Investigating Gp93 Biology Via Cloning *Drosophila* Toll and Integrin Genes

University of Wisconsin Eau Claire

Ruby Gravrok, Ashley Walker, Ceana Olson, & Crystal M. Del Valle Ph.D.

Department of Biology, University of Wisconsin – Eau Claire; Eau Claire, WI. 54702

ABSTRACT

In *Drosophila melanogaster* (fruit flies), the protein glycoprotein 93 (gp93) appears to be homologous to the mammalian protein glycoprotein 96 (gp96). As a chaperone, gp96 is required for Toll-like receptor (TLR) and integrin (Itg) expression, two protein families with roles in innate immunity and which also have *Drosophila* homologues. Due to this link with innate immunity, gp96 contributes to cancer, colitis, autoimmunity, and other diseases. Although a better understanding of gp96 biology is needed, this system is unfortunately complex and hard to study. Thus, we aim to characterize the simpler gp93 system to better understand the more complex gp96 system, and thus further development of immunotherapies directed at gp96 for the aforementioned diseases.

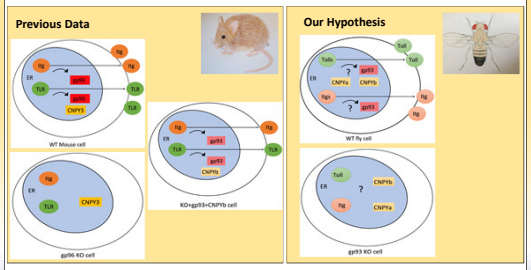
This project aims to clone *Drosophila* Toll and integrin genes with tags . Once cloned, these genes will then be inserted into a *Drosophila* cell line where they can be easily identified by their tags and investigated. This is required as currently no antibodies exist to identify these proteins directly. Thus, we are ultimately creating tools in order to study the gp93 pathway and to ascertain whether gp93 is a chaperone for *Drosophila* Tolls and integrins. Future experimentation with these tools will allow for better understanding of the gp93 and, by extension, the gp96 system which will aid development of gp96 targeted therapies.

INTRODUCTION

Toll-like receptors (TLRs), proteins which recognize infection and initiate an immune response, and integrins (Itg), cell-surface proteins which enable cell-to-cell adhesion, are just two of the many classes of proteins involved in innate immunity (1). In order to function, these proteins must be folded by chaperones. Glycoprotein 96 (gp96) folds (chaperones) mammalian TLRs and integrins, while canopy 3 (CNPY3) co-chaperones only TLRs (Figure 1, Previous Data; 2,3). Due to its role as a chaperone for TLRs and integrins, mammalian gp96 has implications in autoimmunity, sepsis, Bernard-Soulier Syndrome, colitis, and cancer. (4-6)

Given the structural and functional similarities, *Drosophila* gp93 has been identified as the counterpart (ortholog) to mammalian gp96, and *Drosophila* CNPYb as the ortholog to the mammalian CNPY3 (2). Previous research has shown that *Drosophila* gp93 can artificially chaperone mammalian TLRs and integrins while *Drosophila* CNPYb appears to co-chaperone mammalian TLRs (Figure 1, Previous Data; 2), but it has yet to be researched whether or not gp93 and CNPYb can chaperone *Drosophila* tolls and integrins (Figure 1, Our Hypothesis).

Figure 1: Left; Diagram of previous data regarding mammalian chaperones and client interaction and expression in mouse cells. Right; Diagram of our hypothesis regarding *Drosophila* chaperones and client interaction and expression in fly cells.
ER = endoplasmic reticulum, KO = knock out, Itg = integrin, TLR = Toll-like receptor



Although related, the mammalian gp96 system is markedly more complex than the *Drosophila* gp93 system with more genes in each protein family (Table 1). The relative simplicity of the *Drosophila* system makes it more efficient for preliminary research into the development of immunotherapies directed at the aforementioned diseases. investigation which can then be used to direct research of the mammalian system thereby advancing

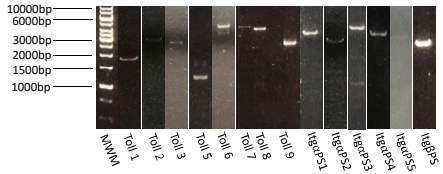
Table 1: TLR/Toll, integrin, and CNPY family members in mammals and *Drosophila*

	Mammals	Drosophila
TLRs/Toll	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12, TLR13	Toll, Toll-2, Toll-3, Toll-4, Toll-5, Toll-6, Toll-7, Toll-8, Toll-9
Alpha Integrins	α1, α2, α3, α4, α5, α6, α7, α8, α9, α10, α11, αIIb, αD, αE, αL, αM, αV, αX	α_{PS1}, α_{PS2}, α_{PS3}, α_{PS4}, α_{PS5}
Beta Integrins	β1, β2, β3, β4, β5, β6, β7, β8	β_{PS}, β_{v}
CNPYs	CNPY1, CNPY2, CNPY3*, CNPY4	CNPYa, CNPYb

Underlined genes are known gp96 client proteins. * gp96 co-chaperone

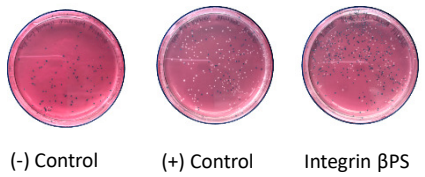
RESULTS

Figure 2:
DNA agarose gel electrophoresis of PCR amplified *Drosophila* Toll and Integrin genes



Representative pictures of successful polymerase chain reaction (PCR) amplification of the indicated *Drosophila* Toll and integrin (Itg) genes ran on DNA gels. A molecular weight marker (MWM) with DNA of known base pair (bp) lengths was ran alongside samples to verify size of the PCR amplified genes of interest.

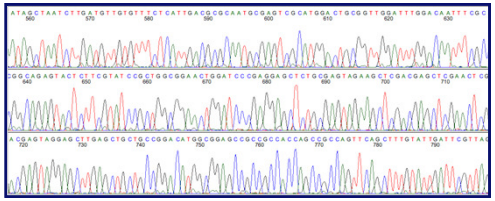
Figure 3:
***E. coli* cells transformed with vector containing Integrin βPS cultured on bacterial agar plates**



After ligating the genes of interest into the pGEM-Teasy cloning vector, competent *E. coli* cells were then transformed with the vector and spread on Ampicillin + IPTG + X-Gal LB agar plates. Blue/White color screening was used to identify colonies that contained vector and insert. White colonies were selected for sequencing to validate the presence of the inserted gene of interest. Representative plates are shown for integrin βPS with negative (-) and positive (+) controls.

Figure 4:
Sequencing Trace and Alignment of Integrin βPS

A.



B.

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ItgBPST7    gagggatatccggtggatctctactacttgatggatctctccaaatccatggaggacgac
ItgBPS      gagggatatccggtggatctctactacttgatggatctctccaaatctatggaggacgac
            ************************************************.***********

ItgBPST7    aaggccaaattgtctacgctgggcgacaagttgtccgagaccatgaaacgcatcaccaac
ItgBPS      aaggccaaattgtctacgctgggcgacaagttgtccgagaccatgaaacgcatcaccaac
            ************************************************************

ItgBPST7    aatttccatcttggtttcggttcgtttgtggacaaggtgctcatgccctatgtctcgacc
ItgBPS      aatttccatcttggtttcggttcgtttgtggacaaggtgctcatgccctatgtctcgacc
            ************************************************************
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pGEM-Teasy vectors with ligated genes of interest were purified from white bacterial colonies after transformation using a miniprep kit. Isolated DNA was then sent for sequencing to confirm the presence of the gene of interest in the vector. A representative sequence trace is shown in 'A', and a representative nucleotide alignment between the sequencing data (ItgβPST7) and the known gene sequence (ItgβPS) is shown in 'B', each for integrin βPS.

METHODS

RNA was isolated from *Drosophila melanogaster* using TRIzol reagent and reverse-transcribed to cDNA using SuperScript III First-Strand Synthesis System (Both from Invitrogen). Genes of interest were amplified from cDNA via polymerase chain reaction (PCR) using Thermo Scientific's Phusion High-Fidelity PCR kit and gel purified via agarose gel electrophoresis. 3' A overhangs were added to the genes before being ligated into the pGEM-T Easy cloning vector (Promega) and transfected into competent JM109 *E. coli* cells. Transfected cells were then selected for on Ampicillin + IPTG + X-Gal LB agar plates using blue/white color screening. White bacterial colonies were grown in LB media from which the vector was isolated using Thermo Scientific's GeneJET Plasmid Miniprep Kit. Sanger sequencing of the isolated vectors at UW – Madison's Biotechnology Center was performed to verify successful ligation of the genes of interest into the cloning vector.

CONCLUSIONS

Table 2: Current PCR amplification and cloning status for all genes of interest

Genes of Interest	PCR Amplification	Cloning
Toll-1	Successful	Successful
Toll-2	Successful	Pending sequencing
Toll-3	Successful	Pending sequencing
Toll-4	Pending	N/A
Toll-5	Successful	Pending sequencing
Toll-6	Successful	Successful
Toll-7	Successful	Pending sequencing
Toll-8	Successful	Pending sequencing
Toll-9	Successful	Pending sequencing
Integrin αPS1	Successful	Pending sequencing
Integrin αPS2	Successful	Pending sequencing
Integrin αPS3	Successful	Pending sequencing
Integrin αPS4	Successful	Pending sequencing
Integrin αPS5	Successful	Successful
Integrin βPS	Successful	Successful

Most *Drosophila* tolls and integrins have been successfully amplified via PCR, and the following genes of interest have been cloned into the pGEM-Teasy vector: Toll-1, Toll-6, ItgαPS5, and ItgβPS. Cloning of other genes is pending sequencing results.

FUTURE DIRECTIONS

The genes of interest in the pGEM-Teasy *Drosophila* cloning vector will be sub-cloned into a *Drosophila* expression vector in order to transfect them into the S2* *Drosophila* cell line. Once the cell lines are created, exploration into whether gp93, CNPYa*, or CNPYb can chaperone *Drosophila* tolls or integrins will proceed. Specifically, we have 3 research questions:

1. Is there loss of toll or integrin expression following gp93, CNPYa or CNPYb knock out?
2. Do gp93, CNPYa and CNPYb physically interact with tolls or integrins?
3. Does knockdown of gp93, CNPYa or CNPYb lead to increased bacterial pathogenesis?

From this data, a greater understanding of gp93 biology will be available to aid in development of gp96-targeted therapies for multiple diseases.

*CNPYa is included to ensure that a possible chaperone of *Drosophila* tolls or integrins is not overlooked.

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