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dUTPase expression correlates with cell division potential in *Drosophila melanogaster*

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Running title

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Abbreviations

dUTPase – dUTP pyrophosphatase, BER – base excision repair, UDG – uracil-DNA glycosylase, DRE – DNA replication related element, DREF – DRE binding factor, CNS – central nervous system

Enzymes

dUTPase

Keywords (up to 5)

dUTPase, proliferation, differentiation, DRE, transcription

Abstract

dUTPase is a dNTP sanitizing enzyme that prevents the appearance of the potentially harmful uracil bases in DNA by hydrolyzing cellular dUTP. This function of dUTPase is found to be essential in many organisms including *Drosophila melanogaster*. Previously we showed that the expression pattern of dUTPase determines the extent of uracil accumulation in the genome of different tissues. We wished to reveal the regulatory mechanism that eventually leaves a set of tissues to have uracil-free and intact genome. We found that the expression pattern established by the promoter of *Drosophila* dUTPase overlaps with mRNA and protein expression pattern, excluding the involvement of other posttranscriptional contribution. This promoter was found to be active in primordial tissues, such as in imaginal discs of the larvae, in the larval brain and in reproductive organs. In the case of brain and imaginal tissues, we observed that the promoter activity depends on DRE motifs, the docking site of DREF, which is known as a transcriptional activator of genes involved in replication and proliferation. These results suggest that dUTPase expression is fine-tuned to meet the requirements of DNA synthesis, in tissues where the maintenance of genome integrity is of high importance.

Introduction

Effective and accurate cellular DNA synthesis requires a well regulated deoxynucleotide triphosphate (dNTP) pool. Both the concentration and the ratio of dNTP components affect replication fidelity and robustness. Abnormalities in the dNTP pool can bias DNA synthesis, resulting in DNA damage or replication arrest [1,2]. Physiological dNTP pool consisting of modified nucleotides can also result in lesions in DNA after incorporation. Most common modified dNTPs can be 8-oxo-deoxyguanosine triphosphate (8-OH-dGTP), formed upon oxidation, deoxyinositol triphosphate (dITP) and deoxyuridine triphosphate (dUTP), formed upon deamination. Enzymatic machineries have been evolved to get rid of such modified moieties sanitizing the dNTP pool. MTH1 and MTH2, homologues of the *Escherichia coli* MutT, selectively hydrolyse 8-OH-dGTP [3,4]; dITPase, the homologue of the *E. coli* RdgB eliminates dITP [5]. dUTPase selectively hydrolyzes dUTP thereby eliminating it from the dNTP pool [6–11]. In addition, it contributes to thymidylate biosynthesis since its hydrolysis product; deoxyuridine monophosphate (dUMP) is the precursor of deoxythymidylate monophosphate (dTMP) [12].

dUTP elimination was found to be essential in several organisms such as *E. coli*, *Saccharomyces cerevisiae*, *Trypanosoma brucei*, *Arabidopsis thaliana*, *Caenorhabditis elegans* and *Drosophila melanogaster* [13–18]. In most cases, lethal consequences of

dUTPase were shown to depend at least partially on the status of Base Excision Repair (BER) component, UNG. UNG is the most effective member of the Uracil-DNA Glycosylase (UDG) family, specialized to remove uracil bases from DNA regardless to the sequence context while promoting further repair processes [13–15,17,19]. The UNG-encoding gene is almost ubiquitous among free-living organisms, however, as an exception, the *D. melanogaster* genome lacks this gene, but still requires dUTPase in some tissues and specific developmental stages [18]. dUTPase deficiency was shown to allow uracil enrichment in *E. coli*, *S. cerevisiae* and *D. melanogaster* genome [14,18,20].

D. melanogaster expresses two isoforms of dUTPase, achieved by alternative splicing. The short isoform is excluded from the nucleus while the long isoform, possessing a nuclear localization signal, accumulates there [21,22]. The expression of dUTPase in fruit fly is restricted to certain developmental stages and tissues. The highest expression was observed in embryo and adult ovaries, furthermore, a significant source of dUTPase mRNA in embryo is maternal. During larval and further stages, dUTPase expression is restricted to particular tissues. Larvae express dUTPase only in imaginal tissues and the central nervous system (CNS). According to previously presented immunoblot analysis on adult animals, dUTPase expression was shown only in females in which ovaries maintain a high expression level [18,23].

We showed previously that dUTPase expression correlates with a decreased genomic uracil content of the fruit fly. [18]. The reason for the tolerance of increased genomic uracil in dUTPase non-expressing tissues is proposed to be the absence of UNG. From this correlation however, we can conclude that the regulatory mechanism that affects and fine-tunes dUTPase transcription, determines the extent of uracil accumulation in the genome. The importance of this regulation is emphasized by the fact that depletion of dUTPase by siRNA causes lethality in prepupal and pupal stages and coincides with γ H2Av signaling and cell death in imaginal discs of the larvae [18]. In the present study, we aimed to characterize the factors that regulate dUTPase transcription and might be responsible for the observed spatio-temporal expression pattern. Our current results and previous knowledge indicates that dUTPase expression and consequently uracil-free genome is generally typical for proliferating cells while absence of dUTPase and increased genomic uracil accumulation is a hallmark of differentiated cells even if they undergo endoreplication. We propose that this regulation is strongly associated with cell cycle and proliferation.

Results

dUTPase expression coincides with proliferation

The dUTPase protein level was previously shown to reach its maximum in the embryo through the developmental stages [18,23]. This is consistent with mRNA expression levels measured by real-time PCR and high throughput data obtained from modENCODE RNA-seq database [24][25]. The latter one also showed that the expression is the highest in the 4 – 8. embryonic stages (Figure 1A). The dUTPase expression reaches its minimum during the larval stages both at protein and mRNA level and starts to increase slightly during pupal stages [18,23]. Our previous data indicated that dUTPase protein is only detectable in females [23]. Accordingly, high throughput mRNA expression data from modENCODE also indicates that only females have increased dUTPase expression, while expression in males is at minimum level (Figure 1A).

We analyzed the dUTPase expression pattern in some embryonic stages by immunofluorescence using antibody raised against *Drosophila* dUTPase [23]. In stage 8, dUTPase co-localizes with nuclear staining indicating that nearly all the cells have a significant pool of the protein (Figure 2A). We observed the same in stages 11 and 16, namely that dUTPase and nuclear staining showed coincidence. However, in stage 16, we observed a relatively increased dUTPase staining in the germ cells compared to other tissues (cf arrowhead on Figure 2A, E16 panel). RNA *in situ* hybridization data obtained from Berkeley *Drosophila* Genome Project also showed that dUTPase mRNA is ubiquitous in the entire embryo until stage 9-10. From stage 11, it starts to be predominant in some embryonic organ precursors, such as in CNS and germ cells [26,27].

Among larval tissues, dUTPase is predominantly expressed in the imaginal discs, brain, imaginal rings of salivary gland and proventriculus and primordial testis as it was shown previously by immunofluorescence. We could not detect dUTPase in differentiated tissues, such as salivary gland and gut [18,23]. However, within both tissues, dUTPase expression was detected at specific primordial regions such as in imaginal rings in salivary gland and ventriculus and imaginal cells in gut (Figure 2B) [18]. This difference between imaginal and differentiated tissues is also significant at mRNA level showed by us previously and according to modENCODE RNA-Seq data [18] (Figure 1B).

Microarray data obtained from FlyAtlas shows that in adult animals, dUTPase encoding mRNA level is remarkably high only in the ovaries compared to the minimum level in other tissues (Figure 1C). Our previous observation by immunofluorescence also shows that only ovaries produce dUTPase which explains why dUTPase expression was only seen in females [23]. We were also interested in the detailed dUTPase expression pattern of the testes. Using

the polyclonal antibodies raised against dUTPase in our routine staining protocol did not indicate any dUTPase expression. However, after the depletion of the antiserum (with embryos silenced maternally for dUTPase) to enhance specificity, we show clear dUTPase expression in the nuclei of proliferating germinal cells, predominantly in the apical tip of testis (Figures 3). These results suggest that while testes globally express remarkably low level of dUTPase, some cells still show protein expression. The alternative staining method could allow us to decrease the background and to differentiate among dUTPase expressing and non-expressing cells. We used the improved antibody for western blot to analyze dUTPase expression in isolated adult gonads and adult animals. We detected the presence of the protein only in ovaries or intact females with this approach (Figure 4). This expression pattern agrees with our previous observation using the original antibody [23]. We suppose that adult testes express dUTPase only in a limited number of cells at a moderate or low rate. In contrast, ovaries show an abundant expression that might be one of the highest relative to tissue size.

Mapping the regulatory elements in the promoter of Drosophila dUTPase

In all the twelve *Drosophila* species with entirely known genomic sequences, a gene with opposite orientation is located upstream to the gene of dUTPase. In the *Melanogaster* subgroup, namely in *Drosophila melanogaster*, *simulans*, *sechellia*, *yacuba* and *erecta*, this gene encodes an arginine methyltransferase (Art8). In the other *Drosophila* species with known genome (*Drosophila ananassae*, *pseudoobscura*, *persimilis*, *willistoni*, *mojavensis* and *virilis*), a RAN GTPase activating protein is located at this position, except from *Drosophila grimshawi*, in which a gene encoding a predicted oxidoreductase is upstream to the dUTPase gene. In the five species in which Art8 gene is located upstream dUTPase, the 5' UTR and a 48 base pairs long sequence next to the transcription start site (TSS) show high similarity; suggesting important function in transcriptional regulation. We also identified motifs that show similarity to DNA-replication related elements (DRE) in the upstream region of dUTPase gene in the case of ten *Drosophila* species [28]. The species from the *Melanogaster* subgroup have this motif in a tandem repeat (Table 1).

As DRE sequences are palindromic, possible extending their regulatory range covering both the dUTPase and the Art8 gene. However, the latter one has a more evenly distributed level of expression in various tissues and developmental stages according to modENCODE RNA-Seq and FlyAtlas microarray expression data. This indicates a distinct regulatory mechanism for

the two genes, even though both showing their expression peaks in embryonic stages and in ovaries (Figure 1A and B).

In order to identify sequences that have impact on the transcriptional regulation of dUTPase, we mostly analyzed the upstream segment of dUTPase encoding gene spanning from -857 to 67 bases relative to the transcriptional start (dutP). This segment was examined with a luciferase encoding promoter – a reporter system in *Drosophila* S2 cells. We observed that the deletion of the distal parts of this segment (dutPb, dutP2) did not cause decrease in reporter activity compared to the full (-857 – 67 bases) segment unless the potential DRE motifs at the position -221 - -205, compared to TSS, were affected (dutP3). On the contrary, the reporter activity somewhat increased after the removal of the -857 - -257 bases segment (Figure 5A).

Deleting the 21 – 67 bases segment in the proximal region of the promoter that also functions as 5' UTR in dUTPase gene decreased the reporter activity almost by half (dutPr2). Removing further segments (-33 – 67 bases, dutPr3) resulted in dramatical loss of transcription activation capability. (Figure 5B).

In order to test the functionality of the DRE motifs in tandem repeat, we inactivated them by mutagenesis, transforming the consensus TATCGATA sequence to TATCGAGC. This mutation is reported to abolish the transactivation by DRE binding factor (DREF) [29]. Inactivation of the distal DRE motif (DRE1) led to a more dramatic decrease (down to one fifth) in reporter activity as compared to the mutation of the proximal one (DRE2, decrease by half). Mutation of both motifs (DRE12) decreased the reporter activity even more (one twentieth of the activity) (Figure 5C). These results indicate that both DRE motifs are functional in the promoter.

DRE-dependent transcriptional regulation in larval imaginal tissues

In order to analyze transcriptional regulatory features of the upstream segment of dUTPase gene in various tissues and developmental stages, we introduced it into a beta-galactosidase reporter system (pP{CaSper-AUG-betaGal}) that can be integrated into the *Drosophila* genome by transposon mediated translocation. Reporter constructs with dutP, dutP2 and DRE12 segments were microinjected into embryos and transgenic flies were generated (Figure 6A). In the tissues, reporter activity was analyzed following X-gal staining, and activity was measured based on intensities. In order to observe reporter activity in embryonic stages without the influence of the maternal effect, we stained embryos collected from crossing reporter containing males with wild type virgin females. In embryos, we could not observe any difference between dutP, dutP2 and DRE12 segments regarding transcriptional

activation. Staining did not appear in most of the strains, but even if it appeared, the staining was visible only from stage 13 in the case of all three constructs (Figure 6B, Table 2). This observation suggests that the major source and ubiquitous presence of dUTPase in embryonic stages derives from the maternal effect. The staining showed a segmental pattern along the dorsal midline, overlapping with the primordial CNS (Figure 6B).

In 3rd larval stage reporter activity, regulated by the dutP segment, was observed in the CNS, imaginal discs, imaginal ring of proventriculus and primordial testes. Deletion of the distal segment, resulting in dutP2, allowed the appearance of the same pattern, but the signal was less intense. Furthermore, mutation of the DRE elements abolished reporter activity in imaginal discs, in the imaginal ring of the ventriculus and CNS, but staining was still observed in the primordial testes (Figure 6B, Table 2). This indicates that DRE motifs have their major regulatory role in promoting dUTPase expression in imaginal tissues and brain.

Among adult tissues, reporter activity was observed in ovaries and in testes. In these tissues, the reporter activity was not even decreased upon the deletion of the distal segment (-846 - -257 bases) if the DRE motifs were mutated (Figure 6B, Table 2). In testes, reporter activity was the strongest in the apical region where immunocytochemistry indicated the presence of dUTPase protein (cf. Figure 3, Figure 6B). Taken together, our promoter reporter system indicated that DRE independent regulatory mechanisms are responsible for transcriptional expression of dUTPase in both larval primordial or adult gonads.

Physiological competence of upstream region of dUTPase gene with endogenous dUTPase expression

In order to test whether dutP segment is able to reproduce the required transcriptional pattern and expression level of dUTPase, we introduced this segment into a complement cassette including a 3xFLAG-tagged dUTPase encoding cDNA. Transgenic flies were generated possessing this cassette on chromosome 2. We showed previously that RNAi against dUTPase causes a severe lethal phenotype in prepupal, pupal stages if expression was induced by a ubiquitous driver, actin-Gal4 [18]. We combined UAS-RNAi alleles with dUTPase complementing cassette and Gal4 source. The complementing cassette could partially rescue the lethal phenotype caused by RNAi against dUTPase. We hypothesized that RNAi against dUTPase might have been able to silence the expression from complement cassette besides endogenous dUTPase. Therefore, our intention was to establish strains in which dUTPase can be silenced by short hairpin RNA (shRNA) spanning only limited parts of the UTRs which is absent from the complementing cassette. Therefore we generated strains possessing UAS-

shRNA alleles inserted into chromosomes 2 and 3. Upon induction by the actin-Gal4 driver, these alleles were able to cause 100% lethality (Figure 7A and B). If complementing cassette driven by the promoter segment *dutP* was present in the silenced flies, the lethal phenotype of dUTPase silencing was completely rescued (Figure 7A and B). The complete suppression of the lethal phenotype was achieved by two independent complementing alleles. Therefore, we concluded that the expression pattern established by the *dutP* segment was able to reproduce the physiologically relevant levels of dUTPase during developmental stages.

Downregulation of dUTPase transcription upon 20-hydroxyecdysone treatment

As presented above, dUTPase expression is repressed mostly in differentiated tissues even in larval and adult developmental stages. The mechanism of the repression might depend on the binding of downregulating factors to some regulatory regions of the dUTPase encoding gene. The upstream sequence of the dUTPase encoding gene also showed a similar functionality as a promoter in both luciferase and β -galactosidase reporter systems; since in imaginal tissues and S2 cell line, DRE motifs were shown to be important for promoter activity. Cut is a repressor, which antagonizes the DRE binding factor, DREF after ecdysone treatment and thought to be involved in differentiation signaling [28]. Therefore we intended to test how dUTPase expression and its transcriptional regulation are affected by ecdysone treatment. In order to perform such an analysis we created a reporter system in which short lifetime YFP was used as a reporter. The -857 – 67 bases segment upstream to the dUTPase encoding gene (*dutP*) was introduced in such a reporter system. The DRE12 segment in which DRE motifs are mutated was also introduced into the reporter system in order to test a potential antagonistic effect. We expected that the promoter with disrupted DRE motifs shows a decreased or abolished response to the ecdysone treatment. *Drosophila* S2 cells were transfected with a *dutP* or DRE12 segment driven reporter system and after a cell division cycle, cells were treated with 20-hydroxyecdysone using various concentrations. dUTPase and destabilized YFP reporter levels were analyzed by western blot. We observed that 20-hydroxyecdysone decreased dUTPase and YFP levels in a concentration-dependent manner (Figure 8A). Reporter protein levels were also decreased if it was driven by the DRE mutant segment, indicating that ecdysone mediated repression does not involve DRE motifs (Figure 8B).

Discussion

By eliminating dUTP from the dNTP pool, dUTPase is suggested to be indispensable for faithful replication process. mRNA expression, immunoblot and immunocytochemistry results

presented by us or obtained from databases, indicate that dUTPase is mostly present in proliferative tissues, in which faithful replication is essential, such as in embryonic tissues, in imaginal discs of larvae and reproductive organs (Figures 1, 2 and 3) [18,23]. Proliferation linked expression of the nuclear isoform of dUTPase was observed in humans as well; since the presence of the protein was shown predominantly in proliferating B cells in germinal center; the replicating zone located in the bottom half of crypts of Lieberkühn and the stratum basale layer of associated stratified squamous epithelium. In addition, increase in dUTPase expression was observed in peripheral blood lymphocytes after mitogenic activation by phytohaemagglutinin. [30,31]. Therefore we propose that dUTPase fulfills its predominant function in proliferating cells.

We analyzed the genomic sequence upstream to the dUTPase encoding gene with transcriptional reporter systems (Figures 5 and 6). We found that the sequence of interest was capable to establish a reporter activity which overlapped with the pattern formed by the dUTPase encoding mRNA or protein itself. Namely dUTPase is present in embryos, in the imaginal tissues and brain of the larvae and in reproductive organs. Applying this segment as promoter for dUTPase cDNA encoding cassette could entirely complement the lethal phenotype of shRNA mediated dUTPase depletion. Further dissection of the promoter revealed that the sequence in the proximity of the transcriptional start site (-33 – 67) is inevitable for expression (Figure 5B). This segment could provide docking sites for general transcriptional factors. Furthermore, within the promoter, we identified two DRE motifs in a tandem repeat potentially providing binding sites for DRE binding factor, DREF. DREF was discovered as a major transcriptional regulator that promotes expression of a set of genes in proliferating cells [28,32–38]. We suspect that regulation through this motif could be conserved among *Drosophila* species (Table 1). Removal or mutation of these motifs decreased promoter activity remarkably in embryo derived S2 cells, imaginal tissues and the brain of larvae (Figures 5C and 6B, Table 2). We found that both dUTPase expression and transcriptional activity of the promoter is downregulated upon 20-hydroxyecdysone treatment in a concentration dependent manner in S2 cells; but this decay did not depend on the DRE motifs (Figure 8B). However we could not exclude that such a downregulation exists in other tissues.

Proliferation dependent regulation was also observed in the case of the nuclear isoform of human dUTPase (hN-dUTPase). In human cell lines, hN-dUTPase expression was found to increase after G0 cell cycle release and decrease upon serum starvation [30]. For this regulation, the binding of transcription factors E2F-1 and Sp1 might be responsible [39].

Proliferation dependent transcriptional regulation is also peculiar in the case of other enzymes of dNTP pool maintenance. The gene of *Drosophila* IMP dehydrogenase, involved in cellular nucleotide metabolism and biosynthesis, also possess DRE motifs [40]. Cellular dNTP concentrations show a fluctuation during the cell cycle in order to meet the requirements of replication [41]. This fluctuation is achieved through the cell cycle dependent transcriptional regulation of ribonucleotide reductase (RNR). Expression of the R1 subunit of the mouse RNR is regulated by the S-phase specific YY transcription factor. Cell cycle dependent fluctuation of the R2 subunit of RNR is determined by retinoblastoma (Rb) repression – E2F4 derepression cycles [42]. Such a regulation is observed even in the case of *Drosophila* RNR as well [43]. Enzymes of thymidylate metabolism are also regulated in a proliferation-dependent matter, as dihydrofolate reductase (DHFR) and thymidylate kinase expression is regulated by E2F1 [44]. Furthermore, thymidylate synthase and deoxynucleotide kinase in *Drosophila* is regulated by E2F2 [43]. Coexistent expression of UNG and dUTPase is expected for genome integrity maintenance, since loss of dUTPase function is reported to interfere with viability in the presence of UNG. This might be the reason for the proliferation-dependent and S-phase specific regulation of UNG expression by transcription factors Sp1, AP-2, E2F1 and c-Myc in mammalian cells [45–47].

In two groups of tissues, the examined promoter segment did not require the DRE motifs for transcriptional activation; in embryos and in reproductive organs or in their precursors (Figure 6B, Table 2). dUTPase protein and mRNA are both present in embryos, but their origin is mainly maternal. We found that the examined promoter segment was activated only from stage 13 (Figure 6B). Interestingly, expression data showed that dUTPase expression starts to decrease in stages 9-11 (Figure 1A). We suspect that endogenous dUTPase transcription is not necessary, until maternal source of the enzyme is abundant, and this is why transcription is activated only after maternal dUTPase starts to decrease.

The other group of tissues in which the examined promoter segment did not require DRE motifs includes adult gonads and primordial testes of larvae (Figure 6B, Table 2). In these tissues, other gonad-specific regulatory elements might be responsible for the transcriptional activation, which possibly reside in the region -257 to 67 bases relative to TSS. *In silico* sequence analysis did not result in any potential transcription factor binding site in this region. However, the expression pattern of dUTPase in adult ovaries and testes seems to overlap with the pattern established by Ovo B transcription factor and the expression pattern of Vasa [48–50]. Ovo B recognizes a motif in which a GTT sequence core is inevitable. GTT motifs frequently occur in the promoter of dUTPase, we identified 10 instances of this triplet within

the -257 to 67 bases long range of the promoter. Expression pattern of Vasa is supposed to be regulated by TBP related factor, TRF. Within -257 – 67 bases region relative to the TSS of dUTPase encoding gene, a putative TC box is also present at -16 position that might be the recognition site of TRF. Further investigation is required to inspect the potential involvement of these factors in the regulation of dUTPase expression. However, it can be stated that a distinct regulatory mechanism maintains expression in gonads inferring that dUTPase expression might be exceptionally important in these tissues. Maintenance of intact genome of germ cells throughout generations is a primary mission of the homeostasis of an organism. Thus giving an evolutionary reason for expressing dNTP pool sanitizing dUTPase in these cell types. We suspect that dUTPase might be also vital in reproductive capacity and its expression could be predominant in reproductive organs of other organisms as well.

Interestingly, our studies on *Drosophila* dUTPase expression and promoter activity concluded to the result that dUTPase encoding gene is active predominantly in precursor or proliferative cells. Our previous study also revealed that such proliferative precursor cells such as imaginal discs of the wing are sensitive to dUTPase silencing [18]. Differentiated tissues of the larvae also replicate their genome in order to multiply cell mass and meet the requirements of larval development [51]. In spite of this mass increasing replication, larval differentiated cells does not express dUTPase expression and not even require sanitizing from deoxyuridin incorporation. For instance, we showed previously that larval salivary gland possess an elevated level of uracil in its genome [18]. Probably, the transient functionality of these tissues does not require such an efficient dNTP pool sanitizing capacity. Furthermore, differentiated polyploid cells were reported to maintain an increased DNA instability with stalled replication forks [52].

Therefore, we can conclude that dUTPase expression pattern is evolved to prevent uracil incorporation into DNA in tissues, whose genome requires a special care as being replicated for special developmental or reproductive cell fates. dUTPase might be also important in order to preserve replicative potential, as its expression is also upregulated in tumorous cells [31,53–55]. Further studies focusing on other dNTP sanitizing or DNA damage preventing enzyme machineries might reveal more examples for the synchronicity of genome protection and proliferation. This knowledge together with our presented results about dUTPase could help predict the effects of genotoxic agents perturbing genome metabolism.

Materials and methods

Sequence analysis and alignments

dUTPase encoding genes were determined by BLAST in the genomic sequence of different *Drosophila* species. Upstream sequences of dUTPase encoding genes were obtained from FlyBase. MAFFT version 7 was applied for alignments of promoter candidates using E-INS-I parameter [56].

Construction of reporter plasmids

As a promoter-less reporter system, we used the pGL3 plasmid from Promega (Madison, WI, USA). To amplify different regions of upstream region of the *Drosophila* dUTPase gene, we used the following primer combinations: dFw (5' - CGT GCA GAA GAT CTT GCG GAT TCA GC - 3') – dRev (5' – CGG GAT CCG CAG AAT TCT GGT CTG AAA ATA ACG CGG - 3'); d2Fw (5' - GGG GTA CCC GTG CTA AAT AGA GGT GTG TTA ATC AAC TAC - 3') - dRev; d3Fw (5' - GGG GTA CCG TTG CTT ATC AGG GTT GGT TGT GAT TGG - 3') - dRev; dFw - d2Rev (5' - CGG GAT CCC TAC CAA AAA ATC TTA AGT CAG CTT TGC -3') and dFw - d3Rev (5' - CGG GAT CCA ATT GGC GGA CTT CCA GTG TTG C -3') on genomic DNA of W1118 *Drosophila melanogaster* strain to produce dutP, dutP2, dutP3, dutPr2 and dutPr3 fragments respectively. These DNA fragments were digested by KpnI and BamHI to ligate into the multicloning site of pGL3 digested by KpnI and BglII. pGL3 plasmid containing the dutPb fragment was produced from pGL3-dutP after cutting by BglII and religation. Inactivation of the two DRE elements were carried out by site directed mutagenesis using the primer pairs DRE1Fw - DRE1Rev (5' - CAA CTA CAA TAG GCT CGA TAT ATC GAT AGG GTT GCT TAT C - 3' and its reverse complement); DRE2Fw - DRE2Rev (5' - CAA CTA CAA TAG TAT CGA TAG CTC GAT AGG GTT GCT TAT C - 3' and its reverse complement) and DRE12Fw - DRE12Rev (5' - CAA CTA CAA TAG GCT CGA TAG CTC GAT AGG GTT GCT TAT C - 3' and its reverse complement) to generate the mutant versions of pGL3-dutP containing the DRE1, DRE2 and DRE12 fragments by site directed mutagenesis respectively. As reference construct for luciferase assay, we used the pRL-OpiE2 plasmid, in which the OpiE2 promoter was derived from the pIZ/V5-His plasmid (Invitrogen, Carlsbad, CA, USA). Insert was generated by digestion with BamHI and Sall, and was ligated into the BglII and Sall sites of pRL-TK plasmid (Promega) replacing the original HSV TK promoter.

Beta-galactosidase reporter constructs with dutP and DRE12 fragments were produced after PCR amplification from the corresponding pGL3 reporter constructs using the primers bgaldFw (5' - CGG GAT CCG GGT GCC ACG AAA ATT GTG CAC - 3') and bgaldRev (5' - GGG GTA CCG CAG AAT TCT GGT CTG AAA ATA ACG CGG - 3'). For the dutP2

fragment, containing beta-galactosidase reporter construct, we used the d2Fw - bgaldRev primer set for amplification from the pGL3-dutP2 construct. The resulting fragments were digested by BamHI and KpnI and ligated in the corresponding sites of the p{CaSpeR-AUG-betaGal} plasmid.

Destabilized YFP reporter constructs were produced from the pGL3-dutP or pGL3-DRE12 plasmids using the dFw - dRev primers where the amplicons were digested by KpnI and BamHI. The obtained fragments were ligated into the KpnI and BamHI site of pd2EYFP-N1 plasmid (Clontech, Mountain View, CA, USA). The original CMV promoter of this plasmid was removed by NdeI and NheI digestion and religation.

Cell lines and luciferase assay

Drosophila S2 cells were propagated in Schneider's insect medium (Sigma-Aldrich, St. Louis, MO, USA) in the presence of 10% heat inactivated FBS (Gibco, Life Technologies, Carlsbad, CA, USA) and 1% Pen/Strep (Gibco) at 27°C. Transfection was carried out using the calcium phosphate method. 2×10^5 cells were seeded into a 24-well plate a day before the transfection. 3 hours before the transfection, the culture medium was replaced with 280 μ l fresh medium. 18 μ l HBS solution (21 mM HEPES, 137 mM NaCl, 5.5 mM dextrose, 49.5 mM KCl, 0.7 mM Na_2HPO_4 , pH=7.1) and 1.1 μ g plasmid / well was mixed in a microcentrifuge tube. Then, 1.1 μ l 2.5 mM CaCl_2 solution was added to the tubes. After 20 minutes complexation, the mixture was added to the cells.

Before the luciferase assay, firefly luciferase encoding pGL3 and renilla luciferase encoding pRL constructs were cotransfected in triplicates. The assay was performed using the Promega Dual-Luciferase Reporter Assay kit following the instructions of the manufacturers. Each transfectant was assayed three times in a Greiner Lumitrac 600 High binding 96 well plate. Luminescence was detected by a PerkinElmer Wallac-VICTOR² 1420 plate reader. Renilla luciferase activity driven by the OpIE2 promoter served as internal control. Average activity and standard error of mean (s.e.m.) was calculated from the normalized values for each construct.

20-hydroxyecdysone treatment and western blot

Drosophila S2 cells were transfected with pd2EYFP constructs containing the dutP fragment as promoter. A day after the transfection, 0.1, 0.5, or 2.4 μ g/ml 20-hydroxyecdysone (Sigma-Aldrich) was added to the cell culture. The cells were harvested after 16 hours of treatment and were processed for western blotting. Harvested cells were extracted in

denaturing and reducing conditions. The extracts were loaded onto a 12% polyacrylamide gel and transferred to PDVF membrane (Immobilon-P, Millipore). The blots were developed with polyclonal primary antiserum against *Drosophila melanogaster* dUTPase (1:100000) [23], and polyclonal anti-GFP (1:1000) (Sigma-Aldrich). As loading control, monoclonal anti-actin (1:300) (Sigma-Aldrich) was used. As secondary antibodies, horseradish peroxidase conjugated anti-rabbit (GE Healthcare) or anti-mouse (Sigma-Aldrich) antibodies were used.

Immunofluorescence

w¹¹¹⁸ embryos were collected at various stages and fixed in 4% PFA, 2.5 % Tween-20, 1 mM MgCl₂, 1 mM EGTA, 100 mM PIPES, pH=6.9 after dechorionization and devitellinization in heptane : ice cold methanol (1:1) emulsion. Then embryos were washed for 10 minutes three times in 150 mM NaCl, 0.5% Tween-20, 50 mM TRIS, pH=7.4 and blocked for 1 hour in blocking buffer (1% BSA, 5% Fetal Bovine Serum in PBS) in the presence of 0.1% Tween-20 and 1% Triton X-100. Staining with polyclonal antibody raised against *Drosophila* dUTPase (1:10000) [23] was performed in blocking buffer complemented with 0.05% Tween-20 and 0.5% TritonX-100 overnight, at 4°C. After washing in blocking buffer with 0.1% Tween-20, 1% TritonX-100 for 4 hours, the secondary antibody was applied: Alexa-488 labeled antibody (1:1000, Life Technologies, Carlsbad, CA, USA) in blocking buffer, 0.01% Tween-20, 0.1% TritonX-100 for 2 hours, RT. After washing with blocking buffer, 0.1% Tween-20, 1% TritonX-100 for 3x30minutes, nucleus was counterstained with Hoechst (1µg/ml).

Adult testes were stained as described for embryos, except that fixation was performed in the presence of 50% heptane in order to get rid of fatty components. dUTPase antibody was depleted in the presence of maternally silenced *dUTPaseV22-4(attP40)*; *dUTPaseV22-4(attP2)* embryos, in which dUTPase expression was not detectable (cf. *Drosophila* transgenic animals in this section). Secondary antibody control experiment was performed by immunocytochemistry without the use of primary antibody. Images of the stained tissues were obtained by Zeiss LSCM 710 confocal microscope.

Drosophila transgenic animals

Beta-galactosidase reporter constructs were microinjected into *w¹¹¹⁸* embryos (BestGene, Chino Hills, CA, USA). Eight dutP-reporter, nine dutP2-reporter and seven DRE12-reporter strains were isolated. To analyze the transgenic expression pattern in embryonic phase excluding the maternal import of the reporter, we crossed transgene carrying males with *w¹¹¹⁸* virgin females.

For constructing a complementing cassette for dUTPase silencing (dUTPase_complement), we removed the UAS promoter by NheI and XhoI from pUAS-K10attB plasmid. Into this plasmid, we introduced a cassette possessing the -846 – 67 bases segment upstream to the dUTPase encoding gene (*dutP*) as promoter bordered by BamHI and KpnI sites; the long (nuclear) isoform dUTPase encoding cDNA equipped with Kozak (agccacc) bordered with KpnI and SphI restriction sites and as a C-terminal fusion tag, 3xFLAG bordered by SphI and XhoI sites. The construct was inserted in the genome by transposon mediated translocation after microinjection into *w¹¹¹⁸* embryos resulting alleles on the chromosome 3.

To generate *Drosophila* strains encoding a shRNA construct targeting dUTPase mRNA, we designed complementary RNA segments that are able to hybridize to the 3'-UTR to the mRNA. The dUTPase 3'UTR specific shRNA sequence (5'-CTTCATGGTCACTATCAAAGA-3') was inserted into Valium22 vector and the transgene was introduced into the 2nd and 3rd chromosomal docking sites (*attP40*, *attP2*): *dUTPaseV22-4(attP40)*; *dUTPaseV22-4(attP2)* following the flyRNAi protocol [57]. This allows the silencing of endogenous dUTPase source while expression of dUTPase from a complementing cassette is unaffected. We obtained flies having Gal4 inducible dUTPaseV22-4cassette on chromosome 2 (*dUTPaseV22-4(attP40)*) and chromosome 3 (*dUTPaseV22-4(attP2)*).

Besides shRNA constructs, we also used the UAS-IR strains 21883 and 21884 obtained from Vienna *Drosophila* RNAi Centre (VDRC, Vienna, Austria) possessing dUTPase RNAi encoding transgenes on chromosome 2 [18]. For complementation analysis, homozygous RNAi or shRNA (*dUTPaseV22-4(attP40)*; *dUTPaseV22-4(attP2)*) virgin females were crossed with *actGal4/CyO*; *dUTPase_complement/Tm3* males. Progeny with different allele combinations were determined according to balancer phenotypes.

Beta-galactosidase assay

Beta-galactosidase assay was carried out as described in [58]. After dechorionization, embryos were fixed in 18.81 mM NaH₂PO₄, 81.17 mM Na₂HPO₄, 4% formaldehyde solution: heptane emulsion (1:1) for 20 minutes RT while continuous shaking. Then the embryos were washed twice with NaCl-Triton buffer (0.7% NaCl, 0.04% Triton-X100). After 5 minutes rehydration, embryos were placed into 37°C X-gal staining solution (3.16 mM NaH₂PO₄, 6.84 mM Na₂HPO₄, 150 mM NaCl, 1 mM MgCl₂, 3 mM K₄[Fe(CN)₆], 3 mM K₃[Fe(CN)₆], 0.3% Triton X-100). 2 mg/ml X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) substrate

was mixed to the solution. The staining was performed for 1 hour. Devitellinization was carried out in ice cold methanol : heptane emulsion (1:1). Embryos were rehydrated in serial dilution of ethanol in phosphate buffered saline (PBS).

To stain larval wing discs, CNS, midgut, and testis primordium tissues, adult ovaries and testes were collected in ice cold PBS. The tissues were fixed in PBS containing 1% glutaraldehyde for 15 minutes RT. The fixed tissues were washed twice in Na-P/0.2TX solution (72 mM Na₂HPO₄, 28 mM NaH₂PO₄, 0.2% Triton-X 100; pH=7.2). The tissues were placed into 37°C X-gal staining solution (7.2 mM Na₂HPO₄, 2.8 mM NaH₂PO₄, 150 mM NaCl, 1 mM MgCl₂, 3 mM K₃[Fe(CN)₆], 3 mM K₄[Fe(CN)₆]). The reaction was performed in 37°C for 15 minutes in the presence of 2 mg/ml X-gal substrate. After the staining, the tissues were washed with PBS.

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Author Contributions

Specific categories of contribution: Planned experiments: AH, LH, ME, BGV; Performed experiments AH, JB, LH, TL, GR; Analyzed data: AH, JB, LH, ME; Wrote the paper: AH, JB, LH, ME, BGV .

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Tables**Table 1. Alignment of genomic segments located upstream to dUTPase encoding gene, in which subsequences showed similarity with the canonical DRE motif, TATCGATA.**

Canonical DRE motif was identified in the putative promoter fragment of *Drosophila melanogaster*, *simulans*, *sechellia*, *yakuba*, *erecta* and *mojavensis* dUTPase. In the melanogaster subgroup (first five on the table), the putative DRE motifs are arranged in duplicates.

Table2. Detection of in vivo promoter activity of modified versions of the upstream segment of dUTPase encoding gene

Expression pattern of beta-galactosidase was mapped through developmental stages in different tissues of independent strains of transgenic reporter allele possessing animals (cf. Figure 3B). Activities were determined according to staining intensity that is shown by the darkness of the indicated color. White fields indicate no staining.

Table 1.

Species	Position from start codon	Sequence
<i>D. melanogaster</i>	-350	---tatcgata----tatcgatagggttg
<i>D. simulans</i>	-207	---tattgata----tatcgataa-----
<i>D. sechellia</i>	-208	---tattgata----tatcgataa-----
<i>D. yakuba</i>	-356	---tatcgata----tatcgatag---tg
<i>D. erecta</i>	-345	---tatcgataacatatcgat--gaatg
<i>D. pseudoobscura</i>	-278	attgagatggcagtgcttcgataa-----
<i>D. persimilis</i>	-278	attgagatggcagtgcttcgataa-----
<i>D. mojavensis</i>	-351	-----gtggcggcttatcgataa-----
<i>D. virilis</i>	-408	-----gtggtggtccaccgatat-----
<i>D. grimshawi</i>	-344	-----gtggtggtccgccgatat-----

Table 2.

		embryo	3. stage larva				adult		
			brain	disc	foregut	testis	ovary	testis	
dutP	2/1	++	+	++	++	++	+++	+++	
	2/3	++	++	++	++	++	+++	++	
	2/4	++	+++	++	+++	+++	+++	++	
	2/5	++	+++	+++	++	++	++	+	
	2/6	0	+++	0	++	++	++	++	
	2/7	+	++	+++	+++	++	+++	+++	
	2/8	0	0	0	0	+	++	++	
	2/10	+++	+++	+++	+++	+++	+++	+++	
	dutP2	1/1	++	+	0	+	+	++	+
		1/2	0	+	0	0	+++	+++	++
1/3		0	0	0	0	0	+	0	
1/4		0	++	++	+	++	++	+	
1/5		++	++	++	++	++	+++	++	
1/6		+	+	++	++	+	++	+	
1/7		0	0	0	0	++	++	+	
1/8		++	++	++	+++	+	++	++	
1/9		0	0	0	0	+++	++	++	
DRE12	3/1	++	++	0	0	++	+++	+++	
	3/2	0	0	0	0	0	0	0	
	3/3	+	0	0	0	++	+++	++	
	3/4	++	0	0	0	++	+++	++	
	3/7	0	0	0	0	0	++	+	
	3/8	0	0	0	0	++	+++	++	
	3/9	0	0	0	0	0	+	+	

+++	+++
++	++
+	+
0	0

Figure legends

Figure 1. High throughput expression data of *Drosophila* dUTPase (A-C) and Art8 (D-F) mRNA obtained from modENCODE RNA-seq or FlyAtlas microarray projects.

(A and D) modENCODE RNA-seq data in developmental stages. E 1-4 to 15-17: Embryonic stages; L1, L2, L3: first, second and third stages of larvae; PF: puparium formation, P: pupae, males and females 5 days after hatching. (B and E) modEncode RNA-seq data in larval tissues. Img. disc: imaginal discs; CNS: central nervous system; Sal. gland: salivary gland; Digestive sys.: digestive system. (C and F) FlyAtlas microarray data in adult tissues.

Figure 2. Distribution of dUTPase expression in embryos and larval gut determined by immunocytochemistry.

(A) Nuclear staining and dUTPase expression pattern in 8, 11, and 16. embryonic stages. White arrowhead indicates intense staining for dUTPase at germ cells. (B) Nuclear staining and dUTPase expression pattern in larval gut. dUTPase is expressed in small imaginal cells. Scale bar: 100 μ m.

Figure 3. dUTPase expression in adult testes detected by immunocytochemistry and secondary antibody control.

dUTPase expression was detected in testes by immunocytochemistry using improved anisera in order to decrease background staining. Secondary antibody control is shown in the panel, *ab-control* for which no primary antibody was applied. Staining with the enhanced antibody reveals dUTPase expressing cells at the apical part of the testis. Scale bar: 100 μ m.

Figure 4. dUTPase expression in adult animals and gonads detected by western blot.

Enhanced antibody was employed to detect dUTPase in dissected adult ovaries (O), dissected adult testes (T), adult males (M), adult males without testes (M-), adult females (F) and adult females without ovaries (F-) using western blot. dUTPase isoforms were only detected in the isolated ovaries and intact females. Protein load was determined by comassie staining.

Figure 5. Mapping of the putative promoter of dUTPase in *Drosophila* S2 cells.

Upstream segment of dUTPase encoding gene was examined in luciferase reporter system. *Drosophila* S2 cells were transfected with reporter constructs containing the indicated segment of the promoter. pGL3 vector without promoter was used as negative control.

Promoter activity was read out from firefly luciferase activity. As an internal control, OpiE2 constitutive promoter driven renilla luciferase was used. Effect of deletions or mutations on the putative dUTPase promoter activity was determined by the firefly luciferase luminescence relative to the renilla luciferase activity. The bars show the relative average promoter activity of the individual segments compared to the -857 to 67 bases segment of the dUTPase encoding gene, which promoter activity was regarded as one. Error bars represent the standard error of mean (s.e.m.). (A) Deletions of the distal elements of the putative promoter. Major decrease in promoter activity occurred after the removal of DRE motifs. (B) Deletions of proximal elements of the putative promoter. (C) Effect of the inactivation of the first, second or both DRE motifs on the promoter activity. Mutations of either the first or second individual motifs result in significant decrease in activity. Double mutants show the greatest decrease in activity.

Figure 6. In vivo promoter activity in Drosophila tissues.

Multiple independent transgenic *Drosophila* strains were developed having a beta-galactosidase reporter with the putative dUTPase promoter. Promoter activity was determined by measuring the staining intensities that correlates with the expression level of the beta-galactosidase reporter. Embryos were obtained from crossing wild type females with males having the promoter-reporter alleles in order to exclude reporter expression from maternal source. (A) Number of independent strains developed for examining the activity of -846 to 67 bases segment (dutP) or a truncated version (dutP2) of the putative promoter, and the promoter segment with inactivated DRE motifs (DRE12). (B) Typical pattern of reporter activity in embryos and larval or adult tissues determined by the corresponding promoter if staining was detected.

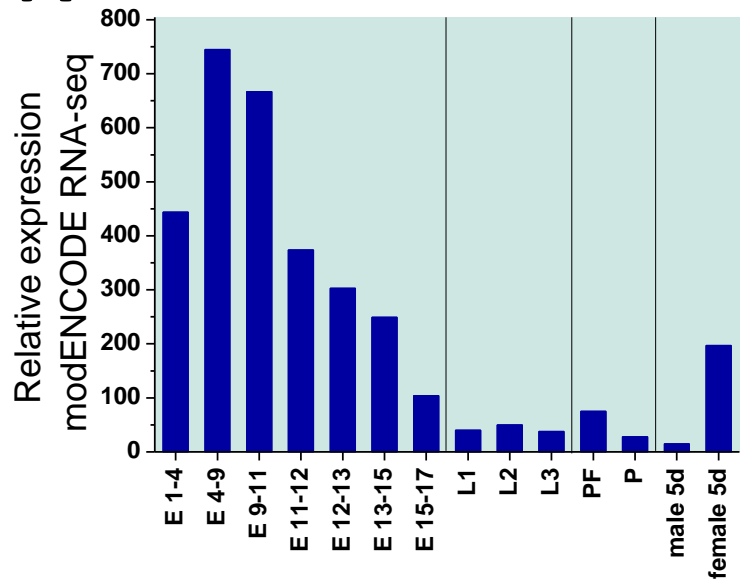
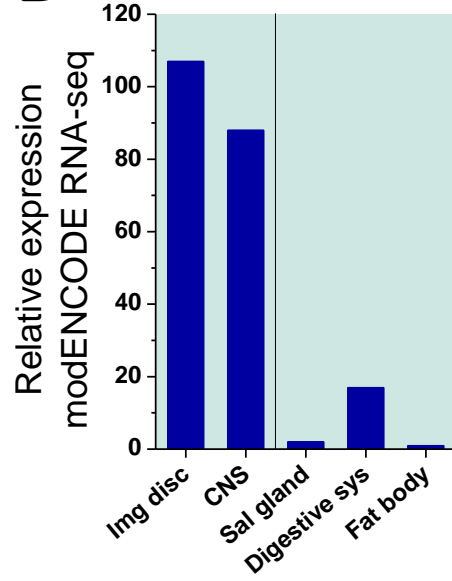
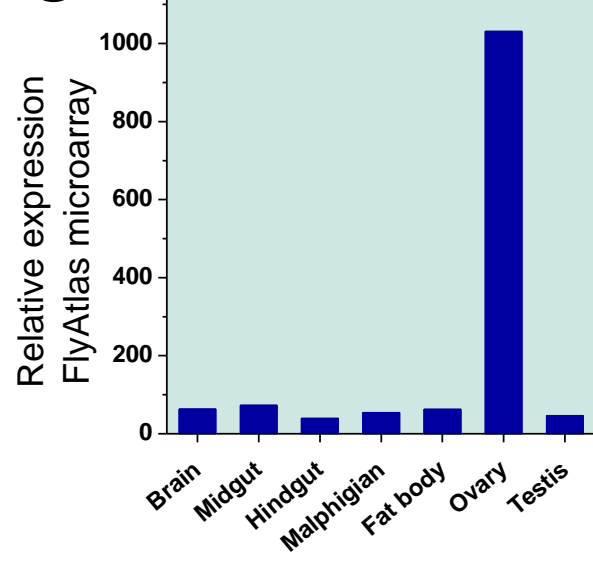
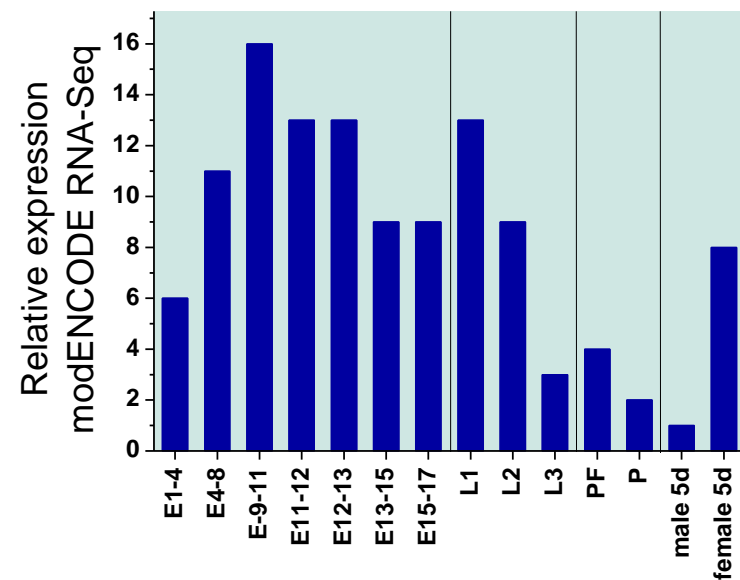
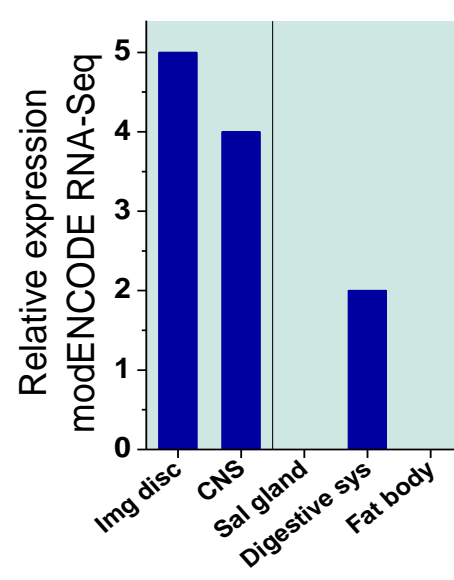
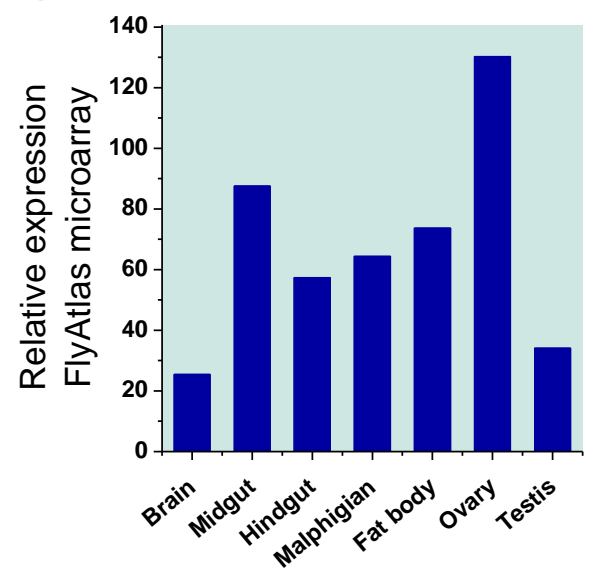
Figure 7. Complementing experiment on dUTPase depleted animals using Drosophila dUTPase cDNA driven by the examined promoter segment of dUTPase encoding gene.

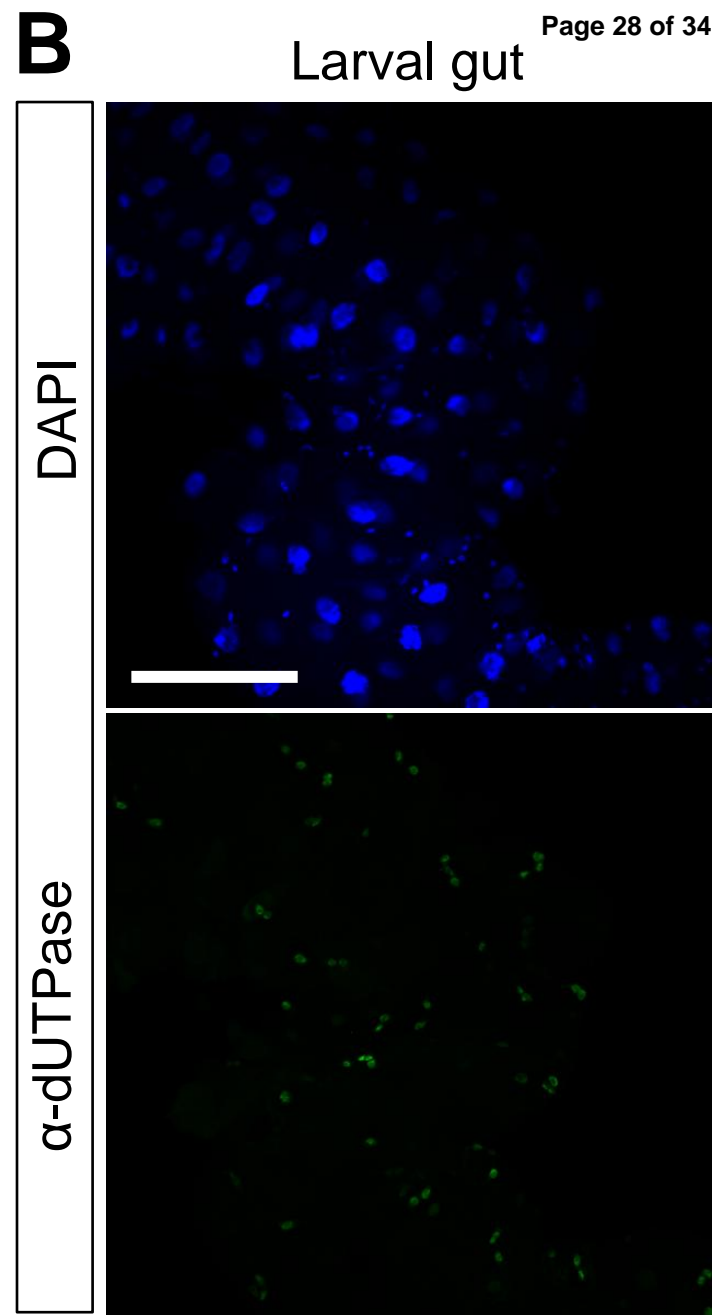
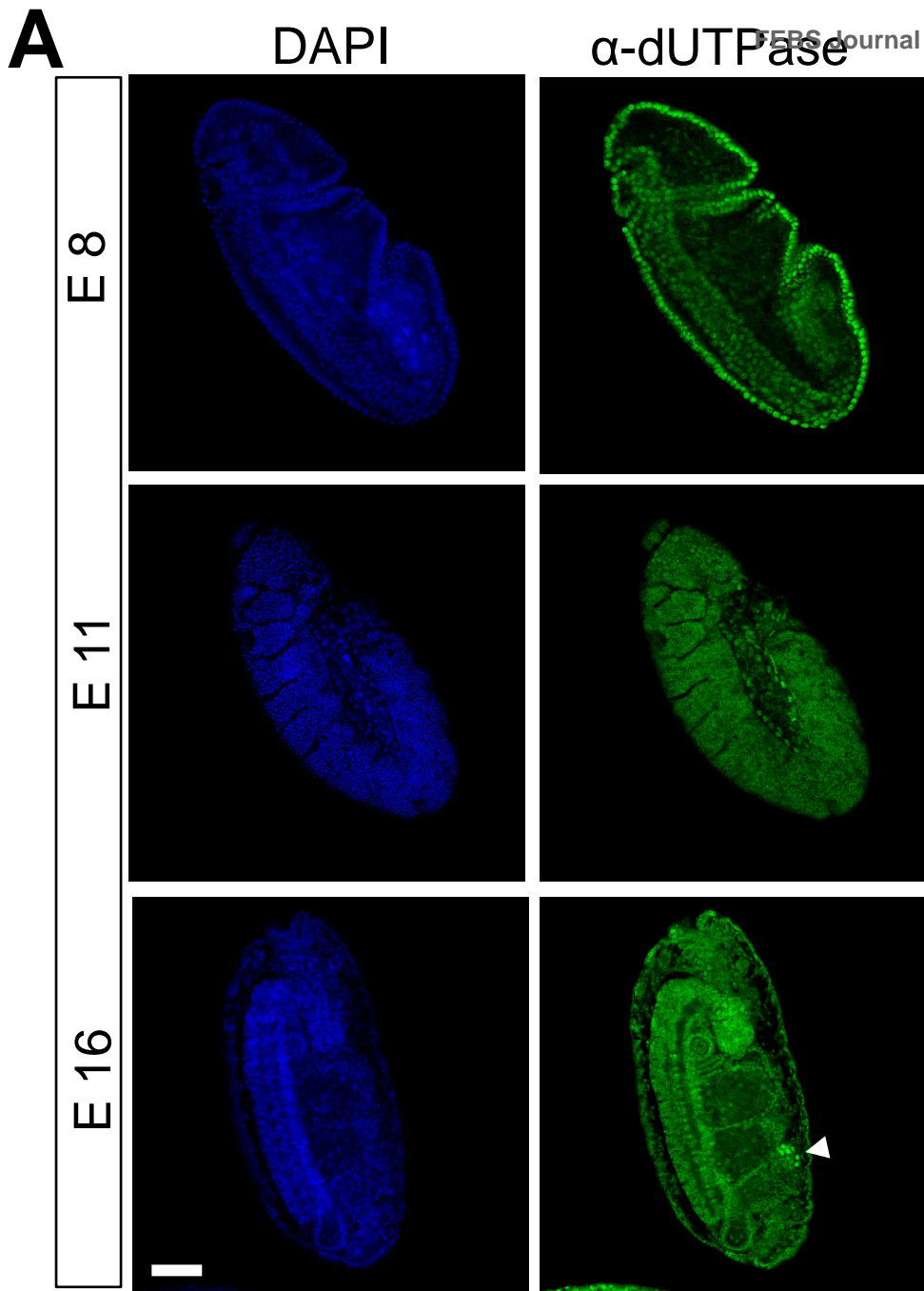
shRNA (*dUTPaseV22-4(attP40)*; *dUTPaseV22-4(attP2)*) virgin females were crossed with *actGal4/CyO*; *dUTPase_complement/Tm3* males. Progeny with CyO and TM3 balancer chromosomes does not have shRNA or dUTPase_complement alleles and served as control. In the progeny with *act-Gal4*; *TM3* genotype, shRNA expression is induced, that causes nearly 100% lethal phenotype up to the pupal stages. In the progeny with *act-Gal4*; *dUTPase_complement* alleles (*act-Gal4*; *compl*), lethal phenotype was completely suppressed. Progeny with CyO balancer chromosomes and dUTPase_complement allele (*CyO*; *compl*)

have the complementing cassette on wild type background. Two complementing alleles was used: (A) dUTPase_complement (8); (B) dUTPase_complement (10).

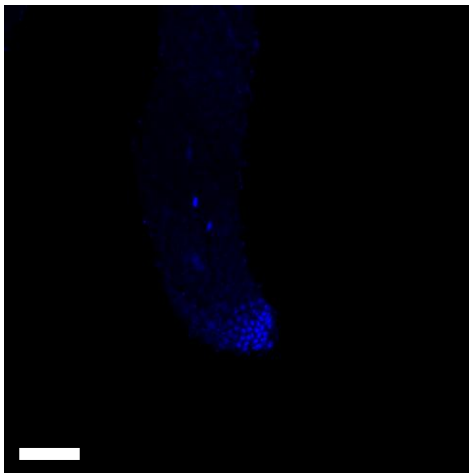
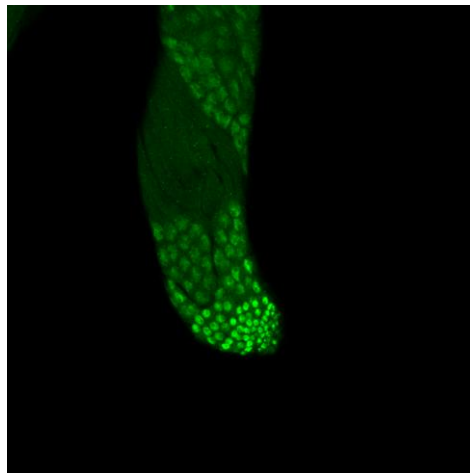
Figure 8. Effect of 20-hydroxyecdysone on dUTPase expression and its promoter activity.

S2 cells were transfected with destabilized YFP reporter driven by the dUTPase promoter or its DRE mutant version. dUTPase or YFP levels were determined by western blot a day after ecdysone treatment. (A) S2 cells expressing the reporter in a dUTPase promoter dependent manner. (B) S2 cells expressing the reporter driven by the DRE mutant dUTPase promoter. dUTPase and reporter protein levels decreased in a concentration dependent manner in both cases.

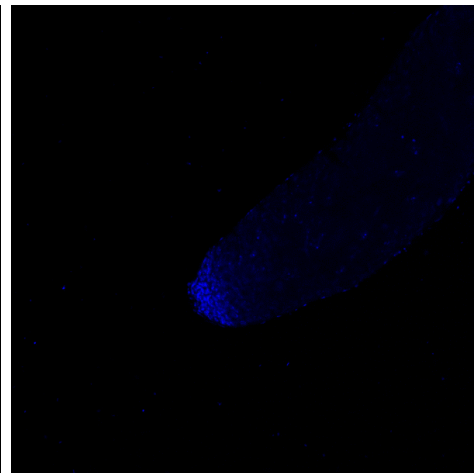
A e 27 of 34**B** FEBS Journal**C****D****E****F**



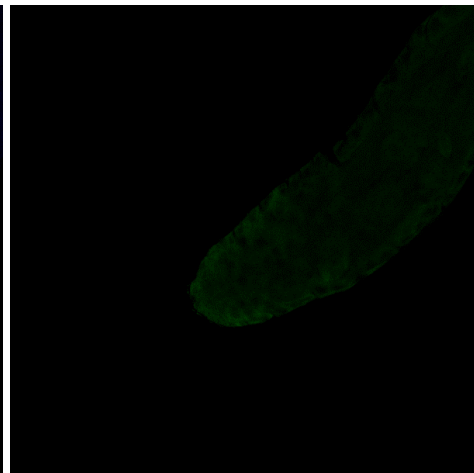
DAPI

 α -dUTPase

DAPI



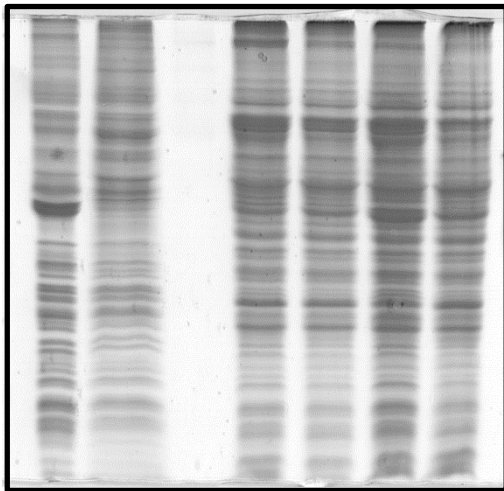
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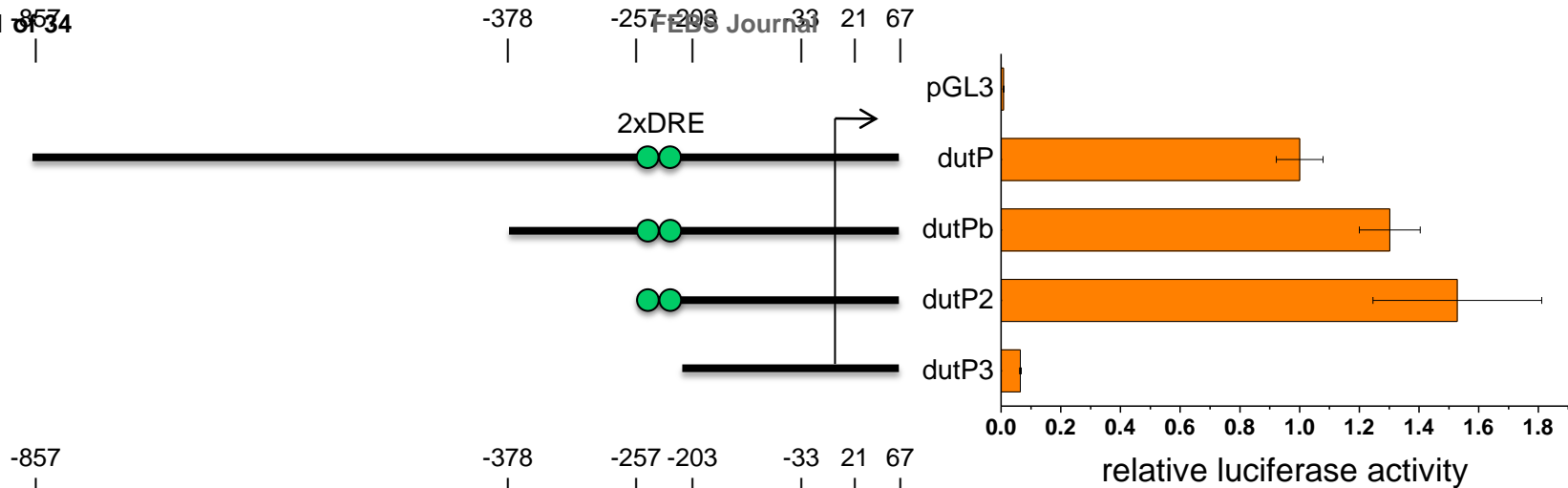
dUTPase 23 kDa
21 kDa



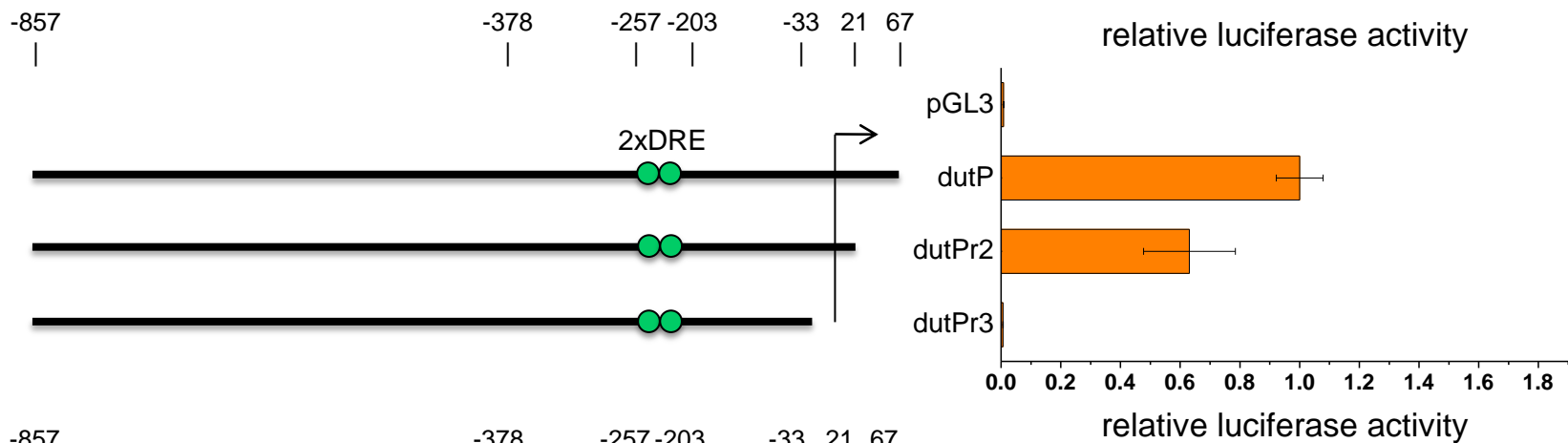
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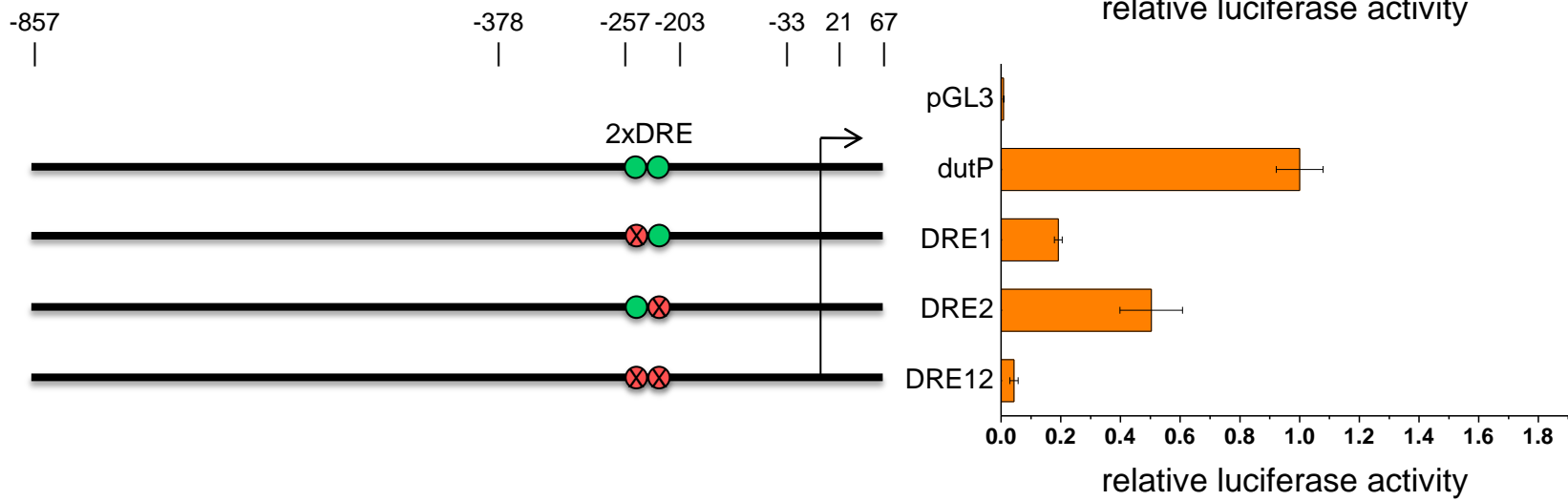
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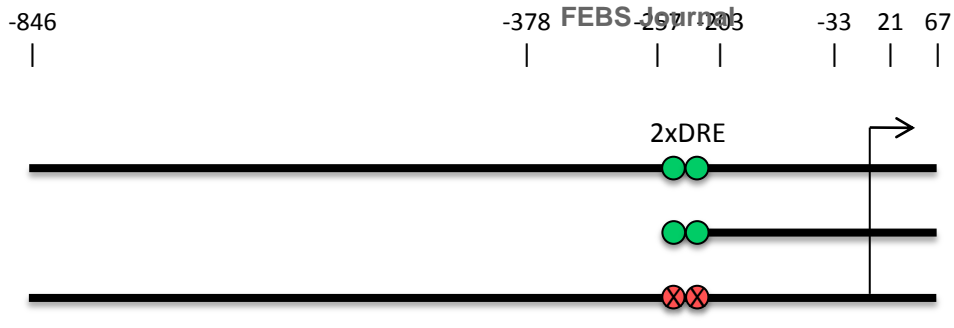


B



C



A

Number of strains

<i>dutP</i>	8
<i>dutP2</i>	9
DRE12	6

B3rd stage larvae

adult

embryo

CNS

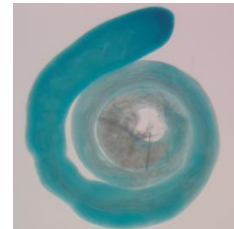
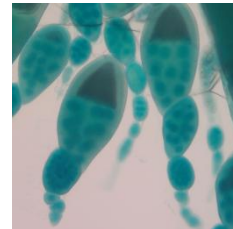
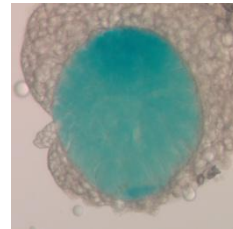
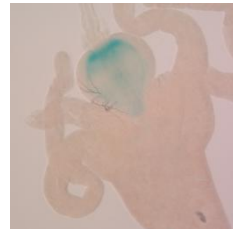
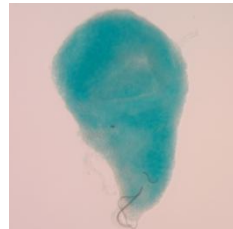
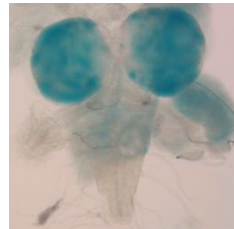
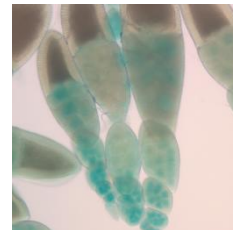
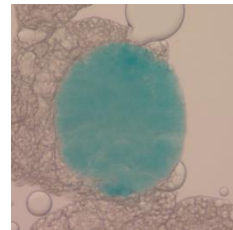
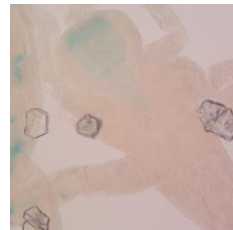
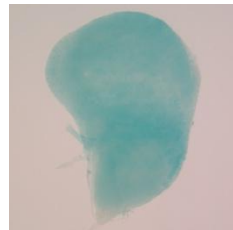
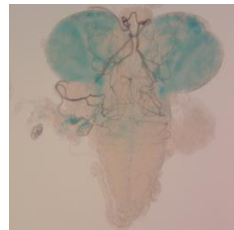
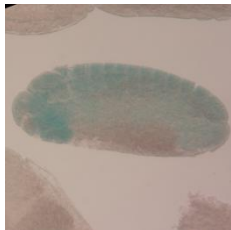
imaginal disc

ventriculus

testis
primordium

ovary

testis

dutP*dutP2*

DRE12

