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Characterizing the *Drosophila melanogaster lethal(2)denticleless* gene in InsP3R background

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Abstract- Protein containing WD-repeats participate in a wide range of cellular functions, they are generally involved in regulatory mechanisms concerning chromatin assembly, cell cycle control, signal transduction, RNA processing, apoptosis and vesicular trafficking. *lethal(2)denticleless* is a recently identified novel class of Protein containing WD-repeats. Bioinformatic analysis has shown conservation of critical functional motifs among the human L2DTL, mouse L2dtl and *Drosophila* l(2)dtl proteins. The function of l(2)dtl is still under speculation but the studies suggest that L2DTL and PCNA are involved in CDT1 degradation after DNA damage and cell cycle regulation. The gene expression studies have shown that *l(2)dtl* levels are altered in different condition and even found up-regulated in under galactose challenge conditions in human. *l(2)dtl* was chosen for its potential regulation by $InsP_3R$ mediated intracellular Ca^{2+} signaling. This study indicates a possible role of *l(2)dtl* in $InsP_3R$ mediated intracellular Ca^{2+} signaling.

Index Terms- *lethal(2)denticleless* (*l(2)dtl*), Inositol 1,4,5 trisphosphate receptor ($InsP_3R$), signal transduction and WD-repeats.

I. INTRODUCTION

lethal(2)denticleless gene was identified while analyzing the cell cycle gene *morula (mr)* by complementation analysis of embryonic-lethal mutations and the mutants found lacking ventral denticle belts named *l(2)dtl* [1]. The human L2DTL, a conserved WD40 domain-containing protein was found to be part of CUL4 ubiquitin E3 ligase complex identified by mass-spectrometry [2]. The proteins containing WD-repeats participate in a wide range of cellular functions, they are generally involved in regulatory mechanisms concerning chromatin assembly, cell cycle control, signal transduction, RNA processing, apoptosis and vesicular trafficking [2]. In *Drosophila* it is expressed at a very high levels in the early embryo and in adults shown as by in-situ hybridization and northern blotting respectively [1]. The reason for high level expression in the early embryo is maternal contribution confirmed by *in situ* hybridization in the female germ line. The human ortholog when down-regulated by RNAi in hepatocellular carcinoma (HCC) leads to reduced cancer cell growth and invasion capability *in vitro* and in which microarray analysis disclosed dysregulation of genes involved in cell cycle regulation, chromosome segregation, and cell division [3]. In mouse the *l(2)dtl* homolog is known as CTD2. L2DTL knockout mice show embryonic lethality which is also reported in *Drosophila* and morphological studies of early embryos have shown fragmentation of mitotic chromosomes and chromosomal lagging indicating its involvement in nuclear division [3]. As of now the Drosophila *l(2)dtl* gene had not been annotated as a cell cycle regulator but data from both *Drosophila* and human cells suggests that the L2DTL protein is a targeting subunit of the CUL4/DDB1 ubiquitin ligase complex that targets critical cell cycle regulators for degradation [4]. RNAi targeting in the human ortholog has been shown to cause growth arrest and an increase in both p53 protein and the DNA replication licensing factor CDT1 [5, 6]. Additionally, human *l(2)dtl* has been shown to oscillate during the cell cycle with peak expression occurring at the G1/S transition, consistent with a role in regulating G1/S transition [7]. Other independent studies have shown that it plays an important role in cell proliferation through regulation of a G2/M checkpoint [8]. Some recent reports also indicate its involvement in differentially regulating histone methylation at K4, K9 and K27 through interaction with CUL4–DDB1 E3 ligase complexes which target CDT1 for proteolysis in response to DNA damage [2]. Very recently *l(2)dtl* was reported as one of the hits whose function might be linked to blood cell development or function in *Drosophila* and its involvement in transcription through DREF involving *ddb1* [9]. Other reports, report role in regulation of endocycles in *Drosophila* through E2F1 transcription factor [10, 11].

$InsP_3R$ is a calcium channel on ER. $InsP_3R$ functions, differentially in specific tissues or at specific stages of development. This has been shown using EMS generated single point mutations in the *itpr* gene [12]. The phenotypes include defective recovery from olfactory adaptation [13], altered adult wing posture, defective flight and flight physiology and defective nerve branching at neuromuscular junctions in adults [14] and smaller larval body size due to a feeding defect and reduced insulin signaling [15]. Also gene expression study in IP_3R mutant larvae shows altered regulation of transcripts related to carbohydrate and amine metabolism [16]. A micrroarray study was performed to study the feeding defect leading to developmental arrest of second instar larvae [16]. *l(2)dtl* genes was found differentially regulated in the microarray and was selected for functional validation.

II. MATERIALS AND METHODS

A. Drosophila melanogaster strains

The *UAS-RNAi-l(2)dtl* was procured from VDRC [17]. $Elav^{C155}GAL4$ (a stronger pan-neuronal driver) [18]. $itpr^{Ka1091/ug3}$ is a heteroallelic combination of single point mutants in the *itpr* gene generated in an ethyl methanesulfonate (EMS) screen. Detailed molecular information on these alleles has been published [12]. The wild-type Drosophila strain used in all experiments is *Canton-S* (*CS*). Fly strains were generated by

standard genetic methods using individual mutant and transgenic fly lines described above. Flies were grown at 25°C in standard cornmeal medium containing agar, corn flour, sucrose and yeast extract along with anti-bacterial and anti-fungal agents.

B. Larval staging and lethality measurements

Staging experiments for obtaining molting profiles of heteroallelic mutant larvae were carried out as described previously with minor modifications [12]. Briefly, flies were allowed to lay eggs for a period of 8 hrs at 25°C. Embryos were allowed to hatch and grow further at this temperature. Larvae of the desired genotype were selected from these cultures at 56–64 h after egg laying (AEL) and transferred into vials of standard cornmeal medium lacking agar (agar less medium). Larvae were grown in agar less medium at 25°C and screened at the indicated time points for number of survivors and their phase of growth and development. For each time interval, a minimum of 75 larvae were screened in batches of 25 larvae each. Computation of means, SEM, and *t*-tests were performed using Origin 7.5 software (Origin Lab, Northampton, MA, USA).

C. RNA isolation

For isolation of total RNA, larvae of the requisite genotypes were selected at 120-124 hr AEL and snap frozen in liquid nitrogen. Total RNA was isolated using TRI Reagent (Sigma, St. Louis, USA). The frozen larvae were crushed with a baked homogenizer after adding 250µl of the TRI Reagent (Sigma, St. Louis, USA). The pestle was washed with another 250µl of TRI Reagent, mixed properly and transferred onto a clean eppendorf. 250µl each of equilibrated RNAse free phenol and chloroform were added and vortexed for 60 seconds. The tube was then centrifuged at 14,000 rpm for 5 min at 4^0C. The aqueous layer was removed into a fresh tube and re-extracted with the same amount of phenol and chloroform, repeating the above spin. The aqueous layer was again put into a fresh tube and extracted with 500µl of chloroform and spun at 14,000 rpm for 5 min at 4^0C. The upper aqueous layer was then precipitated with two volumes of RNAse-free 100% ethanol by keeping it at -80^0C for at least 30 minutes. The sample was spun at 14,000 rpm for 30 min at 4^0C. The supernatant was removed and the pellet washed with 500µl of 70% ethanol. The pellet was air dried and finally dissolved in appropriate amount of DEPC treated water. After quantification, RNA with a ratio of $OD_{260}/OD_{280} > 1.8$ was taken for further experiments. Integrity of the isolated RNA was confirmed by the presence of full length rRNA bands on a 1.2% formaldehyde agarose gel. RNA was isolated from *CS*, female *UAS-RNAi-l(2)dtl/Elav^{C155}GAL4.*

D. Reverse Transcription-PCR (RT-PCR)

Total RNA (1µg) was reverse transcribed in a volume of 20µl with 1µl (200U) Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen Technologies, Carlsbad, CA, USA) using 1µl (200ng) random hexaprimers (MBI Fermentas, Glen Burnie, MD, USA) containing 1mM dithiothreitol (DTT) (Invitrogen Technologies, Carlsbad, CA, USA), 2mM of a dNTP mix (GE HealthCare, Buckinghamshire, UK) and 20U of RNase Inhibitor (Promega, Madison, WI, USA) for 1h at 42°C. The polymerase chain reactions (PCRs) were performed using 1µl of cDNA as a template in a 25µl reaction volume under appropriate conditions.

E. TOPO TA Cloning

Full length cDNA of *l(2)dtl* was PCR amplified by using *l(2)dtl* specific primers by Taq polymerase and was cloned into pCRII-TOPO vector. The TOPO vector is linearized and has single 3' – thymidine (T) overhangs and topoisomerase I that is covalently bound to it. The ligation reaction takes place with the help of the Topoisomerase I enzyme within 5 minutes. The ligation reaction was carried out using TOPO TA Cloning kit (Invitrogen, California, USA). A 4:1 ratio of PCR product to vector was used. 2µl of the ligation reaction was used for transformation.

F. Transformation

Transformation was performed using One Shot TOP 10 competent *E. coli* cells (Invitrogen, California, USA). 1-2µl of the ligated DNA was added to 100µl of TOP 10 competent cells thawed on ice and mixed gently and allowed to incubate on ice for 30 minutes. A heat shock was given at 42^0C water bath for 30 seconds and placed them on ice for 2 minutes.250 µl of LB medium was added to the solution and incubated at 37^0C water bath for one hour. After an hour, 50µl was plated out on an LB agar plate containing 50µg/ml ampicillin and rest was store at 4^0C. The plate was incubated at 37^0C overnight. The colonies obtained were inoculated in 2-5 ml of liquid LB medium + 50µg/ml ampicillin and cultured overnight. DNA was extracted following standard protocols as described below.

G. Plasmid DNA Extraction (Mini prep)

After overnight incubation, the cultures were transferred to an eppendorf, spun at 8,000 rpm for 3 minutes at 4^0C and supernatant discarded. The pellet was re-suspended in 150µl of Solution I (GTE, 50mM glucose, 25mM Tris-Cl, pH 8.0 and 10mM EDTA, pH 8.0) by vortexing. 250µl of Solution II (0.2N NaOH and 1% SDS) was added and mixed by inverting the tube 5-6 times and incubated on ice for 3 minutes 30 seconds. 150µl of Solution III (3M Potassium acetate and 5M glacial acetic acid) was added, mixed by inverting and kept in ice for 10 minutes. The solution was then centrifuged at 14,000 rpm at 4^0C for 10 minutes and supernatant transferred into a fresh tube avoiding any cell debris. An equal volume of equilibrated Phenol: Chloroform was added to the supernatant and spun at 14,000 rpm for 15 minutes at room temperature. The upper aqueous phase was removed carefully and 1ml of cold 100% ethanol along with 50µl of Sodium acetate was added, mixed and then spun at 14,000 rpm, 4^0C for 10 minutes. The supernatant was discarded and the pellet washed with 500µl of 70% ethanol and spun again at 14,000 rpm, 4^0C for 5 minutes. The supernatant was discarded and the pellet was allowed to air dry. The pellet was then re-suspended in 40-50µl of autoclaved double distilled water or TE having RNAseA (10µl of 20mg/ml RNAseA in 1ml double distilled water or TE) and incubated at 37^0C for 30 minutes. 1µl of the DNA was then run on a 0.8% agarose gel along with the vector DNA as the control and another 1µl was used on nanodrop for quantification. Plasmid DNA that migrated slower than vector DNA was selected for further analysis.

Restriction endonuclease (RE) digestions were done with specific enzymes at 37^0C for 3 hours to confirm the presence of the required fragment.

H. PEG precipitation of plasmid DNA

To the confirmed positive clones from RE digestions, 2.5µl of 4M NaCl and 40 µl of 13% PEG8000 was added to linearised plasmid by BamHI RE and mixed vortexing and incubation on ice for 20 minutes, the solution was centrifuged for 15 minutes at 14,000 rpm at 4^0C. The supernatant was removed and the pellet was washed with 70% ethanol and air-dried. The pellet was dissolved in 15-20 µl of PCR quality water and 1µl of the DNA was then run on a 0.8% agarose gel along with the vector DNA as the control and another 1µl was used on nanodrope for quantification. Approximately 200 ng of the PEG precipitated DNA was used for sequencing.

H. Sequencing Analysis

The cloned DNA fragments were sequenced with gene specific and SP6/T7 specific primers using the Big Dye terminator cycle sequencing kit on the 301 Genetic Analyser Sequencer (ABI Prism). The gene sequence obtained was then compared with the published *Drosophila* sequence from flybase and analysed on DNAMAN.

I. Phenol Chloroform Extraction of DNA

In order to clone the positive full length cDNA of *l(2)dtl* into the multiple cloning site of the vector pUAST in 5' -3' orientation, a restriction digest of both the purified PCR fragment and the vector with XbaI and EcoRI was done separately (single digest). To 100 µl of the PCR reaction, 100 µl of phenol: chloroform was added and mixed by vortexing and centrifuged at 14,000 rpm for 5 minutes at RT. The upper aqueous layer was transferred to a fresh eppendorf and an equal volume of chloroform:isoamylalcohol (24:1) added and centrifuged again at 14,000 rpm for 5 minutes. The above step was repeated once again. To the upper aqueous layer that was transferred into a fresh tube, one-tenth volume of 3M sodium acetate (pH 5.0) and 2.5 volumes of double distilled alcohol was added and kept at -80^0C for 30 minutes or -20^0C for two hours or more. The solution was spun at 14,000 rpm for 15 minutes and supernatant discarded. The pellet was then washed with 70% ethanol and spun for 5 minutes at 14,000 rpm. The pellet was then air dried and dissolved in 20 µl of PCR quality water. 1µl of the DNA was then run on a 0.8% agarose gel along with the vector DNA as the control and another 1µl was used on nanodrop for quantification and was further used for ligation.

J. Gel Extraction

The required full length cDNA of *l(2)dtl* fragment from pCRII-TOPO vector and linearised pUAST vector was digested with EcoRI RE and was gel purified using the Qiagen Gel Extraction Kit (Qiagen, Venlo, Netherlands). The restriction-digested fragment was run on a 0.8% agarose gel. The area of the gel containing the DNA fragment was cut under minimum UV exposure, using a clean and sharp blade and put into an eppendorf. The gel was weighed and 300 µl of QG buffer was added for every 100mg of gel. The tube was incubated at 50°C for 10 min (or until the gel slice has completely dissolved) was vortexed thoroughly. After the gel slice has dissolved completely, 1 gel volume of isopropanol (agarose gel slice is 100 mg, add 100 µl isopropanol) was added to the sample and mix. The tube was then placed in a QIAquick spin column provided with 2 ml collection tube and centrifuged at 13,000 rpm for 30 seconds and the flow-through was discarded. The QIAquick spin column was then washed with 0.75 ml of Buffer PE and centrifuged at 12,000 rpm for 1 minute and flow-through was discarded. The QIAquick column was centrifuged for an additional 1 min and flow-through was discarded. QIAquick column was placed into a clean 1.5 ml microcentrifuge tube to elute DNA, 30 µl of autoclaved double distilled water was added to the center of the QIAquick membrane and centrifuged for 2 min at maximum speed. 1µl of the DNA was then run on a 0.8% agarose gel and another 1µl was used on nanodrop for quantification and was further used for ligation.

K. Ligation

The ligation reaction was carried out using the Quick Ligase kit (NEB, Ipswich, Massachusetts, USA). A 1:3 ratio of restriction digested gel purified vector and PCR fragment was used along with 1µl of the quick ligase enzyme and 10µl of the buffer. The reaction mixture was kept at room temperature for 15 minutes. For transformation 10µl reaction mix was used into One Shot TOP 10 competent *E. coli* cells (Invitrogen, California, USA).

L. DIG-labeled l(2)dtl RNA probe preparation for in situ hybridization

Digoxigenin labeled probes were made by random-primed labeling of DNA fragments (Cat.No.11175025910, Roche Applied Science). Probes for *Drosophila l(2)dtl* were designed to the common region and specific exons. Probes were amplified from gene specific PCR fragment cloned in PCRII-TOPO (#K460001, Invitrogen, California, USA) and sequenced to verify orientation and identity of the fragment. DIG-labeled RNA probes were generated from plasmid DNA using the Roche DIG-RNA Labeling Kit (#11175025910, Indianapolis, Indiana, USA) and the labeling efficiency was determined following the manufacturer's protocol. Sense and antisense probes were generated from the same plasmid, taking advantage of the SP6 and T7 dual promoter flanked PCRII-TOPO cloning site. Probes were diluted to10ng/µl and stored at -80°C for up to 6 months.

M. Primers used

1. For RT-PCR and for preparartion of *l(2)dtl* RNA probes
forward AAGGAGAAGGTGGACTGGCTGA
reverse GGATTGGGAATGGGAGTGCGA
2. For amplification of ful length cDNA clone
forward CACCGAATTCACGGTCACACTGCCCAAG
reverse GCTCTAGATGTACAGAGGGCGGGGAG
3. For sequencing of ful length cDNA clone
Oligo 1 CGATCACACTGCCAGACTGTGG
Oligo 2 CATCAAATCCTGCCTCAGCCCG
Oligo 3 CTCCTTCGCCTTCCGCTTGG
T7 TAATACGACTCACTATAGGG
Sp6 ATTTAGGTGACACTATAG

III. RESULTS AND DISCUSSION

A. Bioinformatic analysis of l(2)dtl sequence

l(2)dtl gene has a coding sequence of 2274 bp, consisting two exons 8 bp and 2266 bp long which is separated by a 274 bp intron (Figure 1A). The promoter region has three typical heat shock elements (HSE) and three thermal stress responsive elements (TSE, Figure 1A). Bioinformatic analysis revealed conservation of critical functional motifs among the human L2DTL, mouse L2dtl, and *Drosophila* l(2)dtl proteins. Sequence analysis from Clustal-W reveals that the 769 amino acid protein consists of highly conserved region at the C-terminus and a poorly conserved region at the N-terminus (Figure 1B). The protein motif analysis from PROSITE and P-fam shows multiple Protein kinase C and Casien Kinase II phosphorylation sites, multiple N-myristolytion sites, three each

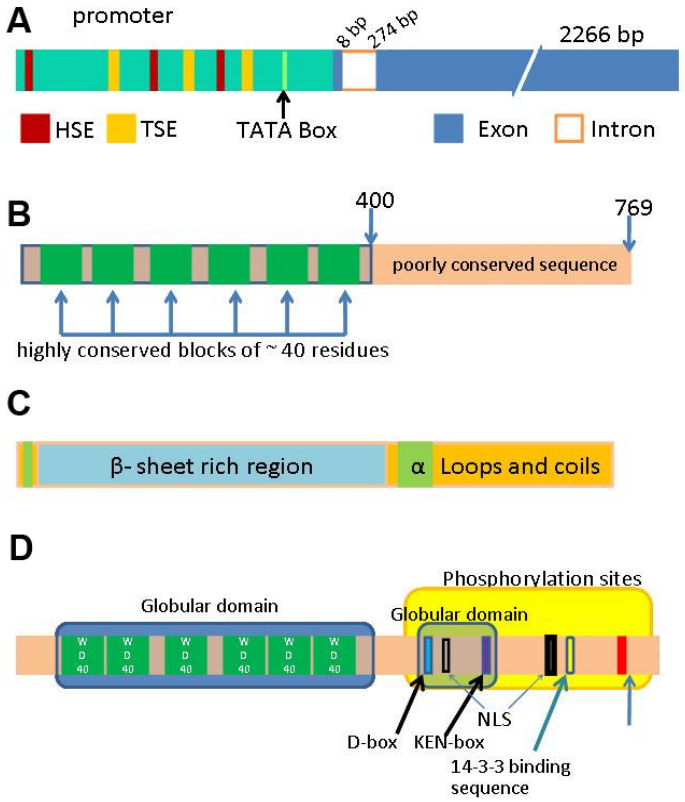

Figure 1: Schematic representations and Bioinformatic analysis of *Drosophila l(2)dtl* (A) *l(2)dtl* gene contains 2 exons (blue boxes) and one intron (white boxes) (B) Clustal-W analysis (C) secondary structure prediction (D) protein architecture search-Elm showing different domains.

of N-glycosylation and cAMP-dependent protein kinase phosphorylation sites, one tyrosine kinase phosphorylation and one DNA methylase site. The protein is 49% and 45 % similar to its human and mouse homolog respectively. Secondary structure prediction shows the presence of 3.51% α-helices, 41.22% β-sheets, and 55.27% loops and coils (Figure 1C).

The protein contains six WD40 repeats, two nuclear localization signal (NLS) at the N-terminus, one potential PEST sequence, one KEN box signal, and a D-box signal (Figure 1D). WD40 (Tryptophan-Aspartate) repeat proteins are involved in several important biological functions including transmembrane signaling, cytoskeletal dynamics, vesicle fusion, protein trafficking, nuclear export, RNA processing, chromatin modification, and transcriptional mechanisms and cell division [19, 20]. KEN box and D-box are involved in regulation of cell cycle through ubiquitin-mediated protein degradation [21] and PEST-like sequences are required for phosphorylation and ubiquitination [22].

B. Characterization of l(2)dtl in InsP$_3$R background (itpr$^{ka1091/ug3}$)

To elucidate the possible role of *l(2)dtl* in mediating calcium release from InsP$_3$R, the flies were analysed with *l(2)dtl* mRNA knocked down by using specific GAL4 driver. A pan-

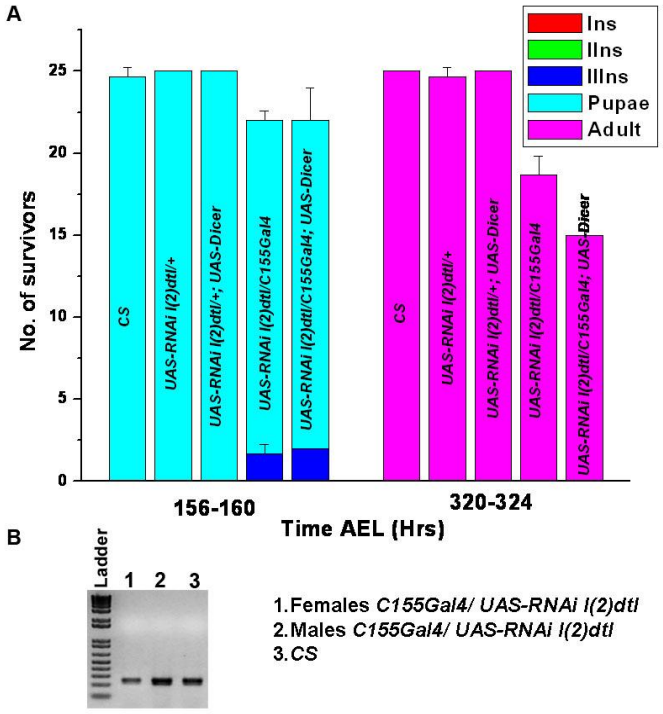

Figure 2: (A) Stage profiling of *UAS-RNAi-l(2)dtl* driven by pan-neuronal GAL4 in the wild type background with and without dicer shows decrease in number of survivors at 320-324 hrs compared to wild type and other controls conditions grown at 25°C AEL (B) RT-PCR showing decrease level of RNA in animals with *UAS-RNAi-l(2)dtl* driven by pan-neuronal GAL4 compared to controls. Since both the GAL4 and the UAS line are on X, only female progeny will show the knockdown.

neuronal GAL4 (*Elav^{C155}GAL4*) was used to drive UAS-RNAi against *l(2)dtl*. It was found that *UAS-RNAi-l(2)dtl* by itself or with *UAS-Dicer* does not have a very profound effect at 156-160 hrs after AEL compared to wild type. But it does show an effect at 320-324 hrs AEL (adult stage) by increasing lethality both by itself or with dicer as compared to wild type (Figure 2A). The RNA level was checked to find out the efficiency of RNAi in brains isolated from third instar larvae (120-124 hrs AEL) expressing RNAi against *l(2)dtl*. The level of *l(2)dtl* was found to be low compared to controls (Figure 2B). As it is evident that

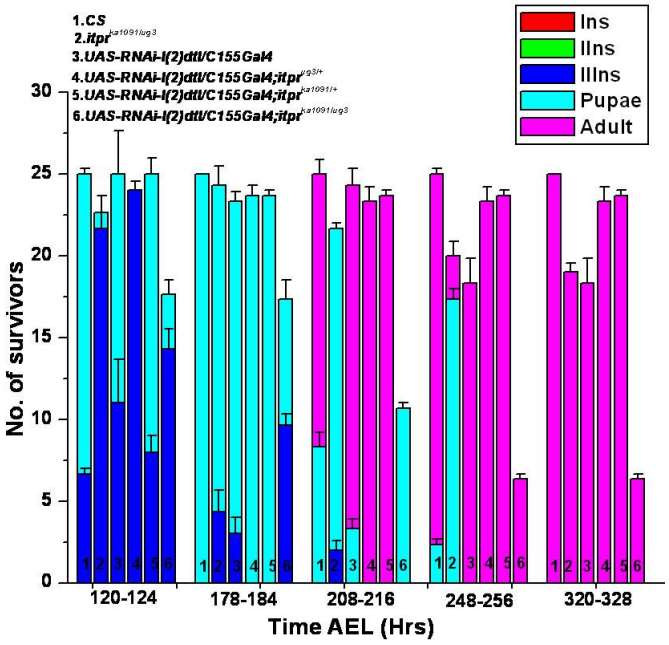

Figure 3: Lethality assessment.
Pan-neuronally driven *UAS-RNAi-l(2)dtl* in the background of *itpr* mutant (*itpr*$^{ka1091/ug3}$) animals shows a decrease in the number of survivors compared to wild type and other controls conditions including *itpr* mutant (*itpr*$^{ka1091/ug3}$) grown at 25°C and same conditions at later times AEL.

low level of l(2)dtl RNA enhances lethality, the well characterized weaker heteroallelic combination (*itpr*$^{ka1091/ug3}$) was used to assess its functional effect. As shown in Figure 3, animals expressing RNAi against *l(2)dtl* pan-neuronally in wild type background in terms of number of viable organisms mimics *itpr*$^{ka1091/ug3}$ animals, which are 80% viable at 25^{0}C under normal conditions. The lethality however, is further enhanced significantly when it was expressed in *itpr*$^{ka1091/ug3}$ background. This shows there is some kind of direct or indirect interaction happening at the molecular level between *l(2)dtl* and InsP_3R mediated calcium signaling. There was no other obvious enhancement or suppression of *itpr*$^{ka1091/ug3}$ phenotype observed.

C. Full length cDNA cloning of l(2)dtl for making UAS- l(2)dtl transgenic flies

The idea behind generation of full length cDNA clone of *l(2)dtl* in an expression vector was to make *UAS-l(2)dtl* transgenic flies and use them to revert back the phenotype of lethality seen by *UAS-RNAi-l(2)dtl* and to address other questions keeping them as positive control. Full length cDNA clone of *l(2)dtl* (LD21681) was obtained in pOT2 vector from BDGP. The expression vector used was pUAST whose intactness and restriction endonuclease (RE) sites were checked (Figure 4A-C). RE sites present in pOT2 with the clone were also checked but because of the incompatibility with pUAST RE sites, the full length cDNA of *l(2)dtl* was PCR amplified by using *l(2)dtl* specific primers by Taq polymerase (4D). *Taq* polymerase has a tendency to incorporate single point mutations and generate A overhang products. So the full length cDNA was cloned into

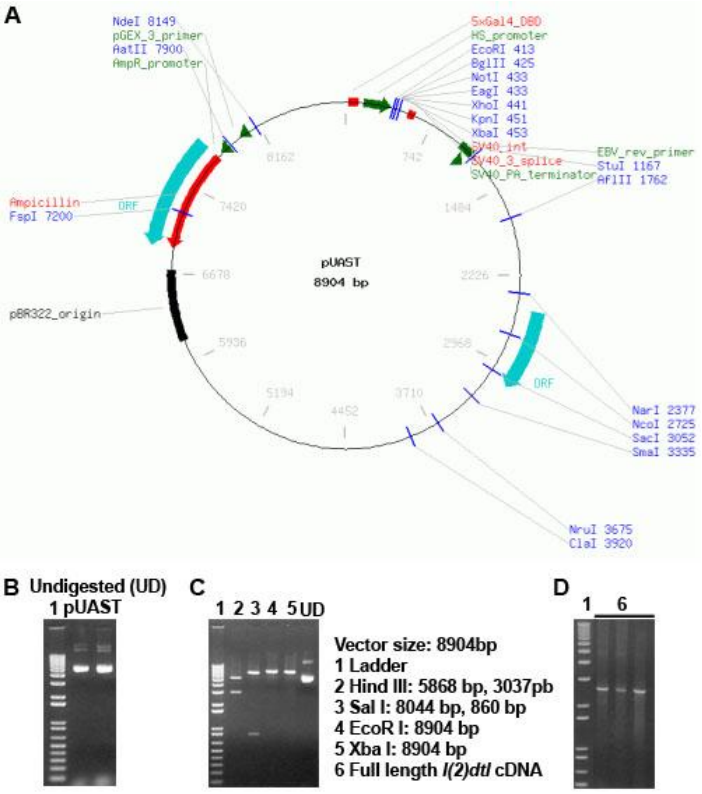

Figure 4: (A) Schematic representations of pUAST expression vector (B) and (C) Integrity analysis of pUAST by restriction endonclease digest (D) PCR amplification of *l(2)dtl* full length cDNA.

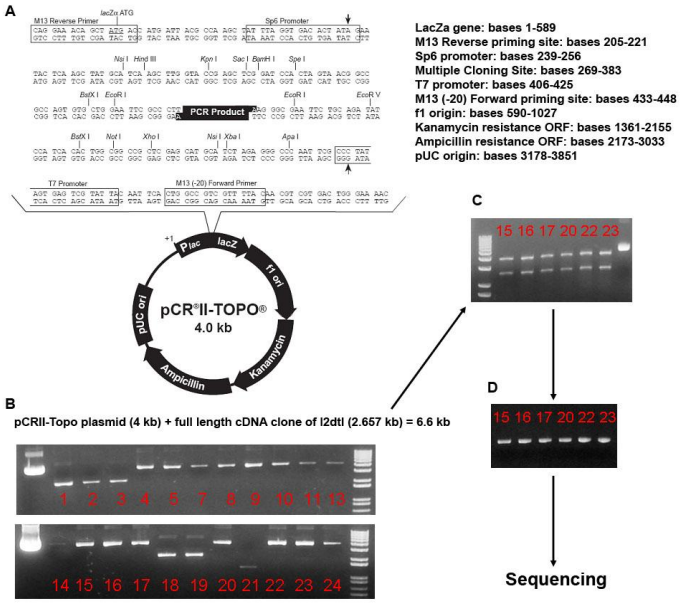

Figure 5: (A) Schematic representations of pCRII-TOPO cloning vector (B) Screening of positive clones for *l(2)dtl* full length cDNA plus vector (C) EcoRI digest of positive plasimds (D) purified BamHI digest linearised positive plasmid for sequencing.

pCRII-TOPO vector (Figure 5A) in order to add pUAST-compatible RE sites. Obtained clones were checked for the presence of full length cDNA by running them on agarose gel ((Figure 5B)) and also by RE digestion of the clones by using appropriate RE (Figure 5C). The positive clones then were sequenced to check for any point mutations incorporated by *Taq* polymerase. The sequencing strategy (Figure 6A) and sequence analysis (Figure 6B-E) was done by using DNAMAN software. The analysis shows incorporation of a single point mutation at position 1778, a transition of T to C nucleotide (Figure 6B and E). This was further analyzed to find out if this mutation caused any change in amino acid sequence. The point mutation obtained leads to a silent mutation as CCT (wild type) and CTC (clone) both coding for leucine. So clone 17 was used in making of *UAS-l(2)dtl* transgenic construct as the other clones had more mutations. The transgenic flies with this construct will be generated and used for further experiments.

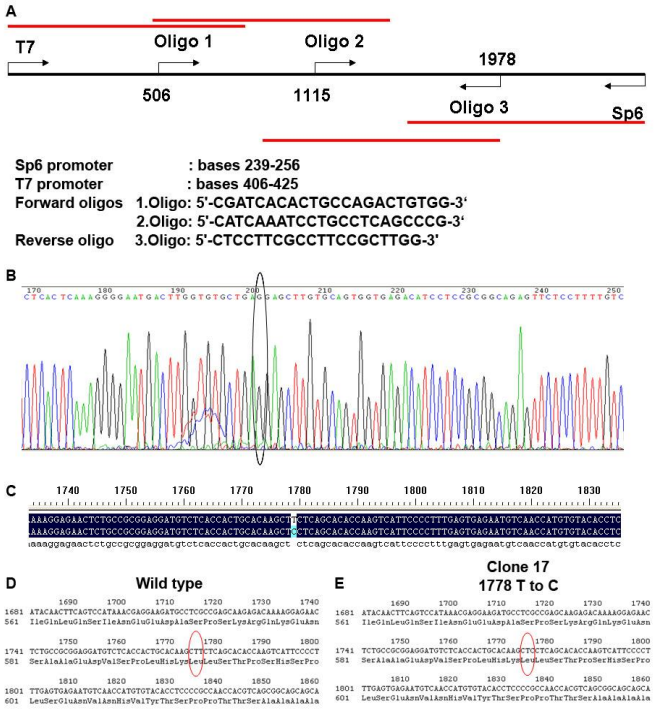

Figure 6: (A) Sequencing strategy showing position of overlapping primers (B) Read out sequences of positive clones obtained from Chromas lite (C) Sequences alignment of the positive clone (second row) to wild type (first row) and third row represent consensus sequence show transition from T to C and amino acid prediction of coding sequences (D) wild type and (E) positive clone 17 obtained from DNAMAN.

D. Preparation of DIG-labeled l(2)dtl RNA probes for RNA in situ

As *l(2)dtl* levels in the brain are very high compared to its level in fat body, it critical to find out which part or domains of the brain require more of it. So to look into the location of enriched compartments in greater details, RNA probes for *l(2)dtl* were generated. A probe size ranging between 150-400 bp is more effective than the larger fragments and hence *l(2)dtl* primers were designed to generate a 232bp amplicon (Figure 7A) and were purified and cloned into the polylinker site of pCRII-TOPO transcription vector (Figure 5A), which contains promoters for SP6 and T7 RNA polymerase. Positive clones were obtained and confirmed for the presence of *l(2)dtl* fragment (Figure 7B).Even though cloning the probe is more work intensive, it is useful because it gives enough pure starting material for amplification and avoids non-specific amplification. Cloning into the dual promoter vector allows generation of sense and antisense probes from the starting material. These positive clones were linearized and then purified (Figure 7C). To get specific probes without the TOPO vector sequence, the vectors were RE digested at the the polylinker site that is absent in the probe. This linearization of the vector was done by using BamHI and NotI REs. Then these vectors were used as a template for PCR amplification of *l(2)dtl* fragment and used for sequencing to find the orientation in the vector (Figure 7D). After orientation mapping it became clear that SP6 promoter leads to antisense and

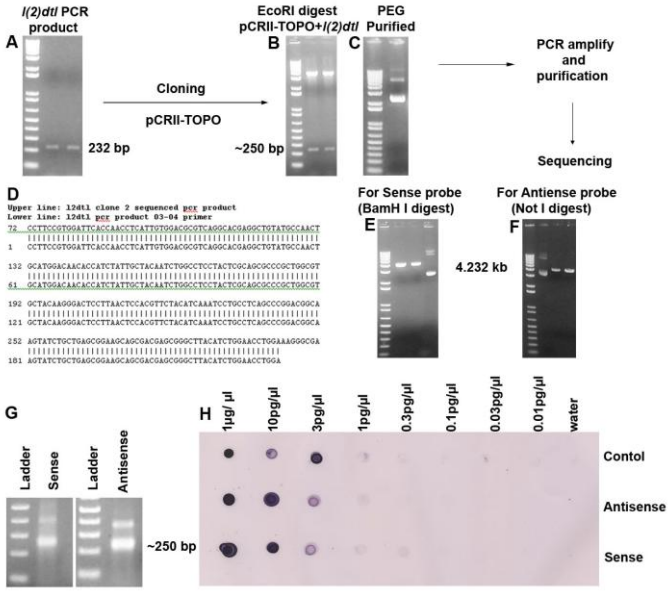

Figure 7: Generation of DIG-labeled *l(2)dtl* RNA probes (A) PCR amplification of *l(2)dtl* fragment (B) and (C) Screening by EcoRI digest and purification of positive clones (D) Sequencing read out of positive clone for *l(2)dtl* to check orientation of insert (E) and (F) Linearised vector with BamHI and NotI ERs for sense and antisense probe generation respectively (G) Integrity analysis of sense and antisense RNA probes and (H) Quantification of RNA probes by spot test.

T7 polymerase to sense probes (Figure 7E-F). SP6 and T7 polymerases were used respectively to generate probes. DIG-labeled probes were generated by using Roche RNA *in vitro* transcription and labeling kit and their integrity was assayed by running them on RNase free agarose gel (Figure 7G). An accurate quantification of DIG-labeled RNA obtained by *in vitro* transcription is most important for optimal and reproducible results *in situ* hybridization. Very high concentration of probe usually causes high background while too low concentration leads to weak signals. So to estimate the yield of DIG-labeling, a dilution series of labeling reaction and also of control RNA were spotted on nylon membrane and colorimetric detection procedure

using NBT/BCIP was performed as per Roche RNA labeling kit protocol. The DIG-labeled probe spots were compared to that of control spots and the amount of DIG-labeled RNA was calculated (Figure 7H).

IV. CONCLUSIONS

l(2)dtl mutants in *Drosophila* are lethal as embryos and l(2)dtl transcripts are up-regulated under heat-shock [1]. The molecular function of *l(2)dtl* has not been investigated in *Drosophila*. A functional analysis of *l(2)dtl* gene by using *l(2)dtl* RNAi was carried out in *Drosophila itpr* mutants. The results show additive effect. The present study indicates that *l(2)dtl* has a potential role in $InsP_3$ mediated intracellular Ca^{2+} signaling pathway. Down-regulation of this gene enhances the lethality of the weak $Insp_3R$ heteroallelic mutant combination. As this gene is highly enriched in brain [16], RNA *in situ* will provide a better picture about its involvement of neuronal domains. Using *UAS-l(2)dtl* can enhance the reliability as compared to *l(2)dtl* RNAi. This will help validate their potential regulation by $InsP_3$ mediated intracellular Ca^{2+} signaling and also in understanding how developmental pathways impact growth and metabolism.

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