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THE DOWNREGULATION OF THE *MINIATURE* GENE DOES NOT REPLICATE *MINIATURE* LOSS-OF-FUNCTION PHENOTYPES IN *DROSOPHILA MELANOGASTER* WING TO THE FULL EXTENT

During maturation Drosophila wing epithelial cells undergo number of changes due to processes, which take place in the wing of the newly emerged fly, among which epithelial-to-mesenchymal transition (EMT) and apoptosis are pivotal. It is considered that neurohormone bursicon is responsible for their triggering. In turn, extracellular matrix protein Miniature is also essential for proper progress of apoptosis and, presumably, EMT. In accordance with our previously proposed hypothesis, Miniature and bursicon form stabilizing/accumulative complexes, which are able to diffuse freely within Drosophila wing, in such a way constitutively promoting enough concentrations of the maturation triggering signal. Here we tried to come to confirmation of our hypothesis from the other side, using UAS/GAL4 system and RNAi-silencing techniques.

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Introduction. The wing of an adult *Drosophila* fruit fly is the mostly dead structure with only vein and sensory cells remaining alive [1]. This firm and flexible flight organ is a result of maturation processes which take place in *Drosophila* wing soon after the fly eclosion from a pupal case. Epithelial-to-mesenchymal transition (EMT) and apoptosis are the two major events of the wing maturation: epithelial cells lose their contacts with each other, become round shaped and undergo programmed cell death respectively [2, 3]. The hemolymph current, generated and promoted by pumping contractions of bilateral «wing hearts», washes out cell debris from the wing cavity between dorsal and ventral layers of cuticule, secreted by underlying epidermis, and provides then unfolding and expansion of the previously folded and immature *Drosophila* wing [4]. Further fusion of these two cuticules, theirs subsequent sclerotization and melanization finalize maturation processes [1].

Binding of the neurohormone bursicon to its cognate G protein-coupled receptor Rickets on wing epithelia is considered to be the trigger of all these cellular events happening during the first two hours of insect's adult life [5, 6]. Rickets receptor activates the heterotrimeric Gs protein [3, 7], producing the Gαs-GTP subunit and the Gβγ heterodimer [8]. GTP-charged Gαs then activates the cAMP-PKA pathway responsible for induction of apoptosis [3], while the Gβγ part appears to regulate the signaling branch controlling EMT and wing expansion [9]. Additionally, tissue inhibitor of metalloproteinases, integrins, and β-catenin are implicated in *Drosophila* wing maturation [10, 11].

On the other hand, the X chromosome-linked *miniature* gene seems to play not the last role only in all these processes indicated above, but it is also involved in wing morphogenesis in general. Thus, *miniature* mutant wings are 1.5 fold smaller than wild-type ones, but the number of epithelial cells is definitely still the same in both [12, 13]. This phenomenon can be explained by the fact that in mutants at early developmental stages, initially columnar epithelial cells of the wing do not expand in horizontal plane and so do not flatten, in contrast to what they should normally do. Among other phenotypes, also wing hairs disorientation and presence of visible cell outlines are described [13, 14], moreover in different *Drosophila* species [15].

In our previous research it was found, that the extracellular Miniature protein is also important for proper progress of the wing maturation of *D. melanogaster*. This protein is definitely involved in apoptosis of wing epithelial cells, and presumably also in EMT: different *miniature* mutants (including m^1 loss-of-function allele) showed a delay of apoptosis in their wings normally triggered by bursicon after eclosion of the fly from a pupal case, and they also showed different deviations from normal wing expansion [16], which is used for the indirect investigation of EMT [9].

A hypothesis in which Miniature traps bursicon in extracellular matrix (ECM), increasing its concentration or longevity of its presence for higher activation of wing maturation processes was proposed: Miniature protein creates a «sink» of the neurohormone in ECM, which can then diffuse freely triggering signaling in any possible direction [17]. So Miniature acts cell-autonomously, but also to a certain extent in a non-autonomous manner within the wing [16].

Trying to make our proposed hypothesis more relevant and more attractive, in this particular research we decided to address the question from the other side.

Materials and methods. The following *Drosophila* lines were used: *hh-Gal4* [18], *UAS-RNAi-miniature* and *UAS-Dicer* from Vienna *Drosophila* RNAi Center, Canton-S as a control (Bloomington *Drosophila* Stock Center). All crosses were performed at 25 °C.

Adult wings were mounted as described [19]. For fluorescence labeling, 2h post-eclosion wings were fixed, stained and mounted as described [16].

Results and discussion. To manipulate Miniature expression in *Drosophila* wing we used *UAS-RNAi-miniature* [20] construct and *hh-Gal4* driver to downregulate *miniature* function in the posterior compartment of the wing, in contrast to our previous attempts to overexpress it in the same area.

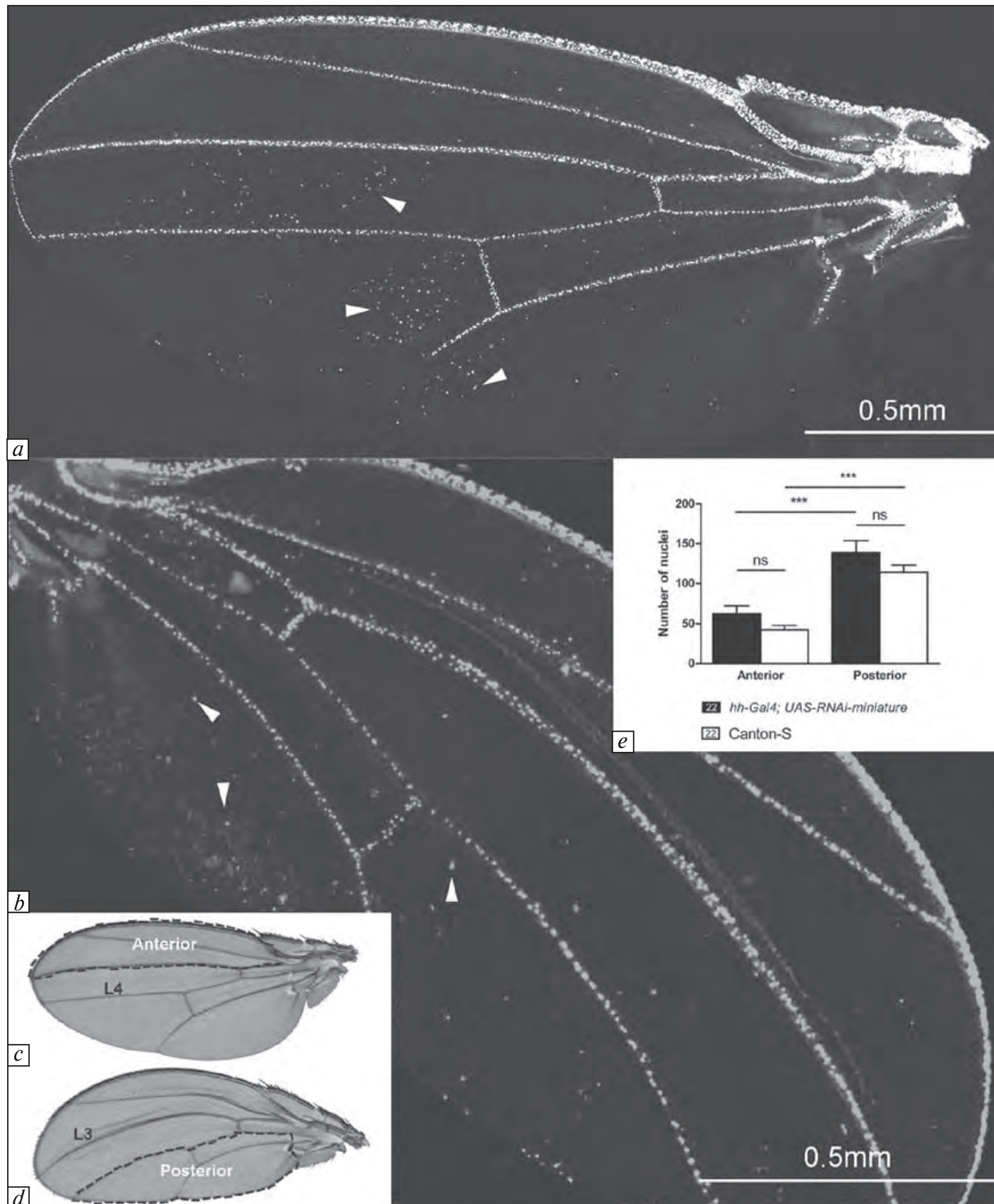
This time we did not try to rescue *miniature* phenotype, but mimic it [16]. Such local expression of *UAS*-constructs permitted us to compare resulting phenotypes between two compartments (one of which served as the internal control) within one single wing.

Obtained phenotype was weaker than that of the null m^1 allele (for example, cell outlines visible under light microscope in m^1 wings could not be seen in the *hh-Gal4; UAS-RNAi-miniature* wings). However, certain *miniature* phenotypes were eventually replicated by the expression of the *RNAi-miniature* construct. Specifically, wings of these flies were curved in the anterior-posterior direction apparently due to cross-sectional size reduction of posterior cells (Figure, *b*, *d*), orientation of wing hairs was also disturbed in that region (data not shown).

Next, we stained *hh-Gal4; UAS-RNAi-miniature* and Canton-S (control) wings at 2 h post-eclosion time point with DAPI and separately analyzed the anterior and posterior compartments by fluorescence microscopy (Figure, *a*, *b*). To do this we visually divided each wing into two rather certain areas: an anterior compartment – the region between veins L1 and L3, and a posterior compartment – the region between vein L4 and the posterior margin of the wing. So the region between veins L3 and L4 (middle-wing area) was excluded from the evaluation analysis to avoid overlapping of DAPI staining during calculations (Figure, *c*, *d*).

The number of remaining nuclei in the posterior region of the *hh-Gal4; UAS-RNAi-miniature* wings was ca. two-fold higher than that of the anterior compartment. This difference was extremely statistically significant ($P << 0.0001$) by the Student *t*-test. But more or less the same proportion of remaining nuclei between these two compartments could be also observed in wings of the control flies (Figure, *e*) and was also extremely statistically significant ($P << 0.0001$). Later it became clear that, this obtained proportion was due to almost the same difference in sizes of analyzed areas of *Drosophila* wing: the posterior compartment was ca. two-fold bigger than the anterior one as well. But in case of *hh-Gal4; UAS-RNAi-miniature* wings you should not be confused by their appearance, just keep in mind that we observe the reduction of the cell size, but not of their number [12, 13].

So then, we compared anterior and posterior compartments separately in pairs *hh-Gal4; UAS-RNAi-miniature* – Canton-S. This operation revealed no statistically significant differences between analyzed pairs (Figure, *e*) that forced us to find the possibility to enhance targeted expression of the *RNAi-miniature* construct.



Apoptosis levels in *hh-Gal4; UAS-RNAi-miniature* and wild-type wings. Wings were stained by DAPI (the signal is indicated by arrowheads) at 2 h post-eclosion time point (*a, b*): Canton-S (*a, c*) and *hh-Gal4; UAS-RNAi-miniature* (*b, d*). Each wing was visually divided into two compartments (indicated by the dashed line as an example, and the name of the nearest vein as a landmark) and then analyzed (*c, d*). Number of nuclei was calculated and then compared between two wing compartments within one analyzed group (the level of the statistical significance is indicated by asterisks) and between corresponding compartments of each analyzed group (n.s. means non-significant: $P > 0.05$). Number of analyzed wings is indicated in squares below the graph. *P*-value was evaluated by the Student *t*-test (*e*)

Drosophila Dicer is important molecule in different silencing pathways [21]. It, for instance, cleaves double-stranded RNAs into small interfering RNAs, thus increasing efficiency of RNAi silencing altogether [22]. Simultaneous targeted expression of *UAS-RNAi-miniature* and *UAS-Dicer* could help us to reach a desired result: the replication of the m^1 loss-of-function allele phenotype in a particular wing compartment. But the only one creature who survived expressing these both structures could not say us anything.

Summarizing all obtained information we can conclude that the downregulation of the *miniature* gene does not replicate *miniature* loss-of-function phenotypes in *D. melanogaster* in the full extent. But if we try to analyze why, we can come to exciting inferences. Of course, the one explanation of the occurrence of this failure could be because RNAi silencing approach is not ideal and some RNAi constructs can have a low efficiency of the binding to the mRNA of the targeted gene. But anyway we still had at least partial replication of the m^1 mutant phenotype in the region downregulating *miniature* expression, which means that this particular RNAi construct is effective. It allowed us to make careful, but a much more interesting assumption.

If we address to our previous hypothesis again which propose that Miniature can serve as stabilizer of the neurohormone bursicon maintaining its active concentration and providing its diffusion in and through the wing tissue during its maturation [16], this last observation could confirm the «sink» hypothesis in some extent. In this particular experiment *miniature* expression was downregulated in the posterior wing compartment, while in anterior its levels is considered to be normal. According to our hypothesis, Miniature-bursicon expressed-produced complexes in the anterior compartment could freely diffuse into the posterior one thus providing partially normal progress of apoptosis also in that region. And that's why apoptosis in *hh-Gal4; UAS-RNAi-miniature* wings eventually reaches almost the same levels as those in control ones.

Regarding that fact that every RNAi-constructs obtained from VDRC is previously carefully tested and though presumed to work well, we inclined to the last assumption confirming our «sink» hypothesis.

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СНИЖЕНИЕ УРОВНЯ ЭКСПРЕССИИ ГЕНА *MINIATURE* НЕ ВОСПРОИЗВОДИТ ФЕНОТИПЫ СВОЕГО НУЛЕВОГО АЛЛЕЛЯ В КРЫЛЕ *DROSOPHILA MELANOGASTER* В ПОЛНОЙ МЕРЕ

Клетки эпителия крыла дрозофилы во время матурации претерпевают ряд изменений как следствие происходящих в это время в крыле мухи процессов, ключевыми из которых являются эпителиально-мезенхимальный переход (ЭМП) и апоптоз. Считается, что нейрогормон бурсикон ответствен за их запуск. В свою очередь белок внеклеточного матрикса Miniature также необходим для успешного прохождения апоптоза и, вероятно, ЭМП. Согласно предложенной нами ранее гипотезе Miniature и бурсикон формируют стабилизирующие/накопительные комплексы, которые способны свободно диффундировать в плоскости крыла, постоянно поддерживая тем самым достаточную для запуска процессов матурации концентрацию сигнала.

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ЗНИЖЕННЯ РІВНЯ ЕКСПРЕСІЇ ГЕНА *MINIATURE* НЕ ВІДТВОРЮЄ ФЕНОТИПІВ СВОГО НУЛЬОВОГО АЛЕЛЯ В КРИЛІ *DROSOPHILA MELANOGASTER* У ПОВНІЙ МІРІ

Під час матурації клітини епітелію крила дрозофіли зазнають ряд змін за рахунок процесів, які відбуваються в цей час в крилі мухи, ключовими з яких є епітеліально-мезенхімальний перехід (ЕМП) та апоптоз. Вважається, що нейрогормон бурсикон відповідальний за їхній запуск. В свою чергу білок позаклітинного матриксу Miniature також є необхідним для успішного проходження апоптозу і, ймовірно, ЕМП. Відповідно до запропонованої нами раніше гіпотези Miniature та бурсикон формують стабілізуючі/накопичувальні комплекси, які здатні вільно дифундувати в площі крила, постійно підтримуючи тим самим достатню для запуску процесів матурації концентрацію сигналу.

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