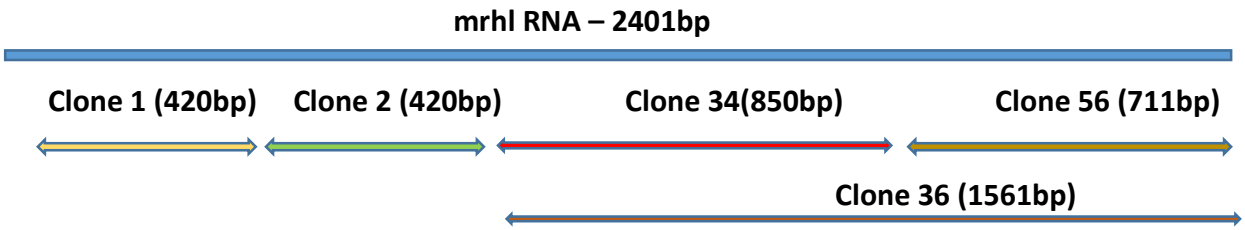
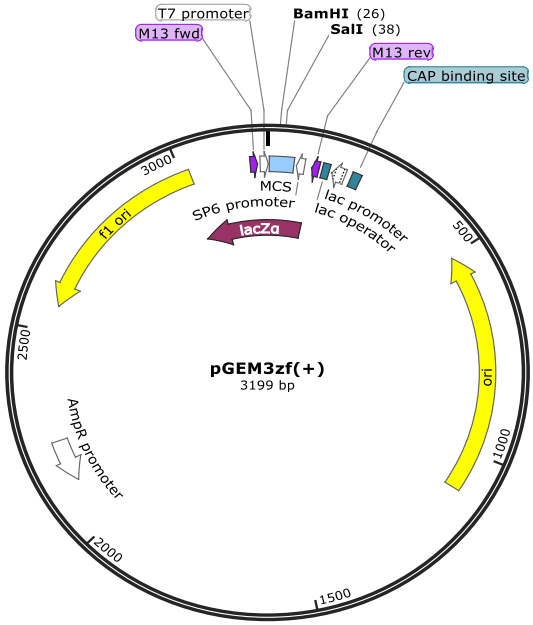


Fig 4.3 – A schematic representation of trucation clones of mrhl RNA. The full lenth 2401 base mrhl RNA gene was truncated into regions as indicated in the figure based on the results from the prediction software catRAPID and BLAST results oh mrhl v/s hmrhl.

The clones were made in the vector pGEM3zf(+) to support *in vitro* transcription between the restriction sites for BamHI and SalI



A

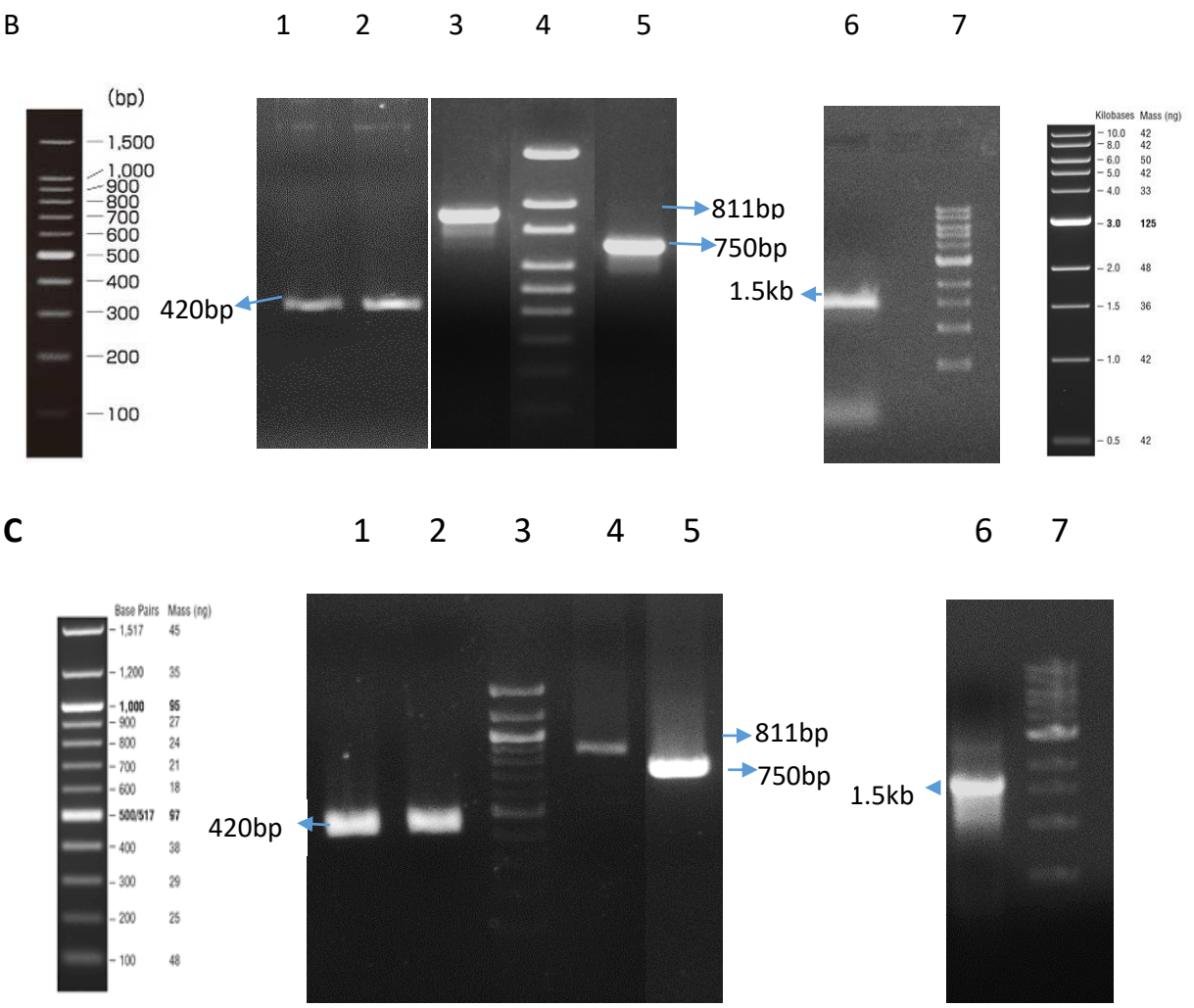
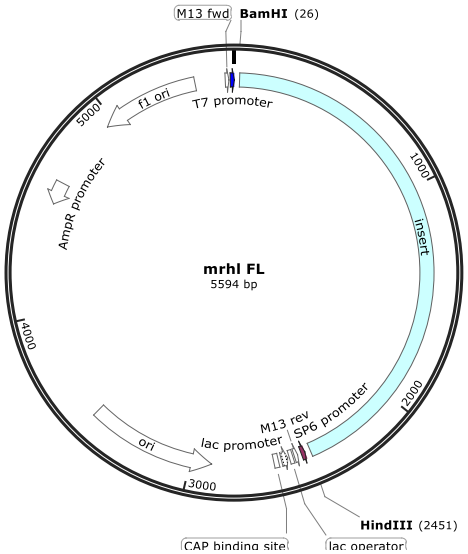


Fig 4.4- Cloning of mrhl truncations in pGEM3zf (+) vector. A – Vector map of pGEM3zf (+), B – PCR amplification of fragments for cloning, Lane 1 PCR amplified product for 1 to 420. Lane 2 – PCR amplified product for 420 to 840, Lane 3 – PCR amplified product 840 – 1690,Lane 4 – NEB 100bp ladder, Lane 5 – PCR amplified product for 1690 – 2401, Lane 6 – PCR amplified product for 840 to 2401, Lane 7 – NEB ladder 1 kb ladder -. C – Confirmation of clones by PCR, Lane 1 – PCR confirmation of clone 1, Lane 2 – PCR confirmation for clone 2, Lane 3 –NEB 100bpb ladder, Lane 4 – PCR confirmation of clone 34, Lane 5 – PCR confirmation of clone 56, Lane 6 – PCR confirmation of clone 36, Lane 7- NEB 1kb ladder .

4.3 *In vitro* pulldown assay to determine region of interaction

Sequence confirmed clones of mrhl truncations were linearized down stream of the gene with HindIII enzyme for the synthesis of sense transcripts with T7 RNA polymerase and upstream of gene with BamHI enzyme for synthesis of anti-sense transcripts with SP6 polymerase. *In vitro* transcription was set up with 1µg of vector as template. Transcripts were body labelled with biotin by using Biotin-14-CTP along with unlabeled CTP in a ratio of 1:3.

A



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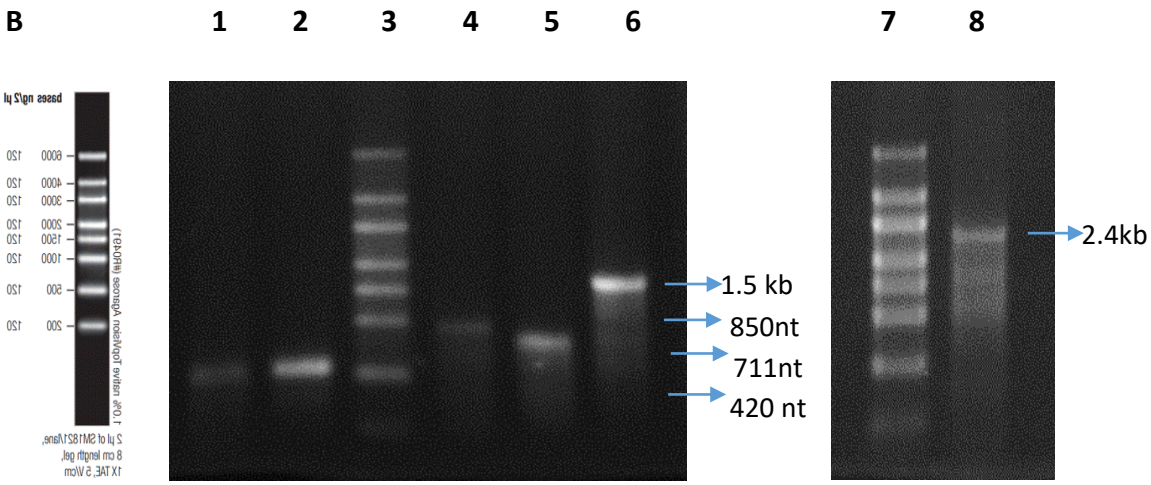


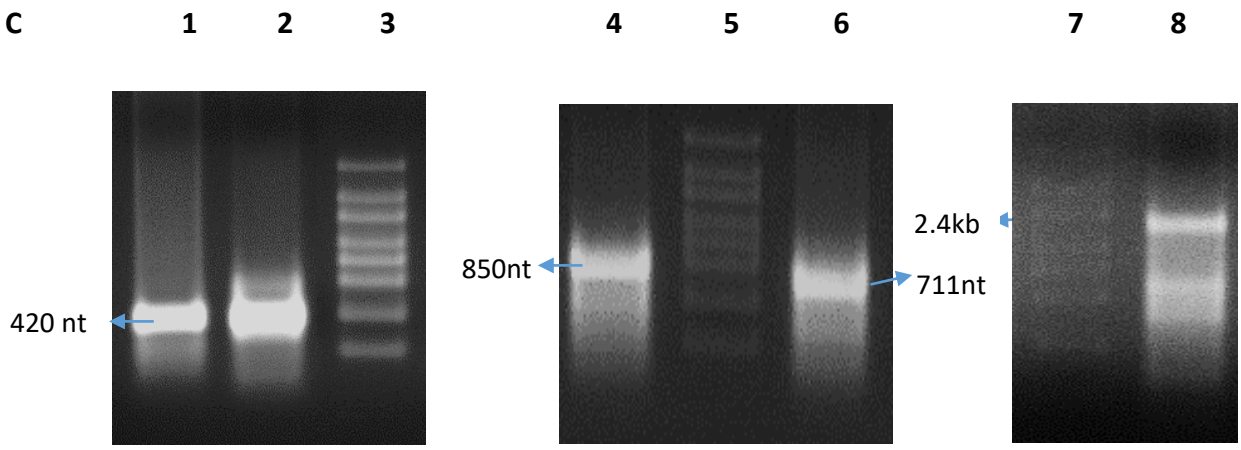
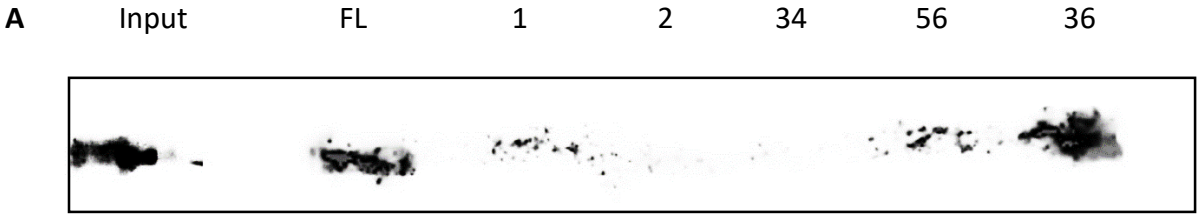


Fig 4.5 – *In vitro* transcription of mrhl Full lenth and truncated gene. A – Vector map of pGEM3zf (+) with cloned insert. B – Sense transcripts of mrhl generated by *in vitro* transcription with T7 polymerase, Lane 1 – Transcript of clone 1, Lane 2 – Transcript of clone 2, Lane 3 – Thermoscientific RNA high range ladder, Lane 4 – Transcript of clone 34, Lane 5 – Transcript of clone 56, Lane 6- Transcript of clone 36, Lane 7 - Thermoscientific RNA high range ladder. Lane 8 – Transcript of full length clone. C – Anti-sense transcripts generated by *in vitro* transcription with SP6 polymerase, Lane 1 – Transcript of clone 1, Lane 2 – Transcript of clone 2, Lane 3 – Thermoscientific RNA high range ladder, Lane 4 – Transcript of clone 34, Lane 5 - Thermoscientific RNA high range ladder, Lane 6 - Transcript of clone 56, Lane 7 - Thermoscientific RNA high range ladder, Lane 8 – Transcript of full length clone.

Transcripts were incubated with precleared nuclear lysates overnight and proteins bound to the transcripts were resolved by SDS-PAGE. Western blot was performed to detect binding of p68 to different regions of mrhl. It was observed that the last 1.5kb (36) showing homology to hmrhl showed binding equivalent to the full length mrhl gene as can be seen in Fig. 4.6.



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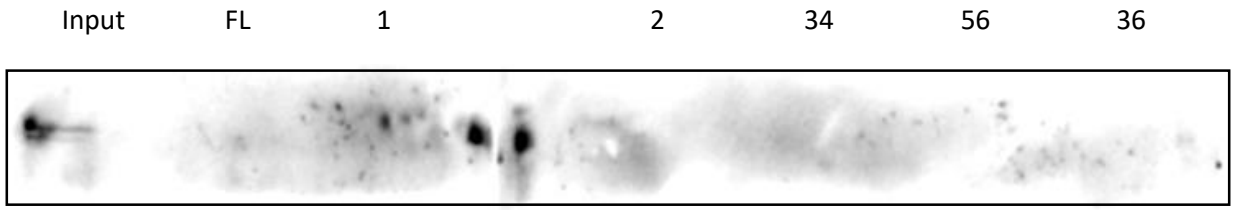
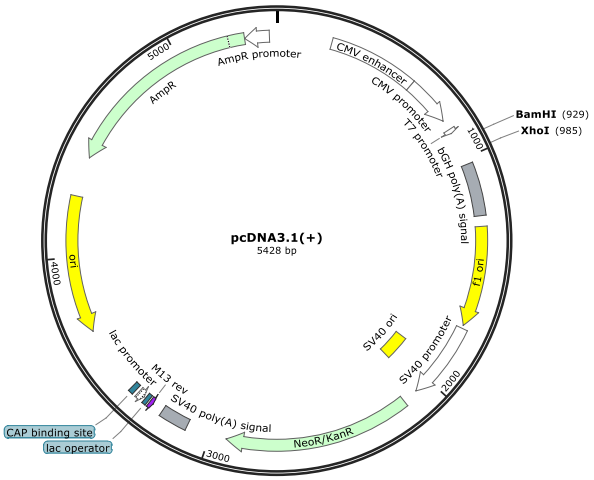


Fig 4.6- *In vitro* pulldown assay. A- Western blot for detection of p68 protein for invitro pulldown with sense transcripts. 5% Input was loaded along with pulldown fraction from full length mrhl, truncations 1, 2, 34, 56 and 36**, B-** Western blot for p68 detection for Control pulldown set up with antisense transcripts of full length and truncated mrhl .

4.4 Mapping the region of mrhl iteracting with p68 *In vivo*

The different regions of mrhl were cloned into pCDNA3.1 mammalian expression vector for the *in vivo* assay. In addition, the S1 aptamer was attached to the 3' end of all the clones by means of adapter introduced in primers to facilitate affinity pull down of RNA transcripts. The inserts were cloned into BamHI and XhoI sites of the vector. Clone of full length mrhl tagged to S1 aptamer was available in the laboratory.

A



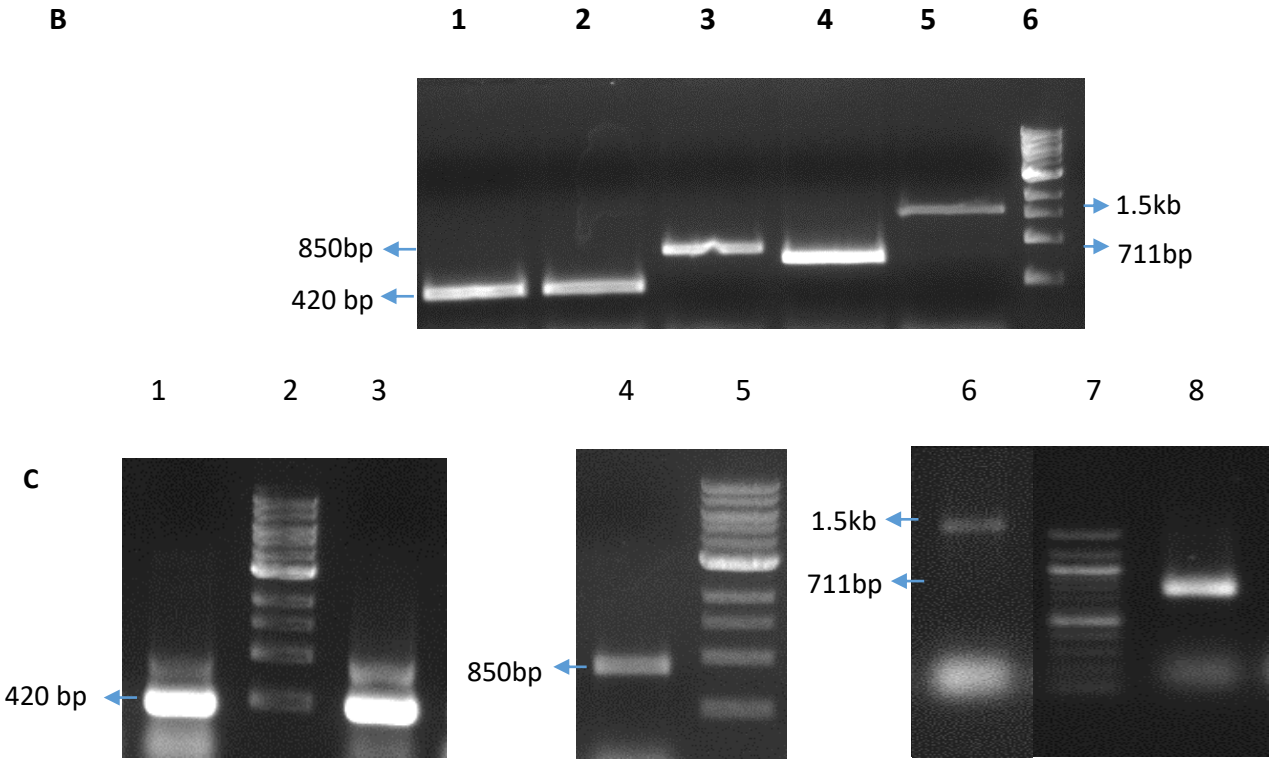


Fig 4.7 – Cloning of mrhl full lenth and truncations in pCDNA3.1, A – Vector map of pCDNA3.1, B – PCR amplification of inserts to be cloned, Lane 1 – PCR amplification of region 1 to 420, Lane 2 – PCR amplification of region 420 to 840, Lane 3 – PCR amplification of region 840 to 1690, Lane 5 – PCR amplification of region 1690 to 2401,Lane 6 – NEB 1kb ladder. C – Confirmation of clones by PCR, Lane 1 – PCR confirmation of clone 1, Lane 2 – NEB 1kb ladder, Lane 3 – PCR confirmation of clone 3, Lane 4 – PCR confirmation of clone 34, Lane 5 – NEB 1kb ladder, Lane 6 – PCR confirmation of clone 36, Lane 7 – TaKaRa 100bp ladder, Lane 8 – PCR confirmation of clone 56.

The sequence confirmed clones were transfected into Gc1 spg cells grown to about 60% confluency. 48 hours post transfection, cells were lysed and RNA transcripts were pulled down with streptavidin beads. Interacting proteins were resolved by SDS-PAGE and Western blot was performed to analyze p68 interacting region of mrhl. It was seen that similar to the results of *in vitro* pulldown assay, the last 1.5 kb region showed interaction with p68 *in vivo* as shown in Fig 4.8.

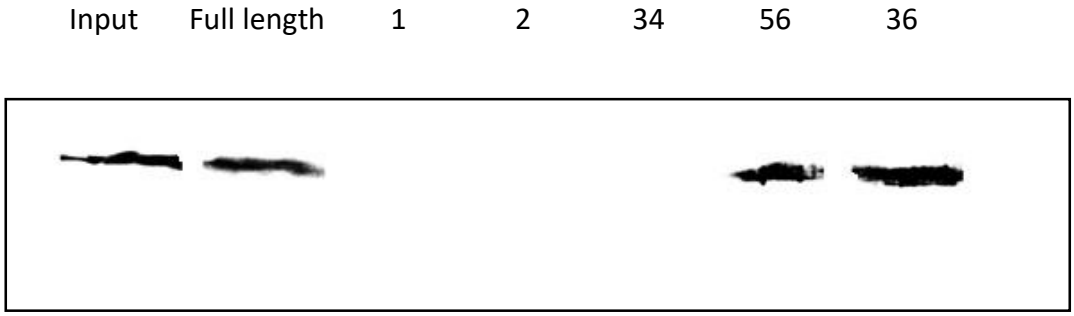


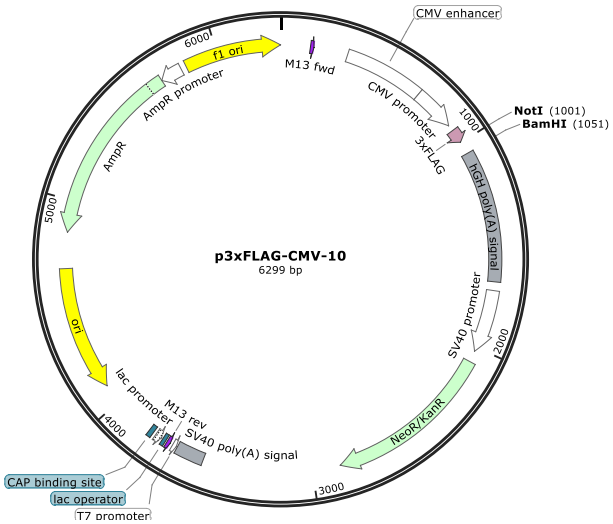
Fig 4.8 – Western blot for p68 for *in vivo* pulldown assay. 5% input was loaded along with pull down fractions from full length mrhl and the various truncations.

5. Mapping the RNA interacting region on p68.

5.1 Cloning of internal deletion constructs of p68

The amino acids involved in RNA binding present in DEAD box helicases have beem reported in literature. To analyze if the same regions encompassing these residues present in p68 were responsible for mrhl interaction, internal deletion constructs of p68 were made in the vector p3xFLAG CMV vector such that all constructs were FLAG tagged at the N terminus. Along with the full length p68 gene,two internal deletion constructs of the gene between residues 161-235, which is located within the first of the two identical RecA domain, and residues 312-444, which is located within the second RecA domain were made. The fragments were amplified by overlap extension PCR and cloned into the NotI and BamHI sites of the vector.

A



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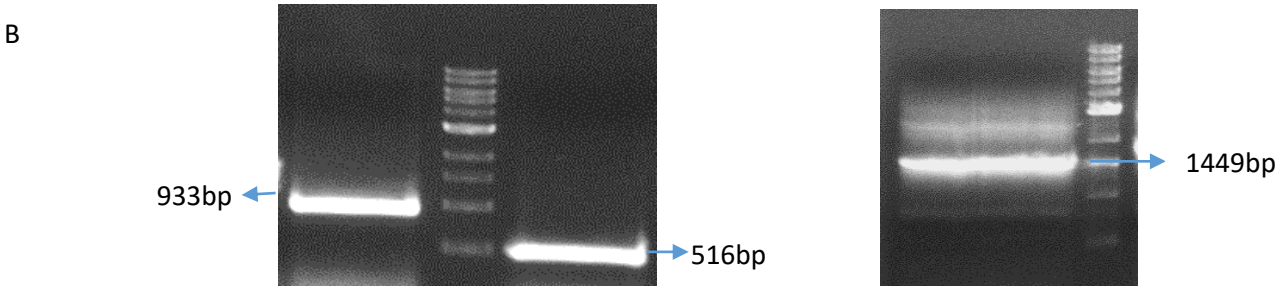


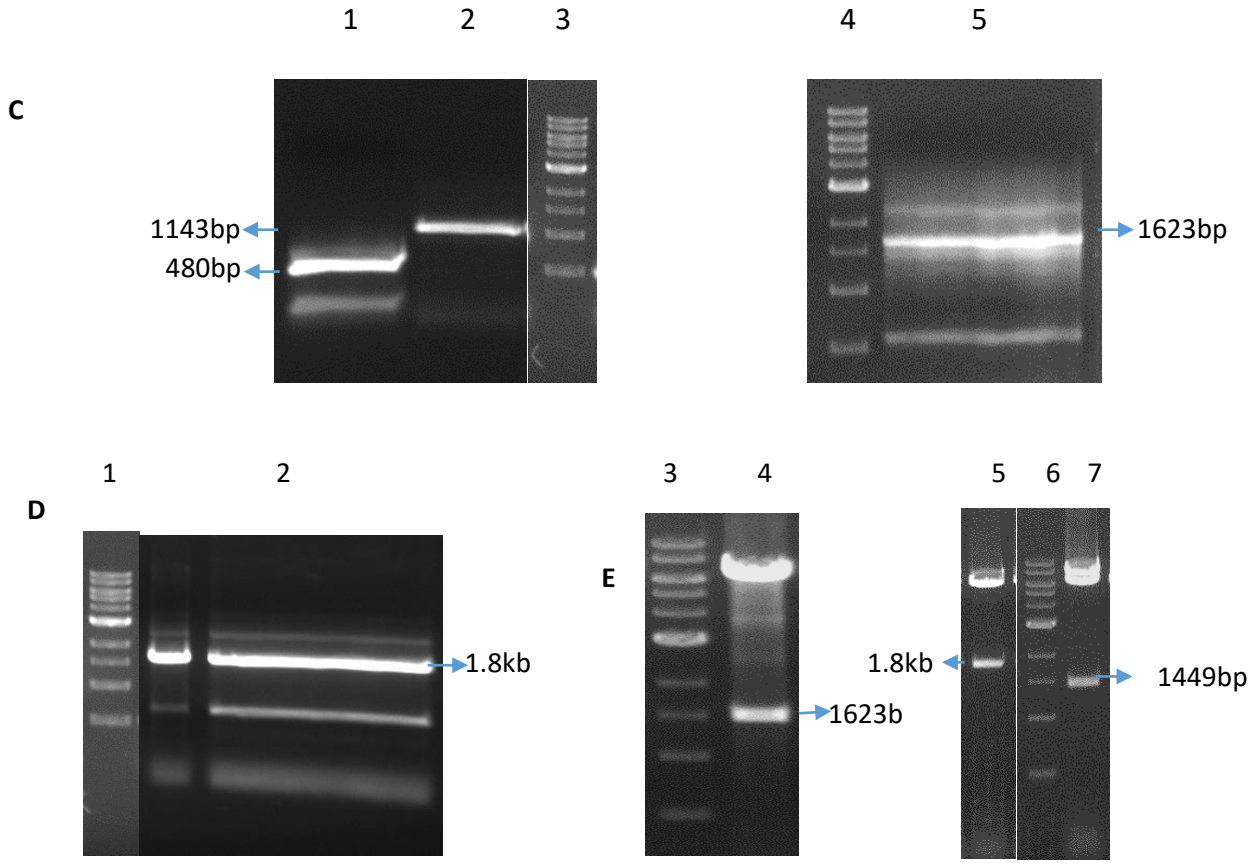


Fig 5.1- Cloning of 68 gene full length and internal deletions into p3X FLAG CMV vector. A – Vector map of p3XFLAG – CMV, B – Amplification of individual fragments and overlap fragment for cloning internal deletion residues 312-444, Lane 1 – Amplification of N terminal for deletion of region 312 – 444, Lane 2 – NEB 1kb ladder, Lane 3 – Amplification of C terminal for deletion of region 312 – 444, Lane 4 – PCR amplification by overlap of N- and C- terminal sequences for deletion of region 312-444 , C – Amplification of individual fragments and overlap for deletion of residues 161-235 Lane 1 – Amplification of N terminal for deletion of region amino acids 161-235, Lane 2- Amplification of C terminal for deletion of region 161-235, Lane 3 – NEB 1 kb ladder, Lane 4 – Amplification by overlap of N terminal and C terminal sequences for deletion of region 161-235, D – Amplification of full length p68, Lane 1 – NEB 1kb ladder, Lane 2 – PCR amplified full length p68 gene, E – Confirmation of clones by insert release, Lane 1 – NEB 1kb ladder, Lane 2 – insert release for internal deletion clone for region 161-235, Lane 3 – Insert release for full length p68 clone, Lane 4 – NEB 1kb ladder, Lane 5 – Insert release for internal deletion clone for region 312-444 .

The clones were confirmed by DNA sequencing and expression of proteins was confirmed by Western blotting.

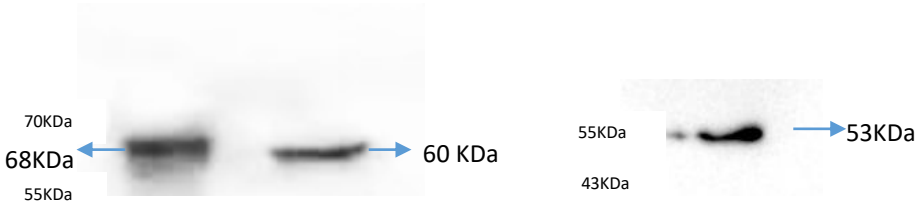
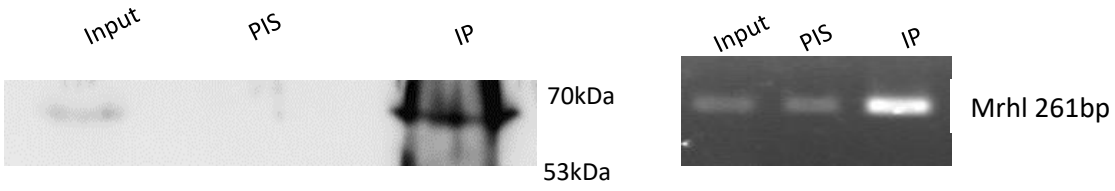


Fig 5.2- Western blotting for confirmation of expression of full length and truncated p68. Lane1- Full length, Lane 2 – Internal deletion 161-235, Lane 3 – internal deletion 312-444.

5.2 RNA Immunoprecipitation to understand RNA binding region of p68

Immunoprecipitation of p68 protein with Anti-FLAG antibody was carried out to understand which residues on the protein are important for its interaction with mrhl RNA. Negative control pulldown reactions were set up with mouse pre immune serum . Western blotting was performed to confirm that immunoprecipitation had worked. PCR was set up for mrhl amplification to check for binding to full length and internal deletion constructs of p68.

A



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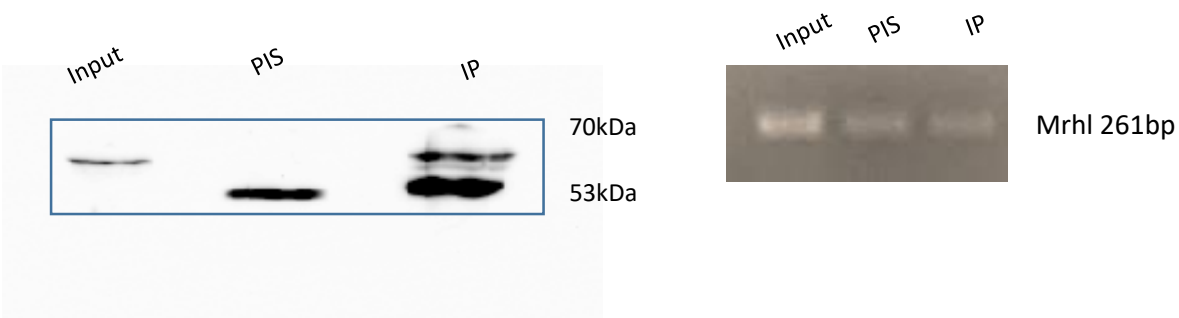

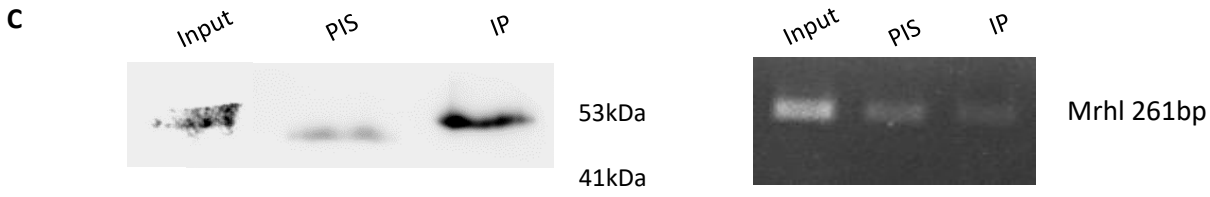

Fig 5.3- RNA immunoprecipitation to understand the residues in p68 nivolved in mrhl binding. **A**- Western blot with FLAG antibody for confirmation of Immunoprecipitation with full length p68 and PCR to show binding of mrhl, **B** – Western blot for internal deletion 161-235 of p68 and mrhl PCR, **C**- Western blot for internal deletion 312-444 of p68 and mrhl PCR.

It was observed that mrhl was showing interaction with full length p68 as there was enrichment of the RNA in IP fraction over pre immune serum in full length p68 IP (Fig 5.3 A). However, this interaction seemed to be abolished in both the internal deletion constructs as can be seen by the lack of enrichment of mrhl in IP fraction over pre immune serum (Fig 5.3 B,C). This result indicates that the regions implicated in RNA interaction in DEAD box helicases as per literature maybe the same regions involved in mrhl interaction in p68 as well.

Mrhl is a 2.4kb long non coding RNA found in mouse kidney, liver, spleen and testis. Earlier work from the laboratory has characterized mrhl RNA in the mouse spermatogonial cells and found it to be nuclear localized in these cells. P68 DEAD box helicase is an interacting partner of mrhl. This protein is essential for the nuclear translocation of beta catenin from cytoplasm upon Wnt signaling activation. It is also essential for physical association of mrhl with 27 of the 37 target genes which it regulates.

The aim of the current work was to analyze the interaction between mrhl and p68.

Though p68 is known to interact with mrhl, it was not known whether this interaction is direct or mediated by bridging molecules. Results from the electrophoretic mobility shift assay indicate that p68 directly interacts with mrhl and that this interaction is enhanced in the presence of ATP.

Bioinformatically, three possible regions of interaction on mrhl with p68 was predicted. In addition, Mrhl shares 65% homology with the human homolog hmrhl towards the 3' end. There is a possibility that the p68 binding region may be present in the conserved region. Experiments carried out *in vitro* and *in vivo* show that the conserved region of mrhl indeed shows interaction with p68.

DEAD box helicases are involved in most steps of RNA metabolism. The residues of DEAD box helicases that are involved in their interaction with RNA have been characterized for the protein VASA in *Drosophila melanogaster* by crystallography studies.

Experiments done with internal deletion constructs of the DEAD box helicase p68 in mouse spermatogonial cells indicate that the same region the protein is involved in mrhl interaction of p68. It would be interesting to explore the role of the RGG motif present in p68, if any, in its interaction with mrhl.

How mrhl interacts with its target genes and the contribution of p68 to this interaction is yet to be understood.

6: Future directions

- Now that the region involved in interaction with p68 has been determined, the functional relevance has to be addressed and this can be done by studying if the p68 interacting region of mrhl is sufficient to carry out the functions of the full length RNA. This can be done by providing the p68 interacting region of mrhl *in trans* under mrhl down regulated (Wnt up regulated) conditions. It is known that pre meiotic and meiotic markers are up regulated in mouse spermatogonial cells when Wnt signaling is active and mrhl is down regulated. The assay would aim at observing if the expression of these markers can be reverted to similar levels as when full length mrhl is being expressed when Wnt ligand is being exogenously supplied.
- The exact nucleotides in mrhl involved in p68 binding is to be analysed by RNase protection assay.
- The experiments indicate that ATP binding to p68 increases p68 binding to mrhl. It would be interesting to see if ATP binding is sufficient or ATPase activity is required for this interaction.
- The amino acid residues involved in p68 in interacting with mrhl can be identified by site directed mutagenesis of suspected residues and then scoring for mrhl binding.

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