Discerning ctpsyn localization patterns in mosquitoes and rna extraction, cdna sy...

Engineering

Embryos were dechorionated prior to immunostaining (Juhn & James, 2012). A protocol described for zebrafish juveniles was adapted for both embryonic and larval tissue due to perceived similarities in their compositions (Goody & Henry, 2013). Tissues were dissected in PBST, then fixed in 4% PFA for 15 to 20 minutes. Succeeding steps followed details for immunohistochemistry in drosophilid tissues. Cells should be processed according to the protocol. Multiple anti-CTPsyn primaries were tested for their ability to stain CTPsyn around these tissues as no mosquito-specific variants are available in production. Where necessary, non-goat serum was replaced with bovine serum albumin (BSA) to ensure effectiveness of the staining procedure. Mounting was always done in SlowFade as mosquito tissues tolerate prolonged storage and visualization in secondary nutation mix very poorly. Both UV and laser-confocal microscopy were employed for image capture. A fluorescence-enabled BX53 Light Microscope platform (Olympus, Netherlands) was utilized with magnifications of 10X, 20X, 40X, 75X, and 130X with immersion oil. Confocal visualization on the LSM710 platform (Zeiss, Germany) involved 10X, 20X, 40X and 63X lenses, with additional magnifications manually specified on the accompanying software. These will be annotated individually on images throughout the Results Section.

RNA extraction, cDNA synthesis, and standard curve generation

Samples collected throughout rearing were immediately stored in TRIzol® reagent (Invitrogen™, Ambion™, Life Technologies) at -20°C. Total RNA extraction was done within two days of collection with a protocol previously described for mosquito tissue samples (Kafatos, 2009). Cells were

centrifuged at minimal speed, then as much of the culturing media was aspirated away. RNA extraction proceeded in accordance to the protocol described by TRIzol® manufacturers Abcam®. Extracts were quantified on the Hellma® Analytics TrayCell system in the SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, California). Three criterial points were considered before cDNA synthesis: (a) extracts must have an A260: A280 value between 1. 75 and 2. 05, (b) extracts must be of a reasonable ng/µl concentration, and (c) extracts must show clear 18S banding and minimal smearing in 1. 0% agarose gel. All were kept at -20°C for the duration of the experiment.

Quantitative PCR for CTP Synthase expression levels

Reverse transcription i. e. cDNA synthesis and qPCR were conducted according to the protoco. A combination of ACT/RPS17 was utilized for normalizing CTPsyn expression in Ae. aegypti (Dzaki et al., 2017). The combination of reference genes applied for Ae. albopictus were more varied, and chosen based on the recommendations put forth in a previous publication (Dzaki & Azzam, 2018). Primers for aaeCTPsyn and aalCTPsyn can be found within the same table. For each species, these are designed to be degenerate and mRNA-specific. Fold changes were calculated according to the Livak method i. e. $2-\triangle\triangle Cq$ (Livak & Schmittgen, 2001).

Results

Antibody suitability consideration and optimization of protocol

No commercially produced antibodies specific against mosquito CTPsyn (aeCTPsyn) are presently readily available. Many variants catalogued by

companies elicit Saccharomyces cerevisiae i. e. baker’s yeast or human epitopes instead. In Ae. aegypti, two isozymes of the protein have been described, translated from two isoforms. Ae. albopictus has had four mRNA isoforms identified for its singular variation of CTPsyn protein. Prior to testing, protein sequences of CTPsyn from Ae. aegypti, Ae. albopictus¸ humans and yeast were aligned to observe their level of homology as well as ancestry. Whilst there is a 91. 6% similarity between the cousin specie, this number decreased significantly to 70. 7% with human CTPS1, and further down to 58. 7% with the yeast homologue.

We endeavored to obtain more specifics i. e. the specific stretch of amino acids which make up the epitope used for generation of seven in-hand primaries, but these were only disclosed for the y-88 antibody. BLASTp indicated that the particular region of yeast CTP synthase used to produce y-88 i. e. the amino acids 38-118 near the N-terminus of the protein, has a degree of homology of 70% and 62% with Ae. aegypti and Ae. albopictus, respectively. Though this is a relatively poor indication of similarity, previous studies (Wang et al., 2015) had utilized y-88 against CTPsyn in cells of the model insect organism D. melanogaster to great effect. Given the ancestral-descendant relationship between the CTPsyn paralogues of the fruit fly to both Ae. aegypti’s and Ae. albopictus’, we were therefore optimistic that if any of the primary antibodies were to work against aeCTPsyn, it would be this one.

The greater challenge was to optimize existing immunohistochemistry protocols for Aedes mosquitoes specifically. As the nature of tissues to be

stained would vary, multiple methods were considered. We drew parallels between tissue-type and a most suitable protocol; as a result, adult internal organs were stained in accordance to the Drosophila ovaries protocol, whereas embryos and larvae were treated in a manner similar to zebrafish. After applying multiple modifications to the protocols, some showed potential as a positive antibody against aeCTPsyn. However, low signal intensities mean that results obtained were neither of high quality nor confidence. To further determine the effectiveness of these antibodies, we proceeded to repeat the process on fixed cells. Like S2 cells, neither Aag2 (Ae. aegypti cell line) nor C6/36 (Ae. albopictus cell line) have endogenous cytoophidia, and therefore staining was once again inconclusive. However, upon treatment with a non-toxic amount of the glutamine analogue drug 6-Diazo-5-oxo-L-norleucine (DON), cytoophidia-like structures began appearing in the cell cytoplasm. These were very visibly stained by y-88 antibody, showing in high confidence that the antibody is also able to detect the mosquito CTPsyn protein. Cytoophidia density was also greater in C6/36 cells than they were in Aag2. This inherent usability of y-88 against aeCTPsyn meant that for all in vivo staining undertakings herewith, this primary was used as the default antibody, unless stated otherwise.

Discerning CTPsyn localization patterns in adult mosquitoes

Adult Ae. aegypti

Multiple organs in adult mosquitoes were isolated and stained for CTP synthase. These were classifiable into three main tissue classes: (a) components of the reproductive system including testes, accessory gland,

and seminal vesicles from males, and the ovaries, ovary ducts, and accessory glands from females; (b) the excretory system i. e. midgut and the hindgut, which comprises of the small intestine and rectum; and (c) channels for liquid transport and ionic exchange i. e. Malpighian tubules.

Male Ae. aegypti

All reproductive organs appeared completely void of CTPsyn. Instead, localization of the protein in male Ae. aegypti tissues appeared exclusive to the Malpighian tubules and hindgut. Nuclearly-contained CTPsyn (n-CTPsyn) was very clearly observed in most if not all hindgut cells, both along the trunk of its multinucleated small intestine and the lobal cells of its rectum. Localization patterns of the protein within Malphigian tubules is more varied in nature. For young individuals aged below 15DAE, CTPsyn was dispersed across the large nuclei of the osmoregulation-organ. Older males instead displayed CTPsyn as punctuates which aggregate around the periphery of the nucleus, forming an encapsulating ring. This trait is largely retained in males through to the age of 40DAE.

Non-blood fed female Ae. aegypti

For females, distinctions were made between blood fed and non-blood fed adults. A population is bred solely on sucrose as food, whereas a separate population was fed blood once sexual maturity is established three days post-emergence (Oliva et al., 2011). The ovaries of non-blood fed female Ae. aegypti. It displays a low-level presence of CTPsyn in ovaries which are under a ‘ stagnated’ developmental phase i. e. under conditions where blood was unavailable. Aggregation of CTPsyn into either punctuates or cytoophidia

was not observed. The protein is minimally detectable in the nurse cells of some egg chambers, and appear to localize cytoplasmically. Ovarian follicle cells of Ae. aegypti, unlike the follicle cells of Drosophila, seemed to be devoid of significant amounts of CTPsyn.

Similar to their male counterparts, CTPsyn was also detected in Malphigian tubules and hindgut of the female Ae. aegypti. Patterns seen in male organs are mirrored in females. N-CTPsyn was abundant and easily observable in small intestine and rectal tissue cells. CTPsyn protein aggregation into distinct punctuates in Malphigian tubules was also found to be age-dependent here; however, these structures were only detectable in females aged 20DAE and beyond. Distribution was evenly scattered around the nuclear envelope, although aeCTPsyn still does not appear to polymerize into cytoophidia.

It was within these older mosquitoes that we found a noteworthy peculiarity. Although we had expected there to be no change in CTPsyn localization and distribution patterns in ovaries as long as the female was deprived of a complete blood or protein meal, we detected signs of perinuclear aggregation occurring around its nurse cell nuclei instead. The ovarioles here were obtained from 20DAE females; this trait was consistently seen in females up to 30DAE, after which naturally-occurring oogenesis reactivation was usually observed in well-fed individuals regardless of blood-feeding status (Baldini et al., 2013).

Blood-fed Ae. aegypti females

The anautogenous nature of most mosquito species is attributed to them lacking the faculty to produce certain types of amino-acids required for egg production. Without a blood meal, factors and hormones which drive the process are also unreleased, and hence the development of oocytes remain under arrest (Baldini et al., 2013). Acquisition of blood is therefore a monumental time point in a female's life cycle. As the transmission of viruses and other opportunistic pathogens from an infected host to the mosquito do occur via blood, we therefore thought it necessary to attempt to track CTPsyn movement on an hour to hour basis after such a meal has been partaken.

A large population of 5 to 7DAE females were blood-fed per bioreplicates. Five to six individuals were captured at each specific point of time, dissected, and immuno-stained. Accumulation of CTPsyn within the nuclei of nurse cells occur as quickly as within one to two hours of a blood meal. Highly-positive signals for the protein is retained as oogenesis continues. Staining intensity and the number of nurse cells displaying moderate to high levels of nuclear CTPsyn increased as time after blood-meal progresses into four, then eight and twelve hours.

Nurse cell nuclei capacity for n-CTPsyn slowly diminished from the fourteenth hour onwards. Throughout oogenesis the protein appeared completely limited to nurse cells; stained regions receded and shrunk along with these group of cells as they made way for the growing oocyte. CTPsyn levels within

the cell remained low enough to be undetectable even as it has developed into a fully mature egg at 36 hours post-blood meal.

We had initially expected there to be a gradual increment of CTPsyn protein within the oocyte, as embryogenesis typically entailed the deposition of nurse cell content into its cytoplasm. As this was not observed, it might be an indication that maternal factors secreted into the developing oocyte does not include CTPsyn.