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

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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 (Schaletzky 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 chromosome[1].

$\frac{w}{w}; \frac{Driver}{(Sm,Cy)}; \frac{TTLL5^{PBac}}{TM,Sb}$

$\frac{w}{w}; \frac{Driver}{(Sm,Cy)}; \frac{TTLL5^{Minos}}{TM,Sb}$

$\frac{w}{w}; \frac{Driver}{(Sm,Cy)}; \frac{TTLL5^{MI-Ex}}{TM,Sb}$

$\frac{w}{w}; \frac{venus-TTLL}{(Sm,Cy)}; \frac{Df(TTLL)}{TM,Sb}$

$\frac{w}{w}; \frac{Driver}{(Sm,Cy)}; \frac{PrDr}{TM,Sb}$

$\frac{w}{w}; \frac{venus-TTLL}{(Sm,Cy)}; \frac{venus-TTLL}{TM,Sb}$

$\frac{w}{w}; \frac{msps-mcherry}{(TM,Sb)}$

$\frac{w}{w}; \frac{TACC-mcherry}{(TM,Sb)}$

$\frac{w}{w}$

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)

[1] Information available on *FlyBase*

Experiment 1: Fertility test

Material and methods

Flies used for Fertility Test:

$\frac{w}{w}$; $\frac{venus-TTLL}{Driver}$; $\frac{venus-TTLL}{(Tm,Sb)}$

$\frac{w}{w}$; $\frac{venus-TTLL}{(Sm,Cy)}$; $\frac{venus-TTLL}{TM,Sb}$

$\frac{w}{w}$; $\frac{+}{SM,Cy}$; $\frac{msps-mcherry}{Driver}$

$\frac{w}{w}$; $\frac{+}{SM,Cy}$; $\frac{TACC-mcherry}{Driver}$

$\frac{w}{w}$; $\frac{msps-mcherry}{msps-mcherry}$

$\frac{w}{w}$; $\frac{TACC-mcherry}{TACC-mcherry}$

$\frac{w}{w}$

Crosses for Fertility Test:

4) $\frac{w}{w}$; $\frac{Driver}{(SM,Cy)}$; $\frac{PrDr}{TM,Sb}$ x $\frac{w}{w}$; $\frac{venus-TTLL}{(SM,Cy)}$; $\frac{venus-TTLL}{TM,Sb}$

5) $\frac{w}{w}$; $\frac{+}{+}$; $\frac{msps-mcherry}{(TM,Sb)}$ x $\frac{w}{w}$; $\frac{Driver}{(SM,Cy)}$; $\frac{+}{+}$

6) $\frac{w}{w}$; $\frac{+}{+}$; $\frac{TACC-mcherry}{(TM,Sb)}$ x $\frac{w}{w}$; $\frac{Driver}{(SM,Cy)}$; $\frac{+}{+}$

Procedure

Material

- Apple juice plates
- Yeast

Preparation

For the apple juice plates, disolve 1 L boiling tap water with 30 g agar. Mix with 35 g white table sugar and 2 g Nipagin (Methyl-4-hydroxy-benzoate) disolved 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:

	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
	$w; \frac{Driver}{+} \frac{msps-mcherry}{+}$	25°C	100	67	33
	$w; \frac{Driver}{+} \frac{msps-mcherry}{+}$	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
	$w; \frac{Driver}{+}; \frac{TACC-mcherry}{+}$	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

Experiment 2: Ovary stainings of Staufen - Confocal microscopy

Material and methods

Flies used for Western blot and ovary stainings:

$\frac{w}{w}$; $\frac{Driver}{venus-TTLL}$; $\frac{TTLL5^{PBac}}{Df(TTLL)}$

$\frac{w}{w}$; $\frac{Driver}{venus-TTLL}$; $\frac{TTLL5^{Minos}}{Df(TTLL)}$

$\frac{w}{w}$; $\frac{Driver}{venus-TTLL}$; $\frac{TTLL5^{Mi-Ex}}{Df(TTLL)}$

$\frac{w}{w}$

$\frac{w}{w}$; $\frac{SM,Cy}{venus-TTLL|Driver}$; $\frac{TTLL5^{PBac}}{Df(TTLL)}$

$\frac{w}{w}$; $\frac{SM,Cy}{venus-TTLL|Driver}$; $\frac{TTLL5^{Minos}}{Df(TTLL)}$

$\frac{w}{w}$; $\frac{SM,Cy}{venus-TTLL|Driver}$; $\frac{TTLL5^{Mi-Ex}}{Df(TTLL)}$

$\frac{w}{w}$; $\frac{venus-TTLL}{Driver}$; $\frac{venus-TTLL}{TM,Sb}$

Crosses for Western blot and ovary staining:

1) $\frac{w}{w}$; $\frac{venus-TTLL}{(SM,Cy)}$; $\frac{Df(TTLL)}{TM,Sb}$ x $\frac{w}{w}$; $\frac{Driver}{(SM,Cy)}$; $\frac{TTLL5^{PBac}}{TM,Sb}$

2) $\frac{w}{w}$; $\frac{venus-TTLL}{(SM,Cy)}$; $\frac{Df(TTLL)}{TM,Sb}$ x $\frac{w}{w}$; $\frac{Driver}{(SM,Cy)}$; $\frac{TTLL5^{Minos}}{TM,Sb}$

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

4) $\frac{w}{w}$; $\frac{Driver}{(SM,Cy)}$; $\frac{PrDr}{TM,Sb}$ x $\frac{w}{w}$; $\frac{venus-TTLL}{(SM,Cy)}$; $\frac{venus-TTLL}{TM,Sb}$

$\frac{w}{w}$

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	$w; \frac{Driverorvenus}{SMCy}; \frac{PBac}{Df}$	2	10
2a	$w; \frac{Driver}{venus-TTLL}; \frac{Minos}{Df}$	14	16
2b	$w; \frac{venus-TTLL}{SMCy}; \frac{Minos}{Df}$	∼15	16
3a	$w; \frac{Driver}{venus-TTLL}; \frac{Ex128}{Df}$	∼16	21
3b	$w; \frac{Driver}{SMCy}; \frac{Ex128}{Df}$	∼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

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	
Heptane	
DMSO	
PBS	
PBST	PBS + 0.1% Tween-20
Triton X-100	
Tween-20	
Milk powder (or BSA)	
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 transfered in Eppendorf tubes (Eppendorf AG, Hamburg, Deutschland) containing : 200 µL 4% PFA, 600 µL Heptane and 20 µL 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 µL 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 µL of primary antibody containing mouse anti-GFP (1:200) and rabit 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 µL Hoechst 33528 to en end concentration of 2.5 µL/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 transfered 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

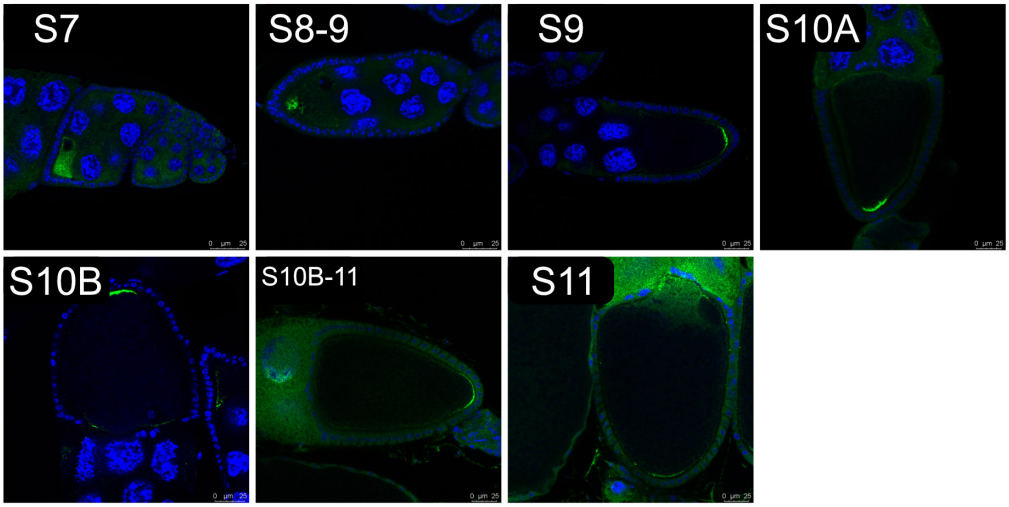

Figure 2: White

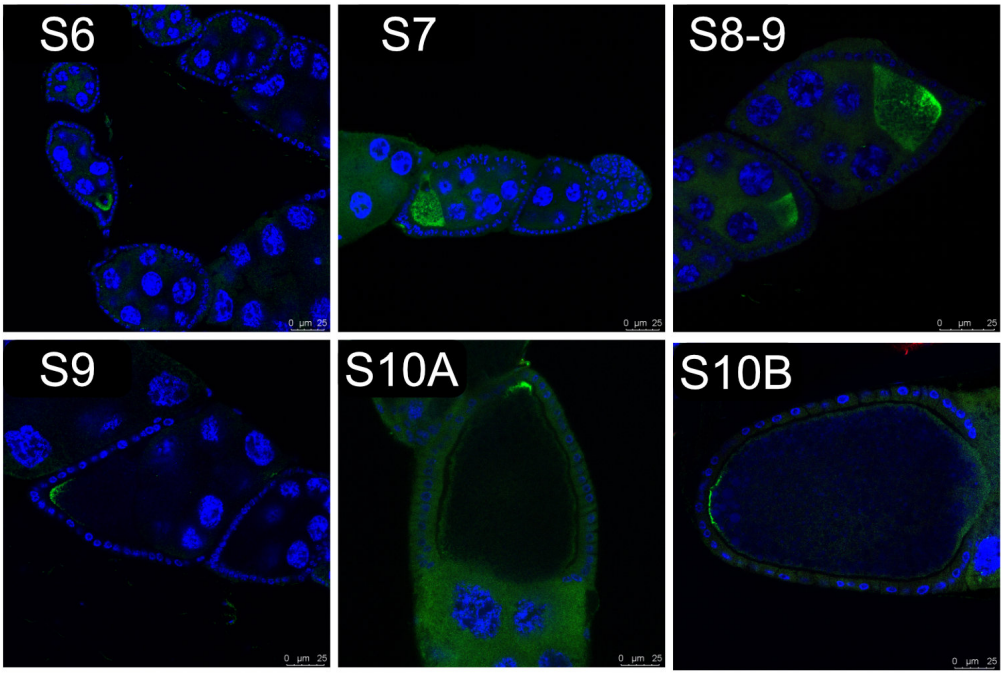

Figure 3: Ex128 over Df

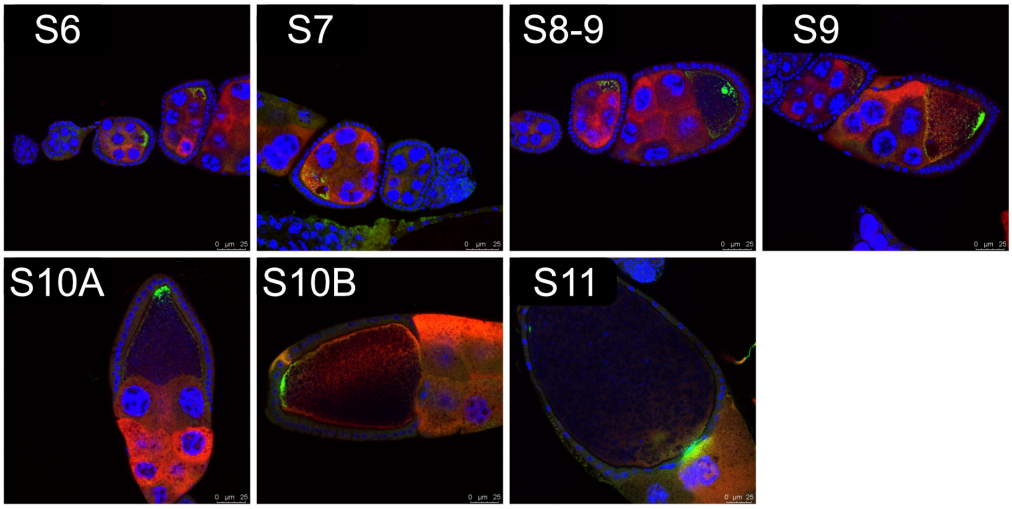

Figure 4: Ex128 over Df rescue

Experiment 3: Western blot

Material and methods

Flies and ovaries number described in Experiment 2.

Experiment 4: CRISPR/Cas9

Conclusion and discussion

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.

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