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The *Drosophila* nuclear receptors EcR and ERR jointly regulate the expression of genes involved in carbohydrate metabolism

Elena V. Kovalenko[1], Marina Yu Mazina[1], Aleksey N. Krasnov, Nadezhda E. Vorobyeva*

Institute of Gene Biology, Russian Academy of Sciences, Moscow, 119334, Russia

ABSTRACT

The rate of carbohydrate metabolism is tightly coordinated with developmental transitions in *Drosophila*, and fluctuates depending on the requirements of a particular developmental stage. These successive metabolic switches result from changes in the expression levels of genes encoding glycolytic, tricarboxylic acid cycle (TCA), and oxidative phosphorylation enzymes. In this report, we describe a repressive action of ecdysone signaling on the expression of glycolytic genes and enzymes of glycogen metabolism in *Drosophila* development. The basis of this effect is an interaction between the ecdysone receptor (EcR) and the estrogen-related receptor (ERR), a specific regulator of the *Drosophila* glycolysis. We found an overlapping DNA-binding pattern for the EcR and ERR in the *Drosophila* S2 cells. EcR was detected at a subset of the ERR target genes responsible for carbohydrate metabolism. The 20-hydroxyecdysone treatment of both the *Drosophila* larvae and the S2 cells decreased transcriptional levels of ERR targets. We propose a joint action mode for both the EcR and ERR, for at least a subset of the glycolytic genes. We find that both receptors bind to the same regulatory regions and may form or be part of a joint transcriptional regulatory complex in the *Drosophila* S2 cells.

1. Introduction

Maintaining an appropriate level of glucose in the body is a necessary requirement of homeostasis. An organism reacts acutely to any change in the glucose concentration, regulating the efficiency of its metabolism by various organs. For instance, during a prolonged hypoglycemia, the human body reduces glucose consumption by the peripheral tissues, suppressing glycolytic flux in them, to conserve glucose for the brain (Mergenthaler et al., 2013; Youn and Buchanan, 1993). For a coordinated regulation of the carbohydrate metabolism in various tissues, animal body uses several signaling pathways, the components of which are evolutionarily well conserved (Guo et al., 2012; Mattila and Hietakangas, 2017).

A distinctive feature of the carbohydrate metabolism regulation in *Drosophila* is that during development, the metabolic state of the animal changes dramatically. In the first hours of development, the embryo is in a fairly frugal state of metabolism, using the maternally provided glycogen stock. At the very end of embryogenesis, there is a strong transcriptional activation of enzymes responsible for glycolysis, the TCA cycle, and oxidative phosphorylation. This event was termed the embryonic metabolic transition (EmbMT) (Tennessen et al., 2014). Active growth and locomotor activity of larvae require an increased rate of carbohydrate metabolism, with a special role for glycolysis at this stage of development. During this stage, all carbohydrate metabolic enzymes are expressed at high levels, and the increased activity of glycolytic enzymes results in the accumulation of lactate in the body (Tennessen et al., 2011). Later on, during metamorphosis, *Drosophila* has to go through another switch, this time to a more economical metabolic mode, with a substantial transcriptional repression of all glycolytic enzymes, and at this stage the fly gets most of its energy from lipids (Merkey et al., 2011). *Drosophila* metamorphosis ends with the adult eclosion, accompanied by another round of activation of carbohydrate metabolism (Barry and Thummel, 2016).

A long-term change in the intensity of carbohydrate metabolism requires a robust cellular response, including changes in the activity of many genes. In *Drosophila*, two DNA-binding protein complexes are known that primarily control transcriptional regulation of the carbohydrate metabolism: the Mondo-Mlx and HNF4 complexes (Mattila and Hietakangas, 2017). The first is responsible for the ability of *Drosophila* to withstand an increased dietary intake of carbohydrates, affecting the transcription of glycolytic enzymes in the fat body (Havula et al., 2013). The latter, in addition to regulating glycolysis, is able to activate transcription of genes responsible for the TCA (tricarboxylic acid) cycle and expression of oxidative phosphorylation enzymes in the fat body and insulin-producing cells (Barry and Thummel, 2016). Recently, it was found that the estrogen-related receptor (ERR) can specifically regulate transcription of the glycolytic genes (Tennessen et al., 2011). The tissue specificity of this transcriptional regulator is distinct from the Mondo-Mlx and HNF4 complexes. Tissue-specific overexpression of the ERR protein in *err* mutants restores the expression of glycolytic

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* Corresponding author.

E-mail address: vorobyeva@genebiology.ru (N.E. Vorobyeva).

[1] These authors contributed equally to this work.

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Table 1

ERR binds to regulatory regions of glycolytic genes and enzymes of carbohydrate metabolism in *Drosophila* S2 cells. Genes with peaks of ERR in their promoters or inside coding regions identified by ChIP-Seq from *Drosophila* S2 cells stably expressing 3xFLAG-ERR. Genes are listed according to the intensity of ERR binding signal. ChIP-Seq FLAG-ERR values are given as a number of reads at peak position. Enrichment fold was calculated in relation to the ChIP-Seq signal obtained with the anti-FLAG antibody in untreated *Drosophila* S2 cells. Only ERR binding sites with an enrichment of 3-fold and higher are shown. The right-most column presents the decrease in transcription of the corresponding genes in ERR-deficient second instar larvae relative to controls [data from (Tennessen et al., 2011)]. Genes are color-coded as green (glycolytic), orange (glycogen metabolism), blue (other metabolic pathways), and grey (an insulator-binding protein).

	ChIP ERR-FLAG peak position in S2 cells (dm6 genome release)	ChIP-Seq FLAG-ERR value	The gene corresponding to the peak position	The symbol of the gene	Function of the gene	ChIP-Seq ERR-FLAG enrichment fold in S2 cells	ChIP EcR-FLAG enrichment fold in S2 cells	Transcription decrease fold in dERR(-) mutant larvae (from Tennessen et al, 2011)
1	3R:29148302	560.23	*phosphoglyceromutase 78*	*pglym78*	glycolysis	9.8	2.9	3.3
2	2L:2130368	530.25	*glycogen phosphorylase*	*glyp*	glycogen metabolism	6.8	2.4	2.2
3	2L:2748584	397.22	*phosphoglycerate kinase*	*pgk*	glycolysis	16.8	-	3.7
4	2R:16139078	327.90	*glutamate oxaloacetate transaminase 1*	*got1*	glutamate biosynthesis	3.3	3.2	-
5	X:15868134	292.30	*glyceraldehyde 3 phosphate dehydrogenase 2*	*gapdh2*	glycolysis	21.7	-	3.1
6	3R:30134233	281.05	*triose phosphate isomerase*	*tpi*	glycolysis	7.0	-	3.1
7	3R:26261509	266.06	*aldolase 1*	*ald1*	glycolysis	11.3	9.8	3.5
8	2R:8913993	221.10	*phosphoglucose isomerase*	*pgi*	glycolysis	7.3	-	5.3
9	2R:7530934	215.47	*death resistor Adh domain containing target*	*drat*	NADH binding	3.6	2.7	-
10	2L:1727562	211.73	*enolase*	*eno*	glycolysis	3.9	-	1.9
11	2R:10111033	198.61	*phosphofructokinase*	*pfk*	glycolysis	5.9	5.2	7
12	3R:26259191	192.99	*aldolase 1*	*ald1*	glycolysis	4.4	-	-
13	2R:13171745	191.12	*1,4-Alpha-Glucan Branching Enzyme*	*AGBE*	glycogen metabolism	5.2	4.9	2.2
14	2R:7791407	174.25	*glyceraldehyde 3 phosphate dehydrogenase 1*	*gapdh1*	glycolysis	12.9	-	2.1
15	X:9585723	157.39	*hexokinase A*	*hexA*	glycolysis	5.2	4.7	1.7
16	2R:21312620	153.64	*CG9485*	*CG9485*	glycogen metabolism	15.2	-	4.1
17	2L:1728228	149.90	*enolase*	*eno*	glycolysis	5.6	-	-
18	2L:18974198	146.15	*L-2-hydroxyglutarate dehydrogenase*	*L2HGDH*	glycolysis	14.5	-	1.8
19	2R:7457663	138.65	*myo-inositol-1-phosphate synthase*	*inos*	lipid signaling	3.4	4.9	-
20	2L:4197673	133.03	*nicotinate phosphoribosyltransferase*	*naprt*	NAD biosynthesis	5.6	-	1.9
21	3L:16128703	127.41	*phosphoglucose mutase 1*	*pgm1*	glycogen metab	3.4	-	3.9
22	3L:5188852	125.54	*alan shepard*	*shep*	insulators activity	3.1	4.3	-
23	X:10784443	121.79	*stress-sensitive B*	*sesB*	metabolic carrier	3.3	7.2	-

enzymes in muscles and epidermis, but has only a minor effect on their expression in the fat body. It is the ERR that bridges the regulation of carbohydrate metabolism and the response to hypoxia (Li et al., 2013).

The presence of several metabolic switch points during *Drosophila* development suggests an existence of dedicated regulatory control systems (Tennessen and Thummel, 2011). The best known and best studied system for controlling *Drosophila* development is the ecdysone signaling pathway (Yamanaka et al., 2013). Recently, ERR has been shown to counter ecdysone signaling by reducing vitellogenin expression in females of the silkworm *Bombyx mori* (Shen et al., 2018). However, in other tissues and stages of insect development, the relationship between ecdysone and ERR signaling remains poorly understood. In this work, we investigate interactions between the ecdysone receptor EcR and ERR of *Drosophila* in transcriptional regulation of common target genes. We identify a possible role of ecdysone signaling in the transcription of carbohydrate metabolism genes which are targets of ERR.

2. Materials and methods

2.1. Treatment of Drosophila S2 cells and larvae

Drosophila Schneider cell line 2 (S2) cells were maintained at 25 °C in ecdysone-free Schneider's insect medium (Sigma) containing 10% FBS (HyClone). The treatment of S2 cells with 20-hydroxyecdysone (20E) (H5142, Sigma-Aldrich) was performed at a final concentration of 0.3 μM, as was described previously (Mazina et al., 2015, 2018). Treatment of *Drosophila* larvae was performed in PBS/0.1% NP40 containing 20E at 1 μM final concentration, or an equal volume of DMSO solvent (1 μM 20E concentration was used to compensate for a less effective hormone penetration into the living tissue, comparatively to the cell culture). For the treatment, L3 larvae corresponding to the puff stage 1 (dark blue gut) were collected by culturing larvae in the fly media supplemented with 0.05% bromophenol blue to mark the guts of feeding animals. Collected animals were rinsed three times with PBS and cut along the body axis with a dissecting needle before incubation with 20E or solvent (DMSO).

For the stable expression of 3xFLAG-ERR, ERR-HA, and 3xFLAG-EcR proteins, S2 cells were transfected with the corresponding expression plasmids (all nuclear receptors were placed under the Act5C promoter) and the pCoBlast plasmid, taken at a 20 to 1 M ratio using the Effectene Transfection Reagent (Qiagen). Polyclonal cell lines bearing stably integrated expression constructs were selected and maintained subsequently in Schneider medium supplemented with Blasticidin (Sigma-Aldrich) at a final concentration of 20 μg/mL.

2.2. ChIP-Seq

The chromatin immunoprecipitation (ChIP) was performed exactly as previously described (Vorobyeva et al., 2013). All primers used for the analytical amplification with RT-PCR are listed in Table S1. ChIP-Seq libraries were obtained using the NEBNext DNA library preparation kit (New England Biolabs). Only the library fragments of 200–500 bp were subjected to NGS sequencing. Bowtie2 was used to map the reads on the Release 6 of the *Drosophila* genome (Langmead and Salzberg, 2012). For each of the ChIP-Seq libraries, we obtained approximately 10 million mappable reads. The Galaxy-P platform was used for analysis of ChIP-Seq data (Afgan et al., 2018). Genome-wide profiles (WIG files) of ERR and EcR were generated with Spp program. Genome-wide profile of ChIP-Seq with an FLAG antibody (F1804, Sigma-Aldrich) on the untreated *Drosophila* S2 cells was used as an input. Peak calling was performed on WIG files with a custom C# program. A peak was considered to be at the maximal value for ERR or EcR signal in 500bp window, if it satisfied the following conditions: signal/input ratio greater than 1.5, and signal value exceeded the Input mean + Input stddev. BigWig files were generated using wigToBigWig program. Tables S2 and S3 contain lists of FLAG-ERR and FLAG-EcR peaks with enrichment relative to FLAG S2 ChIP-Seq over 2. Peak list in Table 1 contains FLAG-ERR ChIP-Seq peaks with enrichment over 3. All obtained ChIP-Seq data were deposited to the Gene Expression Omnibus (GEO accession GSE122810).

2.3. Nuclear extracts and immunoprecipitation

The nuclear fraction was obtained after a 15-min incubation of cells in a low-salt buffer (20 mM Hepes KOH, pH 7.9, 5 mM $MgCl_2$, 10 mM

KCl, protease inhibitor cocktail (Roche)), followed by centrifugation at 700*g* for 7 min, as described previously (Mazina et al., 2018). The nuclear fraction was incubated in the Lysis buffer (20 mM Hepes KOH, pH 7.9, 5 mM $MgCl_2$, 100 mM KCl, 0.1% NP40, DNase I (ThermoScientific), protease inhibitor cocktail (Roche)), for 30 min at 4 °C and subjected to two rounds of ultrasonication, 10 s each. Nuclear extracts were centrifuged for 20 min at 14,000 g and used for immunoprecipitation with antibodies bound to MabSelect sepharose (GE Healthcare). After 2 h of incubation at 4 °C on a rotating platform, IPs were washed three times with the Lysis buffer, eluted with the PAGE loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol), and analyzed by western blots. Antibodies against FLAG (F3165) and HA (H6908) epitopes were purchased from Sigma-Aldrich. An anti-EcR antibody was raised in rabbits against an antigen corresponding to amino acids Ile-691 to Ala-878 of the EcR-B isoform (NM 165465) and were affinity purified with the antigen prior to use. The anti-EcR antibody was tested in immunoprecipitation experiments with or without EcR depletion by RNA interference (Fig. S1). We found that this anti-EcR antibody specifically precipitated only the EcR-B isoform from the *Drosophila* S2 cell protein extract (Fig. S1).

3. Results

3.1. The ERR nuclear receptor is a specific regulator of glycolysis and glycogen metabolism

The role of ERR in regulating the expression of genes that control carbohydrate metabolism in *Drosophila* has been demonstrated previously (Tennessen et al., 2011). It was shown that in *err* mutant larvae there is a very strong decrease in the transcription levels of genes responsible for glycolysis and carbohydrate metabolism. To study the precise role of ERR in the transcriptional regulation of these genes, we performed a search for the binding sites of ERR in the *Drosophila* S2 cells. We expressed the ERR protein tagged with a triple FLAG epitope, and performed ChIP with an anti-FLAG antibody, followed by whole genome sequencing. As a control experiment, we carried out immunoprecipitation in the control S2 cell line, which had no FLAG-tagged ERR protein, using the anti-FLAG antibody. The obtained data were analyzed, and the enrichment of the ChIP FLAG-ERR signal was calculated relative to ChIP FLAG in the control S2 cells. The calculated peaks are listed in the Table S2. The peaks with the highest enrichment scores are given in Table 1, they are listed in the order of decreasing ChIP value. Table 1 shows that ERR peaks with the highest ChIP enrichment value are located on promoters or in the coding regions of glycolytic and glycogen metabolism genes. Interestingly, the FLAG-ERR peaks are present in the regulatory regions of 10 enzymes involved in glycolysis (out of the 12 glycolytic enzymes). These results confirm previously obtained data on the role of ERR in the transcriptional regulation of genes involved in carbohydrate metabolism (Table 1, right column) (Tennessen et al., 2011). We have not found any ERR peaks at the regulatory regions of the glycolytic genes *lactate dehydrogenase* (*ldh*) and *pyruvate kinase* (*pyk*), which may be due to limitations of the ChIP-Seq analysis based on FLAG-tag precipitation. We indeed found a high level of FLAG-ERR binding at the *ldh* promoter, but ChIP-Seq data with the anti-FLAG antibody in the control *Drosophila* S2 cells also showed an increase in the signal at the same region.

Interestingly, transcription of genes responsible for carbohydrate metabolism is upregulated several times during *Drosophila* development. As an example, Fig. 1A shows the transcriptional pattern of the *aldolase 1* (*ald1*) gene. Other glycolysis genes have similar transcriptional patterns (Graveley et al., 2011). The initial transcriptional activation of glycolysis genes during embryogenesis is determined by the appearance of their regulator, the ERR protein at the corresponding stages of development (Tennessen et al., 2011). At present, it is not clear what regulates the decrease in the transcriptional levels of these genes during transition of larvae to metamorphosis.

In our previous work, we have found that ERR is involved in the transcriptional regulation of the *hormone receptor* genes *hr3* and *hr4* of the ecdysone cascade in *Drosophila* S2 cells (Mazina et al., 2017). We have shown that overexpression of these receptors leads to repression of the ecdysone response genes in the absence of 20E, as well as to a higher level of activation after 20E treatment (Mazina et al., 2017). Here, we hypothesize that EcR could participate in regulation of the ERR targets, which are genes involved in carbohydrate metabolism. To test this hypothesis, we expressed the FLAG-tagged EcR protein in *Drosophila* S2 cells and performed ChIP, followed by the whole genome sequencing. The EcR binding sites we have identified in S2 cells are given in Table S3. We compared the obtained FLAG-EcR ChIP-Seq data with the peaks determined by the FLAG-ERR ChIP-Seq (Table 1). Only the FLAG-ERR and FLAG-EcR peaks showing 3-fold and higher enrichment were taken for analysis. We found that about one-third of the FLAG-ERR binding sites also contained FLAG-EcR peaks (Fig. 1B–D). The FLAG-EcR ChIP-Seq signal revealed a significant accumulation at FLAG-ERR sites (Fig. 1C). It appears that many of the ERR-dependent genes can indeed be regulated by ecdysone signaling as well.

3.2. 20E represses transcription of ERR-dependent genes

To identify the role of ecdysone signaling in the regulation of transcription of the ERR-dependent genes, we induced *Drosophila* S2 cells with 0.3 μM 20E and measured transcription of the corresponding genes 1 h and 14 h after treatment with 20E (Fig. 2A). We found that incubation of the cells with 20E led to a decrease in the level of transcription of 10 out of 11 top ERR target genes listed in Table 1. For two genes, *death resistor Adh domain containing target* (*drat*) and *ald1*, the level of repression was 2-fold and more. Previously, Stoiber with colleagues studied the ecdysone response in 41 different *Drosophila* cell lines (Stoiber et al., 2016). We used their data to investigate the role of 20E in the regulation of the 11 top ERR-dependent genes from the list provided in Table 1. The transcription of all 11 top ERR-dependent genes was found to be repressed after the treatment with 20E, in various cell lines studied by the authors (Stoiber et al., 2016). Transcription of genes encoding *Phosphoglyceromutase 78 (pglym78)*, *Glycogen phosphorylase* (*glyp*), *Phosphofructokinase* (*pfk*), *Phosphoglucose isomerase* (*pgi*), *Glyceraldehyde 3 phosphate dehydrogenase 2* (*gapdh2*), and *ald1* was reduced in 11, 25, 7, 5, 6, and 12 *Drosophila* cell lines, respectively, upon 20E treatment. None of these genes showed an increase in their transcription after addition of 20E. Only 1 out of 11 top ERR-dependent genes showed an increased transcription level upon 20E treatment, and only in 1 out of the 41 tested *Drosophila* cell lines. Transcription of the *err* gene itself did not change upon 20E treatment in any of the 41 tested *Drosophila* cell lines (Stoiber et al., 2016). Based on these data, we conclude that the repression of carbohydrate metabolism genes following treatment with 20E, is a common effect not limited to only one type of *Drosophila* cell lines.

3.3. EcR and ERR co-immunoprecipitate from Drosophila S2 cells

After confirming our hypothesis about participation of ecdysone signaling in the regulation of transcription of the ERR target genes, we speculated that EcR could act as an ERR functional partner, working in a joint protein complex. To test this assumption, we generated a polyclonal *Drosophila* S2 cell line stably expressing EcR and ERR proteins tagged with FLAG and HA epitopes, respectively. In immunoprecipitation experiments, we were able to demonstrate an interaction between FLAG-EcR and ERR-HA (Fig. 2B). We found that this interaction does not depend on the presence of 20E in the cell medium. Since DNaseI was added to the proteins precipitated from the nuclear extract, we believe that EcR-ERR interaction does not depend on DNA, and propose an existence of a multiprotein complex containing both EcR and ERR. Using an antibody against EcR, prepared in our laboratory, we were also able to perform a reciprocal experiment, demonstrating the

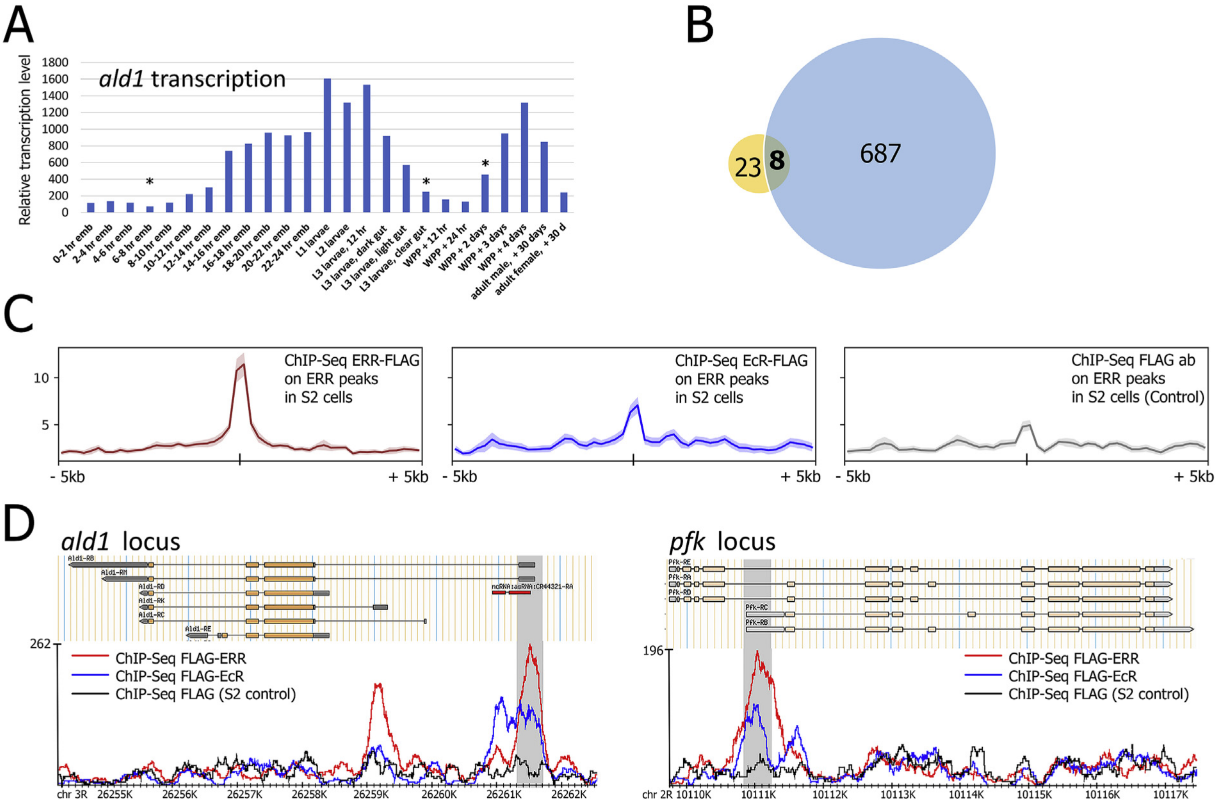

Fig. 1. EcR binds part of the ERR target sites in *Drosophila* S2 cells.

(A) Developmental transcription profile of the *ald1* gene [data from (Graveley et al., 2011)]. Asterisks mark developmental stages with high 20E titer (Lavrynenko et al., 2015).

(B) Venn diagram showing overlap between peaks obtained using ChIP-Seq in the FLAG-ERR and FLAG-EcR experiments. Only peaks with enrichment exceeding values in control S2 cells by more than 3-fold are shown.

(C) Binding profiles of FLAG-EcR and FLAG-ERR proteins at genomic loci representing ERR-binding sites listed in Table 1 (median levels are shown). Shadows of the lines represent statistical error, which was calculated as a standard deviation. The coordinates of the X-axis represent genomic region 5000 bp up- and downstream of the ERR-binding sites. The coordinates of the Y-axis represent the number of reads (normalized to the size of the *Drosophila* genome).

(D) Binding profile of FLAG-ERR (red) and FLAG-EcR (blue) proteins at *ald1* and *pfk* genomic loci obtained in ChIP-Seq experiments. The coordinates of the X-axis correspond to the dm6 release of the *Drosophila* genome. The Y-axis represents the number of reads. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

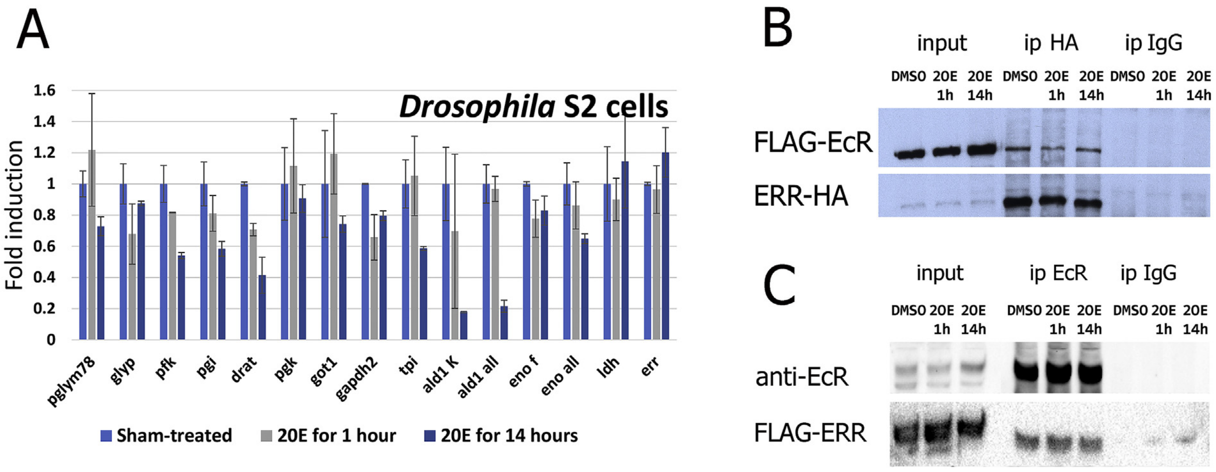

Fig. 2. 20E represses transcription of ERR target genes, and EcR is part of an ERR-containing protein complex

(A) Transcription levels of the ERR target genes in *Drosophila* S2 cells after treatment with 20E as assessed by qRT-PCR. S2 cells were sham-treated with DMSO or exposed to 0.3 μM 20E for 1 or 14 h. The Y-axis units represent fold induction relative to the sham-treated state. Data are mean values from three independent experiments, error bars represent standard deviations.

(B) Immunoprecipitation from nuclear lysates of *Drosophila* S2 cells stably co-expressing FLAG-EcR and ERR-HA after 1 and 14 h of treatment with 20E. Sham-treated (DMSO) S2 cells were taken as a control. Immunoprecipitations were performed with an anti-HA (ip HA) antibody or a serum of non-immunized rabbits (ip IgG).

(C) Immunoprecipitation from nuclear lysates of *Drosophila* S2 cells stably expressing FLAG-ERR after 1 and 14 h of treatment with 20E. Sham-treated (DMSO) S2 cells were taken as a control. Immunoprecipitations were performed with an anti-EcR (ip EcR) antibody or a serum of non-immunized rabbits (ip IgG).

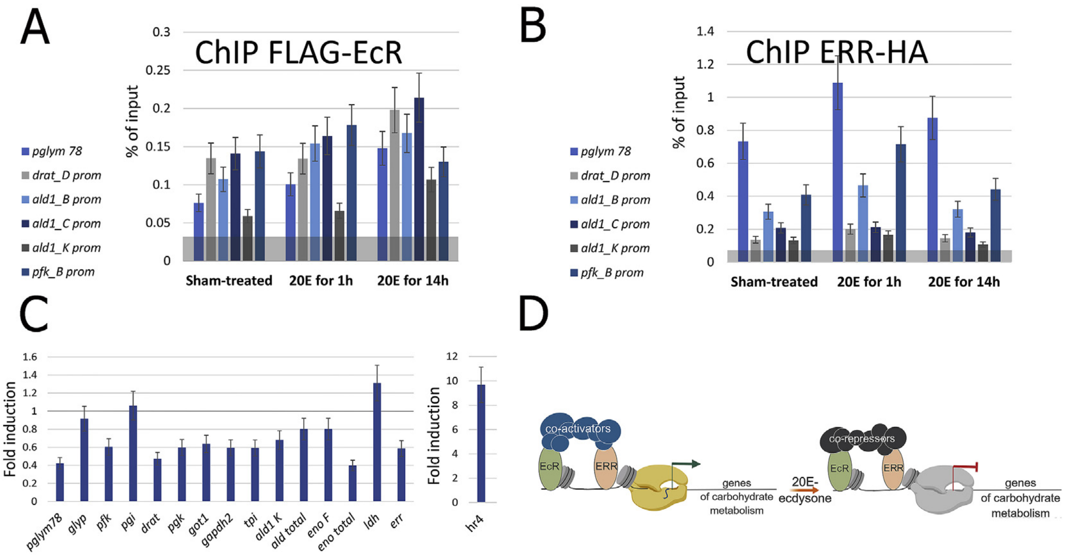

Fig. 3. 20E represses ERR-dependent genes but does not substantially affect binding of EcR or ERR to the ERR target sites
(A) FLAG-EcR and **(B)** ERR-HA binding of ERR target promoters, as estimated by chromatin immunoprecipitation from *Drosophila* S2 cells stably expressing the corresponding proteins. ChIP experiments were performed on DMSO (sham)- or 20E-treated cells. Mean values were calculated from technical repeats of the experiment; error bars represent standard deviations. All experiments were repeated independently three times. The grey area on a plot represents the background of the ChIP experiment, which was measured at the negative 1a6 genome locus.
(C) Levels of ERR-dependent gene transcription in *Drosophila* L3 larvae after treatment with 20E, as assessed by qRT-PCR. Larvae were dissected and incubated for 2 h in PBS/0.1% NP40 containing 1 μM 20E (DMSO was used for control). The Y-axis units represent fold induction relative to the sham-treated state. Mean values were calculated from technical repeats of the experiment; error bars represent standard deviations. All experiments were repeated independently three times. **(D)** Proposed model describing a mechanism of EcR participation in the regulation of ERR target gene transcription.

immunoprecipitation of FLAG-tagged ERR with the endogenous EcR protein (Fig. 2C).

3.4. *EcR and ERR bind the ERR target sites independently of 20E*

To clarify the role of EcR in the regulation of transcription of the ERR target genes (carbohydrate metabolism genes), we investigated the strength of binding of EcR and ERR in ChIP experiments, before and after treatment of the *Drosophila* S2 cells with 20E. For this experiment, we utilized the polyclonal *Drosophila* S2 cell line stably expressing FLAG-EcR and ERR-HA proteins (Fig. 3A and B). We found a significant level of binding of FLAG-EcR and ERR-HA to promoters of ERR target genes from the list given in Table 1 (*pglym78, drat, ald1,* and *pfk)* before and after treatment of *Drosophila* S2 cells with 20E. We observed a slight increase in the level of EcR binding to the studied promoters after exposure to the hormone (Fig. 3A and B). This increase in EcR binding was statistically significant, but the level of this increase did not exceed the amount initially bound to the studied sites in the S2 cells untreated with 20E. Both EcR and ERR were found to occupy promoters of the ERR target genes (*pglym78, drat, ald1,* and *pfk*) before and after the 20E treatment. This result is in agreement with our immunoprecipitation experiments, which demonstrated that EcR and ERR are working in a joint multiprotein complex, both at low and high titer of 20E. Thus, the effect of 20E on the reduction of transcription of the ERR-dependent genes may not be associated with changes in the ability of EcR or ERR to bind their targets in the genome.

3.5. *20E treatment represses transcription of the ERR target genes in larval organs*

To demonstrate that the transcriptional repression of the ERR target genes by 20E is not limited to cultured cells of embryonic origin, we investigated the effect of 20E on the tissues of the *Drosophila* 3rd instar larvae (L3 stage) (Fig. 3C). Dissected L3 stage larvae were incubated in PBS containing 1 μM 20E or equal amount of DMSO solvent. After 2 h of incubation, we collected the larvae and analyzed the total transcription level of 11 top ERR-dependent genes from the list provided in Table 1. The degree of transcriptional induction was calculated as the ratio of the transcriptional induction level in larvae treated with the hormone, to the untreated control larvae. As a positive control for our experiment, we used the *hr4* gene, which is a well-known 20E target (King-Jones et al., 2005; Mazina et al., 2018) (Fig. 3C, right panel). Transcription of 9 out of 11 tested top ERR target genes was found to be repressed in larvae by the 20E treatment (Fig. 3C). Transcription of the *glyp* and *pgi* genes remained unchanged upon the treatment. The transcriptional level of the *err* gene was reduced in larvae treated with 20E (Fig. 3C). We believe that decrease in the transcriptional level of gene coding for ERR, upon 20E treatment, cannot be the cause of repression of the ERR target genes, since the duration of the 20E treatment of larvae was rather short (2 h). The results of this experiment are consistent with the repressive effect of ecdysone signaling on the carbohydrate metabolism genes in various tissues.

4. Discussion

An equilibrium between pathways of carbohydrate metabolism (glycolysis, the Krebs cycle, and oxidative phosphorylation) is an important condition for providing the cell with energy. But in the course of malignant transformation and at certain stages of development this balance can be violated and the glycolytic activity can prevail over the Krebs cycle and oxidative phosphorylation (Chen et al., 2007; Lopaschuk and Jaswal, 2010). This phenomenon is called the Warburg effect. *Drosophila melanogaster*, at the larval stage of development, actively uses glycolysis for its rapid growth (Tennessen and Thummel, 2011). The change in the efficacy of glycolysis at individual developmental stages implies a possibility of its specific regulation (Tennessen and Thummel, 2011). For the *Drosophila* neural tissues, it was previously shown that the action of 20E during metamorphosis reduces the excessive glycolytic activity exhibited at the larval stage, by repressing the transcription of glycolytic enzymes (Homem et al., 2014). Here, we demonstrate that the observed effect is not limited to the nervous system, but is also present in cells of embryonic origin. We propose a model where EcR participates in the transcriptional repression of some of the glycolytic genes by means of interaction with ERR, a previously established regulator of the carbohydrate metabolism in *Drosophila* (Tennessen et al., 2011). While our data do not directly reveal the precise nature of the interaction between EcR and ERR, they suggest that the two nuclear receptor proteins are part of a larger transcriptional complex.

Using ChIP-Seq analysis, we have found that in the *Drosophila* S2 cells, ERR binds to the regulatory regions of 10 out of 12 genes coding for glycolytic enzymes, and to several genes involved in the glycogen metabolism. These data reinforce previous findings of ERR importance for the transcriptional control of the glycolytic genes in *Drosophila* larvae (Tennessen et al., 2011). In order to investigate participation of the ecdysone signaling in the transcriptional regulation of the ERR target genes, we performed ChIP-Seq analysis of the EcR binding in *Drosophila* S2 cells. We have found high levels of EcR binding at 4 out of 10 ERR target sites located at genes coding for glycolytic enzymes. Both EcR and ERR occupy the ERR sites before and after the 20E treatment of the *Drosophila* S2 cells. In co-immunoprecipitation experiments, we have detected interaction between EcR and ERR. While this interaction may be indirect, it appears not to depend on DNA but rather it suggests a multiprotein complex containing both EcR and ERR. Our findings are supported by experiments that demonstrate transcriptional repression of most of the ERR target glycolytic genes upon 20E treatment, both in *Drosophila* S2 cells and in larvae (Fig. 3D).

What role does 20E play in the regulation of ERR-dependent genes? It has been shown that the start of transcription of glycolytic genes in embryogenesis coincides with the appearance of the protein product of the *err* gene, which turned out to be their specific transcriptional regulator (Tennessen et al., 2011). Interestingly, the expression of glycolytic genes also correlates with a decline in the concentration of 20E that in turn induces expression of some early-late ecdysone cascade genes such as *ftz-f1* (Vorobyeva et al., 2012; Woodard et al., 1994). Based on the data presented here, we believe that EcR can act as an additional regulator of ERR target genes, by coordinating their transcription in response to 20E. This coordination can be achieved by a temporary transcriptional repression of these genes during periods of high 20E titer. In this context, it would be interesting to know if ecdysone might be important for repressing glycolytic genes during metamorphosis.

The response of different *Drosophila* tissues to 20E can vary considerably. A possible mechanism of the 20E tissue-specific action could involve cross-talk of EcR with other transcription factors. The presence of binding sites for tissue-specific transcription factors, e.g., Traffic jam and Serpent, are important for the activation of 20E-dependent enhancers in the *Drosophila* OSC and S2 cells, respectively (Shlyueva et al., 2014). It has been shown that EcR can act cooperatively with various transcriptional factors, and we suppose that EcR can interact with ERR in a tissue-specific manner. It is known that 20E represses insulin-signaling pathway (IIS) in the fat body during metamorphosis, stimulating autophagy of the organ (Colombani et al., 2005; Yamanaka et al., 2013). Based on our results, we propose a possibility of a greater involvement of EcR in metabolic control of the larval fat body, by affecting not only IIS but also ERR signaling.

In summary, our data increase the knowledge on ERR function as a specific regulator of glycolysis in *Drosophila* by providing new evidence that the ERR protein is present at the regulatory regions of most glycolytic genes. In addition, we have found the steroid hormone receptor EcR to jointly regulate glycolytic genes together with ERR. Both nuclear receptors bind the same regulatory regions of many glycolytic genes and may potentially engage in a common regulatory protein complex as suggested by their mutual immunoprecipitation in the *Drosophila* S2 cells. It will be of great interest to find out in which particular *Drosophila* tissues the EcR-ERR functional interaction takes place. Considering the tissue specificity of ERR effects on various glycolytic genes (Tennessen et al., 2011), the repressive action of ecdysone signaling on ERR targets can be restricted to specific organs or cell types. It would also be interesting to investigate a possible EcR participation in a recently discovered HIF-independent response to hypoxia, which has been found to depend on ERR during the late larval stage of *Drosophila* (Li et al., 2013).

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6. Conflicts of interest

Authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103184.

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