

The *Tubulin Tyrosine Ligase Like 5* Gene of *Drosophila melanogaster*

Thibault SCHOWING

January 28, 2020

Date Performed:	HS 2019
Course:	Molecular Biology for non-biologists
Professor:	Ruth Doerig
Institution:	University of Bern

Objective

Microtubules are cytoskeletal filaments involved in movement, transport and structure of the cell. Many of these functions require post-translational modifications that regulate the activity, localization or stability of the microtubules, e.g. polyglutamylation (Schaetzky and Rape (2016)). Altering the functional property of microtubules can also alter the complex cell architecture and thus alter its functionality.

The *TTLL5* gene encode for a polyglutamylase that modifies α -tubulin. Mutated *TTLL5* is known to be involved in cone-rod degeneration and reduced male fertility in human (Bedoni et al. (2016)). The aim of this practical is to find more information about the human *TTLL5* (Q6EMB2) homolog in *D. melanogaster*. Therefore, four experiments were performed.

Experiments

Experiment 1: Fertility test Does the overexpression of *TTLL5* result in female sterility?

Experiment 2: Confocal microscopy Is the Staufen protein localization in early oocytes dependent on a functional TTLL5 protein?

Experiment 3: Western blot Does the glutamylation of α -tubulin depend on a functional TTLL5 protein?

Experiment 4: CRISPR/Cas9 Introduce a point mutation into the *TTLL5* gene by using the CRISPR/Cas9 system.

Fly stocks

In wild type Drosophila, *TTLL5* is located on the third chromosome¹.

$\frac{w}{w}; \frac{\text{Driver}}{(Sm,Cy)}; \frac{\text{TTLL5}^{PBac}}{TM,Sb}$	$\frac{w}{w}; \frac{\text{venus-TTLL}}{(Sm,Cy)}; \frac{\text{venus-TTLL}}{TM,Sb}$
$\frac{w}{w}; \frac{\text{Driver}}{(Sm,Cy)}; \frac{\text{TTLL5}^{Minos}}{TM,Sb}$	$\frac{w}{w}; \frac{\text{msps-mcherry}}{(TM,Sb)}$
$\frac{w}{w}; \frac{\text{Driver}}{(Sm,Cy)}; \frac{\text{TTLL5}^{MI-Ex}}{TM,Sb}$	$\frac{w}{w}; \frac{\text{TACC-mcherry}}{(TM,Sb)}$
$\frac{w}{w}; \frac{\text{venus-TTLL}}{(Sm,Cy)}; \frac{Df(\text{TTLL})}{TM,Sb}$	$\frac{w}{w}$
$\frac{w}{w}; \frac{\text{Driver}}{(Sm,Cy)}; \frac{PrDr}{TM,Sb}$	

Genes and their full names:

- *TTLL5* = tyrosin tubulin ligase like 5
- Mutant alleles *TTLL*^{PBac}, *TTLL*^{Minos}, *TTLL*^{Minos-Ex128}
- *venus* = gene encoding a yellow fluorescent protein (variant of GFP)
- *mcherry* = gene encoding a red fluorescent protein
- *TACC* = tumor associated coiled coil protein (Used as control for fertility test)
- *msps* = mini spindles (Used as control for fertility test)

¹Information available on *FlyBase*

Experiment 1: Fertility test

The disruption of the TTLL5 gene in *Mus musculus* causes sperm malformation and infertility in males Lee et al. (2013). The aim of this experiment is to test whether the TTLL5 deficient *D. melanogaster* experience fertility decline.

Material and methods

Flies used for Fertility Test:

$\underline{\underline{w}}; \underline{\underline{venus-TTLL}}$	$\underline{\underline{Driver}}$	$\underline{\underline{venus-TTLL}}$	$\underline{\underline{w}}; \underline{\underline{msps-mcherry}}$	$\underline{\underline{w}}; \underline{\underline{msps-mcherry}}$
$\underline{\underline{w}}; \underline{\underline{venus-TTLL}}$	$\underline{\underline{(Sm,Cy)}}$	$\underline{\underline{venus-TTLL}}$	$\underline{\underline{w}}; \underline{\underline{TACC-mcherry}}$	$\underline{\underline{w}}; \underline{\underline{TACC-mcherry}}$
$\underline{\underline{w}}; \underline{\underline{+}}$	$\underline{\underline{SM,Cy}}$	$\underline{\underline{msps-mcherry}}$	$\underline{\underline{w}}$	$\underline{\underline{w}}$
$\underline{\underline{w}}; \underline{\underline{+}}$	$\underline{\underline{SM,Cy}}$	$\underline{\underline{TACC-mcherry}}$	$\underline{\underline{w}}$	$\underline{\underline{w}}$
$\underline{\underline{w}}; \underline{\underline{Driver}}$				

Crosses for Fertility Test:

- 4) $\underline{\underline{w}}; \underline{\underline{Driver}}$; $\underline{\underline{PrDr}}$ x $\underline{\underline{w}}; \underline{\underline{venus-TTLL}}$; $\underline{\underline{venus-TTLL}}$
- 5) $\underline{\underline{w}}; \underline{\underline{+}}$; $\underline{\underline{msps-mcherry}}$ (TM,Sb) x $\underline{\underline{w}}; \underline{\underline{Driver}}$; $\underline{\underline{+}}$
- 6) $\underline{\underline{w}}; \underline{\underline{+}}$; $\underline{\underline{TACC-mcherry}}$ (TM,Sb) x $\underline{\underline{w}}; \underline{\underline{Driver}}$; $\underline{\underline{+}}$

Procedure

Material

- Apple juice plates
- Yeast

Preparation

For the apple juice plates, dissolve 1 L boiling tap water with 30 g agar. Mix with 35 g white table sugar and 2 g Nipagin (Methyl-4-hydroxy-benzoate) dissolved in 350 mL apple juice. Pour about 100 small or 30 medium sized plates. Store at 4 °C. Prior use, add some yeast paste.

Flies

- Collect females once a day
- Cross three 2-4 days old females with three *white* males and place flies into a fresh vial containing few grains of dried yeast
- Remove adult flies after 2-3 days and wait for larvae to crawl up the glass wall
- Use the removed females for egg laying.

Results



Figure 1: No sign of infertility is shown here except for the PBac line.

	Flies	Hatching temp of female	Layed eggs	Unhatched eggs	Hatching rate
4)	$w; \frac{venus-TTLL}{venus-TTLL}; \frac{venus-TTLL}{TM,Sb}$	25°C	100	7	93
	$w; \frac{venus-TTLL}{Driver}; \frac{venus-TTLL}{TM,Sb}$	25°C	97	9	90.72
5)	$w; \frac{msps-mcherry}{msps-mcherry}$	25°C	94	49	48
	$w; \frac{msps-mcherry}{TM,Sb}$	25°C	100	48	52
	$w; \frac{Driver\ msps-mcherry}{+ + +}$	25°C	100	67	33
	$w; \frac{Driver\ msps-mcherry}{+ + +}$	25°C	9	4	(56)
6)	$w; \frac{TACC-mcherry}{TACC-mcherry}$	25°C	91	29	68
	$w; \frac{TACC-mcherry}{TM,Sb}$	25°C	88	66	25
	$w; \frac{Driver\ TACC-mcherry}{+ +}$	25°C	111	79	29
2)	$w; \frac{venus-TTLL}{SM,Cy}; \frac{Minos}{Df(TTLL)}$	29°C	30	9	70
	$w; \frac{venus-TTLL}{Driver}; \frac{Minos}{Df(TTLL)}$	29°C	73	6	92
4)	$w; \frac{venus-TTLL}{venus-TTLL}; \frac{venus-TTLL}{TM,Sb}$	29°C	86	17	80
	$w; \frac{venus-TTLL}{Driver}; \frac{venus-TTLL}{TM,Sb}$	29°C	85	14	84

Discussion

The fertility does not look affected by the deficiency. However, the PBac line shows an abnormally small number of offspring compared to the others. As the TTLL5 deficiency is present in the other lines, an unknown problem might be the cause and further research would be necessary to understand what is happening.

Experiment 2: Ovary stainings of Staufen - Confocal microscopy

Material and methods

Flies used for Western blot and ovary stainings:

$\frac{w}{w}; \frac{\text{Driver}}{\text{venus-TTLL}}, \frac{\text{TTLL5}^{PBac}}{\text{Df(TTLL)}}$	$\frac{w}{w}; \frac{\text{SM,Cy}}{\text{venus-TTLL Driver}}, \frac{\text{TTLL5}^{PBac}}{\text{Df(TTLL)}}$
$\frac{w}{w}; \frac{\text{Driver}}{\text{venus-TTLL}}, \frac{\text{TTLL5}^{Minos}}{\text{Df(TTLL)}}$	$\frac{w}{w}; \frac{\text{SM,Cy}}{\text{venus-TTLL Driver}}, \frac{\text{TTLL5}^{Minos}}{\text{Df(TTLL)}}$
$\frac{w}{w}; \frac{\text{Driver}}{\text{venus-TTLL}}, \frac{\text{TTLL5}^{Mi-Ex}}{\text{Df(TTLL)}}$	$\frac{w}{w}; \frac{\text{SM,Cy}}{\text{venus-TTLL Driver}}, \frac{\text{TTLL5}^{Mi-Ex}}{\text{Df(TTLL)}}$
$\frac{w}{w}$	$\frac{w}{w}; \frac{\text{venus-TTLL}}{\text{Driver}}, \frac{\text{venus-TTLL}}{\text{TM,Sb}}$

Crosses for Western blot and ovary staining:

- 1) $\frac{w}{w}; \frac{\text{venus-TTLL}}{(\text{SM,Cy})}, \frac{\text{Df(TTLL)}}{\text{TM,Sb}} \times \frac{w}{w}; \frac{\text{Driver}}{(\text{SM,Cy})}, \frac{\text{TTLL5}^{PBac}}{\text{TM,Sb}}$
 - 2) $\frac{w}{w}; \frac{\text{venus-TTLL}}{(\text{SM,Cy})}, \frac{\text{Df(TTLL)}}{\text{TM,Sb}} \times \frac{w}{w}; \frac{\text{Driver}}{(\text{SM,Cy})}, \frac{\text{TTLL5}^{Minos}}{\text{TM,Sb}}$
 - 3) $\frac{w}{w}; \frac{\text{venus-TTLL}}{(\text{SM,Cy})}, \frac{\text{Df(TTLL)}}{\text{TM,Sb}} \times \frac{w}{w}; \frac{\text{Driver}}{(\text{SM,Cy})}, \frac{\text{TTLL5}^{Mi-Ex}}{\text{TM,Sb}}$
 - 4) $\frac{w}{w}; \frac{\text{Driver}}{(\text{SM,Cy})}, \frac{\text{PrDr}}{\text{TM,Sb}} \times \frac{w}{w}; \frac{\text{venus-TTLL}}{(\text{SM,Cy})}, \frac{\text{venus-TTLL}}{\text{TM,Sb}}$
- $\frac{w}{w}$

Crosses offsprings and number of ovaries selected for Western and Confocal:

	Flies	Confocal Nb ovaries	Western Nb ovaries
1a	$w; \frac{Driver}{venus-TTLL}; \frac{PBac}{Df}$	2	8
1b	$w; \frac{DriverOrVenus}{SMC_y}; \frac{PBac}{Df}$	2	10
2a	$w; \frac{Driver}{venus-TTLL}; \frac{Minos}{Df}$	14	16
2b	$w; \frac{venus-TTLL}{SMC_y}; \frac{Minos}{Df}$	~15	16
3a	$w; \frac{Driver}{venus-TTLL}; \frac{Ex128}{Df}$	~16	21
3b	$w; \frac{Driver}{SMC_y}; \frac{Ex128}{Df}$	~18	18
4a	$w; \frac{venus-TTLL}{Driver}; \frac{venus-TTLL}{TMSb}$	16	16
4b	$w; \frac{venus-TTLL}{venus-TTLL}; \frac{venus-TTLL}{TMSb}$	~14	16
5	w	16	25

Table 1: Fly crosses used for Western blot and ovary stainings.

Procedure

Solutions

Antibodies: polyclonal rabbit antibody anti Staufen protein
monoclonal mouse antibody anti GFP
anti-mouse antibody, fluorophore coupled (Alexa Fluor 647)
anti-rabbit antibody, fluorophore coupled (Alexa Fluor 488)

Hoechst DNA coloration

Rhodamine Phalloidin Actin coloration

Ringer's Isotonic solution containing 6.5 g $NaCl$, 0.42 g KCl , 0.25 g $CaCl_2$, 0.2 g $NaHCO_3$ in 1 L H_2O .

PFA Paraformaldehyd - Cell fixation

Heptane Solvent

DMSO Solvent

PBS Phosphate-buffered saline

PBST PBS + 0.1% Tween-20

Triton X-100 Detergent

Tween-20 Polysorbate-type non-ionic surfactant

Milk powder (or BSA) Blocking agent

Aquamount

Procedure

To obtain nice ovaries, 3 days old females were kept with males on food containing dry yeast in non-crowding condition at 25°C.

Dissection and fixation The ovaries were dissected in PBS and transferred in Eppendorf tubes (Eppendorf AG, Hamburg, Deutschland) containing : 200 μ L 4% PFA, 600 μ L Heptane and 20 μ L DMSO. They were fixed during 20 minutes on a rotation wheel, rinsed 3 times with PBST and rehydrated in PBST during 20 minutes. Still in PBST, the ovaries were opened (not completely separate the ovarioles) and transferred in a 500 μ L reaction vial for blocking.

Blocking Ovaries were permeabilized and blocked for 45 minutes in PBS with 0.1% Tween-20, 0.1% Triton-X100 and 5% milk powder. Then the blocking buffer has been carefully removed and the ovaries transferred into PCR tubes for primary antibody. To better see the ovaries to transfer, the blocking buffer can be diluted with PBST.

Primary antibody Ovaries were incubated overnight on a rotation wheel at 4°C in 100 μ L of primary antibody containing mouse anti-GFP (1:200) and rabbit anti Staufen (1:200) in PBS with 0.1% Tween-20 and 5% milk powder.

Secondary antibody Ovaries were rinsed 3 times with PBST and washed 3 times in PBST during 20 minutes. They were then incubated 3 hours at room temperature on a rotation wheel in the dark in goat anti-mouse 647 (1:200) and goat anti-rabbit 488 (1:200) in PBS with 0.1% Tween-20 and 5% milk powder.

Hoechst Ovaries were rinsed 3 times with PBST and washed 3 times in PBST during 20 minutes. They were then incubated during 20 minutes in 1 mL PBST, 0.5 μ L Hoechst 33528 to an end concentration of 2.5 μ L/mL. At this stage, ovaries can be stored in the dark at 4°C during a couple of days before mounting.

Mounting Ovaries were rinsed 3 times with PBST and washed 3 times in PBST during 20 minutes. To be mounted in Aquamount, ovaries were transferred onto labelled slides with a bit of PBST. Then, the ovarioles were separated before adding the Aquamout, covering with the coverslip and let settling before storage in the dark at 4°C.

Results

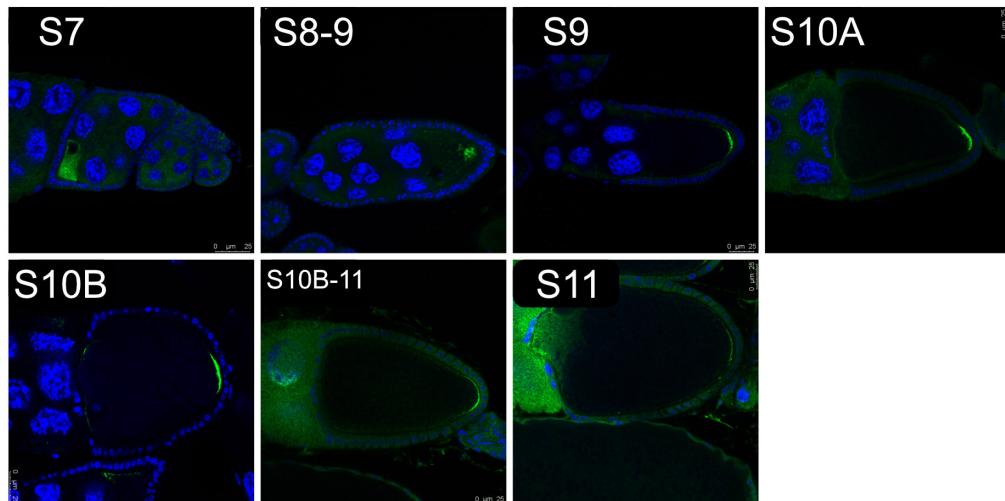


Figure 2: White. DNA in blue (Hoechst), Staufen in green.

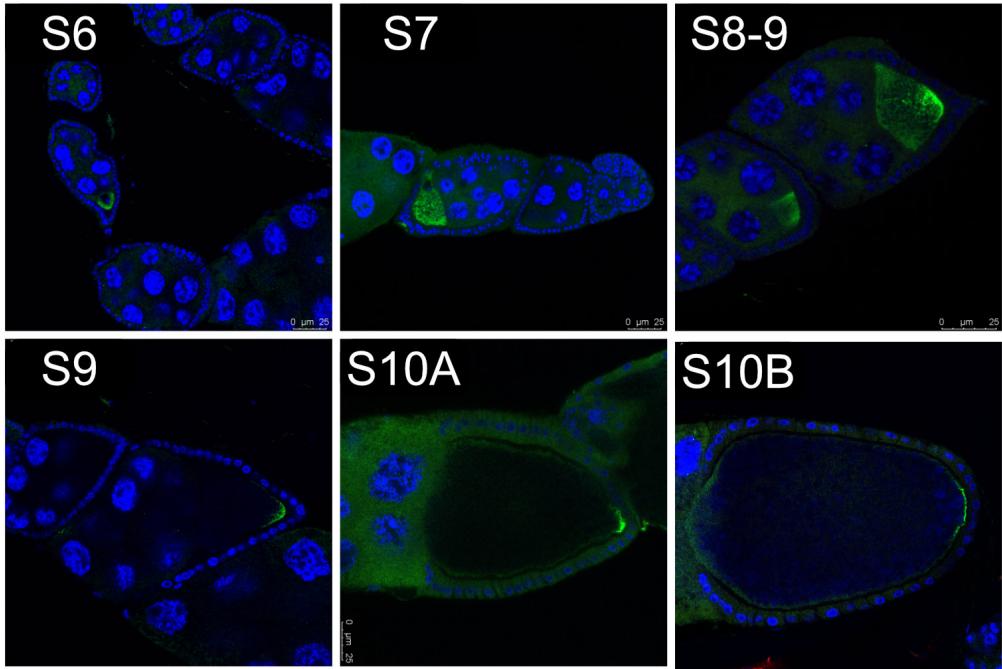


Figure 3: Ex128 over Df, DNA in blue (Hoechst), Staufen in green.

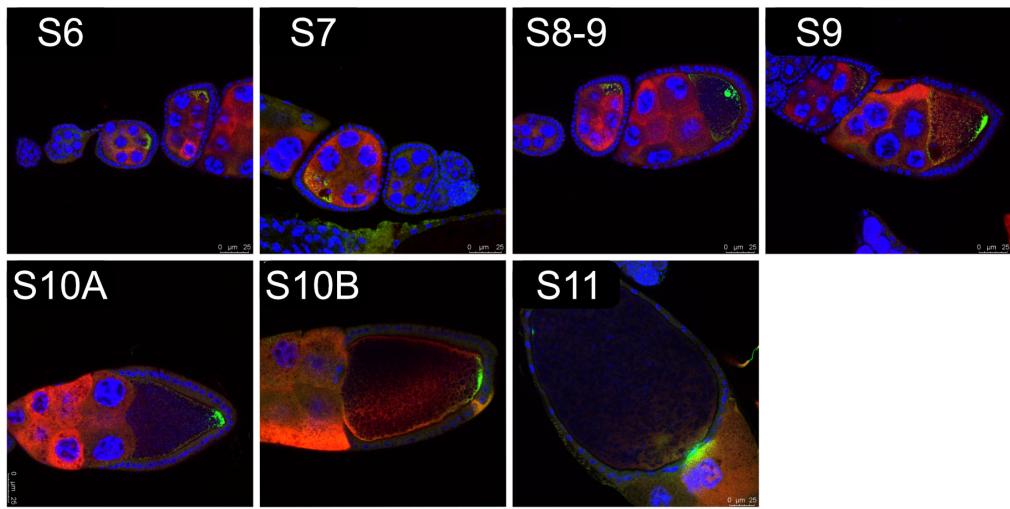


Figure 4: Ex128 over Df rescue, DNA in blue (Hoechst), Staufen in green, GFP against venus in red.

Experiment 3: Western blot

Material and methods

Ovary sample preparation The ovaries were stored at -80°C after dissection. Add 2.5 µL of 2x loading buffer (Laemmli buffer) per ovary to denature the proteins while successively boiling and spinning.

Gel electrophoresis on 10 % SDS PAGE (Polyacrylamide gel) Once the gels (running and stacking) are assembled in the running tank, load 10 µL per sample and 10 µL of prestained protein ladder (NEB). Run the gel at low voltage (80 V) as long as samples are in the stacking gel and at 100 V for the resolving (running) gel during 2-3h until the blue stain (bromophenol blue) exits the gel. Then semi-dry transfer onto nitrocellulose membrane.

Ponceau staining To detect the presence of protein on the membranes reversible Ponceau S stain was used: 1g Ponceau S, 50 mL acetic acid made up to 1L with H_2O . Bands were cut between 36 and 55 kDa and between 72 and 95 kDa to be stained separately.

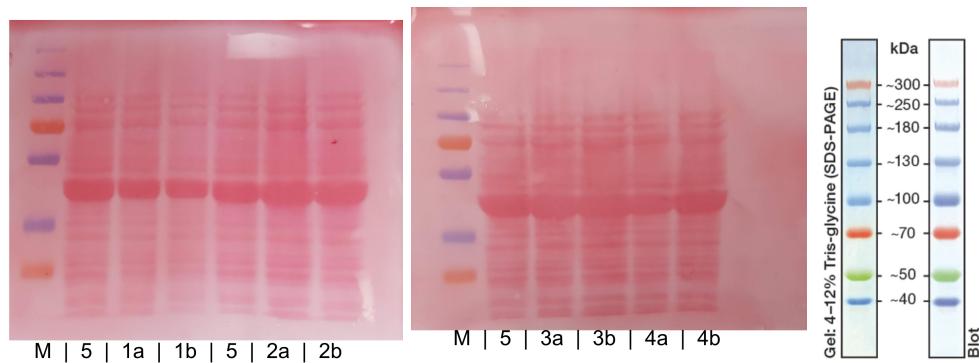


Figure 5: Ponceau staining shows a good load of proteins

Stainings For the further steps the following filters were used with the specific solutions and antibodies:

- 1.1 and 1.2 (**0 - 40 kDa**) to detect GAPDH (37 kDa) in **TBST** with
- 2.1 and 2.2 (**40 - 80 kDa**) to detect glutamylated α -tubulin (55 kDa) in **PBS**
- 3.1 and 3.2 (**80 - 250 kDa**) to detect venus-TTLL5 (125 kDa) in **TBST**

Incubation with first antibody The membrane were first blocked with 5% milk powder in PBS or TBST and incubated for at least 1h at room temperature. 10 mL of primary antibody were added like bellow and incubated overnight:

- 5% milk powder
- PBS or TBST
- antibodies:
 - mouse anti glutamylated alpha tubulin 1D5 (1:400)
 - rabbit anti GFP (1:2000)
 - rabbit anti GAPDH (1:1000)

Incubation with second antibody The membranes were washed with PBS or TBST to retrieve the primary antibodies. Small containers were prepared with:

- 2nd antibody (coupled to horse radish peroxidase)
 - HRP coupled antibody α mouse (1:10000)
 - or HRP coupled antibody α rabbit (1:10000)
- 5% milk powder in PBS (0.5 g in 10 mL) or TBST (1 g in 20 mL)

Pictures were then taken with Enhanced Chemiluminescence at 428 nm with horse radish peroxidase and Luminol.



Figure 6: Western blot. Staining against alpha-tubulin, GAPDH and venus-TTLL5.

	Flies	Confocal Nb ovaries	Western Nb ovaries
1a	$w; \frac{Driver}{venus-TTLL}; \frac{PBac}{Df}$	2	8
1b	$w; \frac{DriverOrVenus}{SMC_y}; \frac{PBac}{Df}$	2	10
2a	$w; \frac{Driver}{venus-TTLL}; \frac{Minos}{Df}$	14	16
2b	$w; \frac{venus-TTLL}{SMC_y}; \frac{Minos}{Df}$	~ 15	16
3a	$w; \frac{Driver}{venus-TTLL}; \frac{Ex128}{Df}$	~ 16	21
3b	$w; \frac{Driver}{SMC_y}; \frac{Ex128}{Df}$	~ 18	18
4a	$w; \frac{venus-TTLL}{Driver}; \frac{venus-TTLL}{TMSb}$	16	16
4b	$w; \frac{venus-TTLL}{venus-TTLL}; \frac{venus-TTLL}{TMSb}$	~ 14	16
5	w	16	25

Table 2: Fly crosses used for Western blot and ovary stainings.

Results

The Ponceau staining shows that the protein have correctly been loaded and allowed to separate GAPDH, glutamylated α -tubulin and venus-TTLL5. The control chemiluminescence with Rabbit anti-GAPDH showed a good staining in all samples. Rescue flies (1a, 2a and 3a) all showed an increased expression of venus-TTLL5. Surprisingly, only 2a and 3a showed an increased expression of glutamylated α -tubulin but not 1a (*PBac*) and also not 4a (Driver - Double venus).

Discussion

The experiment showed a good rescue of the TTLL5 function in the *Minos* and *Ex128* mutant lines but remains unclear why this effect was not observed in the *PBac* line. Notice that the *PBac* mutant line also had a really low amount of offspring in the fertility test which could indicate a problem in the line in general. It is also still unclear why the 4a (Driver - double venus) do not express glutamylated α -tubulin.

Experiment 4: CRISPR/Cas9

To use the CRISPR/Cas9 system to introduce a point mutation, it is necessary to integrate to the flies both the guide RNA and the Cas9 protein. In this experiment, four different plasmids containing specific guide RNA have been integrated in flies embryos the goal being to have a stable line that produce the guide RNA that can be later crossed with a Cas9 producing line. These final CRISPR/Cas9 lines should have one point mutation each at a different site of TTLL5.

Material, methods and partial results

Flies used for CRISPR/Cas9

1	y v integrase; attP40	$\frac{yv\{y^+, phiC31 \text{ integrase}\}}{yv\{y^+, phiC31 \text{ integrase}\}}; \frac{\overline{attP40}}{\overline{attP40}}$
2	w nos-Cas9	$\frac{w\{w^+, nos-Cas9\}}{w\{w^+, nos-Cas9\}}$
3	v; Sco/CyO	$\frac{v}{v}; \frac{\overline{Sco}}{\overline{CyO}}$
4	w; Ly/TM3, Sb	$\frac{w}{w}; \frac{\overline{Ly}}{\overline{TM3, Sb}}$

Note: all the steps below have been performed with all four strains. Each student were working with one of the four when separate steps had to be made.

Prepare genomic DNA from single flies for PCR One fly was placed in a 1.5 mL tube with 50 μ L SB (Squishing Buffer: 10 mM Tris pH8.2, 10 mM EDTA, 25 mM NaCl and 200 μ g/mL Proteinase K). It was incubated at 37°C during 30 minutes before inactivating Proteinase K twice at 95°C. The supernatant was then frozen at 20°C for later use.

Set up PCR reactions - Taq Polymerase For each four sample (+1 safety) prepare PCR vials containing the elements below. Add DNA last after thawing.

- DNA 0.8 μ L
- Primer 1 [10 μ M] (=RED-TTLL5) 1.25 μ L (total: 6.25)
- Primer 2 [10 μ M] (=RED-TTLL8) 1.25 μ L (total: 6.25)
- dNTP [10 μ M] 0.5 μ L (total: 2.5)
- 10x Thermopol buffer 2.5 μ L (total: 12.5)
- Taq DNA Polymerase [5 U/ μ L] 0.1 μ L (total: 0.5)
- H_2O to a final volume of 25 μ L

PCR settings with Taq Polymerase

- Initial denaturing 95°C: 10 minutes
- Cyclic denaturing 95 °C: 30 seconds
- Annealing 50 °C: 30 seconds
- Elongation 68 °C: 90 seconds
- Nb. of cycles: 40
- Final elongation 68 °C: 5 minutes
- Holding temperature 10 °C

Gel electrophoresis The gel electrophoresis has been prepared with:

- 1% agarose gel prepared with 0.8 g agarose, 80 mL 0.5x TBE (= Tris, Borat, EDTA) and 4 µL of peqGreen stain (carefull, dangerous).
- Running buffer (TBE)

4 µL of peqGreen have been added to the lower gel chamber and for each sample, one slot has been filled with:

- 2 µL PCR reaction
- 10 µL H_2O
- 2 µL 6x loading buffer

As length marker, 4 µL of 1kb DNA ladder [0.5 µg] *ready to use* were added in a slot. The gel was run at 100V for 30 minutes.

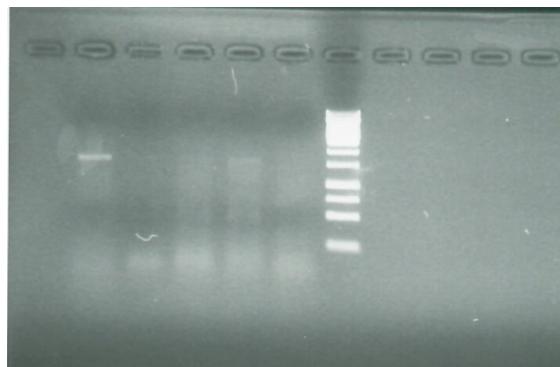


Figure 7: Taq Polymerase Gel electrophoresis: the DNA content is really low.

Set up PCR reactions - Phusion For each four sample (+1 safety) a PCR vial containing the elements below was prepared. Add DNA last after thawing.

- DNA 0.8 µL
- Primer 1 [10 µM] (=RED-TTLL5) 1.25 µL (total: 6.25)
- Primer 2 [10 µM] (=RED-TTLL8) 1.25 µL (total: 6.25)
- dNTP [10 µM] 0.5 µL (total: 2.5)
- HF 5x (High Fidelity Buffer) 5 µL (total: 15)
- Phusion 0.2 µL (total: 0.5)
- H_2O to a final volume of 25 µL 16 µL (total: 80)

PCR settings with Phusion Due to a poor PCR result with Taq Polymerase, the PCR has been done a second time with Phusion as below.

- Initial denaturing 98°C: 10 minutes
- Cyclic denaturing 98 °C: 30 seconds
- Annealing 61 °C: 30 seconds
- Elongation 72 °C: 1 minute
- Nb. of cycles: 40
- Final elongation 72 °C: 5 minutes
- Holding temperature 10 °C

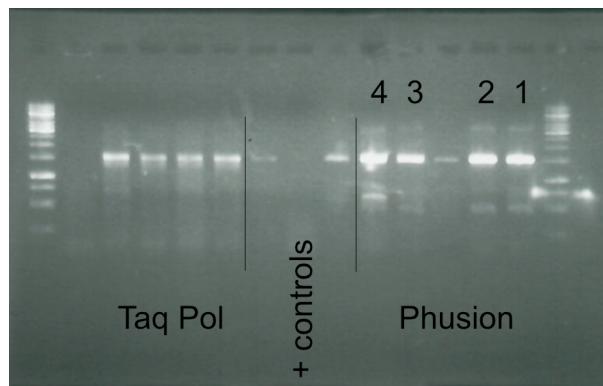


Figure 8: Even if the Taq Polymerase reaction has improved, Phusion still has a higher amount of DNA.

Purification and sequencing The PCR fragments have been purified using the Promega Kit A2893 and their concentration determined with Nanodrop. The primers and fragments have been prepared as below and sent to Mycrosynth for sequencing in order to be certain of the sequence of the TTLL5 gene.

Sample	Concentration	DNA 280 ng	H_2O	TTLL5	TTLL6	TTLL25
1	178 ng/µL	1.6 µL	10.4 µL	3 µL	3 µL	3 µL
2	109 ng/µL	2.6 µL	9.4 µL	3 µL	3 µL	3 µL
3	185 ng/µL	1.5 µL	10.5 µL	3 µL	3 µL	3 µL
4	188 ng/µL	1.5 µL	10.5 µL	3 µL	3 µL	3 µL

Isolate vector DNA for cloning Bacteria carrying pCFD5 vector were incubated overnight in 4 mL LB-medium containing Amipcillin for 16-18 hours at 37°C and 225 rpm. The plasmid DNA was isolated with the DNA miniprep kit from Sigma Aldrich with the corresponding protocol (GenElute HP Plasmid Miniprep Kit, NA0160-1KT). Concentration was then determined with Nanodrop for the five replicates.

Sample	Concentration
1	665 ng/ μ L
2	579 ng/ μ L
3	541 ng/ μ L
4	267 ng/ μ L
5	559 ng/ μ L

Prepare the vector pCFD5 for cloning pCFD5 was digested with Bbs1 like following and incubated for 2-4 hours at 37°C.

- 8 μ g pCFD5
- 4 μ L 10 x Bbs1 restriction buffer (Cutsmart buffer was used instead)
- 1.3 μ L Bbs1
- H_2O to 40 μ L

The vectors were then purified in a 1% agarose gel containing Ethidium Bromide (EtBr, 1:10000) instead of peqGreen. The 9.6 kb fragments were then recovered using long wave UV light (365 nm) and recovered using the Promega Kit A2893 (ReliaPrep DNA - Clean Up and Concentration System). The vectors were then eluted with 25 μ L H_2O and their concentrations were determined with Nanodrop.

Sample	Concentration
2	245 ng/ μ L
3	167 ng/ μ L
4	148 ng/ μ L
5	115 ng/ μ L

Preparation of primers to insert into pCFD5 The following phosphorylation and annealing reaction was performed on each sample (2,3,4 and 5), each one with it's corresponding designed oligos targeting a specific element on TTLL5.

- 1 μ L top oligo [100 μ M]
- 1 μ L reverse oligo [100 μ M]
- 1 μ L 10 x T4 Ligation Buffer (NEB)
- 6.5 μ L H_2O
- 0.5 μ L T4 Polynucleotide Kinase

Samples were then incubated and annealed in a thermocycler at 37°C during 30 minutes then at 95°C during 5 minutes and then cooled down to 20°C at a speed of 5°C per minute.

Ligation The ligation reaction was performed using

- 60 ng BbsI digested pCFD5
- 1 μL annealed oligos diluted 1:200 in H_2O
- 1.5 μL 10 x T4 Ligation Buffer (NEB)
- 1 μL Ligase
- H_2O to 15 μL

and ligated during 30 minutes at room temperature.

Transformation of *E. Coli* XL1 blue 100 μL of XL1 blue cells have been thawed, mixed with the ligation mix and incubated for 20 minutes on ice. Then the mixes were hit shocked on a preheated heat block at 42°C during 45 seconds and quickly chilled on ice for 5 minutes. After adding 200 μL of LB without antibiotics, the cells were incubated at 37°C for 1h at 220 rpm in liquid culture incubator (or 1100 rpm in heatblock). The cells were then spined at 5000 rpm during 2 minutes. All but 70 μL of the supernatant was removed and two dilution (1:10 and 1:100) were made to further plate the cells with a spatula on agar plates containing ampicillin in order to select single colonies.

Pick colonies for colony PCR and overnight culture 3 colonies were picked and for the control we used 2x 3 μL of the vector (1:300) and the rest of Master Mix as blank. Each colony was diluted in a 1.5 mL reaction tube in 12 μL H_2O . 2 x 3 μL were used for the colony PCR and the remaining 7 μL were added to 200 μL of LB with ampicillin and transferred into 15 mL snap cap tube.

Colony PCR To verify if the pCFD5 vectors contain the inserted sequence for the guide RNA, we set the following PCR reaction with the following primers:

- Primer 1: U63 seqfwd (= OL275)
- Primer 2: pCFD seqref or :
- Specific reverse primer (K131 rev, R188 rev, R225 rev, E366 rev)

For each sample the following PCR reaction was prepared.

- 5 μL 10 x thermopol buffer
- 0.5 μL 10 mM dNTPs
- 1.25 μL 10 μM forward primer 1
- 1.25 μL 10 μM reverse primer 2
- 3 μL resuspended bacteria
- 0.1 μL Taq Polymerase
- H_2O to 25 μL

with the parameters:

- Initial denaturing 95°C: 2 minutes
- Cyclic denaturing 95 °C: 30 seconds

- Annealing 48 °C: 30 seconds
- Elongation 68 °C: 1 minute
- Nb. of cycles: 30
- Final elongation 68 °C: 10 minutes
- Holding temperature 4 °C

Analyse colony PCR reactions The PCR reactions were then separately analysed on an agarose gel with 10 µL of PCR reaction, 2.5 µL of 6x loading buffer and 3 µL of 1kb DNA ladder (Thermo Scientific [0.1 µg/µL]).

For the plasmids with the R188 oligo, the gel was run at 135 V during 30 minutes due to its bigger size.

As we removed 170bp and add 20, there must be a difference of 150bp between the colonies without the insert and the ones with it.

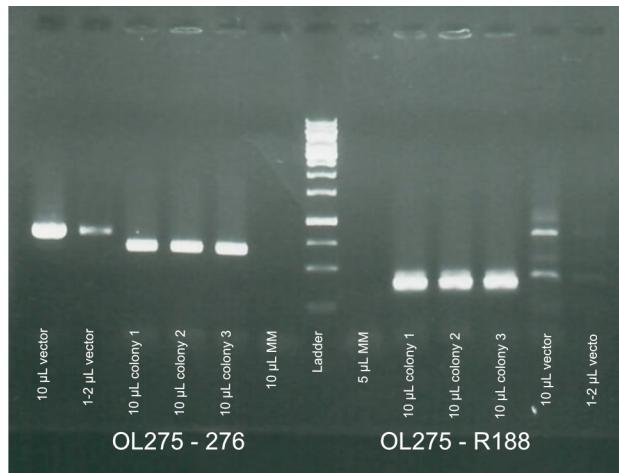


Figure 9: R188 insert check in colonies. It is clearly visible that the 3 colonies with the R188 primer contain the insert.

To set up overnight culture, 4 mL of LB medium with Ampicilin was added to the insert-containing bacteria in SnapCap and incubated for 16-20 hours at 37°C and 250 rpm.

Prepare glycerol stocks The overnight cultures were concentrated in 1 mL of LB/amp and 400 µL of it were added to 1.5 mL tube containing 400 µL of 80% glycerol. These stocks were frozen at -80°C.

Prepare large quantities and clean DNA The Sigma Aldrich Kit NA0200-1KT was used to harvest and purify the DNA from the stocks. After the purification, the concentration of the four samples has been determined.

Sample	Concentration
2-1 R188	300 ng/µL
3-1 K131	400 ng/µL
4-1 E366	373 ng/µL
5-1 R225	361 ng/µL

Each sample was then sent for sequencing with 1.2 µg of plasmid DNA and OL275 (or U63 seqfwd) primer.

Sample	DNA 280 ng	H_2O	OL275
2-1 R188	4 µL	8 µL	3 µL
3-1 K131	3 µL	9 µL	3 µL
4-1 E366	3.2 µL	8.8 µL	3 µL
5-1 R225	303 µL	8.7 µL	3 µL

Prepare DNA for injection into *Drosophila* embryos 25 µg of DNA diluted in up to 100 µL of H_2O have been precipitated in 11 µL of 3M $NaOAc$ and 240 µL of ice cold 70% Ethanol (-20°C) during 1h or longer. The DNA has then been centrifuged at max speed for 10 minutes (and then twice for 2 minutes) and the supernatant has been successfully discarded and replaced with ice cold 70% Ethanol twice before being air-dried. The pellets have been re-suspended in 40 µL of injection buffer (0.1 M phosphate buffer and 5 mM KCl, sterile filtered). The concentration has then been measured and the samples have been diluted to a concentration of 400 ng/µL.

Injection The injections were made in max 20 minutes old embryos under supervision of the lab members.

Intermediate Results

Flies have integrated the genes and are growing

Conclusion and discussion

The goal of this experiment was to find information about the TTLL5 homolog in *D. Melanogaster* in four different experiments.

The first one aimed to analyse whether TTLL5 over-expression has an effect on fertility and the results had no conclusion in this direction and only showed that the PBac line had some troubles. In the second experiment, it has been testes whether the Staufen protein localisation was dependent on a functional TTLL5 protein. The stainings showed a smearer localization of Staufen but no extreme problems. The third experiment tested for the dependence to TTLL5 of α -tubulin glutamylation. Once again, the PBac line can be removed from the experiment due to an extremely low amount of material. The venus-TTLL rescue flies all showed an increased expression of glutamylated α -tubulin but not the Driver-Double venus-TTLL which has to be enquired more deeply. Last but not least, the fourth experiment aimed to introduce a point mutation at four different places in the TTLL5 gene using the CRISPR/Cas9 system. The guide RNA have been integrated to the flies genomes and the lines are growing but the rest, i.e. the cross with a CRISPR line and the analyse of the resulting crosses still has to be pursued.

References

- Bedoni, N., Haer-Wigman, L., Vaclavik, V., Tran, V. H., Farinelli, P., Balzano, S., Royer-Bertrand, B., El-Asrag, M. E., Bonny, O., Ikonomidis, C., Litzistorf, Y., Nikopoulos, K., Yioti, G. G., Stefaniotou, M. I., McKibbin, M., Booth, A. P., Ellingford, J. M., Black, G. C., Toomes, C., Inglehearn, C. F., Hoyng, C. B., Bax, N., Klaver, C. C., Thiadens, A. A., Murisier, F., Schorderet, D. F., Ali, M., Cremers, F. P., Andréasson, S., Munier, F. L., and Rivolta, C. (2016). Mutations in the polyglutamylase gene *TTLL5*, expressed in photoreceptor cells and spermatozoa, are associated with cone-rod degeneration and reduced male fertility. *Human Molecular Genetics*, page ddw282.
- Lee, G.-S., He, Y., Dougherty, E. J., Jimenez-Movilla, M., Avella, M., Grullon, S., Sharlin, D. S., Guo, C., Blackford, J. A., Awasthi, S., Zhang, Z., Armstrong, S. P., London, E. C., Chen, W., Dean, J., and Simons, S. S. (2013). Disruption of *Ttll5/stamp* gene (tubulin tyrosine ligase-like protein 5/SRC-1 and TIF2-associated modulatory protein gene) in male mice causes sperm malformation and infertility. *The Journal of Biological Chemistry*, 288(21):15167–15180.
- Schaetzky, J. and Rape, M. (2016). Getting a Grip on Microtubules. *Cell*, 164(5):836–837.