

JAK/STAT Signaling Promotes Regional Specification by Positively Regulating Cyclin Expression in *Drosophila*

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Abstract-The *Drosophila cyclinD* (*cycD*) gene promoter contains a recognition consensus sequence for *Drosophila* STAT (*stat92E*). Transient luciferase expression assay using S2 cells indicated that the *cycD* gene promoter was activated by cotransfected with HOP and *stat92E* expression plasmid. Furthermore, the S2 cells original protein expression of JAK/STAT signal pathway was inactivated by RNA interference technology, and the *cycD* luciferase expression decreased. The further research with *CycD*-WT, *CycD*-D (deletion with the *stat92E* binding sites) and *CycD*-TM (mutation with the *stat92E* binding sites) indicated that the transient luciferase expression decrease with the deletion and the mutation of all three *stat92E* binding sites. Here we propose that *stat92E* can participate in regulation of the expression of *cycD* through the *stat92E* binding sites.

Keywords-JAK/STAT pathway; *Stat92E*; *Cyclin D*; *CycD*-WT; *CycD*-TM

I. INTRODUCTION

Drosophila serves as an excellent model system to study this pathway, as flies are highly amenable to genetic manipulation and possess only one JAK and one STAT gene, compared with the four JAKs and the seven STATs in mammals. Genetic studies in *Drosophila* have uncovered several components of this pathway, including Upd and two other Upd-like ligands called Upd2 and Upd3; the transmembrane receptor Domeless (Dome), which is distantly related to the gp130 family of cytokine receptors; the JAK Hopscotch (Hop); and the STAT protein Stat92E. The JAK/STAT pathway is crucial for diverse processes in *Drosophila*, including proliferation and planar polarity in the developing eye[1].

To reasearch the function of JAK/STAT pathway, it is a significant work to study the target genes of STAT protein. The former research indicates that the DNA binding sequence of STAT92E is TTCGNNNGNN(N is arbitrary amino acid).When a gene's upstream regulation

sequence contains two or more binding sequences, STAT protein could effectively promote the transcription of this gene.

Mike Levine's lab in California university established a computer program which can find the potential DNA binding sites of the transcription factor. About 2398 genes have been found in the *Drosophila* genome with the program, therefore those genes might be the target genes of STAT92E, which include several important molecules of signal pathway. such as *cycD*, *cdk4* proteins which are the key regulatory proteins in cell cycle, Delta within the Notch pathway, Wingless, Wnt5 and Fz2 within the Wnt pathway.

Our research focuses on the *CycD* which plays an irreplaceable role in animal growth and development.[2,3] If the conclusion that *CycD* is the target gene of STAT92E can be proved, the direct evidence of the crosstalk of those important pathways[4]. The research shows that, the co-expression of the key proteins (hop, *stat92E*) of JAK/STAT pathway and *cycD* in *Drosophila*'s S2 cells would increase the expression of *cycD*. Then, the S2 cell original protein expression in JAK/STAT signal pathway was inactivated using RNA interference technology, and the *cycD* expression decreased. These results suggested the further research with the mechanism with the regulation of the JAK/STAT pathway with *cycD*.

II. MATERIAL AND METHODS

A Material

pGL3-Basic Luciferase Reporter Vector: with a fluorescence reporter gene and without the Promoter (from Promega org.)

B Method

1) Generation of the pGL3-Basic Vector with the upstream regulation sequence of Cyclin D

The sequence between the binding site of STAT92E and the transcription region of Cyclin D was amplified from the genome of the wild type of *Drosophila* (Fig. 1).

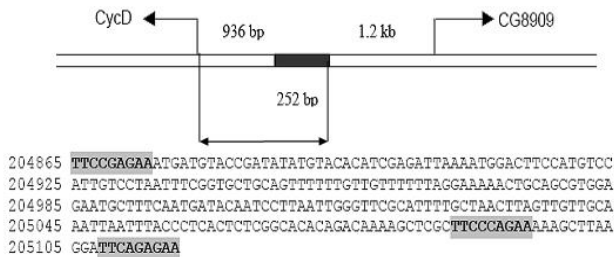


Figure 1. The upstream regulation sequence of *cycD* gene

Primer:F:

5'-CGGAATTCTAAGCACTTGGTTACTTCTGGTC-3';

R: 5'-CATGGTGGCTTTACCAACAG-3'

Reaction system (50 μL) follow the protocol of the *Pyrobest* DNA Polymerase (Takara). Reaction conditions:

Cycle number	Denaturation	Annealing	Polymerization
30 cycles	94°C 5min	60°C 1min	72°C 1min
Last cycle	94°C 5min	60°C 1min	72°C 10min

The PCR fragment after gel purification was digested by *Kpn* I / *Bgl* II, then cloned into the pGL3-Basic vector. Plasmids were qualified both in concentration and purity with Mini Prep Kit (Promega).

2) Generation of the upstream sequence of Cyclin D without the binding sites of STAT92E

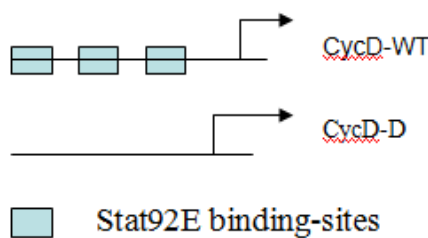


Figure 2. CycD-WT & CycD-D sequence

Primer:F:

5'-CGGGGTACCCGGGGTACCGATCCACAGGTTACTTTTG-3'

R: 5'-CATGGTGGCTTTACCAACAG-3'

Insert CycD-D sequence was obtained with the PCR Reaction conditions of CycD-WT (Fig. 2). The operation followed was same with those in 1.2.1.

3) Generation of the Vector recombinant with mutation in the binding sites of STAT92E (Fig. 3)

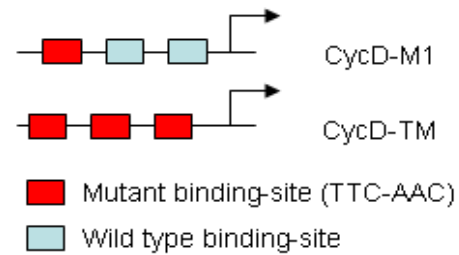


Figure 3. Generation of the binding sites of STAT92E with mutation sequence using PCR

(1) Generation of M1 mutation sequence

Primer F: 5'-ACGTAGTGGGCCATCGCCCTG-3' (The sequence on pGL3 vector about 200bp before the transcription region of Insert sequence)

Primer R: 5'-TAACCTGTGGATCCCAACCGAGAAATGATG-3' (including one mutation binding site of STAT92E)

PCR Reaction system and conditions were same with those in 1.2.3.

(2) Generation of CycD-M1 mutation sequence

Primer F: M1 mutation sequence, Primer R: 5'-CATGGTGGCTTTACCAACAG-3'

Increasing the amount of Primer F.

(3) Generation of M1M2M3 mutation sequence

Amplified from the CycD-M1 mutation sequence.

PCR reaction system and conditions were same with those in 1.2.3.

Primer F: 5'-ACGTAGTGGGCCATCGCCCTG-3'

Primer R: 5'-GCTCGCAACCCAGAAAAAGCTTAAGGAAACAGAGAAAA-3' (including two mutation binding sites of STAT92E)

(4) Generation of CycD-M1M2M3 mutation sequence

Primer F: M1M2M3 mutation sequence,

Primer R: 5'-CATGGTGGCTTTACCAACAG-3'

Increasing the amount of Primer F.

(5) CycD-M1M2M3 mutation sequence was cloned into the pGL3 vector.

The reaction conditions and the operation followed were same with those in 1.2.1.

(6) Detecting fragment length and the mutation by sequencing.

4) Transfection of S2 cells

Seed $3-4 \times 10^6$ cells per well in 35 mm tissue culture plates. And Schneider medium to a total volume of 3 mL. Incubate at 25°C for 1-2 hrs. Prepare the following solutions in 12×75 mm sterile tubes: A Add 1.5 μg DNA to 100 μL Schneider medium (without serum/antibiotics); B Add 6 μL CellFECTIN Reagent to 100 μL Schneider medium. Combine the two solutions, mix by pipettins, incubate at RT for 30min. Remove medium from the plate. Add 0.8ml Schneider medium to each tube containing the DNA-lipid complex. Mix gently, overlay the diluted DNA-lipid complexes onto the cells. Incubate cells at 25°C for 4hrs. Remove the transfection mixtures. Add 3mL UM medium (with 10% FBS and antibiotics). Incubate cells at 25°C for 40-48hrs.

5) *Measurement of luciferase activity in the extract of Drosophila S2 cells*

Renilla Luciferase Assay

(1) Format the luminometer so that the injector dispenses 50µL. Prime the injector with *Renilla Luciferase Assay Solution*.

(2) For each reaction, carefully add 20µL of cell lysate to an individual luminometer tube or to the wells of a multiwell plate.

(3) Add 50 uL *Renilla Luciferase Assay Enhancer* into each reaction.

(4) Place the samples in a luminometer.

(5) Initiate measurement. This action will cause *Renilla Luciferase Assay Solution* to be injected into the reaction vessel and the measurement to be subsequently taken. Luminescence is normally integrated over 10 seconds without pre-read delay. Other integration times may also be used.

(6) Record the *Renilla luciferase activity* measurement.

(7) If using a single tube luminometer, discard the reaction tube, and proceed to the next *Renilla Luciferase Assay* reaction. If using a plate luminometer, the luminometer will automatically begin injecting *Renilla Luciferase Assay Solution* into the next well indicated on the luminometer plate.

III. RESULTS

A The components of the JAK/STAT pathway positively regulate the cycD gene

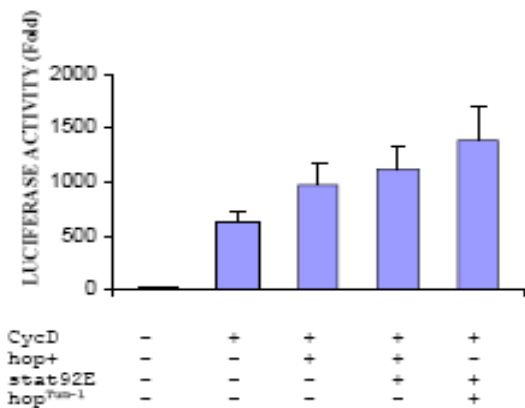


Figure 4. The components of the JAK/STAT pathway positively regulate the *cycD* gene

We therefore assessed the ability of Stat92E to directly regulates *cycD*. A reporter called *CycD*, in which luciferase is driven by a 1188 base pair regulation sequence from the 3' *cis cycD* genomic region, was sufficient to recapitulate *cycD* expression in the S2 cells. Using the empty vector as a blank control, the *CycD* reporter indicates the fluorescent activity much higher than the no-load plasmid (Fig. 4).

This reporter was significant expressed in the co-expression hop in S2 cells which is the kinase of the JAK/STAT pathway. Moreover, the *CycD* reporter was

expressed highly in the co-expression of Stat92E and the hop^{Tm-1} (overexpression with hop) (Fig.4). These data indicate that Stat92E and Hop can regulate *cycD* expression through the upstream regulation sequence of *cycD* which contains three Stat92E binding sites (TTC(N)3GAA), strongly suggesting that Stat92E regulate *cycD* directly through the Stat92E binding sites.

B Repression of the JAK/STAT activating components(hop and stat92E) in S2 cells leads to loss of cycD expression

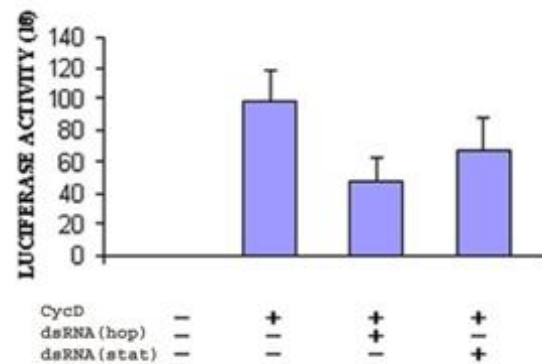


Figure 5. Repression of the JAK/STAT activating components (hop and stat92E) in S2 cells leads to loss of *cycD* expression

In S2 cells, the original protein expression of JAK/STAT pathway was inactivated by the RNA interference technology, therefore the *cycD* expression decreased (Fig. 5). These results suggested that the components of the JAK/STAT pathway regulate the expression of the *cycD* gene.

C Mutation and deletion with the STAT92E binding sites repress the expression of cycD gene

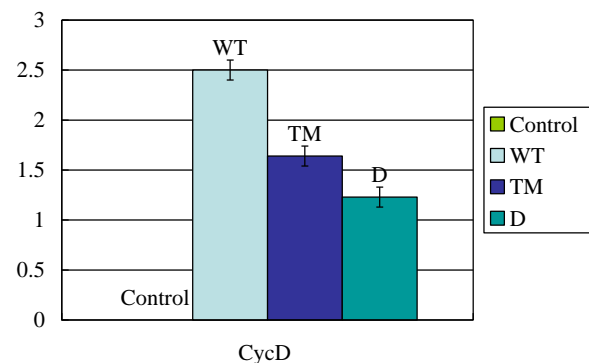


Figure 6. Mutation and deletion with the STAT92E binding sites repress the expression of *cycD* gene

A reporter called *CycD-WT*, in which luciferase is driven by a 1188 base pair regulation sequence from the

3' *cis cycD* genomic region, was sufficient to recapitulate *cycD* expression in the S2 cells. Using the empty vector as a blank control, the CycD-WT reporter indicates the fluorescent activity much higher than the no-load plasmid (Fig. 6). Another reporter called CycD-TM, in which three Stat92E binding sites were mutated, indicate that luciferase activity reduced. The third reporter called CycD-D, in which three Stat92E binding sites were deleted, indicate that luciferase activity reduced more significantly. Here we propose that stat92E can participate in regulation of the positively regulation of *cycD* through the stat92E binding site.

IV. CONCLUSIONS

JAK/STAT pathway is a high evolutionary conserved in many organisms, from *C. elegans* to human beings, and it attracts much attention for its role in conducting many important cellular signalings and cell growth factors. Cyclin D is required for the cell to pass the cell cycle checkpoint, and many tumors grow along with overexpression of Cyclin D[5]. It is generally acknowledged that Cyclin D is an important mediator of cell cycle regulation[6]. Many experimental evidences suggested that CycD reacted with several transcription factors such as: Myb, DMP1, STAT3, Beta2 and influenced the cell growth[7-10]. The expression of *cycD* was much likely regulated by JAK/STAT signal pathway through the STAT92E binding sites before the *cycD*. However, it was reported that CycD-Cdk4 and CycE-Cdk2 directly binded with STAT92E and regulated the JAK/STAT pathway function.

In this experiment, about 1kb upstream regulation sequence of *cycD* (including three STAT92E binding sites) was cloned into the pGL-3 basic vector and expressed in order to study the interaction between *cycD* and JAK/STAT pathway. Finally, it will prove that the expression of *cycD* can be regulated by JAK/STAT pathway through the STAT92E binding sites.

Ectopic activation of this pathway leads to an increase expression of *cycD*. By contrast, loss of activity of this pathway, using RNA interference technology, frequently resulted in the repression of *cycD*. When the mutation

and the deletion reporter did form, which mutated or deleted the Stat92E binding sites, *cycD* was ectopically expressed in S2 cells. Lastly, we showed that *wg* regulation by the JAK/STAT pathway is dependent of the Stat92E binding sites. Future work will be needed to address these issues.

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