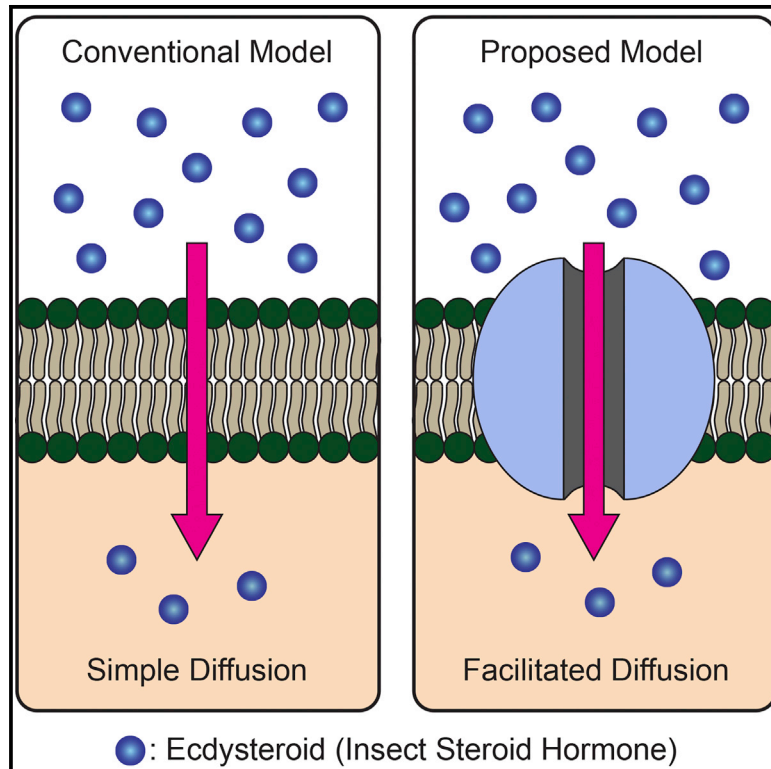


# Developmental Cell

## A Membrane Transporter Is Required for Steroid Hormone Uptake in *Drosophila*

### Graphical Abstract



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### In Brief

Steroid hormones are thought to freely enter cells by simple diffusion across lipid bilayers. Okamoto et al. provide evidence that in *Drosophila*, the insect steroid hormone ecdysone requires a membrane transporter that is a member of the evolutionarily conserved SLCO superfamily to enter receiving cells from circulation.

### Highlights

- Insect steroid hormone ecdysone requires a membrane transporter to enter cells
- Ecdysone Importer (Ecl) is a member of the evolutionarily conserved SLCO superfamily
- Ecl functions cell autonomously to incorporate ecdysone from circulation

# A Membrane Transporter Is Required for Steroid Hormone Uptake in *Drosophila*

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<https://doi.org/10.1016/j.devcel.2018.09.012>

## SUMMARY

Steroid hormones are a group of lipophilic hormones that are believed to enter cells by simple diffusion to regulate diverse physiological processes through intracellular nuclear receptors. Here, we challenge this model in *Drosophila* by demonstrating that Ecdysone Importer (*Ecl*), a membrane transporter identified from two independent genetic screens, is involved in cellular uptake of the steroid hormone ecdysone. *Ecl* encodes an organic anion transporting polypeptide of the evolutionarily conserved solute carrier organic anion superfamily. *In vivo*, *Ecl* loss of function causes phenotypes indistinguishable from ecdysone- or ecdysone receptor (*EcR*)-deficient animals, and *Ecl* knockdown inhibits cellular uptake of ecdysone. Furthermore, *Ecl* regulates ecdysone signaling in a cell-autonomous manner and is both necessary and sufficient for inducing ecdysone-dependent gene expression in culture cells expressing *EcR*. Altogether, our results challenge the simple diffusion model for cellular uptake of ecdysone and may have wide implications for basic and medical aspects of steroid hormone studies.

## INTRODUCTION

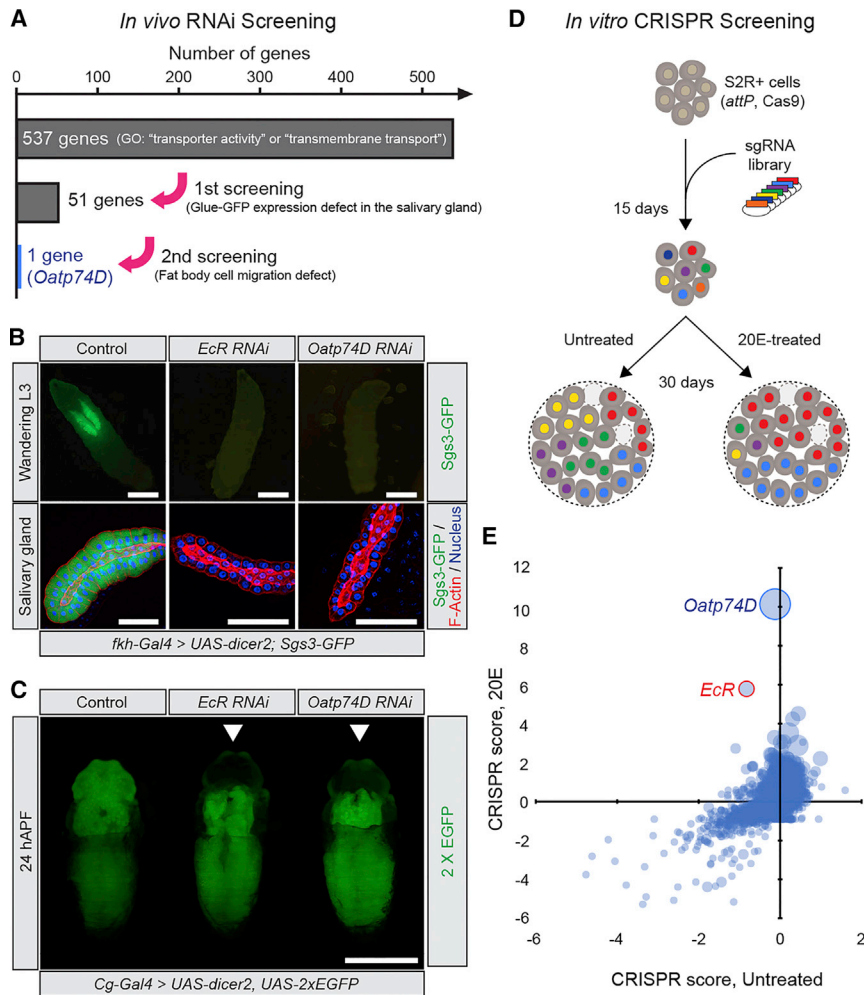
Steroid hormones are a group of lipophilic hormones that regulate diverse biological processes. In mammals, glucocorticoids are primary stress hormones that regulate immune response and energy homeostasis (Sapolsky et al., 2000; Rhen and Cidlowski, 2005; Oakley and Cidlowski, 2011), and gonadal steroids control sexual maturation and reproduction (Sisk and Foster, 2004; Wilson and Davies, 2007). They are also involved in various pathological processes, including inflammatory disorders and multiple types of cancer (Clemons and Goss, 2001; Rhen and Cidlowski, 2005; Attard et al., 2009). Genomic functions of steroid hormones are mediated by intracellular nuclear receptors, which regulate the transcription of target genes in the nucleus upon binding of steroid

ligands (Mangelsdorf et al., 1995; Nilsson et al., 2001; Mckenna and O'Malley, 2002; King-Jones and Thummel, 2005; Evans and Mangelsdorf, 2014). It is therefore important to understand how intracellular concentration of steroid hormones is regulated at the molecular level, both in physiological and pathological conditions.

Steroid hormones are highly conserved in both animal and plant kingdoms. In insects, the primary steroid hormone ecdysone (more specifically, its active form 20-hydroxyecdysone or 20E and related ecdysteroids) enters its target cells and binds to the ecdysone receptor (*EcR*), which forms a heterodimer with another nuclear receptor Ultraspiracle (*Usp*) and activates transcription of multiple genes involved in molting and metamorphosis (Thummel, 1996; Riddiford, 2000; Yamanaka et al., 2013).

It is widely accepted that lipophilic steroid hormones can freely enter and exit cells by simple diffusion across lipid bilayers (Nussey and Whitehead, 2001; Alberts et al., 2015; Urry et al., 2017). This view is supported by previous work on several mammalian steroid hormones (Plagemann and Erbe, 1976; Graff et al., 1977; Giorgi and Stein, 1981). Interestingly, a number of studies also suggested a potential involvement of membrane transporters in cellular uptake of steroid hormones (Milgrom et al., 1973; Rao et al., 1976; Pietras and Szego, 1977). However, a transporter-mediated, facilitated diffusion model of steroid hormone uptake has not been investigated thoroughly.

Recently, we demonstrated in *Drosophila* that ecdysone is released from an endocrine gland through a vesicle-mediated process, not by simple diffusion (Yamanaka et al., 2015). In the proposed model, ecdysone is loaded into vesicles at high concentrations before it is released into circulation through calcium-regulated exocytosis. As this ecdysone transport happens against the concentration gradient through an ATP-binding cassette (ABC) transporter (Yamanaka et al., 2015), our model does not address the simple diffusion model, which argues that no membrane transporter is required for steroid hormones to pass through lipid bilayers down the concentration gradient. Nonetheless, the model indicated that ecdysone can be contained within vesicles, suggesting that ecdysone cannot freely traverse lipid bilayers *in vivo*. This led us to hypothesize that cells receiving ecdysone may also require a membrane transporter(s) for its uptake from circulation, which happens down the concentration gradient.



**Figure 1. Identification of a Putative Ecdysone Importer through Two Independent Genetic Screens**

(A) Scheme of the *in vivo* RNAi screening to identify ecdysone importers. Candidate genes are listed in Table S1.

(B) Knockdown of *EcR* or *Oatp74D* causes the loss of ecdysone-dependent glue-GFP expression in salivary glands in wandering third instar larvae. *fkh-Gal4 > UAS-dicer2* was used to induce RNAi in salivary glands. Scale bars, 1 mm (upper panels), 200  $\mu$ m (lower panels).

(C) Knockdown of *EcR* or *Oatp74D* in the fat body blocks ecdysone-dependent fat body cell migration into the pupal head (arrowheads). *Cg-Gal4 > UAS-dicer2, UAS-2xEGFP* was used to induce RNAi and label cells in the fat body. hAPF, hours after puparium formation. Scale bar, 1 mm.

(D) Schematic of the *in vitro* CRISPR screening in S2R+ cells to identify sgRNAs that render resistance to 20E. Cells with mutations on genes essential for 20E-dependent cell-cycle arrest (shown in red and blue) are expected to be over-represented in the 20E-treated population.

(E) Gene-level CRISPR score distribution for the *in vitro* CRISPR screening. Computed CRISPR score (mean  $\text{Log}_2[\text{fold-change}]$  of all sgRNAs targeting the same gene) for each gene from 20E-treated versus untreated populations is plotted. Size of bubbles represents number of active sgRNAs for each gene, where active sgRNAs are defined as those conferring 20E context-specific resistance in the top 2% of all sgRNAs in the screen.

Here, we report the identification and characterization of a *Drosophila* solute carrier (SLC) transporter, which we named Ecdysone Importer (Ecl), involved in cellular uptake of ecdysone. *Ecl* encodes an organic anion transporting polypeptide (OATP), *Oatp74D*, a member of the solute carrier organic anion (SLCO) superfamily. *Ecl* is highly conserved among insects and other arthropods that utilize ecdysteroids, and its tagged protein localizes to the plasma membrane of the cells in tissues that receive ecdysone. *Ecl* mutants show phenotypes indistinguishable from ecdysone- or *EcR*-deficient animals, which cannot be rescued by 20E application but can be significantly rescued by a non-steroidal ecdysone agonist. *Ecl* is required for cellular uptake of ecdysteroids from the hemolymph *in vivo* and regulates ecdysone-dependent gene expression in a cell-autonomous manner. In *EcR*-expressing cell culture systems, *Ecl* is both necessary and sufficient for the induction of gene expression by 20E added to the media. Collectively, our results challenge the simple diffusion model of ecdysteroid transport across cell membranes, and instead suggest a transporter-mediated, facilitated diffusion mechanism. Evolutionary conservation of SLCO superfamily in metazoans may call for a reconsideration of the simple diffusion model of steroid hormone transport beyond arthropods.

## RESULTS

### Genetic Screens for Potential Ecdysone Importers

Based on the hypothesis that cells receiving ecdysone from circulation need membrane transporters to facilitate its uptake, we conducted two independent genetic screens in *Drosophila*. First, we performed an *in vivo* RNAi screening of putative transporter-encoding genes. Based on the Gene Ontology (GO) terms in FlyBase (Gramates et al., 2017), we selected 537 putative transporter-encoding genes (Figure 1A; Table S1). We specifically knocked down each gene in the salivary glands and examined the effects on ecdysone-dependent expression of a glue gene (*Sgs3-GFP*) (Biyasheve et al., 2001; Costantino et al., 2008). Knockdown of *EcR* eliminates *Sgs3-GFP* expression and reduces the size of salivary gland cells (Figure 1B) (Costantino et al., 2008). From this screen, 51 genes were found to diminish *Sgs3-GFP* expression when knocked down in the salivary glands. Next, these 51 genes were subsequently tested for their effects on ecdysone-induced fat body remodeling during metamorphosis (Cherbas et al., 2003; Bond et al., 2011). Knockdown of *EcR* in the fat body blocks ecdysone-dependent cell migration into the pupal head (Figure 1C). From this screen, we identified a single

gene, *Oatp74D* (CG7571), whose knockdown phenocopies *EcR* knockdown (Figure 1C; Table S1).

Second, as an independent approach, we applied an unbiased *in vitro* screening strategy to identify *Drosophila* genes necessary for the ecdysone response. As *Drosophila* cell lines are known to arrest the cell cycle in response to ecdysone treatment in an *EcR*-dependent manner (Koelle et al., 1991), we used a pooled CRISPR knockout screening platform in *Drosophila* S2R+ cells (Viswanatha et al., 2018) to screen for mutants that allow the cells to grow normally in the presence of 20E (Figure 1D). We screened 25,783 sgRNAs targeting 3,974 genes (group 1 and group 2 libraries in Viswanatha et al., 2018) and compared sgRNA abundance after treatment with a partially inhibitory dose of 20E (15 ng/mL; Figure S1A). Next, using next-generation sequencing, we identified multiple sgRNAs that were enriched in 20E-treated but not in untreated cells, including those targeting *Oatp74D* and *EcR* (Figures 1E, S1B, and S1C). Strikingly, *Oatp74D* was the most enriched gene after 20E treatment, followed by *EcR* (2nd) and *usp* (6th) (Figure S1D).

### **Ecl Is an Evolutionarily Conserved, Ubiquitously Expressed Membrane Transporter**

Identification of the same transporter-encoding gene from the two independent screens strongly suggested its critical role in cellular ecdysone uptake. Based on its putative function, we hereafter call this gene *Ecdysone Importer* (*Ecl*). *Ecl* is a member of the SLCO gene superfamily that comprises membrane-bound transporter proteins called OATPs that are widely present in the animal kingdom (Hagenbuch and Stieger, 2013). Studies on OATPs in fruit flies and other insects are limited, and there are few reports that describe their potential substrates (Torrie et al., 2004; Seabrooke and O'Donnell, 2013; Groen et al., 2017). Phylogenetic analysis indicated that *Ecl* has clear orthologs in insects and other arthropods that utilize ecdysteroids (Table S2; Figures 2A and 2B) (Rewitz and Gilbert, 2008; Niwa and Niwa, 2014; Ogihara et al., 2015).

*Ecl* is expressed ubiquitously with temporal fluctuations during development (Figures 2C and 2D), consistent with the systemic function of ecdysone in controlling stage-specific gene expression (Li and White, 2003; Yamanaka et al., 2013). Compared to the other seven *Drosophila* OATP-encoding genes, *Ecl* showed dominant expression in most tissues except the Malpighian tubules, in which *Oatp58Da*, *58Db* and *58Dc* are dominantly expressed (Figure S2). Silencing the other *Drosophila* OATP-encoding genes in the salivary glands and fat body showed no discernible defect (Table S3), suggesting a specific role of *Ecl* in ecdysone action. Expression of HA-tagged *Ecl* in the salivary glands and fat body confirmed its primary localization to the plasma membrane (Figure 2E), suggesting that *Ecl* indeed has important functions in transporting ecdysone across cell membranes.

### **Ecl Is Essential for the Larval Developmental Transition**

To investigate the loss-of-function phenotype of *Ecl*, we generated two independent *Ecl* null mutants (*Ecl*<sup>1</sup> and *Ecl*<sup>2</sup>) using CRISPR/Cas9-mediated mutagenesis (Figure S3). Although *Ecl* mutants show no discernible defects during embryogenesis and the first 24 hr after hatching (hAH), they show developmental arrest at the end of the first instar larval stage (Figures 3A, 3B,

and S4), suggesting a lack of ecdysone signaling required for normal molting. About 5%–10% of arrested larvae carry both first and second instar mouth hooks (Figures 3C and S4), a phenotype characteristic of defective ecdysone signaling (Schubiger et al., 1998; Gaziouva et al., 2004). Weak ubiquitous expression of *Ecl* using *armadillo* (*arm*)-*Gal4* is sufficient to rescue the developmental arrest phenotype (Figures 3 and S4), whereas strong ubiquitous overexpression of *Ecl* causes lethality during early development (Table S4), suggesting that appropriate levels of *Ecl* expression are important for normal development.

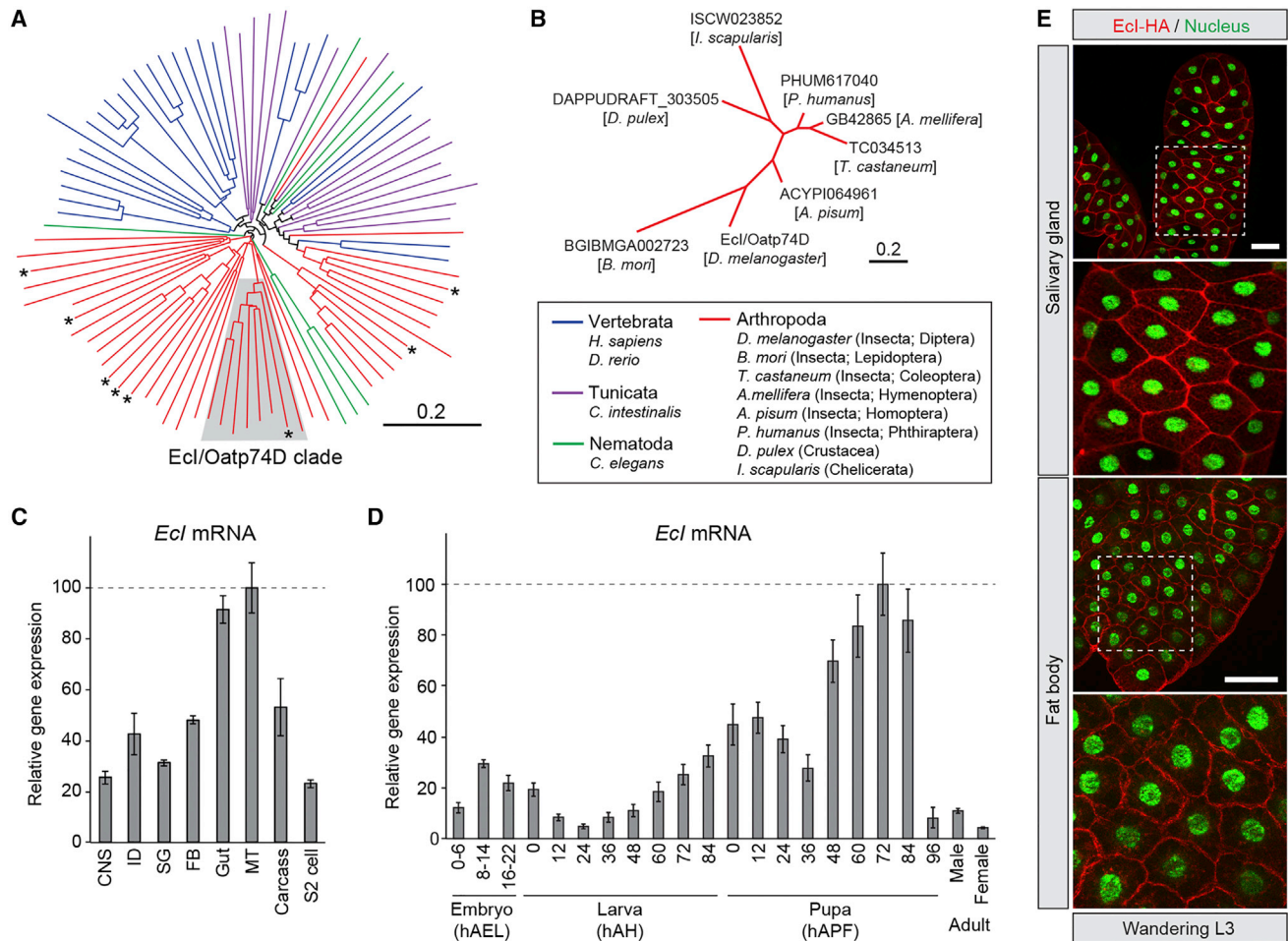
In order to further demonstrate the critical role of *Ecl* in ecdysone signaling, we attempted to rescue the larval arrest phenotype of *Ecl* mutants by oral administration of two different compounds (Figure 4A): an endogenous *EcR* ligand 20E and a non-steroidal ecdysone agonist chromafenozide (CF), which is known to bind directly to *Drosophila* *EcR* and mimic 20E effect (Minakuchi et al., 2005). We developed a transient feeding scheme for the administration of these compounds in order to mimic the endogenous ecdysone pulse during the first instar larval stage (Figure 4B). Consistent with our hypothesis that *Ecl* functions as an ecdysone importer in peripheral tissues, feeding *Ecl* mutants with 20E fails to rescue the first instar arrest (Figures 4C and 4D). We also used CF in an alternative rescue experiment, expecting that this non-steroidal, structurally different ecdysone agonist enters peripheral tissues through molecular machinery distinct from *Ecl*. Indeed, CF significantly rescues the developmental arrest phenotype (Figures 4C and 4D), suggesting that *EcR* function remains intact in *Ecl* mutants.

To confirm the step at which *Ecl* is important for ecdysone signaling, we examined the effects of 20E or CF feeding on the *Ecl* mutant with defective ecdysone production. We used one of the “Halloween” genes, which were initially identified as mutants devoid of ecdysone-dependent cuticle deposition during embryogenesis (Gilbert, 2004). The Halloween gene *phantom* (*phm*) encodes a cytochrome P450 enzyme essential for ecdysone production in the prothoracic gland, a primary steroidogenic tissue in larval insects (Niwa et al., 2004; Warren et al., 2004). Although the *Ecl* mutant phenotype resembles that of *phm* RNAi (Danielsen et al., 2016; Ou et al., 2016), the developmental arrest of *phm* RNAi larvae can be rescued by either 20E or CF application (Figure 4D). In clear contrast, only CF rescues the arrest phenotype of *phm* RNAi larvae in the *Ecl* mutant background (Figure 4D), demonstrating that *Ecl* functions downstream of ecdysone production but upstream of *EcR* action.

### **Ecl Functions Cell Autonomously and Facilitates Cellular Uptake of Ecdysteroids In Vivo**

To further examine the functions of *Ecl* *in vivo*, we analyzed ecdysone-inducible gene expression in the fat body of *Ecl* and *EcR* knockdown animals. We first confirmed that both *Ecl* and *EcR* expression levels were significantly reduced in the fat body of *Ecl* and *EcR* knockdown animals, respectively (Figure S5A). *E74A*, *E75A*, and *E75B* are ecdysone-inducible genes whose expression is directly induced by high levels of ecdysone (Karim and Thummel, 1992). When ecdysone is at its basal level (72 hAH), expression levels of these genes are comparable to controls in the fat body of both *Ecl* and *EcR* knockdown animals (Figure 5A). However, during the white prepupal stage (96 hAH),





**Figure 2. Ecl Is a Conserved Membrane Transporter That Is Expressed Ubiquitously during Development**

(A and B) *Ecl/Oatp74D* has orthologs in a broad range of insects and other arthropods that utilize ecdysteroids as the molting hormone. (A) Neighbor-joining unrooted phylogenetic tree constructed using entire amino acid sequences of 8 *Drosophila melanogaster* Oatps (asterisks) and 82 other OATP proteins from vertebrates, a tunicate, arthropods, and a nematode. Protein names and GenBank accession numbers are listed in Table S2. Shaded area indicates the clade containing *Ecl/Oatp74D*. (B) Neighbor-joining unrooted phylogenetic tree constructed using entire amino acid sequences of *Ecl/Oatp74D* clade proteins that are shaded in (A). Scale bars indicate an evolutionary distance of 0.2 amino acid substitutions per position.

(C and D) *Ecl* is expressed ubiquitously during development. (C) Relative expression levels of *Ecl/Oatp74D* in various tissues and S2 cells, as assessed by qRT-PCR. Tissues were dissected from wandering third instar larvae ( $w^{1118}$ ). CNS, central nervous system; ID, imaginal disc; SG, salivary gland; FB, fat body; MT, Malpighian tubule. (D) Developmental changes in the relative expression level of *Ecl* in the whole body, as assessed by qRT-PCR. Samples were collected from  $w^{1118}$  animals. hAEL, hours after egg laying; hAH, hours after hatching; hAPF, hours after puparium formation. Adult cDNA samples were prepared from flies at 24 hr after eclosion. Values are shown as percentages relative to the maximum level. All values are the means  $\pm$  SD ( $n = 3$ ).

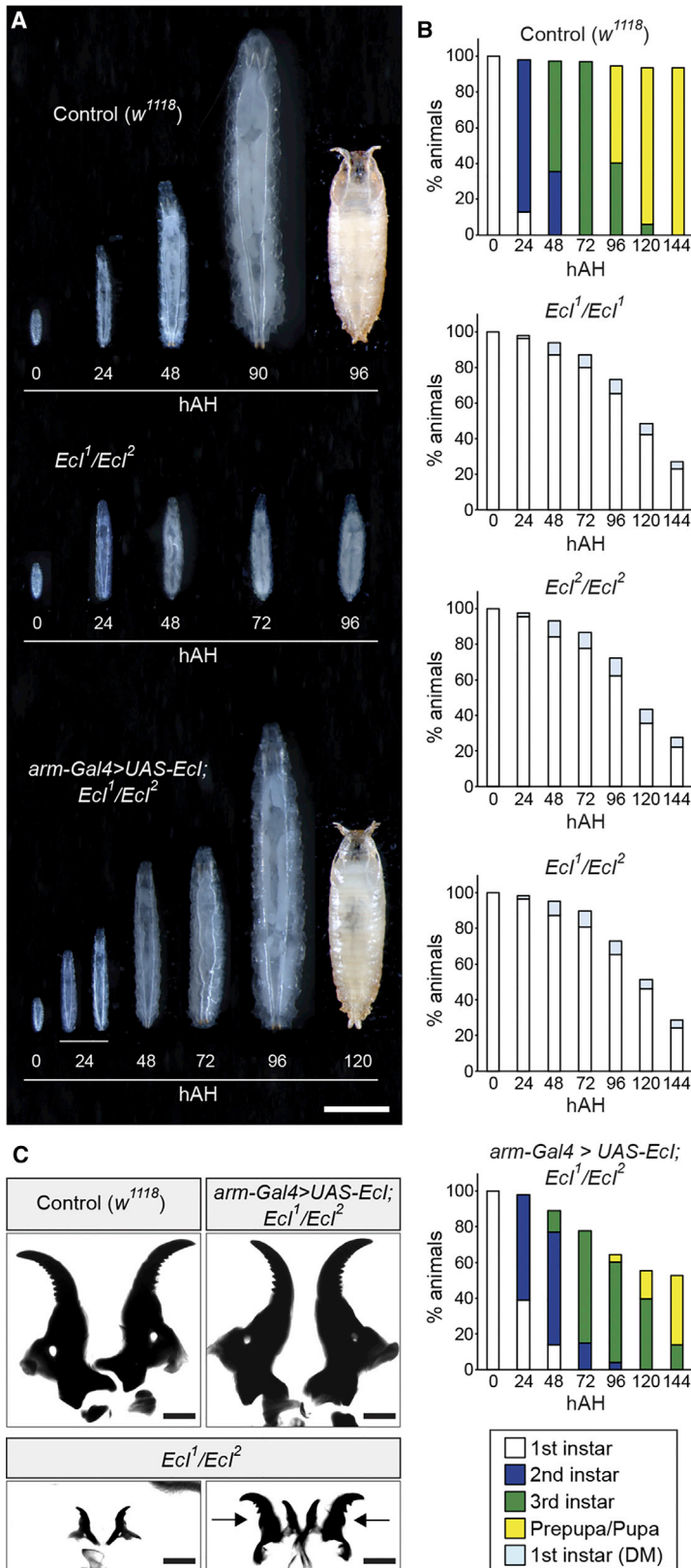
(E) HA-tagged *Ecl* is localized at the plasma membrane. *flkh-Gal4* or *Cg-Gal4* > *UAS-Ecl-HA* wandering third instar larvae were immunostained for HA (red) and nuclei (green). Scale bars, 100  $\mu$ m.

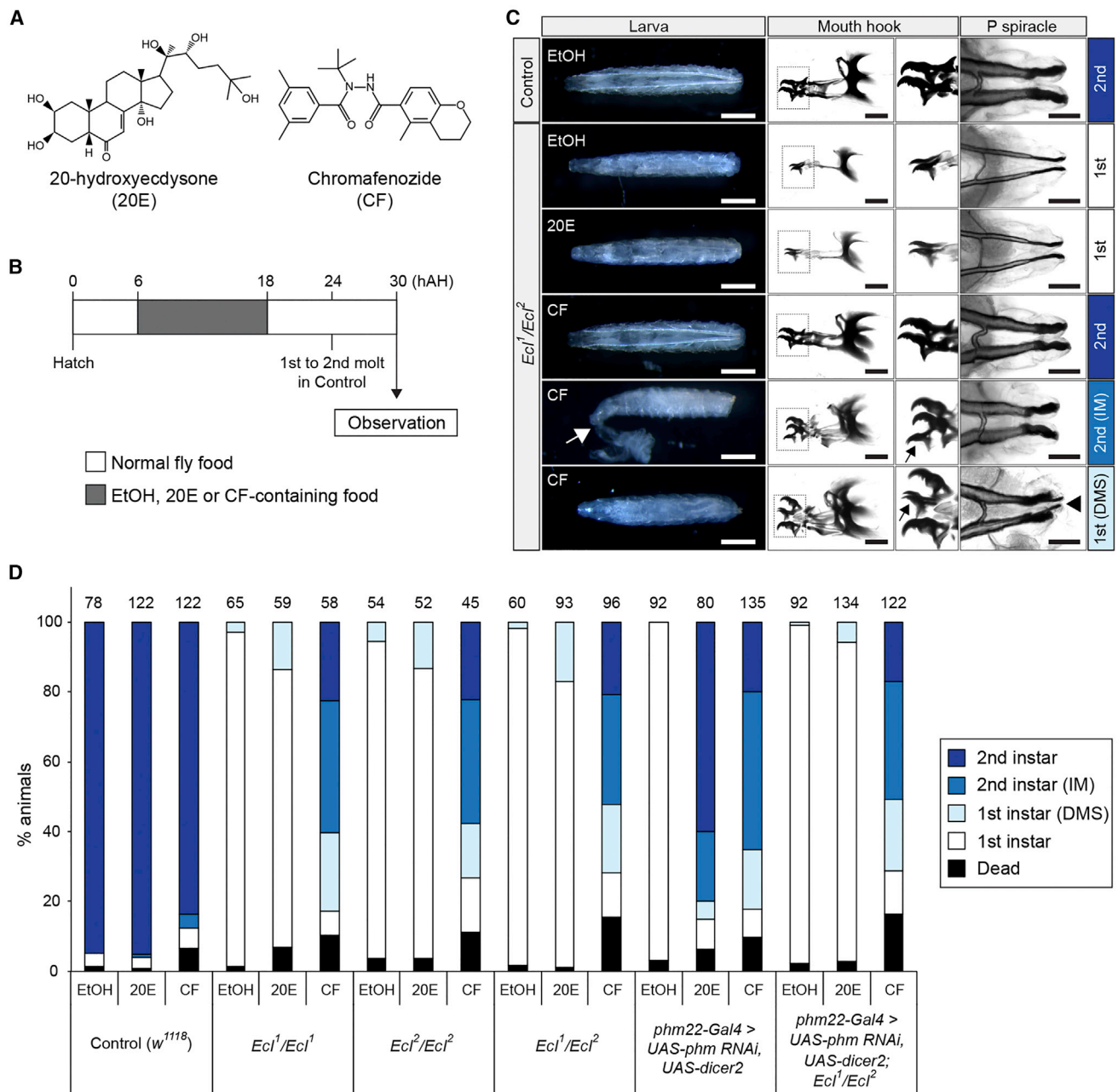
when ecdysone titer is at its peak, their expression levels are significantly reduced (Figure 5A).

We further examined cell-autonomous effects of *Ecl* knockdown using the FLP-out clonal analysis technique. Consistent with the reduced cell size in salivary gland-specific *Ecl* and *EcR* knockdown animals (Figure 1B), we found that salivary gland cell sizes are significantly reduced when *Ecl* or *EcR* levels are reduced by RNAi (Figure S5B). The ecdysone response element (*EcRE*)-driven *LacZ* reporter expression is also clearly reduced in both *Ecl* and *EcR* RNAi clones in the fat body (Figure 5B). These results demonstrate that *Ecl* regulates ecdysone signaling in a cell-autonomous manner. Importantly, neither

*EcR* protein level nor its localization is affected in *Ecl* RNAi clones (Figure S5C).

If *Ecl* is required for cellular uptake of ecdysone from the circulation *in vivo*, ecdysone levels in target cells would be reduced by downregulation of *Ecl*. To test this, we measured the amount of ecdysteroids in both the hemolymph and fat body of fat body-specific *Ecl* knockdown animals. We found that the amount of ecdysteroids in the fat body is significantly reduced in *Ecl* knockdown animals but not in *EcR* knockdown animals (Figure 5C). Ecdysteroid concentration in the hemolymph is not reduced in either *Ecl* or *EcR* knockdown animals but rather is slightly increased in *Ecl* knockdown animals (Figure 5C). Taken





**Figure 4. Developmental Arrest Phenotype of *Ecl* Mutant Can Be Rescued by a Non-Steroidal Ecdysone Agonist**

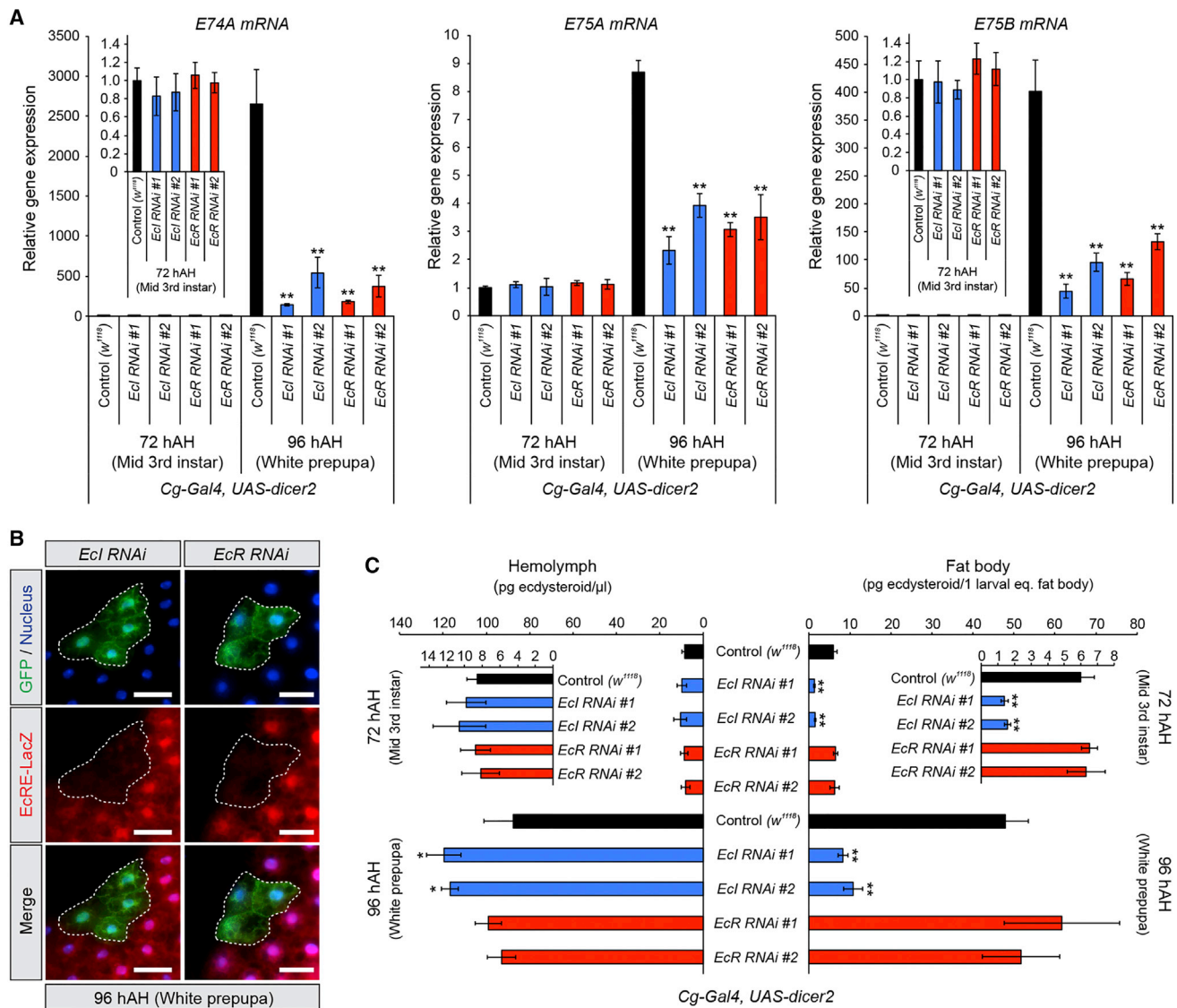
(A) Structures of the endogenous EcR ligand 20-hydroxyecdysone (20E) and a non-steroidal ecdysone agonist chromafenozide (CF).

(B) Transient compound feeding scheme. Larvae were fed on yeast paste with 2% ethanol (EtOH) with or without 1 mM (final concentration) 20E or CF from 6 to 18 hAH. Larvae were then cultured on normal food for an additional 12 hr, and larval morphology was observed at 30 hAH. About 80%–85% of control larvae complete first-to-second instar molting at 24 hAH.

(C) Representative images of mouth hooks and posterior (P) spiracles of control (*w<sup>1118</sup>*) and *Ecl* mutant (*Ecl<sup>1</sup>/Ecl<sup>2</sup>*) larvae at 30 hAH after 20E or CF feeding. Enlarged mouth hook images correspond to the boxed areas. IM, incomplete molting, where the first instar cuticle remains attached (white arrow). DMS, double mouth hooks and spiracles. Black arrows indicate first instar mouth hooks; arrowhead indicates first instar posterior spiracles. Scale bars, 500  $\mu$ m (white) and 50  $\mu$ m (black).

(D) Developmental stages of control (*w<sup>1118</sup>*), *Ecl* mutants (*Ecl<sup>1</sup>/Ecl<sup>1</sup>*, *Ecl<sup>2</sup>/Ecl<sup>2</sup>*, and *Ecl<sup>1</sup>/Ecl<sup>2</sup>*), ecdysone-deficient larvae (*phm22-Gal4 > UAS-phm RNAi*, *UAS-dicer2*), and ecdysone-deficient larvae in *Ecl* mutant background (*phm22-Gal4 > UAS-phm RNAi*, *UAS-dicer2*; *Ecl<sup>1</sup>/Ecl<sup>2</sup>*) after transient feeding of 20E or CF. *phm22-Gal4 > UAS-dicer2* was used as a prothoracic gland-specific *Gal4* driver. Color bars indicate percentages of first instar larvae (white), first instar larvae with the DMS phenotype (light blue), second instar larvae with IM phenotype (blue), and second instar larvae (dark blue). Numbers of animals observed are shown on top of each bar.





**Figure 5. *Ecl* Cell Autonomously Regulates Ecdysone Signaling and Facilitates Cellular Uptake of Ecdysteroids *In Vivo***

(A) Relative expression levels of ecdysone-inducible genes (*E74A*, *E75A*, and *E75B*) in the fat body at 72 and 96 hAH, as assessed by qRT-PCR. Two independent *UAS-RNAi* lines for *Ecl* and *EcR* were used. *Cg-Gal4 > UAS-dicer2* was used as a fat body-specific *Gal4* driver. Values were calculated relative to the expression level of each gene at 72 hAH in control.

(B) Cell-autonomous requirement of *Ecl* and *EcR* for ecdysone signaling in the fat body. Clones of fat body cells expressing *Ecl-RNAi #1* or *EcR-RNAi #1* with *dicer2* were labeled with GFP. *EcRE-LacZ* reporter gene was used to monitor the activity of ecdysone signaling. *hs-flip*; *Act>CD2>GAL4*, *UAS-nlsGFP* was used to generate GFP-marked flip-out clones. Flippase activity was induced by 10 min heat shock. The fat body from white prepupae at 96 hAH was immunostained for LacZ (red), GFP (green), and nuclei (blue). Scale bars, 50  $\mu$ m.

(C) Quantification of ecdysteroids in the hemolymph and fat body at 72 and 96 hAH, as assessed by ELISA. *UAS-RNAi* and *Gal4* lines used were the same as in (A). All values are the means  $\pm$  SD ( $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$  from Student's *t* test compared to control.

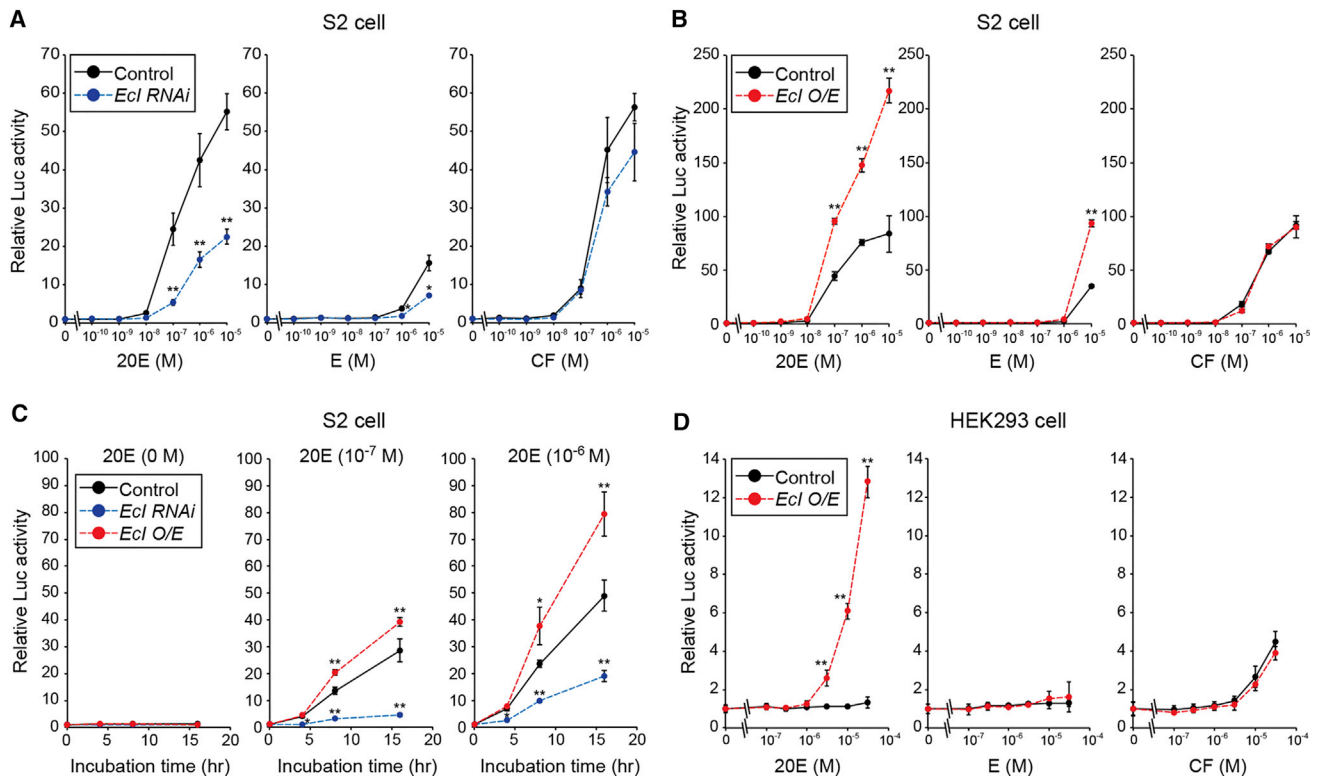
together, these results indicate that *Ecl* functions as an ecdysone importer *in vivo*.

### ***Ecl* Is Both Necessary and Sufficient for *EcR*-Mediated Ecdysone Action in Culture Cells**

In order to further confirm the function of *Ecl* in ecdysone action, a luciferase (*Luc*) reporter construct with *EcRE* (*EcRE-Luc*) and *EcR* were co-transfected into *Drosophila* S2 cells, in which *Ecl* is highly expressed (Figure S2). Altering *Ecl* levels in S2 cells by RNAi or

overexpression significantly changes dose- and incubation time-dependent induction of *Luc* activity by 20E (Figures 6A–6C). Although its ligand activity is much lower than that of 20E (Baker et al., 2000), the effect of the 20E precursor ecdysone is also significantly affected (Figures 6A and 6B), suggesting that both 20E and ecdysone are substrates for *Ecl*. Importantly, neither knockdown nor overexpression of *Ecl* in S2 cells affects *Luc* activity induced by CF (Figures 6A and 6B), consistent with the earlier observation that CF does not require *Ecl* to enter cells (Figure 4).





**Figure 6. *Ecl* Is Both Necessary and Sufficient for Ecdysone Action in *EcR*-Expressing Cells**

(A and B) Luciferase (Luc) reporter activity was monitored in *Drosophila* S2 cells treated with *Ecl* double-stranded RNA (A) or overexpressing *Ecl* (B) after 24-hr treatment with 20E, ecdysone (E), or CF. S2 cells were co-transfected with *EcREx3-Luc* and *EcR*. *usp* is endogenously highly expressed in S2 cells (Baker et al., 2000). O/E, overexpression.

(C) Luc reporter activity in S2 cells treated for 0, 4, 8 or 16 hr with 10<sup>-7</sup> M or 10<sup>-6</sup> M of 20E.

(D) Luc activity in HEK293 cells treated for 24 hr with 20E, E, or CF. *EcREx5-Luc* was used as a reporter construct. HEK293 cells overexpressing modified *EcR* (*VgEcR*) and co-receptor *RXR* were used for the assay.

Values are relative to the control level (0 M or 0 hr). All values are the means  $\pm$  SD (n = 3). \*p < 0.05, \*\*p < 0.01 from Student's t test compared to control.

Lastly, we used an ecdysteroid-inducible gene expression system in HEK293 cells to test whether *Ecl* is sufficient for *EcR*-mediated ecdysone action in a heterologous system (Christopherson et al., 1992; No et al., 1996; Saez et al., 2000). As reported previously (Christopherson et al., 1992; Saez et al., 2000; Dinan and Lafont, 2006), Luc activity is poorly induced by 20E when *EcRE-Luc* and *EcR* alone are transfected (Figure 6D). However, when *Ecl* is co-transfected, 20E-inducible Luc activity is dramatically increased, whereas there is no effect on Luc activity induced by CF (Figure 6D). Taken together, our *in vitro* analyses clearly indicate that *Ecl* is both necessary and sufficient for ecdysone-inducible gene expression in animal cells.

## DISCUSSION

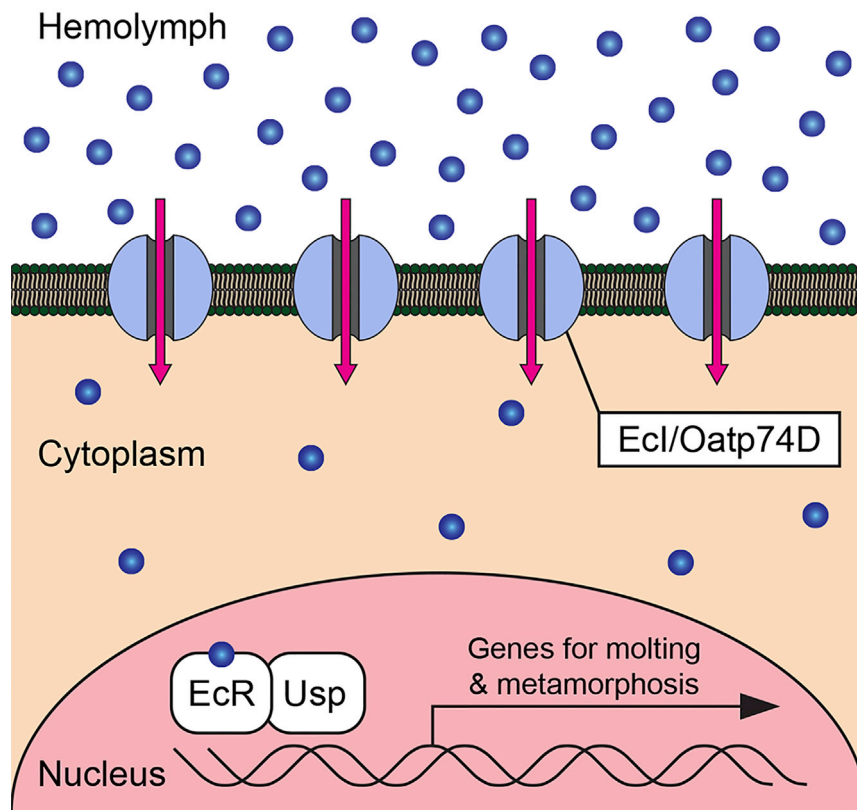
### Genetic Screens Identify an Evolutionarily Conserved Membrane Transporter Necessary for the Ecdysone Response

We conducted two independent *in vivo* and *in vitro* genetic screens in *Drosophila* and identified a single common gene, *Ecl*, as a critical component for ecdysone response. The *in vivo* screen was focused on putative transporter-encoding genes in

the *Drosophila* genome, whereas the *in vitro* screen was an unbiased approach to identify genes involved in the ecdysone response. These two complementary approaches provided compelling evidence that *Ecl* is equally indispensable for ecdysone signaling as the nuclear receptors, *EcR* and *Usp*. *Ecl/Oatp74D* is a member of the evolutionarily conserved SLCO superfamily of SLC transporters, encoding OATPs with 12 predicted transmembrane domains (Hagenbuch and Stieger, 2013). OATPs are membrane influx transporters that mediate ATP-independent cellular uptake of various endogenous substrates, as well as drugs and other xenobiotics. This mode of action of OATPs is consistent with our model (Figure 7), whereby *Ecl* on the plasma membrane transports ecdysone into the cytoplasm in a concentration-dependent manner.

### Facilitated Diffusion Is Necessary for Cellular Uptake of Ecdysone

Biology textbooks state that steroid hormones are lipophilic and therefore do not require membrane transporters to traverse phospholipid bilayers (Nussey and Whitehead, 2001; Alberts et al., 2015; Urry et al., 2017). Although the simple diffusion model of steroid hormone transport has been tested for several mammalian steroid hormones in various cell types (Plagemann



**Figure 7. Facilitated Diffusion Model of Ecdysteroid Uptake by Target Cells**

Ecl/Oatp74D is required for facilitating cellular uptake of ecdysteroids (blue circles) from the hemolymph into cytoplasm down the concentration gradient. The nuclear receptor complex composed of EcR and Usp regulates the transcription of genes for molting and metamorphosis in the nucleus.

transporter-mediated steroid hormone uptake occurs in all arthropod species.

In *Ecl* null mutants, we did not observe any significant embryonic lethality, which is observed in so-called Halloween mutants that cannot produce active ecdysteroids during embryogenesis (Gilbert, 2004; Niwa and Niwa, 2014). Some arrested *Ecl* mutant first instar larvae also showed the double mouth hook phenotype (Figures 3 and S4), indicating defective but low-level ecdysone signaling. Such incomplete block of ecdysone signaling in *Ecl* mutants may either come from maternally loaded *Ecl* and/or ecdysteroids or other unknown ecdysone importers that have partially redundant functions with *Ecl*. Although further studies are warranted to completely elim-

inate the possibility of simple diffusion of ecdysteroids, our results nonetheless clearly demonstrate that facilitated diffusion mechanism is required for most, if not all, physiological functions of ecdysteroids *in vivo*.

and Erbe, 1976; Graff et al., 1977; Giorgi and Stein, 1981), the results were often variable and even controversial, leaving the possibility that membrane transporters may be involved in cellular uptake of steroid hormones (Milgrom et al., 1973; Rao et al., 1976; Pietras and Szego, 1977; Yamamoto, 1985). However, despite these studies, no steroid hormone importers have been shown to be important in regulating the physiological functions of steroid hormones *in vivo*.

It is noteworthy that transporter-mediated steroid hormone trafficking across cell membranes have been demonstrated in many biological systems (Klaassen and Aleksunes, 2010). In most cases, however, transmembrane transport of steroid hormones occurs against their concentration gradient, such as when cells actively eliminate steroid hormones out of the cytoplasm (Kralli et al., 1995; Hock et al., 2000; Karssen et al., 2001; Uhr et al., 2002; Pariente et al., 2004) or load high concentrations of hormones into vesicles (Yamanaka et al., 2015). Such active transport necessarily requires energy, which is often provided by ABC transporters that can couple ATP hydrolysis to substrate transfer (Dean et al., 2001; Rees et al., 2009). These reports do not address the simple diffusion model of steroid hormone transport that involves passive transport of steroid hormones down the concentration gradient across lipid bilayers. In contrast, our current results demonstrate the requirement of *Ecl* for facilitating cellular uptake of ecdysone down the concentration gradient, which clearly challenges the simple diffusion model at least in *Drosophila* (Figure 7). Given that ecdysteroids are ubiquitously employed as signaling molecules in arthropods and *Ecl* is highly conserved in this phylum, we propose that the

Although the simple diffusion model has been predominant for all steroid hormones to date, it is highly desirable from a physiological perspective to regulate their cellular uptake, such that cells can adjust the intracellular concentration of steroid hormones. Consistent with this, complete lethality was observed during early development when *Ecl* was overexpressed using strong ubiquitous drivers (Table S4), suggesting that intracellular concentration of ecdysone needs to be regulated at its initial entry into cells. Transgenic rescue of the *Ecl* mutant with a weak ubiquitous driver was significant but incomplete (Figures 3 and S4), further suggesting that the cellular uptake of ecdysone needs to be precisely controlled in different tissues and/or at distinct stages of development. To further investigate this possibility, it will be important to examine the mechanisms through which expression and subcellular localization of *Ecl* are controlled *in vivo*.

**OATPs and Transmembrane Transport of Mammalian Steroid Hormones**

Do mammalian steroid hormones also require membrane transporters to diffuse across cell membranes? A key point for consideration is the hydrophobicity of steroid hormones: ecdysone has multiple hydroxyl groups on its carbon backbone, making it relatively more hydrophilic than mammalian steroid hormones. From this perspective, it is conceivable that

ecdysone is more similar to other cholesterol-derived substances in mammals, such as bile acids and steroid hormone conjugates. Indeed, mammalian OATPs are known to transport these relatively hydrophilic cholesterol-derivatives across cell membranes (Hagenbuch and Stieger, 2013). Importantly, however, there have been several studies reporting potential functions of OATPs in transmembrane transport of mammalian steroid hormones *per se*, either in heterologous or pathological conditions (Bossuyt et al., 1996; Hamada et al., 2008; Wright et al., 2011; Obaidat et al., 2012; Green et al., 2017). It is intriguing to hypothesize that these OATPs have redundant functions in mammalian steroid hormone transport, which may explain why their roles in physiological functions of steroid hormones *in vivo* have been ignored. As our current study clearly indicates the necessity of a membrane transporter in ecdysone signaling, it will be important to investigate the roles of OATPs in regulating the physiological functions of mammalian steroid hormones *in vivo*.

In conclusion, our results suggest that transporter-mediated, facilitated diffusion is necessary for cellular uptake of steroid hormones in at least one model organism. Our study thus calls for a reconsideration of the simple diffusion model of steroid hormones, which is commonly described in major biology textbooks (Nussey and Whitehead, 2001; Alberts et al., 2015; Urry et al., 2017). It is also noteworthy that OATPs have been recognized as potential drug targets for cancer (Obaidat et al., 2012), and several chemical reagents have already been identified to regulate functions of these membrane transporters (Kalliokoski and Niemi, 2009; El-Gebali et al., 2013). Considering the diverse biological processes regulated by steroid hormones, further investigation of the facilitated diffusion model of steroid hormones may have a tremendous impact on both basic and medical aspects of steroid hormone research.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- Transfection and Luciferase Reporter Assays in S2 Cells
- Transfection and Luciferase Reporter Assays in HEK293 Cells

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and five tables and can be found with this article online at <https://doi.org/10.1016/j.devcel.2018.09.012>.

## ACKNOWLEDGMENTS

We thank Vienna *Drosophila* Resource Center, Bloomington *Drosophila* Stock Center (NIH P40 OD018537), National Institute of Genetics Fly Stock Center, Transgenic RNAi Project at Harvard Medical School (NIH R24 RR032668), T.P. Neufeld, T. Nishimura, and M.B. O'Connor for fly stocks; M.B. O'Connor and M.E. Adams for cell lines; *Drosophila* Genomics Resource Center (NIH P40 OD010949), National Institute of Genetics Fly Stock Center, and D.J. Mangelsdorf for vectors and cDNA clones; and F.M. Sladek, D.S. Straus, C.G. Pontrello, M.B. O'Connor, and M.E. Adams for comments on the manuscript. The monoclonal antibodies developed by J.R. Sanes, C.S. Thummel, and D.S. Hogness were obtained from the Developmental Studies Hybridoma Bank, created by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. This study was supported by a Postdoctoral Fellowship for Research Abroad from the Japan Society for the Promotion of Science to N.O., the Naito Foundation Subsidy for Dispatch of Young Researchers Abroad to N.O., an NIH grant R00 HD073239 from NICHD to N.Y., a research grant from the W.M. Keck Foundation to S.H.-Y. and N.Y., and a Pew Biomedical Scholars Award from the Pew Charitable Trusts to N.Y. N.P. is an Investigator of the Howard Hughes Medical Institute.

## AUTHOR CONTRIBUTIONS

Conceptualization, N.Y.; Methodology, N.O., R.V., S.H.-Y., N.P., and N.Y.; Investigation, N.O., R.V., R.B., Z.L., and S.H.-Y.; Writing – Original Draft, N.O. and N.Y.; Writing – Review & Editing, N.O., R.V., R.B., S.H.-Y., N.P., and N.Y.; Supervision, S.H.-Y., N.P., and N.Y.; Funding Acquisition, N.O., S.H.-Y., N.P., and N.Y.

## DECLARATION OF INTERESTS

N.O., R.B., S.H.-Y., and N.Y. have a patent pending (U.S. Patent App. No. 15/902,948) relevant to this work.

Received: May 9, 2018

Revised: August 6, 2018

Accepted: September 9, 2018

Published: October 4, 2018

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Chicken polyclonal anti-GFP	Abcam	Cat#ab13970; RRID: AB_300798
Goat anti-Chicken IgG, Alexa Fluor 488	Life Technologies	Cat#A-11039; RRID: AB_142924
Goat anti-Mouse IgG, Alexa Fluor 546	Life Technologies	Cat#A-11003; RRID: AB_141370
Goat anti-Rat IgG, Alexa Fluor 546	Life Technologies	Cat#A-11081; RRID: AB_141738
Mouse monoclonal anti-Beta-galactosidase (clone 40-1a)	DSHB	Cat#40-1a; RRID: AB_528100
Mouse monoclonal anti-EcR (clone Ag10.2)	DSHB	Cat#Ag10.2 (EcR common), RRID: AB_528208
Rabbit anti-20-Hydroxyecdysone (EIA Antiserum), Unconjugated	Cayman Chemical	Cat#482202; RRID: AB_328051
Rat monoclonal anti-HA (clone 3F10)	Roche	Cat#11867423001; RRID: AB_390918
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
8% Paraformaldehyde Aqueous Solution	Electron Microscopy Sciences	Cat#157-8
20-Hydroxyecdysone	Sigma-Aldrich	Cat#H5142-10MG
Alexa Fluor 546 Phalloidin	Life Technologies	Cat#A22283
Attractene Transfection Reagent	QIAGEN	Cat#301005
Chromafenozide	Sigma-Aldrich	Cat#32352-50MG
Dimethyldioctadecylammonium Bromide	Avanti	Cat#890810C
Effectene Transfection Reagent	QIAGEN	Cat#301425
Ethanol	Decon	Cat#2716
Hoechst 33342	Life Technologies	Cat#H3570
RNase-Free DNase Set	QIAGEN	Cat#79254
TRIzol Reagent	Invitrogen	Cat#15596026
<b>Critical Commercial Assays</b>		
20-Hydroxyecdysone EIA Kit	Cayman Chemical	Cat#501390
CellTiter-Glo Luminescent Cell Viability Assay	Promega	Cat#G7570
Dual-Glo Luciferase Assay System	Promega	Cat#E2920
MEGAscript T7 Transcription Kit	Ambion	Cat#AM1334
PrimeScript RT Master Mix	TaKaRa Bio.	Cat#RR036Q
RNeasy Mini Kit	QIAGEN	Cat#74104
SYBR Premix Ex Taq™ II (Tli RNaseH Plus)	TaKaRa Bio.	Cat#RR820A
<b>Experimental Models: Cell Lines</b>		
<i>D. melanogaster</i> : Cell line S2	Obtained from Michael B. O'Connor (University of Minnesota)	N/A
<i>D. melanogaster</i> : Cell line S2R+/attP/Cas9 (Cell line S2R+ containing an attP site and expressing Cas9)	Perrimon Lab	<a href="#">Viswanatha et al. (2018)</a>
Human: Cell line HEK293	Obtained from Michael E. Adams (University of California, Riverside)	N/A
<b>Experimental Models: Organisms/Strains</b>		
<i>D. melanogaster</i> : Act5C-Gal4	Bloomington Drosophila Stock Center	BDSC: 3954
<i>D. melanogaster</i> : Act5C-Gal4	Bloomington Drosophila Stock Center	BDSC: 4414
<i>D. melanogaster</i> : arm-Gal4	Bloomington Drosophila Stock Center	BDSC: 1560
<i>D. melanogaster</i> : arm-Gal4	Bloomington Drosophila Stock Center	BDSC: 1561
<i>D. melanogaster</i> : Cg-Gal4	Bloomington Drosophila Stock Center	BDSC: 7011

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>D. melanogaster: da-Gal4</i>	Bloomington Drosophila Stock Center	BDSC: 55850
<i>D. melanogaster: Ecl<sup>1</sup> / TM6B-Dfd-EGFP</i>	This paper	N/A
<i>D. melanogaster: Ecl<sup>2</sup> / TM6B-Dfd-EGFP</i>	This paper	N/A
<i>D. melanogaster: EcRE-LacZ</i>	Bloomington Drosophila Stock Center	BDSC: 4516
<i>D. melanogaster: EcRE-LacZ</i>	Bloomington Drosophila Stock Center	BDSC: 4517
<i>D. melanogaster: fkh-Gal4</i>	Obtained from Michael B. O'Connor (University of Minnesota)	N/A
<i>D. melanogaster: hs-FLP;; Act5c&gt;CD2&gt;Gal4, UAS-nlsGFP</i>	Obtained from Thomas P. Neufeld (University of Minnesota)	N/A
<i>D. melanogaster: phm22-Gal4</i>	Obtained from Michael B. O'Connor (University of Minnesota)	N/A
<i>D. melanogaster: Sgs3-GFP</i>	Bloomington Drosophila Stock Center	BDSC: 5885
<i>D. melanogaster: TM2/TM6B-dfd-EGFP</i>	Obtained from Takashi Nishimura (RIKEN Center for Biosystems Dynamics Research)	N/A
<i>D. melanogaster: TubP-Gal4</i>	Bloomington Drosophila Stock Center	BDSC: 5138
<i>D. melanogaster: UAS-2XEGFP</i>	Bloomington Drosophila Stock Center	BDSC: 6874
<i>D. melanogaster: UAS-dicer2</i>	Vienna Drosophila Resource Center	VDRC: 60008
<i>D. melanogaster: UAS-dicer2</i>	Vienna Drosophila Resource Center	VDRC: 60009
<i>D. melanogaster: UAS-Ecl</i>	This paper	N/A
<i>D. melanogaster: UAS-Ecl-Flag-HA</i>	This paper	N/A
<i>D. melanogaster: UAS-Ecl (Oatp74D) RNAi</i>	National Institute of Genetics	NIG: 7571R-1
<i>D. melanogaster: UAS-Ecl (Oatp74D) RNAi</i>	Vienna Drosophila Resource Center	VDRC: 37295
<i>D. melanogaster: UAS-EcR RNAi</i>	Bloomington Drosophila Stock Center	BDSC: 9327
<i>D. melanogaster: UAS-EcR RNAi</i>	Vienna Drosophila Resource Center	VDRC: 37058
<i>D. melanogaster: UAS-Oatp26F RNAi</i>	Vienna Drosophila Resource Center	VDRC: 2650
<i>D. melanogaster: UAS-Oatp26F RNAi</i>	Vienna Drosophila Resource Center	VDRC: 109633
<i>D. melanogaster: UAS-Oatp30B RNAi</i>	Vienna Drosophila Resource Center	VDRC: 22983
<i>D. melanogaster: UAS-Oatp30B RNAi</i>	Vienna Drosophila Resource Center	VDRC: 110237
<i>D. melanogaster: UAS-Oatp33Ea RNAi</i>	Bloomington Drosophila Stock Center	BDSC: 50736
<i>D. melanogaster: UAS-Oatp33Ea RNAi</i>	Vienna Drosophila Resource Center	VDRC: 105560
<i>D. melanogaster: UAS-Oatp33Eb RNAi</i>	Vienna Drosophila Resource Center	VDRC: 42805
<i>D. melanogaster: UAS-Oatp33Eb RNAi</i>	Vienna Drosophila Resource Center	VDRC: 100431
<i>D. melanogaster: UAS-Oatp58Da RNAi</i>	Vienna Drosophila Resource Center	VDRC: 44122
<i>D. melanogaster: UAS-Oatp58Da RNAi</i>	Vienna Drosophila Resource Center	VDRC: 106377
<i>D. melanogaster: UAS-Oatp58Db RNAi</i>	National Institute of Genetics	NIG: HMJ24090
<i>D. melanogaster: UAS-Oatp58Db RNAi</i>	Vienna Drosophila Resource Center	VDRC: 100348
<i>D. melanogaster: UAS-Oatp58Dc RNAi</i>	Bloomington Drosophila Stock Center	BDSC: 44583
<i>D. melanogaster: UAS-Oatp58Dc RNAi</i>	Vienna Drosophila Resource Center	VDRC: 39469
<i>D. melanogaster: UAS-phm RNAi</i>	Vienna Drosophila Resource Center	VDRC: 108359
<i>D. melanogaster: w<sup>1118</sup></i>	Obtained from Michael B. O'Connor (University of Minnesota)	N/A
<i>D. melanogaster: UAS-RNAi lines for RNAi screening, see Table S1</i>	Bloomington Drosophila Stock Center or Vienna Drosophila Resource Center	N/A
<b>Oligonucleotides</b>		
sgRNA library for CRISPR screening	Perrimon Lab	Viswanatha et al. (2018)
Primers for screening <i>Ecl</i> CRISPR mutants, see Table S5	This paper	N/A
Primers for generating <i>Ecl</i> dsRNA, see Table S5	This paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primers for qRT-PCR, see <a href="#">Table S5</a>	This paper	N/A
Oligonucleotides for generating <i>Ecl</i> mutants, see <a href="#">Table S5</a>	This paper	N/A
<b>Recombinant DNA</b>		
cDNA GH24467	Drosophila Genomics Resource Center	DGRC:18578; FlyBase:FBcl013519
cDNA UFO0685	Drosophila Genomics Resource Center	DGRC:1642000; FlyBase:FBcl013519
<i>pAct5C-EcR</i>	Obtained from David J. Mangelsdorf (UT Southwestern)	N/A
<i>pADH-hspEcRE-LUC</i>	Obtained from David J. Mangelsdorf (UT Southwestern)	N/A
<i>pBFv-U6.2</i>	National Institute of Genetics	<a href="#">Kondo and Ueda (2013)</a>
<i>pBFv-U6.2B</i>	National Institute of Genetics	<a href="#">Kondo and Ueda (2013)</a>
<i>pBRAcPA-Ecl</i>	This paper	N/A
<i>pBS130</i>	Addgene	<a href="#">Gohl et al. (2011)</a>
<i>pcDNA3.1-Ecl</i>	This paper	N/A
<i>pEGSH</i>	Agilent Technologies	Cat#217461
<i>pERV3</i>	Agilent Technologies	Cat#217460
<i>pGEM-T-Ecl</i> (PCR-amplified fragment)	This paper	N/A
<i>pGEM-T-Oatp26F</i> (PCR-amplified fragment)	This paper	N/A
<i>pGEM-T-Oatp30B</i> (PCR-amplified fragment)	This paper	N/A
<i>pGEM-T-Oatp33Ea</i> (PCR-amplified fragment)	This paper	N/A
<i>pGEM-T-Oatp33Eb</i> (PCR-amplified fragment)	This paper	N/A
<i>pGEM-T-Oatp58Da</i> (PCR-amplified fragment)	This paper	N/A
<i>pGEM-T-Oatp58Db</i> (PCR-amplified fragment)	This paper	N/A
<i>pGEM-T-Oatp58Dc</i> (PCR-amplified fragment)	This paper	N/A
<i>pGEM-T-rp49</i> (PCR-amplified fragment)	This paper	N/A
<i>pRL-CMV</i>	Promega	Cat# E2261
<i>pUAST-Ecl</i>	This paper	N/A
<b>Software and Algorithms</b>		
ClustalW	DDBJ	<a href="http://clustalw.ddbj.nig.ac.jp">http://clustalw.ddbj.nig.ac.jp</a>
MAGeCK	N/A	<a href="#">Li et al. (2014)</a>
NIG-FLY Cas9 Target finder	NIG	<a href="https://shigen.nig.ac.jp/fly/nigfly/cas9/cas9TargetFinder.jsp">https://shigen.nig.ac.jp/fly/nigfly/cas9/cas9TargetFinder.jsp</a>
TagDust	N/A	<a href="#">Lassmann et al. (2009)</a>
<b>Other</b>		
Amino acid sequences used for generating phylogenetic tree, see <a href="#">Table S2</a>	NCBI	N/A

**CONTACT FOR REAGENT AND RESOURCE SHARING**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Naoki Yamanaka ([naoki.yamanaka@ucr.edu](mailto:naoki.yamanaka@ucr.edu)).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Flies**

All flies were raised at 25°C under 12 hours/12 hours light/dark cycle. The animals were reared on standard fly food containing 6 g *Drosophila* agar type II (Genesee Scientific, #66-103), 100 g D-(+)-glucose (SIGMA, #G8270-25KG), 50 g inactive dry yeast (Genesee Scientific, #62-106), 70 g yellow corn meal (Genesee Scientific, #62-101), 6 ml propionic acid (SIGMA, #402907-500ML), and 10 ml Tegosept (Genesee Scientific, #20-258) in 1,025 ml of water. All the experiments were conducted under non-crowded conditions.



The control strain was  $w^{1118}$ , and transgenic flies are as follows: *Act5C-Gal4* (#3954 and #4414), *arm-Gal4* (#1560 and #1561), *Cg-Gal4* (#7011), *da-Gal4* (#55850), *TubP-Gal4* (#5138), *UAS-EcR RNAi #1* (#9327), *UAS-Oatp33Ea RNAi* (#50736), *UAS-Oatp58Dc RNAi* (#44583), *UAS-2XEGFP* (#6874), *EcRE-LacZ* (#4516 and #4517) and *Sgs3-GFP* (#5885) were obtained from the Bloomington Drosophila Stock Center (BDSC); *UAS-dicer2* (#60008 and #60009), *UAS-EcI (Oatp74D) RNAi #2* (#37295), *UAS-EcR RNAi #2* (#37058), *UAS-Oatp26F RNAi* (#2650 and #109633), *UAS-Oatp30B RNAi* (#22983 and #110237), *UAS-Oatp33Ea RNAi* (#105560), *UAS-Oatp33Eb RNAi* (#42805 and #100431), *UAS-Oatp58Da RNAi* (#44122 and #106377), *UAS-Oatp58Db RNAi* (#100348), *UAS-Oatp58Dc RNAi* (#39469) and *UAS-phm RNAi* (#108359) were obtained from the Vienna Drosophila Resource Center (VDRC); *UAS-EcI (Oatp74D) RNAi #1* (#7571R-1) and *UAS-Oatp58Db RNAi* (#HMJ24090) were obtained from the National Institute of Genetics Fly Stock Center. *phm22-Gal4* and *fkx-Gal4* were obtained from Michael B. O'Connor. *hs-flp;; Act>CD2>Gal4*, *UAS-nlsGFP* was obtained from Thomas P. Neufeld. All the RNAi lines used for the genome-wide *in vivo* RNAi screening are shown in Table S1.

*UAS-EcI* was generated from cDNA clone GH24467 from the Drosophila Genomics Resource Center (DGRC) and cloned into *pUAST* vector. *UAS-EcI-Flag-HA* was generated from cDNA clone UFO0685 from DGRC. *EcI<sup>1</sup>* and *EcI<sup>2</sup>* were generated by the CRISPR/Cas9 system as described in detail below. All new transgenic flies were generated by BestGene Inc.

### Cell Lines

S2R+ cells containing an *attP* site and expressing Cas9 used for CRISPR screening (Viswanatha et al., 2018) were maintained in a humidified incubator at 25°C in Schneider's *Drosophila* medium (Thermo Fisher Scientific, #21720-024-500ML) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, #10082147) and 1% Penicillin-streptomycin solution (PSS; Thermo Fisher Scientific, #15140-122).

S2 cells used for luciferase reporter assays were obtained from Michael B. O'Connor and maintained in 100 mm x 20 mm Petri dishes (Corning, #430167) in a humidified incubator at 25°C in Shields and Sang M3 (SSM3) insect medium (Sigma-Aldrich, #S3652-500ML) containing 10% Insect Medium Supplement (IMS) (Sigma-Aldrich, #17267-100ML) and 1% PSS (Thermo Fisher Scientific).

HEK293 cells obtained from Michael E. Adams were maintained in 100 mm x 20 mm Petri dishes (Corning) in a humidified incubator at 37°C and 5% CO<sub>2</sub> in Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 mg/ml glucose, L-glutamine, and sodium pyruvate (Corning, #10-013-CV) containing 10% FBS (Corning, #10082-139) and 1% PSS (Thermo Fisher Scientific). Before seeding cells to new plates, they were washed with Dulbecco's phosphate-buffered saline (Thermo Fisher Scientific, #21-030-CV) and treated with 0.05% Trypsin-EDTA (Thermo Fisher Scientific, #25-052-CI).

## METHOD DETAILS

### Genome-wide *In Vivo* RNAi Screening

Seven virgin females from *Gal4* lines (*UAS-dicer2*; *fkx-Gal4*, *Sgs3-GFP* for the first screening, *Cg-Gal4*, *UAS-dicer2*, *UAS-2xEGFP* for the second screening) were crossed with four males from each transgenic *UAS-RNAi* line (see Table S1 for details), and allowed to lay eggs on standard food in vials for 24-48 hours. For *UAS-RNAi* lines on the X chromosome, virgin females were collected from RNAi lines and crossed to males from *Gal4* lines. Two replicates were collected from the offspring of each cross. Each batch of 50 genetic crosses included a negative and positive control: *Gal4* lines crossed to  $w^{1118}$  or *UAS-EcR RNAi* (BDSC #9327), respectively. In the first screening, phenotypes of F1 offspring were analyzed by observing *Sgs3-GFP* expression in the salivary glands of wandering third instar larvae. The number of animals (per 25 animals) that showed defects in GFP expression was recorded, and RNAi lines for 51 genes were found to diminish GFP expression in one or more animals. In the second screening, phenotypes of F1 offspring were analyzed by observing defects in GFP-labeled fat body cell migration at 24-36 hours after puparium formation. In this screening, most crosses showed phenotypes other than or in addition to defects in fat body cell migration, such as growth defects and melanization. Since the positive control pupae (*Gal4* line crossed to *UAS-EcR RNAi*) showed a fat body cell migration defect in all animals without any other discernible defect, we used these criteria to evaluate the RNAi lines. This screen identified only one RNAi line, which targets *Oatp74D*. GFP signals in each larva or pupa on plastic vial walls were observed using a SteREO Discovery.V12 microscope (Zeiss).

### *In Vitro* CRISPR Screening

CRISPR screens were conducted as described (Viswanatha et al., 2018). S2R+ cells containing an *attP* site and expressing Cas9 were transfected with the Group 1 and Group 2 *attB* donor libraries containing 25,783 gene-targeted sgRNAs and 391 control sgRNAs under the Drosophila U6.2 promoter along with an expression vector for the phiC31 integrase (*pBS130* from Addgene; Gohl et al., 2011) at a 1:1 molar ratio to mediate recombination. Transfection of S2R+ cells was performed using Effectene transfection reagent (Qiagen) following the manufacturer's instructions. Approximately 1,500 cells per sgRNA were recombined and >1,500 cells per sgRNA were passaged for 15 days to induce knockout and monitored for percentage of green cells using flow cytometry. Culture was then divided, and half of the cells received 15 ng/ml of 20E (Sigma-Aldrich) while other half was left untreated. Following 30 days, genomic DNA was extracted (Zymo Research) and sgRNA PCR was performed at a sampling of > 1,000 genomes per sgRNA. Next generation sequencing was performed using a NextSeq 500 (Illumina) at the Harvard Biopolymers Facility. Readcounts were demultiplexed and counted using TagDust (Lassmann et al., 2009) and MAGeCK count (Li et al., 2014), respectively. Log<sub>2</sub>

fold-changes were calculated relative to normalized plasmid readcount values for all CRISPR sgRNAs for which initial readcounts were in the top ninety-fifth percentile (Viswanatha et al., 2018). For 20E resistance screen, two technical replicates were performed. To generate sgRNA-level mean, readcounts from each replicate screen were median-adjusted prior to computing means allowing balanced comparisons. The gene-level CRISPR score is defined as the mean of all log<sub>2</sub> fold-changes for all sgRNAs targeting the same gene, as described in Wang et al. (2014).

Cell titer measurements following 20E treatment were conducted using CellTiter-Glo assay (Promega) following the manufacturer's instructions, and analyzed on a Paradigm luminometer (SpectraMax). Luminescence was normalized to untreated wells.

### Phylogenetic Tree Analysis

We generated unrooted neighbor-joining trees using ClustalW (DNA Data Bank of Japan). We aligned the entire amino acid sequences of 8 *Drosophila melanogaster* Oatps, including Ecl/Oatp74D, and 82 OATP proteins from vertebrates (Human, *Homo sapiens*; Zebrafish, *Danio rerio*), tunicate (Sea squirt, *Ciona intestinalis*), arthropods [holometabolous insects (Silk moth, *Bombyx mori*; red flour beetle, *Tribolium castaneum*; western honey bee, *Apis mellifera*), hemimetabolous insects (pea aphid, *Acyrtosiphon pisum*; human body louse, *Pediculus humanus*), crustacean (water flea, *Daphnia pulex*), chelicerate (black-legged tick, *Ixodes scapularis*)] and nematode (round worm, *Caenorhabditis elegans*). The protein names and GenBank accession numbers are listed in Table S2.

### Total RNA Extraction and Quantitative Reverse Transcription (qRT)-PCR

Animals or dissected tissues were collected in 1.5 ml tubes and immediately flash-frozen in liquid nitrogen. Total RNA from animals or tissues was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Extracted RNA was further purified by RNeasy mini kit (Qiagen) following the manufacturer's instructions, combined with treatment with RNase-Free DNase Set (Qiagen). cDNA was generated from purified total RNA using PrimeScript RT Master Mix (Takara Bio). qRT-PCR was performed on the CFX connect real-time PCR detection system (Bio-Rad) using SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio). For absolute quantification of mRNAs, serial dilutions of pGEM-T (Promega) plasmids containing coding sequences of the target genes or *rp49* were used for standards. After the molar amounts were calculated, transcript levels of the target mRNA were normalized to *rp49* levels in the same samples. Three separate samples were collected for each experiment and duplicate measurements were conducted. The primers used are listed in Table S5.

### Immunostaining

Tissues were dissected in 1X phosphate-buffered saline (PBS) (Fisher BioReagents), fixed with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) in PBS (4% PFA/PBS) containing 0.1% TritonX-100 for 20 min at room temperature (RT), and washed multiple times with PBS containing 0.1% TritonX-100 (PBST). Tissues were blocked with 5% normal goat serum (NGS) (Sigma-Aldrich) in PBST for at least 1 hour at RT, incubated overnight at 4°C with the primary antibody mix in PBST containing 5% NGS, washed multiple times with PBST, incubated for 2 hours at RT with the secondary antibody mix in PBST containing 5% NGS, and washed multiple times again with PBST. When needed, DNA was stained with Hoechst 33342 (Life Technologies) at 1:2000 and F-Actin was stained with Alexa Fluor 546 Phalloidin (Life Technologies) at 1:200 for 30 min at RT. After washing, tissues were mounted in Vectashield H-1000 (Vector Laboratories) and observed using a Zeiss Axio Imager M2 equipped with ApoTome.2. Specificity of the signals was established by comparison to appropriate controls.

Primary antibodies used were rat anti-HA (3F10, 1:1000, Roche), mouse anti-beta-galactosidase (40-1a, 1:500, DSHB), mouse anti-EcR (Ag10.2, 1:200, DSHB), and chick anti-GFP (1:500, Abcam). Secondary antibodies used were Alexa Fluor 488 goat anti-chicken (1:500, Life Technologies), Alexa Fluor 546 goat anti-rat (1:500, Life Technologies) and Alexa Fluor 546 goat anti-mouse (1:500, Life Technologies).

### Generation of *Ecl* Mutants

*Ecl* mutant alleles (*Ecl*<sup>1</sup> and *Ecl*<sup>2</sup>) were generated by the CRISPR/Cas9 system. Pairs of gRNA target sequences (20 bp) were designed near the *Ecl* translational start and stop sites using NIG-FLY Cas9 Target finder (NIG). *Ecl*<sup>1</sup> and *Ecl*<sup>2</sup> were generated with target pair-I (T1 and T2) and target pair-II (T3 and T4), respectively (Figure S3). Forward and reverse 24-bp oligonucleotides with 20-bp target sequences (Table S5) were annealed to generate a double-strand DNA with 4-bp overhangs on both ends and inserted into *BbsI*-digested pBFv-U6.2 or pBFv-U6.2B vector provided by the NIG (Kondo and Ueda, 2013). To construct a double-gRNA vector, the first gRNA (T1 or T3) was cloned into pBFv-U6.2 (named pBFv-U6.2-T1 and pBFv-U6.2-T3), whereas the second gRNA (T2 or T4) was cloned into pBFv-U6.2B (named pBFv-U6.2B-T2 and pBFv-U6.2B-T4). A fragment containing the U6 promoter and the first gRNA was cut out from pBFv-U6.2-T1 and pBFv-U6.2-T3, and ligated into pBFv-U6.2B-T2 and pBFv-U6.2B-T4, respectively (named pBFv-U6.2B-T1-T2 and pBFv-U6.2B-T3-T4). These two *Ecl* double-gRNA vectors (pBFv-U6.2B-T1-T2 and pBFv-U6.2B-T3-T4) were independently injected into embryos of *yw; nos-cas9 (Il-attP40)/CyO* flies (BestGene Inc).

For each target pair, surviving G<sub>0</sub> males were divided into 10 groups and crossed *en masse* to *TM2/TM6b-Dfd-EGFP* (obtained from Takashi Nishimura) virgins. From the progeny of each of these ten crosses, 10 single males were isolated and crossed independently to *TM2/TM6b-Dfd-EGFP* virgins to establish independent isogenized lines. Among these 100 lines, those showing homozygous lethality (71 and 27 lethal lines from target pair-I and II, respectively) were isolated. To confirm deletions at the *Ecl* locus, we randomly selected 20 lines from each target pair and performed genome DNA extraction and PCR amplification by using primers

listed in [Table S5](#). Out of 20 lines, 15 lines from target pair-I and 7 lines from target pair-II possessed deletion mutations in *Ecl*. The PCR products from 5 lines from each target pair were sequenced, and we confirmed complete deletions in all lines. We chose 1 line for each target pair for further analyses and named them *Ecl*<sup>1</sup> and *Ecl*<sup>2</sup>. *Ecl*<sup>1</sup> has a 3207-bp deletion including the entire *Ecl* protein coding DNA sequence (CDS), and *Ecl*<sup>2</sup> has a 3858-bp deletion including the 5' untranslated region and almost the entire *Ecl* CDS ([Figure S3](#)).

### Scoring of Developmental Progression of *Ecl* Mutants

Eggs were laid on grape juice plates with yeast paste at 25°C for 6 hours. After 24 hours, early first instar larvae just after hatching were collected (homozygous mutant larvae were collected by selecting those without the balancer chromosome with GFP), and transferred into vials with standard food (less than 50 animals/vial). Every 24 hours, developmental stages were scored by stage-specific morphology of larval mouth hooks and posterior spiracles.

### Pulsed Compound Feeding Assay with 20-Hydroxyecdysone and Chromafenozide

Eggs were laid on grape juice plates with yeast paste at 25°C for 6 hours. After 24 hours, early first instar larvae just after hatching were collected (homozygous mutant larvae were collected by selecting those without the balancer chromosome with GFP), and transferred into vials with standard food (less than 50 animals/vial). After 6 hours of incubation at 25°C, larvae were transferred and cultured on agar plates with yeast paste containing 2% ethanol (Decon) (for control), 1 mM 20E (Sigma-Aldrich), or 1 mM chromafenozide (Sigma-Aldrich) in 2% ethanol for 12 hours at 25°C. After incubation, larvae were washed and transferred into vials with standard food. After 12 hours of incubation at 25°C, developmental stages were scored by stage-specific morphology of larval mouth hooks and posterior spiracles.

### Induction of RNAi Clones in the Fat Body and Salivary Gland

To induce FLP-out RNAi clones, *hs-flp; Act5C>CD2>Gal4, UAS-nlsGFP* flies (obtained from Thomas P. Neufeld) were crossed to relevant *UAS-RNAi* lines, and early first instar larvae were heat shocked at 37°C for either 5 min or 30 min. About 10% of fat body cells and 3-5% of salivary gland cells showed GFP expression after 5-min heat shock, and about 90% of fat body cells and 80% of salivary gland cells showed GFP expression after 30-min heat shock.

### Measurement of Ecdysteroid Concentration

Fat bodies were carefully dissected from 5 mid-third instar larvae (72 hours after hatching, hAH) or white prepupae (96 hAH) in 4% PFA/PBS, briefly rinsed with 4% PFA/PBS, and pooled in 200  $\mu$ l of methanol (Fisher chemical, A452-4) on ice. The fat bodies were thoroughly homogenized using pestles with a micro-tube homogenizer. After centrifugation at 4°C for 10 min, the supernatant was pooled on ice, while the pellet was re-extracted by 100  $\mu$ l of methanol. The resulting extract was stored at -20°C until use. For hemolymph samples, mid-third instar larvae (72 hAH) or white prepupae (96 hAH) were rinsed in PBS, and dried on tissue paper. The cuticle was carefully torn to release the hemolymph onto a parafilm membrane. 2  $\mu$ l of hemolymph were collected from 5 mid-third instar larvae or white prepupae and mixed with 200  $\mu$ l of methanol on ice. After vortexing, samples were centrifuged at 4°C for 10 min, and the resulting supernatant was stored at -20°C until use.

The sample solutions were dried with a CentriVap concentrator (Labconco) and dissolved in EIA buffer (Cayman Chemical). 20E AChE tracer, 20E EIA antiserum, Precoated (Mouse Anti-Rabbit IgG) ELISA 96-well strip plate, Wash buffer, and Ellman's reagent were all purchased from Cayman Chemical. The assay was performed according to the manufacturer's instructions using synthetic 20E (Sigma-Aldrich) as a standard.

### Generation of *Ecl* Double-Strand (ds) RNA

For dsRNA preparation, the 20-bp T7 promoter sequence was attached to the 5' end of PCR primers for *Ecl*, as listed in [Table S5](#). PCR was carried out using *Ecl* cDNA clone GH24467 (DGRC) as a template. PCR product carrying T7 promoter sequences was used as a template for *in vitro* transcription using the MEGAscript T7 kit (Ambion) in accordance with the manufacturer's instructions. The products were incubated at 75°C for 5 min, and then gradually cooled to RT for 2 hours to produce annealed dsRNA. The dsRNA was purified by RNeasy mini kit according to the manufacturer's instructions, combined with treatment with RNase-Free DNase Set.

### Transfection and Luciferase Reporter Assays in S2 Cells

S2 cells (obtained from Michael B. O'Connor) at a density of  $1 \times 10^6$  cells/ml were seeded in 2 ml/well of SSM3 insect medium containing 10% IMS and 1% PSS in a 6-well clear flat bottom multiple well plate (Corning) just before transfection. Transfection of S2 cells was performed using the dimethyldioctadecylammonium bromide (Avanti)-mediated method ([Han, 1996](#)). For *Ecl* RNAi experiments, 0.5  $\mu$ g/well of *pAct5C-EcR* plasmid and 0.5  $\mu$ g/well of *pADH-hspEcRE-LUC* firefly luciferase reporter plasmid (both obtained from David J. Mangelsdorf) were transfected, and 10  $\mu$ g/well of *Ecl dsRNA* was added at the time of transfection and every 24 hours afterward. For *Ecl* overexpression experiments, 1.0  $\mu$ g/well of *pBRACPA* empty vector (control) or *pBRACPA-Ecl* plasmids were transfected along with the 0.5  $\mu$ g/well of *pAct5C-EcR* and 0.5  $\mu$ g/well of *pADH-hspEcRE-LUC* plasmids. For all experiments, 0.1  $\mu$ g/well of *pRL-CMV* Renilla luciferase reporter plasmid (Promega) was co-transfected and used as a reference. The cells were incubated for 3 days after transfection, after which 100  $\mu$ l/well of transfected cells were plated into a 96-well clear flat bottom microplate (Corning). Three hours after incubation, S2 cells were dosed with 25  $\mu$ l of the indicated compounds in SSM3 insect medium

(total 125  $\mu$ l/well) and harvested after the indicated time of treatment. 50  $\mu$ l of the medium in each well was then removed (total 75  $\mu$ l remaining per well), and 75  $\mu$ l/well of Dual-Glo solution (Promega) was added into each well (total 150  $\mu$ l/well). After incubation at RT in the dark for 30 min, 120  $\mu$ l/well of cell lysates were transferred to 96-well solid white flat bottom polystyrene TC-treated plates (Coster). The firefly luciferase activity and co-transfected Renilla luciferase activity were measured subsequently using the Dual-Luciferase Reporter Assay System (Promega) in accordance with the manufacturer's instructions, and analyzed with GloMax-Multi + Microplate Multimode Reader with Instinct (Promega). For each condition, cells were treated independently in 3 wells of a 96-well plate in each experiment, and the same experiment was conducted multiple times on different days. Results from a representative experiment are shown in [Figure 6](#).

#### Transfection and Luciferase Reporter Assays in HEK293 Cells

HEK cells (obtained from Michael E. Adams) at a density of  $4 \times 10^5$  cells/ml were seeded in 100  $\mu$ l/well of Opti-MEM reduced serum media (Thermo Fisher Scientific) containing 5% FBS and 1% MEM non-essential amino acids (NEAA) solution (Thermo Fisher Scientific) in a 96-well clear flat bottom microplate (Corning). Transfection of HEK293 cells was performed using Attractene transfection reagent (Qiagen) by the fast-forward transfection approach following the manufacturer's instructions. 50  $\mu$ l/well of transfection cocktail containing Opti-MEM reduced serum media, Attractene, and DNA plasmids was added to each well, bringing the final volume to 150  $\mu$ l/well. For all experiments, 0.1  $\mu$ g/well of *pcDNA3.1* empty vector (control) or *pcDNA3.1-Ecl* were transfected, along with 60 ng/well of *pERV3* receptor plasmid (Agilent Technologies) containing a modified ecdysone receptor (VgEcR) and RXR, 36 ng/well of *pEGSH-LUC* luciferase reporter plasmid (Agilent Technologies), and 0.9 ng/well of *pRL-CMV* Renilla luciferase reporter plasmid (Promega) as a reference. After 24 hours of incubation at 37°C and 5% CO<sub>2</sub>, transfection medium was removed and replaced with 150  $\mu$ l/well of DMEM with 4.5 mg/ml glucose and sodium pyruvate without L-glutamine and phenol red (w-G-SP, wo-G-PR) (Thermo Fisher Scientific) containing 10% FBS, 1% PSS, and 1% MEM NEAA solution. After 48 hours of incubation at 37°C and 5% CO<sub>2</sub>, medium was removed and replaced with 150  $\mu$ l/well of DMEM (w-G-SP, wo-G-PR) containing 1% PSS, 1% MEM NEAA solution, and the indicated compounds at the indicated concentrations. After 24 hours of incubation at 37°C and 5% CO<sub>2</sub>, 75  $\mu$ l of media was removed (total 75  $\mu$ l remaining per well) and 75  $\mu$ l/well of Dual-Glo Solution was added (total 150  $\mu$ l/well). After 20 min of incubation at RT in the dark, 120  $\mu$ l/well of cell lysates were transferred to 96-well solid white flat bottom polystyrene TC-treated plates. The firefly luciferase activity and co-transfected Renilla luciferase activity were measured subsequently using the Dual-Luciferase Reporter Assay System in accordance with the manufacturer's instructions, and analyzed with GloMax-Multi + Microplate Multimode Reader with Instinct. For each condition, cells were treated independently in 3 wells of a 96-well plate in each experiment, and the same experiment was conducted multiple times on different days. Results from a representative experiment are shown in [Figure 6](#).