Characterization of the effect of the V67 point mutation in dRNase Z in *Drosophila melanogaster* on gene expression

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Abstract

dRNase Z, the ortholog of human ELAC2 in Drosophila melanogaster, is known for participating in transforming growth factor-β (TGF-β) pathway. It functions as a tumor suppressor as well as a scaffold protein by promoting the interaction between Sp1 and Smad2, therefore stimulates TGF-β-induced p21 promoter activation in prostate cancer cells. In this study, I was able to examine the *in vivo* function of dRNase Z by comparison of mRNA expressions of TGF-β pathway downstream target genes Ecdysone Receptor (ECR), Wingless (Wg), and Ultraspiracle (USP) in the point mutation strain V67 and the control strains. I found that both ECR and Wg were considerably down-regulated in V67 and USP was up-regulated, which suggests either a compensatory or a competitive regulatory response between MAD/MED and dSmad2/MED binding.

Keywords: **dRNase Z; V67; TGF-β/Smad pathway**

Introduction

Human ELAC2 is isolated by genome analysis in high-risk prostate cancer families and was grouped as a putative prostate cancer susceptibility gene. [1] *In vitro* biochemical studies showed that ELAC2 is a tRNA processing endonuclease, the RNase Z enzyme, which generates the mature 3'-end of tRNA molecules by removal of the 3'-trailer elements of pre-tRNAs. [2] The *Drosophila* genome contains a single ortholog of human *ELAC2* in the 47A1 region on the 2R chromosome, which was previously identified as *Juvenile hormone-inducible protein 1*, *JhI-1*. Silencing of *JhI-1 in vivo* by RNAi in *Drosophila* S2 cells disrupts tRNA maturation and triggers accumulation of pre-tRNA molecules with 3' extensions. Therefore it's renamed as *dRNase Z*.

Previous studies revealed ELAC2 is involved in TGF-β/Smad signaling mediated growth arrest as an important transcriptional scaffold protein. Furthermore, the DNA microarray data suggested that it is likely participate in the p53 signaling pathway and apoptosis. [1] Therefore in this project, target genes ECR, Wg, USP of TGF-β/Smad pathway were selected to be the candidate genes studies on mRNA expression in V67/ED24 mutant and control larvae.

In order to understand *in vivo* function of dRNase Z, a library of diverse mutant alleles in Drosophila melanogaster was generated. Most point mutations result phenotypes similar to flies with RNAi deletion on dRNase Z, which is early lethal in the beginning of 1st instar. Only V67, which exhibit an Arg to Glu mutation at a conserved region in front of the functioning His-motif, can live until pupation.

However, the mutant eventually dies before pupation because of the deregulated ectopic differentiation of lamellocyte (autoimmune reaction), which gives rise to melanotic tumors at different part of the larva.

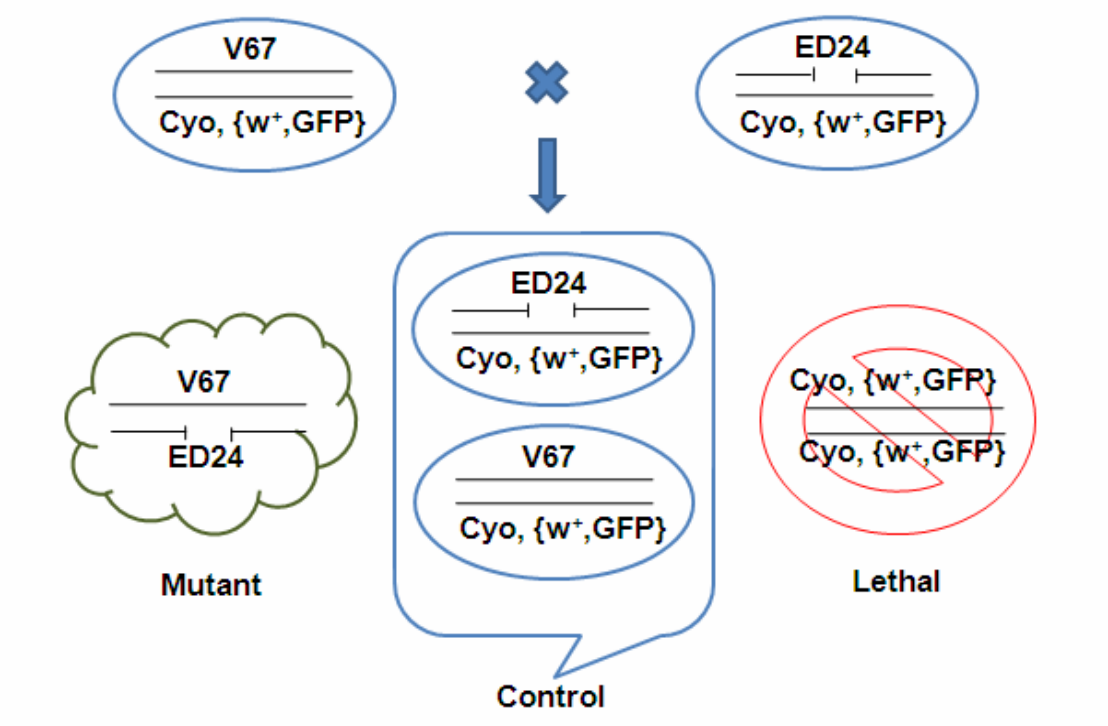

Figure 1. Schematic diagram of study strain and control strains, V67 represents the point mutation of dRNase Z on the second chromosome. ED24 is the null mutation of dRNase Z, which due to chromosome deficiency, cannot produce dRNase Z.

dRNase Z located on the 2nd chromosome. ED24 represents an dRNase Z gene truncated allele, therefore only one mutated allele can be produced in V67/ED24 strain, Cyo, {w^{+}, GFP} is a wildly commercialized allele with a fluorescent protein inserted in. (Figure 1) Daughter strains are early lethal with two Cyo, {w^{+}, GFP} allele or exhibiting the same normal phenotype as heterozygotes, which present one copy of the normal dRNase Z allele, with either one dRNase Z absence allele, one dRNase Z point mutated allele. Both two heterozygotes have no effect on other proteins' expression. Our study strain has one copy of the dRNase Z truncated allele

and one copy of dRNase Z point mutated allele. Point mutation locates in front of the conserved region of a known functioning His – motif and this one basepair change leads to Arg – Glu amino acid mutation.

Genomic-wide microarray analysis has revealed common targets for the *tkv* and *babo* pathways and provided new insights into downstream genes of the two distinct TGF-β-like pathways, which is mostly novel and functioning in growth control. Among the genes regulated by both pathways is *ultraspiracle* (USP), which further connects TGF-β with neuronal remodeling. [2] ECR was selected due to its key regulatory role in introducing Drosophila into pupation stage and its ability of binding ecdysone is greatly stimulated by the addition of USP as a partner in heterodimerization. Furthermore, candidate gene Wg was chosen not only for its key role in Drosophila development, but also due to previous studies which demonstrated that inhibition of Drosophila Wg signaling involves competition between Mad and Armadillo/β-Catenin for dTcf binding.[6]

Materials and methods

Drosophila strains

Mutant (V67/ED24) and Control (V67/GFP & ED24/GFP) 3rd instar larvae were kindly provided by Dr. Dubrovsky's lab.

Total RNA Extraction

Total RNA from the 3rd instar larva samples was isolated using QIAGEN

RNeasy Plus Mini Kit following manufacturer's protocol with minor modifications. The pelleted cells were disrupted by adding twice of 440 µl of Buffer RLT Plus and vortexed to obtain a homogenous lysate. The homogenized lysate was transferred to a gDNA Eliminator spin column placed in a 2 ml collection tube, and was centrifuged for 30s at 13,000 rpm. The column was discarded, and the flow-through was saved. 600ul of 70% ethanol was added to the flow-through and mixed by pipetting. One aliquot of the sample was transferred to an RNeasy spin column placed in a 2 ml collection tube, and was centrifuged for 30s at 13,000 rpm. The flow-through was discarded. The remained aliquot of sample was transferred to the same RNeasy column and centrifuged for 30s at 13,000 rpm. The flow-through was discarded. 700µl of Buffer RW1 was added to the RNeasy spin column and centrifuged at 13,000 rpm for 30 s. 500µl of Buffer RPE was then added and centrifuged at 13,000 rpm for 30s, and this step was repeated but centrifuged for 2min. The RNeasy spin column was placed in a new 2ml collection tube and centrifuged at 13,000 rpm for 1min. Then the spin column was placed in a new 1.5ml collection tube, and was eluted with 30µl deionized water. Extracted RNA was stored at -80℃. The total RNA extracts were digested by DNase I (Invitrogen) as per the manufacturer's protocol.

RT-PCR Primers

Primers		Sequences	Position*	Expected product size	Intron in between the primers
ECR	Forward	CACGGTCCAGTTGATTGTTGAG	Exon 6	361 bp	63 bp
	Reverse	GTAGTAGCTCTGGATCGCTTCG	Exon 7		
Wg	Forward	CGATCAGCAAGATCCATCAC	Exon 3	180 bp	160 bp
	Reverse	GCTCCAGATAGACAAGGTC	Exon 4		
RP49	Forward	CAGTCGGATCGATATGCTAAGC	Exon 2	374 bp	352 bp
	Reverse	CGACCACGTTACAAGAACTC	Exon 3		
USP	Forward	CATGACCAATAGCGTGTCCAG	Exon 1	383 bp	0 bp
	Reverse	GTGATCGTCGCTCAAAGGAG	Exon 1		

* ECR mRNA, GenBank Accession No. NM_165465; Wg mRNA, GenBank Accession No. NM_164746; USP mRNA, GenBank Accession No. NM_057433; RP49 mRNA, GenBank Accession No. NM_001144656

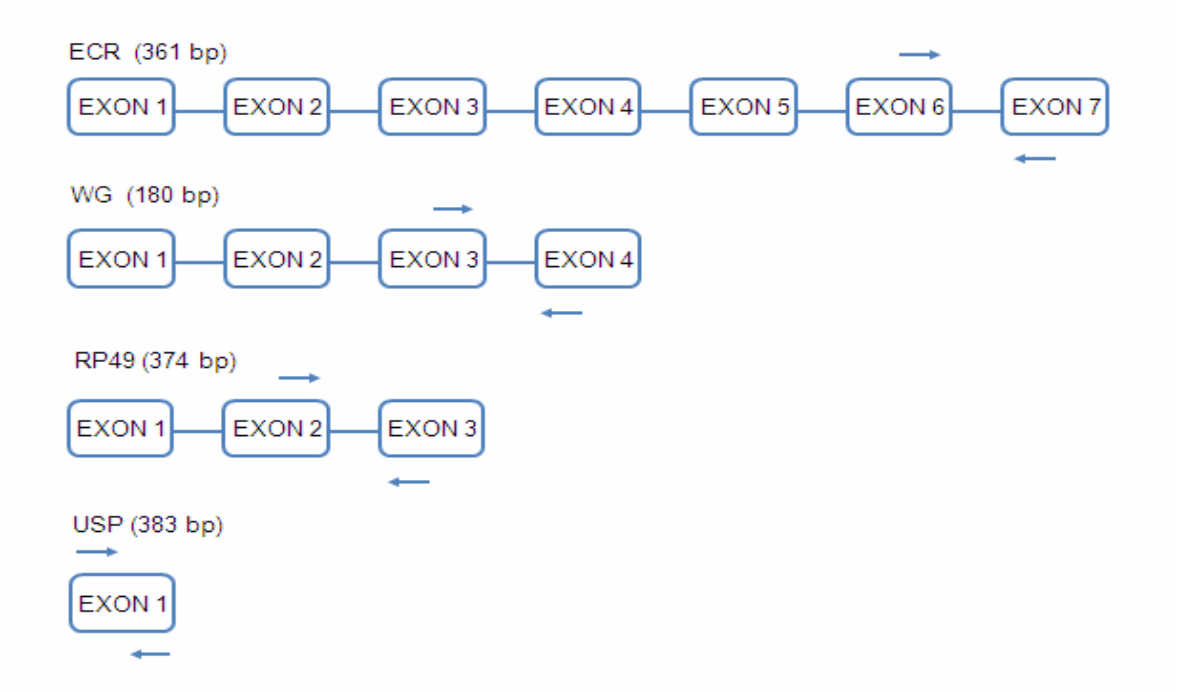

Figure 2. Schematic diagram of ECR, Wg, RP49, USP primer pairs locating regions

RT-PCR

RT-PCR was performed on the DNase I-treated total RNA samples prepared from the larva samples using the Qiagen OneStep RT-PCR kit and primers shown in Table 1 and Figure 2. Primers were synthesized by Integrated DNA Technologies.

Twenty nanograms of RNA was amplified in 25 μl RT-PCRs (5 μl 5×RT buffer, 1 μl 10mM dNTPs, 0.5 μl 10pmol/ μl forward primer, 0.5 μl 10pmol/ μl reverse primer, 1 μl enzyme mix, 4 μl 5ng/ μl RNA and 13 μl ddH2O). One-step RT-PCR was performed according to the following protocol: one cycle of 50℃ for 30min and 95 ℃ for 15min, 94℃ for 30s, 57℃ for 30s, and 72℃ for 30s, and a final extension of 72℃for 10min followed by a final hold at 4°C. For ECR, Wg, USP, amplification was performed under 30 cycles. To control the amount of mRNA present in the samples, RT-PCR amplification of RP49 was performed on all RNA samples under 22 cycles. 5 μl of loading dye was added to each RT-PCR product. 5 μl of each product was then added to a 1% agarose gel, and electrophoresis was performed at 130V. Band intensities were quantified using SigmaGel Scanner.

PCR

To detect possible residue contamination of genomic DNA in the RNA preparations, PCR with USP primers (shown in Table 1 and Figure 2) was performed on DNase I-treated RNA samples using Go-Taq PCR Master Mix (Promega) as per the manufacturer's protocol for 50 cycles.

Sequencing

RT-PCR products were purified using QIAquick PCR Purification Kit (QIAGEN) and then sent out to Genewiz Laboratory for sequencing according to the requirements of the sequencing pre-mix from the company. Sequencing results were analysis using NCBI BLAST program.

Results

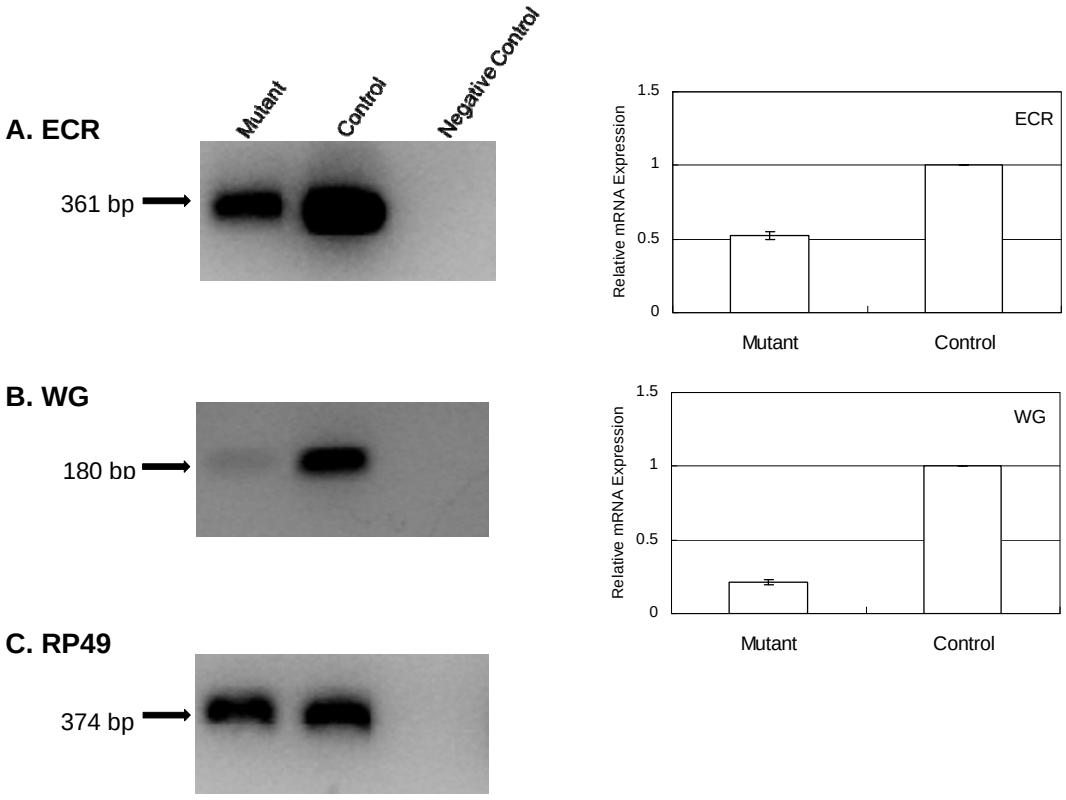

Figure 3. Expression of ECR (A) and Wg (B) in Mutant (V67/ED24) and Control (V67/GFP & ED24/GFP) larvae. RT-PCR products were fractionated on a 1% agarose gel. Internal control RP49 (C) was utilized to monitor the amount of mRNA present in the samples. Band intensity was quantified by Bio-Rad Quantity One and RP49 normalized relative ECR and Wg mRNA expression are shown on the right.

To study the mRNA expression of ECR, Wg and USP genes in the Mutant (V67/ED24) and Control (V67/GFP & ED24/GFP) larvae, RT-PCR using primers shown in Table 1 were performed. Total RNA extracts were digested with DNase I to eliminate genomic DNA contamination in RNA preparation. RT-PCR products are fractionated on 1% agarose gels. PCR products with expected sizes were successfully amplified for all 4 genes and all the PCR products have been confirmed by sequencing. There was no band found on the agarose gel corresponding to the

products that are amplified from genomic DNA. Based on densitometric analysis on gel pictures of triplicate RT-PCR using RP49 as internal control, ECR and Wg were found to be decreased to 52.4% and 21.3% respectively in the mutant compared to the control (Figure 3). USP has no intron in the gene. To verify that the PCR product obtained in the USP RT-PCR was amplified from USP-derived transcripts but not genomic DNA, PCR using same USP primers was performed. The absence of PCR product of 383 bp (Figure 4B) demonstrates that the 383 bp band from RT-PCR was amplified from USP-derived transcripts. Densitometric analysis indicates a 2.1 fold increase of USP mRNA level in the Mutant compared to the control (Figure 4A).

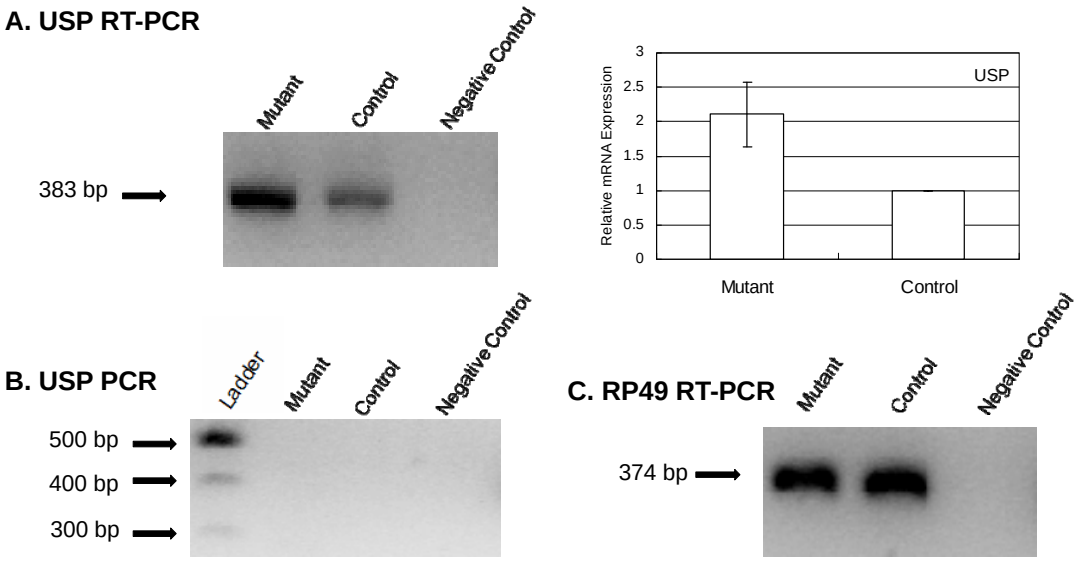

Figure 4. Expression of USP in Mutant (V67/ED24) and Control (V67/GFP & ED24/GFP) larvae. RT-PCR was performed on DNase-treated RNA samples (A). Internal control RP49 (C) was utilized to monitor the amount of mRNA present in the samples. PCR using USP primers on DNase-treated RNA samples (B) was performed to detect possible DNA contamination. RT-PCR and PCR products were fractionated on a 1% agarose gel. Band intensity was quantified by Bio-Rad Quantity One. RP49 normalized relative USP mRNA expression are shown on the right.

Discussion

TGF-β superfamily members, which is a pivotal antimitogenic factor in the normal prostate play a critical role in many cellular processes such as proliferation, differentiation, development, apoptosis, and cancer. [3] (Figure 5) Seven ligands in *Drosophila* are roughly grouped into two major TGF-β pathways, the *dpp*/BMP and activin pathways, mainly signaling through *thick veins (tkv)* and *baboon (babo)*.

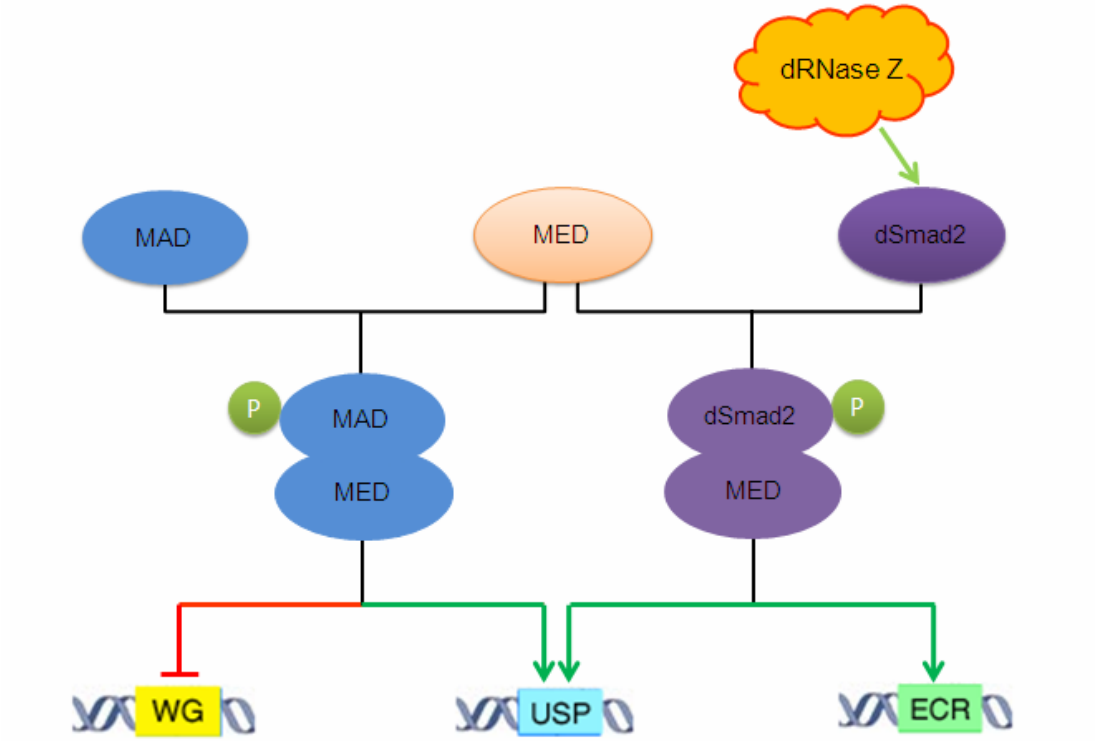

Figure 5. Schematic diagram of dpp/BMP (left) and Activin (right) pathways. Both MAD and dSmad2 are R-Smad, MED is the Co-Smad. MAD and dSmad2 are phosphorylated and form dimmers with MED, respectively, in order to continue the signaling. Dpp/BMP pathway inhibits the expression of Wg through the MAD/MED dimer, whereas ECR expression level is promoted by Activin signaling through the dSmad2/MED dimer.

Although it has been revealed that both TGF-β/BMP and Wnt/Wingless (Wg)

pathways play important roles in organismal patterning and growth and their deregulation can give rise to oncogenesis, fibrosis, immune- and vascular disorders in mammalian, [4] whether they are regulated in the same way in Drosophila was not fully understood. dpp/BMP and Activin pathway compete for the same co–smad called MED. [8]

In this study, downstream target genes of TGF-β pathway ECR, Wg were down-regulated, This observation suggests that dRNase Z in Drosophila elicits TGF-β/Smad induced transcriptional responses by promoting the interaction with activated dSmad2. Disruption of dRNase Z leads to a negative impact on Activin pathway which then causes ECR down-regulated. Since ECR is master regulator of molting in Drosophila development, low level expression of ECR not only leads to underdeveloped larva, but also causes pupation failure, which is consistent with V67/ED24 phenotype. Moreover, it has been reported that Wg expression is antagonized by dpp/BMP signaling. In vivo, high levels of activated dpp/BMP receptor and its effecter Mad can inhibit the expression of Wg. Conversely, loss of mad can induce Wg expression. [5] Function defect of dRNase Z in Activin pathway leaves more MED available for dpp/BMP pathway which gives rise to Wg deregulated. Lacking of Wg leads to a long period of developmental stage in mutant compare to the normal phenotype, which was also consistent in V67/ED24 strain.

However, USP, which is regulated by both dpp/BMP and Activin pathways, is up-regulated in Mutant. This interesting observation may indicates dpp/BMP pathway has a higher efficiency on USP expression than Activin, so when there are more MED

available for dpp/BMP, USP is up-regulated; Or, suggests that dRNase Z has other biological unidentified functions that would affect USP regulation. Moreover, the strong impact of dRNase Z on Wg and ECR might also suggests that there would be possible regulatory mechanism dRNase Z involved to mediate these genes.

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