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Supplemental Information

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

Naoki Okamoto, Raghuvir Viswanatha, Riyan Bittar, Zhongchi Li, Sachiko Haga-Yamanaka, Norbert Perrimon, and Naoki Yamanaka

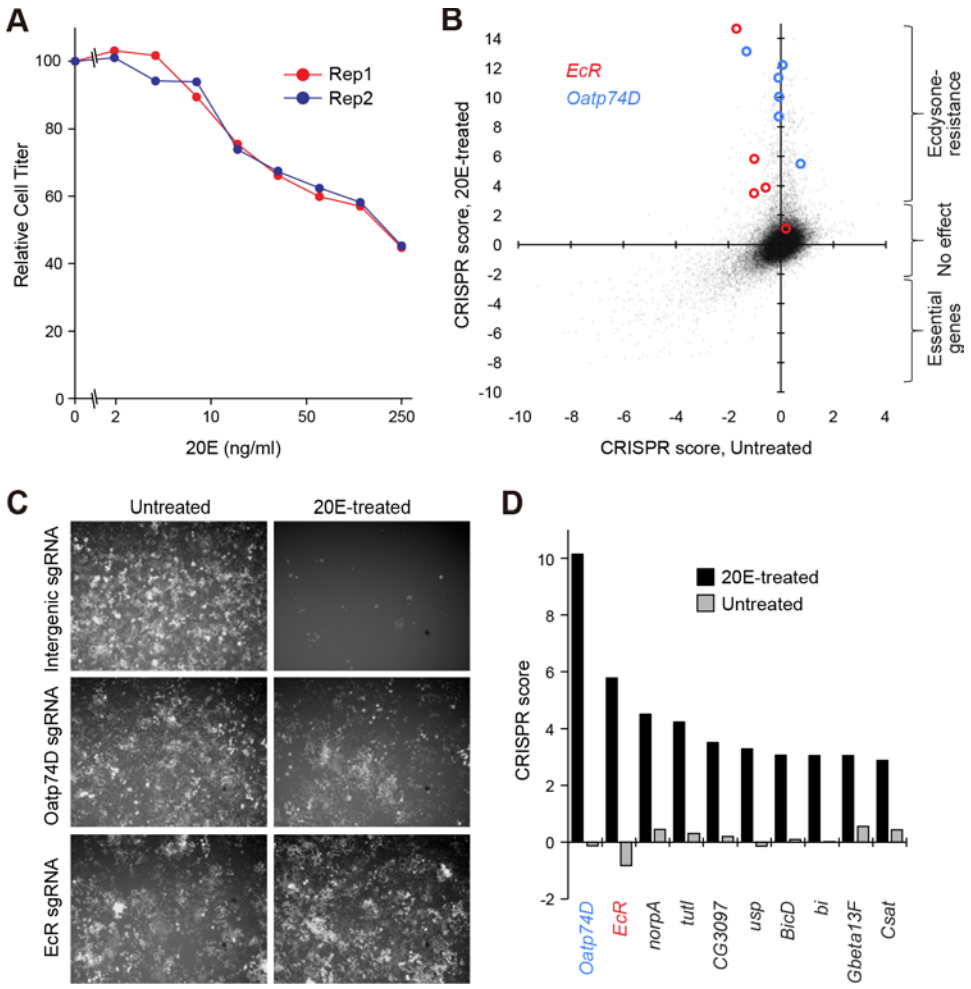

Figure S1. *In Vitro* CRISPR screening in S2R+ Cells to Identify sgRNAs that Render Resistance to 20E, Related to Figure 1.

(A) 20E-dependent inhibition of the cell proliferation in *Drosophila* S2R+ cells. Cell titer was measured after 5 days of treatment with 20E. Values are relative to 20E-untreated (0 ng/ml 20E) cell titer. Two biological replicate measurements are shown.

(B) sgRNA-level CRISPR score distribution for the *in vitro* CRISPR screening. Computed CRISPR score (Log2[fold-change]) for each sgRNA from 20E-treated versus untreated populations is plotted. All sgRNAs targeting *EcR* (red) and *Oatp74D* (blue) are marked with circles. sgRNAs targeting essential genes or having no effect are expected to be near or below zero on the vertical axis, while sgRNAs conferring ecdysone resistance are expected to be higher on the vertical axis.

(C) 20E treatment to cells with mutations on *Oatp74D* or *EcR*. The top scored sgRNA for *Oatp74D* (ACAGCCGAAAAGGCCGCAAC) or *EcR* (GACCGTGAGTATGGTTATCT) from the *in vitro* CRISPR screen was transfected into S2R+ cells. Cells were treated with 20E (15 ng/ml) and cultured for 16 days. sgRNA targeting an intergenic region (AGCGGCTATCGTTTAGTTCC) was used as a negative control. Cells are visualized by fluorescence of GFP encoded by the sgRNA expression vector.

(D) Top ten enriched genes following 20E treatment in the *in vitro* CRISPR screening. Computed CRISPR scores (mean Log2[fold-change] of all sgRNAs targeting the same gene) from each gene for 20E treatment and untreated control are shown as black and gray bars, respectively.

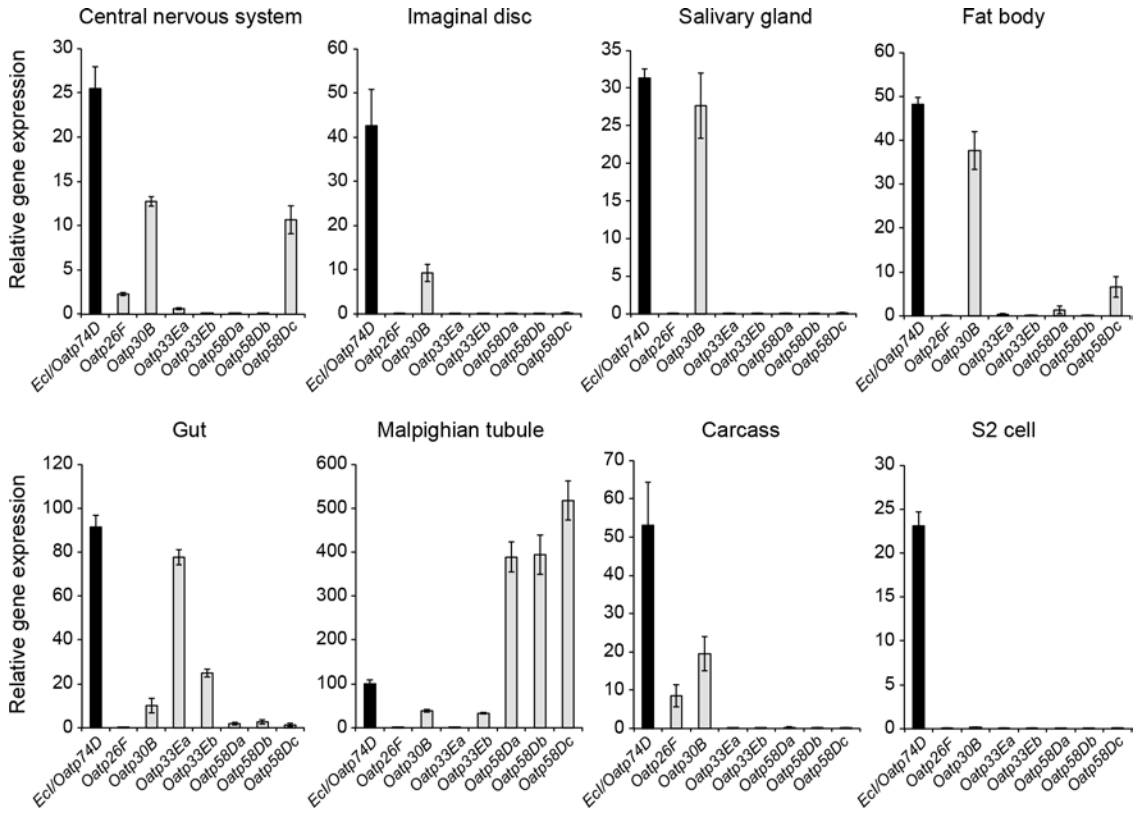

Figure S2. Expression of OATP-Encoding Genes in *Drosophila*, Related to Figure 2.

Relative expression levels of all *Drosophila* OATP-encoding genes in various tissues and S2 cells, as assessed by qRT-PCR. Tissues were dissected from wandering third instar larvae (w^{1118}). For absolute quantification of mRNAs, serial dilutions of plasmids carrying each *Oatp* cDNA sequence were used for standards. After the molar amounts were calculated, transcript levels of *Oatps* were normalized to *rp49* levels in the same samples. Values are shown as percentages relative to the *Ecl/Oatp74D* level in the Malpighian tubule. All values are the means ± SD (n = 3).

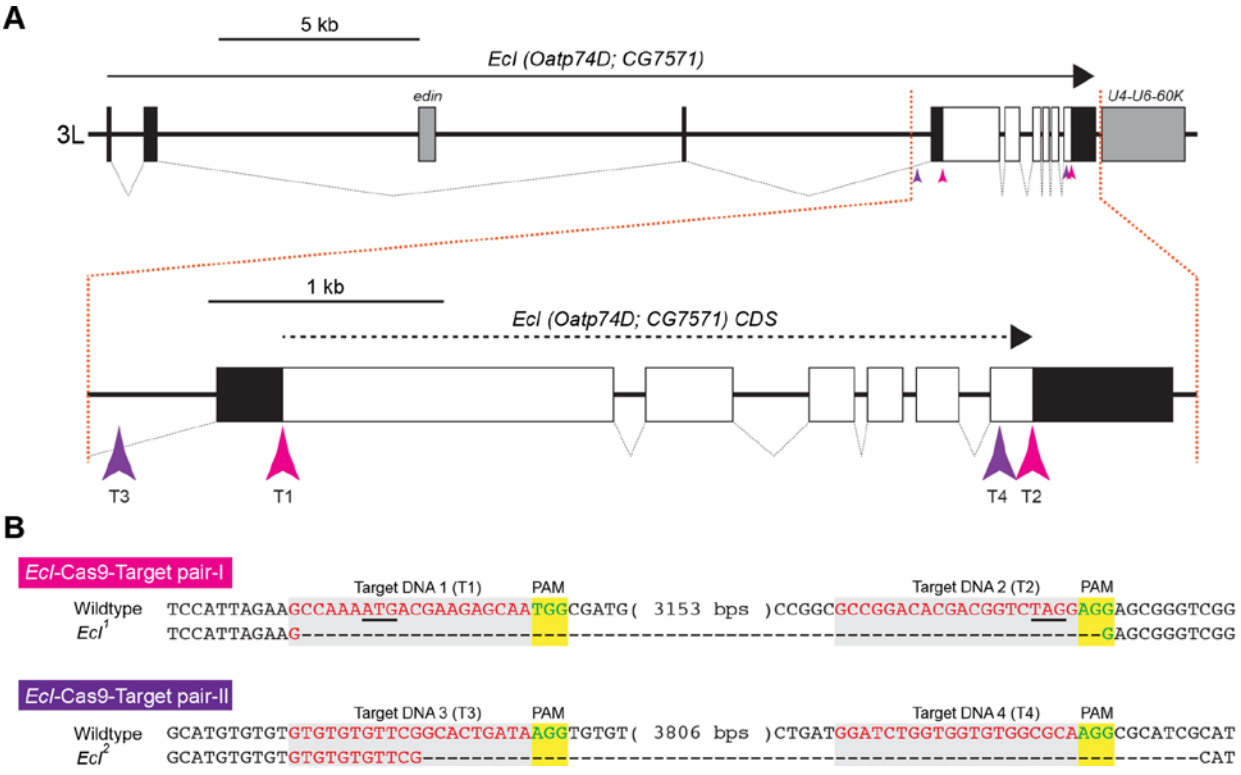

Figure S3. Generation of *EcI* Mutants Using CRISPR/Cas9 Technique, Related to Figure 3.

(A) Schematic representation of the *EcI* locus and guide RNA (gRNA) targets. The protein-coding DNA sequence (CDS) and untranslated regions are represented by open boxes and filled boxes, respectively. Neighboring genes are represented by gray boxes. Arrows indicate the orientation of the *EcI* gene. Each pair of gRNAs was designed to induce double-strand breaks near the *EcI* translational start and stop sites, respectively. T1 and T2 indicate gRNA target pair-I (pink arrowheads), and T3 and T4 indicate gRNA target pair-II (purple arrowheads).

(B) Sequences of *EcI* mutations induced by two independent pairs of gRNAs. The wildtype sequence is shown at the top, with the gRNA target sequences in red and the neighboring NGG protospacer adjacent motif (PAM) sequences in green. Deleted residues are shown as dashes. Underlines in the T1 and T2 sequences indicate *EcI* start and stop codons, respectively. EcI^{1} was generated by Target pair-I (gRNAs against T1 and T2) that caused a 3207 bp deletion, including the entire *EcI* CDS. EcI^{2} was generated by Target pair-II (gRNAs against T3 and T4) that caused a 3858 bp deletion, including the 5' untranslated region and almost the entire *EcI* CDS.

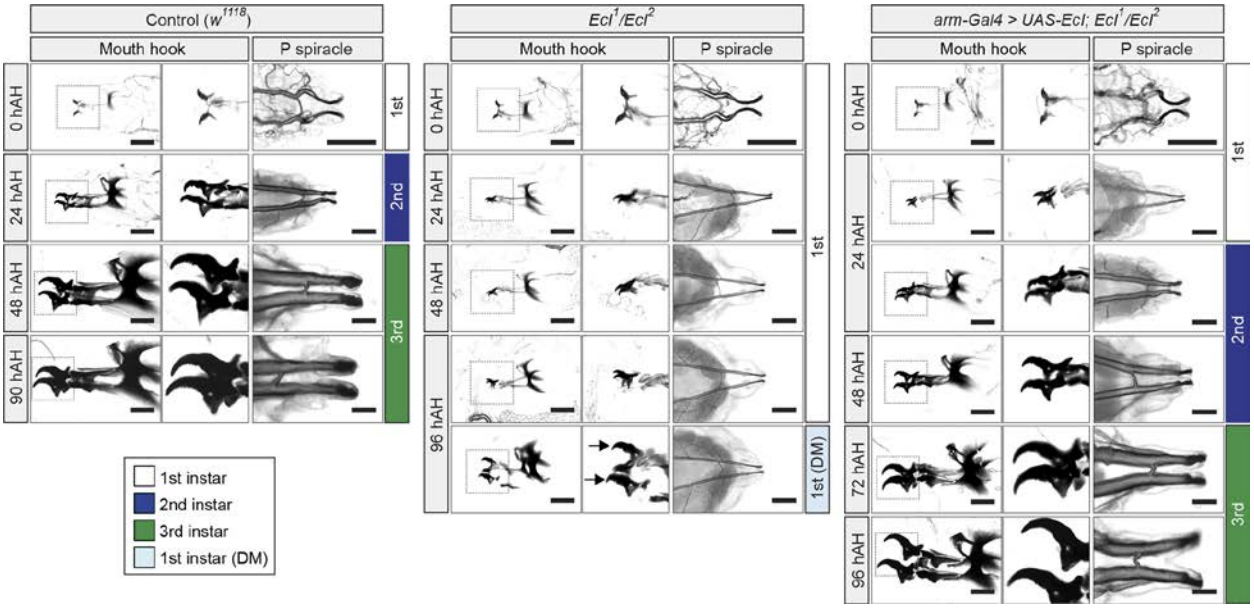

Figure S4. Detailed Developmental Phenotype of *Ecl* Mutants and Rescued Animals, Related to Figure 3.

Developmental changes in larval mouth hook and posterior spiracle morphology of control (w^{1118}), *Ecl* transheterozygous mutant (Ecl^1/Ecl^2), and *Ecl* transheterozygous mutant rescued by weak ubiquitous expression of *Ecl* (*arm-Gal4 > UAS-Ecl;* Ecl^1/Ecl^2). Arrows indicate second instar larval mouth hooks observed in the DM larva. Scale bars, 100 μm.

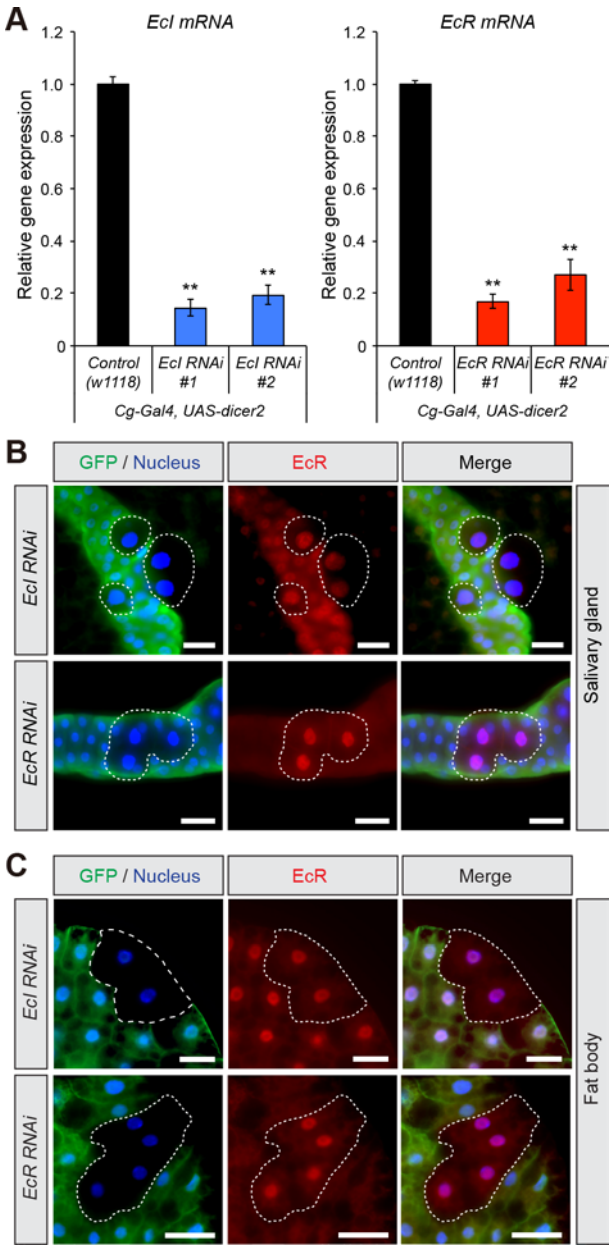

Figure S5. *EcI* Regulates Ecdysone Signaling in a Cell-Autonomous Manner without Disrupting EcR Protein Levels or Localization, Related to Figure 5.

(A) Knockdown efficiency of the two independent *UAS-RNAi* lines for *EcI* and *EcR* in the fat body at 72 hAH, as assessed by qRT-PCR. *Cg-Gal4 > UAS-dicer2* was used as a fat body-specific *Gal4* driver. Values are calculated relative to the control level. Each bar represents mean ± SD of three independent sample preparations. **p < 0.01 from Student's t test compared to control.

(B, C) Clones of salivary gland (B) and fat body (C) cells expressing *EcI-RNAi #1* or *EcR-RNAi #1* with *dicer2*. *hs-flp;; Act>CD2>GAL4, UAS-nlsGFP* was used to generate GFP-marked flip-out clones. Flippase activity was induced by 30 min heat shock. Note that flip-out clones are dominant in both tissues under this condition. The salivary gland and fat body from wandering third instar larvae were immunostained for EcR (red), GFP (green) and nuclei (blue). Knockdown of both *EcI* and *EcR* significantly decreases salivary gland cell size in a cell autonomous manner. Knockdown of *EcR* significantly reduces EcR protein levels, whereas *EcI RNAi* does not affect EcR protein levels or its localization. Scale bars, 50 μm.

Table S2. OATP Proteins Used for Generating the Phylogenetic Tree, Related to Figure 2.

Species	Protein name / Gene ID	GenBank accession number
Homo sapiens	OATP1A2	NP_066580
	OATP1B1	NP_006437
	OATP1B3	NP_062818
	OATP1C1	NP_001139418
	OATP2A1	NP_005621
	OATP2B1	NP_009187
	OATP3A1	NP_037404
	OATP4A1	NP_057438
	OATP4C1	NP_851322
	OATP5A1	NP_112220
	OATP6A1	NP_001275931
	OATP1B7 (Pseudogene)	NP_001009562
Danio rerio	Oatp1c1	NP_001038462
	Oatp1d1	NP_001335015
	Oatp1e1	XP_009299377
	Oatp1f1	NP_998082
	Oatp1f2	NP_001121745
	Oatp1f3	NP_001129156
	Oatp1f4	NP_001074135
	Oatp2a1	NP_001083051
	Oatp2b1	NP_001032767
	Oatp3a1	NP_001038653
	Oatp3a2	XP_699020
	Oatp4a1	NP_001297061
	Oatp5a1	XP_017207576
	Oatp5a2	XP_684701
Ciona intestinalis	LOC100178044	XP_018672246
	LOC100178047	XP_018667361
	LOC100178826	XP_018671846
	LOC100179137	XP_002129833
	LOC100179346	XP_009862505
	LOC100180583	XP_002120104
	LOC100181309	XP_018670607
	LOC100181383	XP_002129378
	LOC100182167	XP_009860360
	LOC100182935	XP_002120038
	LOC100184536	XP_018669740
	LOC101242397	XP_004227423
	LOC101242848	XP_018668290
	LOC104266209	XP_009860177
	LOC108949902	XP_018669460
Drosophila melanogaster	Ecl/Oatp74D*	NP_648989*
	Oatp26F	NP_609055
	Oatp30B	NP_723463
	Oatp33Ea	NP_609568
	Oatp33Eb	NP_609570
	Oatp58Da	NP_611657
	Oatp58Db	NP_611658
	Oatp58Dc	NP_611659

Bombyx mori	BGIBMGA002723*	XP_004932455*
	BGIBMGA003667	XP_012549229
	BGIBMGA008669	XP_012545505
	BGIBMGA013485	XP_012545879
Tribolium castaneum	TC034513*	XP_015832992*
	TC001718	XP_967848
	TC001740	XP_015836575
	TC004793	XP_972698
Apis mellifera	GB42865*	XP_006561855*
	GB50890	XP_016770062
	GB50891	XP_016770061
	GB51165	XP_006566270
	GB55877	XP_016769799
	GB55881	XP_016769795
Acyrthosiphon pisum	ACYPI064961*	XP_003241873*
	ACYPI008520	XP_008182668
	ACYPI50277	XP_003246488
Pediculus humanus	PHUM617040	XP_002433206
	PHUM125290*	XP_002424286*
	PHUM454200	XP_002429870
	PHUM502970	XP_002430869
	PHUM616210	XP_002433182
Daphnia pulex	DAPPUDRAFT_303505*	EFX81434*
	DAPPUDRAFT_46372	EFX84993
	DAPPUDRAFT_54555	EFX77080
Ixodes scapularis	ISCW023852*	XP_002400770*
	ISCW000594	XP_002404592
	ISCW000596	XP_002404594
	ISCW006481	XP_002435666
	ISCW011144	XP_002412159
	ISCW011146	XP_002412161
	ISCW012022	XP_002414101
	ISCW014692	XP_002415171
	ISCW018349	XP_002434179
Caenorhabditis elegans	F21G4.1	NP_509659
	F47E1.2	NP_509531
	F47E1.4	NP_509532
	F53B1.8	NP_001294793
	K02G10.5	NP_508802
	Y32F6B.1	NP_505689
	Y70G10A.3	NP_499267

* (Ecl/Oatp74D subfamily clade proteins)

Table S3. Effect of *Oatp RNAi* and *EcR RNAi* on Ecdysone-Dependent Developmental Events, Related to Figure 2.

Target tissue	*Gal4* driver	Gene name	CG number	Stock ID	Phenotype
Salivary gland	*Fkh-Gal4>UAS-dicer2*	*EcI/Oatp74D*	*CG7571*	NIG: 7571R-1	25 / 25
				VDRC: 37295	25 / 25
		Oatp26F	*CG31634*	VDRC: 2650	0 / 25
				VDRC: 109633	0 / 25
		Oatp30B	*CG3811*	VDRC: 22983	0 / 25
				VDRC: 110237	0 / 25
		Oatp33Ea	*CG5427*	BDSC: 50736	0 / 25
				VDRC: 105560	0 / 25
		Oatp33Eb	*CG6417*	VDRC: 42805	0 / 25
				VDRC: 100431	0 / 25
		Oatp58Da	*CG30277*	VDRC: 44122	0 / 25
				VDRC: 106377	0 / 25
		Oatp58Db	*CG3382*	NIG: HMJ24090	0 / 25
				VDRC: 100348	0 / 25
		Oatp58Dc	*CG3380*	BDSC: 44583	0 / 25
				VDRC: 39469	0 / 25
		EcR	*CG1765*	BDSC: 9327	25 / 25
Fat body	*Cg-Gal4>UAS-dicer2*	*EcI/Oatp74D*	*CG7571*	NIG: 7571R-1	25 / 25
				VDRC: 37295	25 / 25
		Oatp26F	*CG31634*	VDRC: 2650	0 / 25
				VDRC: 109633	0 / 25
		Oatp30B	*CG3811*	VDRC: 22983	0 / 25
				VDRC: 110237	0 / 25
		Oatp33Ea	*CG5427*	BDSC: 50736	0 / 25
				VDRC: 105560	0 / 25
		Oatp33Eb	*CG6417*	VDRC: 42805	0 / 25
				VDRC: 100431	0 / 25
		Oatp58Da	*CG30277*	VDRC: 44122	0 / 25
				VDRC: 106377	0 / 25
		Oatp58Db	*CG3382*	NIG: HMJ24090	0 / 25
				VDRC: 100348	0 / 25
		Oatp58Dc	*CG3380*	BDSC: 44583	0 / 25
				VDRC: 39469	0 / 25
		EcR	*CG1765*	BDSC: 9327	25 / 25

The number of animals (per 25 animals) showed defect in ecdysone-dependent glue-GFP expression in the salivary gland or defect in ecdysone-dependent fat body migration are shown. *UAS-RNAi* lines from National Institute of Genetics (NIG), Vienna *Drosophila* Resource Center (VDRC) and Bloomington *Drosophila* Stock Center (BDSC) were crossed to salivary gland or fat body-specific *Gal4* drivers. *Sgs3-GFP* was used to detect ecdysone-dependent gene expression in the salivary gland and *UAS-2xEGFP* was used to label fat body cells.

Table S4. Lethality of Ubiquitous *EcI* Overexpression, Related to Figure 3.

Target tissue	*Gal4* driver	Stock ID	Phenotype
Ubiquitous	*Act5C-Gal4*	BDSC: 3954	Embryonic lethal (100%)
		BDSC: 4414	Embryonic lethal (100%)
	da-Gal4	BDSC: 55850	Early larval lethal (100%)
	TubP-Gal4	BDSC: 5138	Early larval lethal (100%)
	arm-Gal4	BDSC: 1560	Viable from embryo to adult
		BDSC: 1561	Viable from embryo to adult

Ubiquitous *Gal4* lines from Bloomington *Drosophila* Stock Center (BDSC) were crossed to *UAS-EcI*.

Table S5. Oligonucleotides Used in This Study, Related to STAR Methods.

Primers for qRT-PCR			
Gene Name	CG Number	Forward (5'-3')	Reverse (5'-3')
EcI/Oatp74D	*CG7571*	TGCAGTGCCGCTCTCAACTGTACC	TCACAGTAACCGTTGACCGCCTCC
Oatp26F	*CG31634*	TCAACTCAGCCTGACCAGCGACAG	ATGGGCAAGGCGATGAGCAGACAC
Oatp30B	*CG3811*	GAGGAGGACTTCGATGAGGAGCAG	ATCATCACCAGCAGCGAGAGCAGC
Oatp33Ea	*CG5427*	ATCTACGGAGCTGGTCACGAGGTG	TTGTCCACTCCACAGAGTCGCTCG
Oatp33Eb	*CG6417*	TTGCGTTGGCTTTCGCCTACTGGG	TGGAGGGAATCACAGCCACCACAC
Oatp58Da	*CG30277*	TTGAGACATGACAGAGGAGCGAGG	TGGCAAATCTTTGCATGGAGGGGC
Oatp58Db	*CG3382*	CTACGCTAGTCGAGGACATCGTCC	TGTCAGCCGCAAAGCTTCTTCGCC
Oatp58Dc	*CG3380*	AGAGCGAGAATCCCAGTAGCCTGG	TTCGGAGTGGTCTCTTCACCGTC
EcR (Common)	*CG1765*	TCAACCACAGCCACAGCTCCTTCC	TGATGGGTCCTATGGCCGCACTTC
E74 (IsoformA)	*CG32180*	TGAGACGCGAGGAATACCCTGGAC	AACTGCCAGCGTGTAGCCGTTTCC
E75 (IsoformA)	*CG8127*	TCAGCAGGCCAATCTGCACCACTC	TGATGTACTCGGGAGTCTGGGGAC
E75 (IsoformB)	*CG8127*	AGCAGCACCAGCACCAGCAACAAC	ATTGCCCGCACTGGAGTTGCTCGA
rp49	*CG7939*	AGCTGTCGCACAAATGGCGCAAGC	TTGAATCCGGTGGGCAGCATGTGG
Oligonucleotides for generating *EcI* target gRNAs			
Allele	Target ID	Forward (5'-3')	Reverse (5'-3')
EcI^{1}	Target pair-I (T1)	CTTCGCCAAAATGACGAAGAGCAA	AAACTTGCTCTTCGTCATTTTGGC
	Target pair-I (T2)	CTTCGCCGGACACGACGGTCTAGG	AAACCCTAGACCGTCGTGTCCGGC
EcI^{2}	Target pair-II (T3)	CTTCGTGTGTGTTCGGCACTGATA	AAACTATCAGTGCCGAACACACAC
	Target pair-II (T4)	CTTCGGATCTGGTGGTGTGGCGCA	AAACTGCGCCACACCACCAGATCC
Primers for screening *EcI* CRISPR mutants			
Allele		Forward (5'-3')	Reverse (5'-3')
EcI^{1}		ACCAATCTACCTCGACTTCTGG	ACGCTCGAAGATGCCACTTAAC
EcI^{2}		TCAGCGCTCTTATCATAGTGCC	ACGCTCGAAGATGCCACTTAAC
Primers for generating *EcI* dsRNA			
Forward (5'-3')		TAATACGACTCACTATAGGGTGGCTGAATGCCAGCAGTGAACAGG	
Reverse (5'-3')		TAATACGACTCACTATAGGGTTTAGCTTGGGCTTCTCCTCCGGCT	