Supplementary information

A Halloween gene *noppera-bo* encodes a glutathione *S*-transferase essential for ecdysteroid biosynthesis via regulating the behaviour of cholesterol in *Drosophila*

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Supplementary figure S1. *in situ* RNA hybridisation analysis in embryos.

Lateral (a-f) and ventral (g) views are shown. (a-f) *nobo* mRNA was observed in epidermal cells during stages 5-14. (e) At stage 11, the *nobo* signal was detected in the amnioserosa (arrow), which is thought to synthesise ecdysteroids. (g) At stage 16 and later, the *nobo* signal was detected in the PG cells (arrowheads). An inset with a higher magnification of the PG. Scale bar: 100 μm.

Supplementary figure S2. PCR genotyping analysis of first instar larvae of the wild-type Oregon R, *nobo*^{KO} heterozygous animals (balanced with *CyO*) and *nobo*^{KO} homozygous animals rescued by maternally supplied cholesterol and 7dC.

nobo^{KO} homozygous animals were collected as GFP-negative offspring of *nobo*^{KO}/*CyO-GFP* parents that were fed cholesterol (C) or 7dC. For each genotype, PCR products from three single first instar larvae were used for agarose gel electrophoresis. The PCR primers for genotyping are illustrated in Fig. 2a ('F' and 'R') and described in Supplementary table S3. Black and Magenta arrowheads indicate PCR bands corresponding to the wild-type (2260 bp) and *nobo*^{KO} (4047 bp) alleles, respectively. Lanes 1-3, Oregon R without any sterol supplements; lanes 4-6, *nobo*^{KO}/*CyO* without any sterol supplements; lanes 7-9, *nobo*^{KO}/*nobo*^{KO} first instar larvae that were maternally supplied with cholesterol; and lanes 10-12, *nobo*^{KO}/*nobo*^{KO} first instar larvae that were maternally supplied with 7dC.

Supplementary figure S3. A phylogenetic tree shows the relationship between Nobo and other GST proteins.

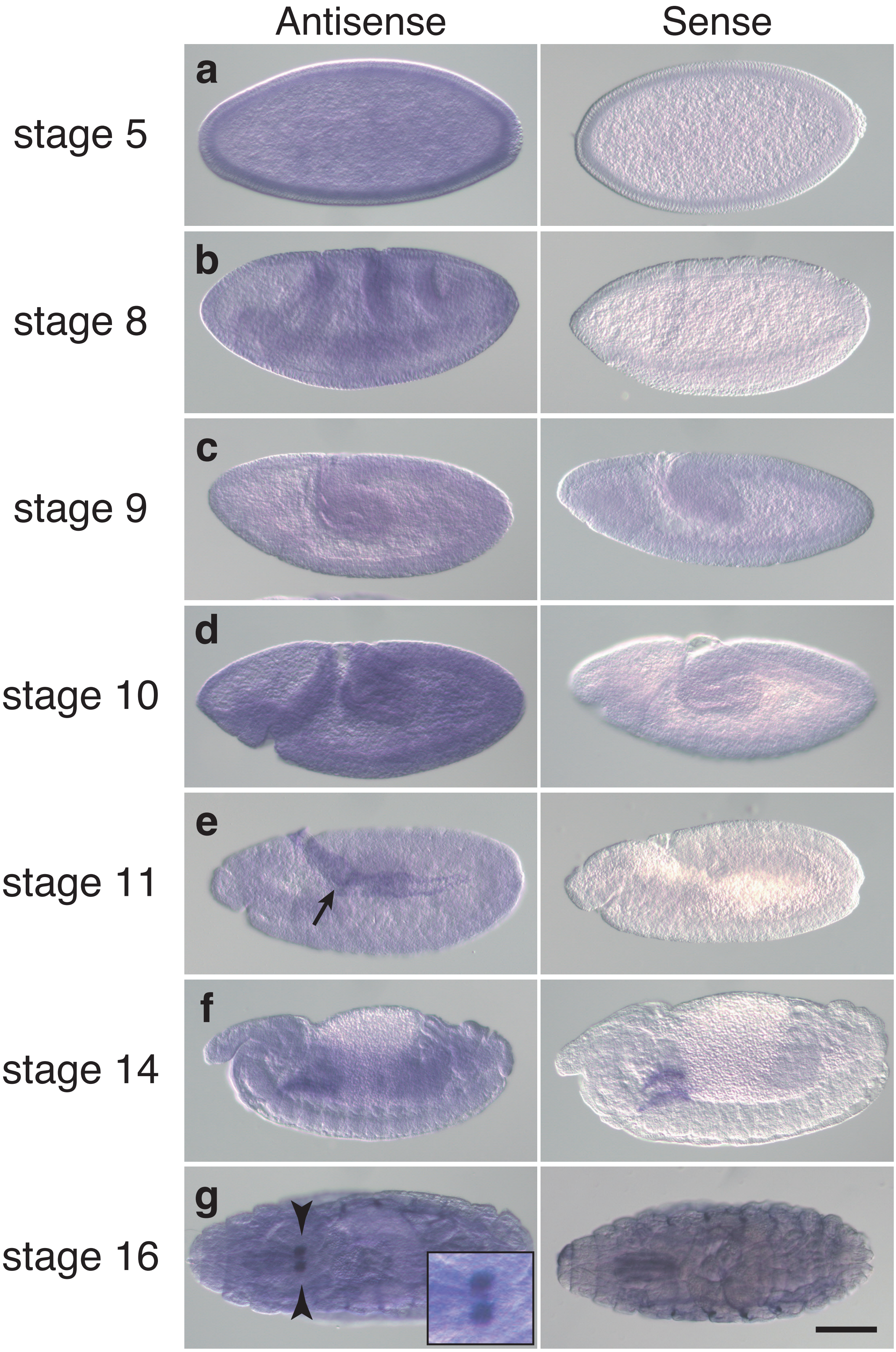
(a) The rootless tree that was generated based on the entire amino acid sequence of *Drosophila melanogaster* Nobo and the other 277 GST proteins by the neighbour-joining method through the MEGA5 program [68]. The names and GenBank accession numbers of GST proteins in this tree are listed in Supplementary table S4. Aa, *Aedes aegypti*; Ag, *Anopheles gambiae*; Tc, *Tribolium castaneum*; Cq, *Culex quinquefasciatus*; Dm, *Drosophila melanogaster*; Bm, *Bombyx mori*; Ph, *Pediculus humanus corporis*; Nv, *Nasonia vitripennis*; Am, *Apis mellifera*; Ap, *Acyrthosiphon pisum*; Dp, *Danaus plexippus*; Ce, *Caenorhabditis elegans*; Hs, *Homo sapiens*. Pink and blue areas indicate clades that include *nobo* subfamily genes and human GSTA3 family genes, respectively.

(b) A phylogenetic clade of *nobo* subfamily genes, which was derived from (a). Each number indicates the bootstrap value for each branch.

A scale bar shows the number of amino acid substitutions per site between two sequences.

Supplementary Reference

68. Tamura, K. *et al.* MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28,** 2731–2739 (2011).



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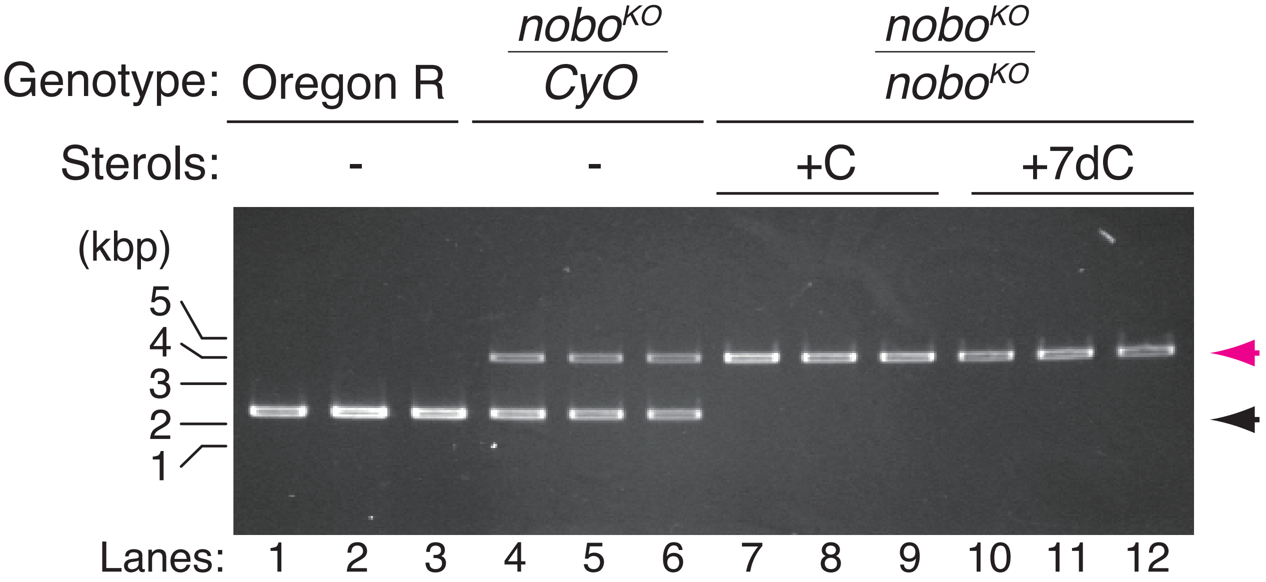

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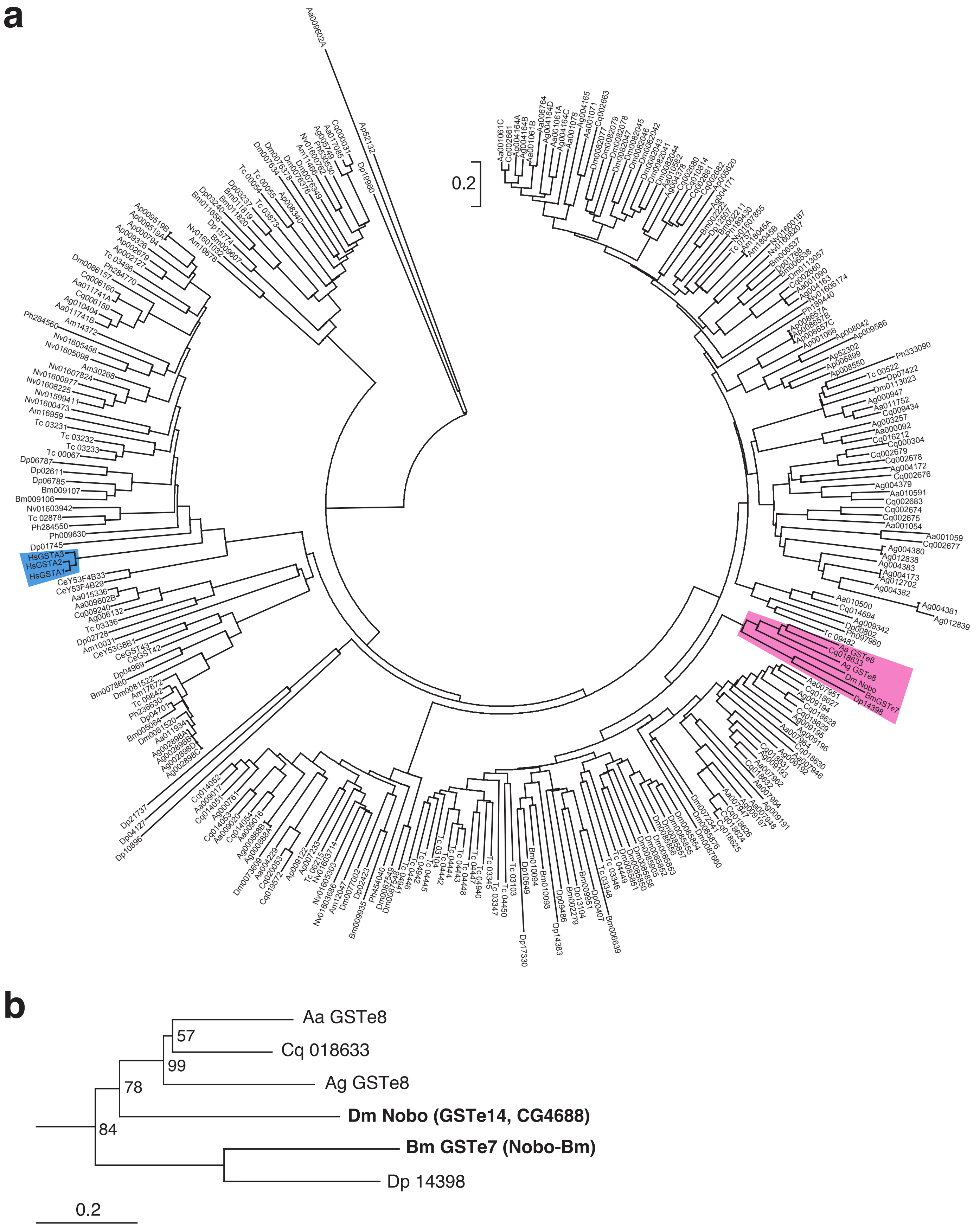
Supplementary figure S2



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Supplementary figure S3

Supplementary table S1. Fly strains used for transgenic rescue for ***nobo*^{KO}**

The lethality of *nobo*^{KO} animals with the expression of *nobo* and other GST genes. Numerical data are the same as shown in Table 1. Each cross of parental males with parental females is indicated. Genetic markers of the *CyO* and *TM6B* balancer chromosomes are *Cy*$^{-}$ and *Hu*$^{-}$, respectively. *nobo*^{KO}/*nobo*^{KO} and viable control *nobo*^{KO}/+ adults were *Cy*$^{+}$ *Hu*$^{+}$ and *Cy*$^{-}$ *Hu*$^{+}$, respectively.

Back-ground	Transgene	Parents (males)	Parents (females)	*Cy*$^{+}$, *Hu*$^{+}$	*Cy*$^{-}$, *Hu*$^{+}$
nobo^{KO}	*nobo#1*	*w; nobo*^{KO}*/CyO; phm-GAL4#22/TM6B*	*w; nobo*^{KO}*/CyO; P{UAS-nobo-HA}#1/TM6B*	101	220
	nobo#2	*w; nobo*^{KO}*/CyO; phm-GAL4#22/TM6B*	*w; nobo*^{KO}*/CyO; P{UAS-nobo-HA}#2/TM6B*	124	255
	nobo#3	*w; nobo*^{KO}*/CyO; P{UAS-nobo-HA}#3/TM6B*	*w; nobo*^{KO}*/CyO; phm-GAL4#22/TM6B*	46	102
	nobo-Bm#1	*w; nobo*^{KO}*/CyO; phm-GAL4#22/TM6B*	*w; nobo*^{KO} *P{UAS-nobo-Bm-HA}#1/CyO*	85	377
	nobo-Bm#5	*w; nobo*^{KO}*/CyO; P{UAS-nobo-Bm-HA}#5/TM6B*	*w; nobo*^{KO}*/CyO; phm-GAL4#22/TM6B*	77	348
	CG6673B	*w; nobo*^{KO}*/CyO; P{UAS-CG6673B}/TM6B*	*w; nobo*^{KO}*/CyO; phm-GAL4#22/TM6B*	0	105
	GSTe4	*w; nobo*^{KO}*/CyO; phm-GAL4#22/TM6B*	*w; P{UAS-GSTe4}attP40 nobo*^{KO}*/CyO*	0	102
	GSTe12	*w; nobo*^{KO}*/CyO; phm-GAL4#22/TM6B*	*w; P{UAS-GSTe12}attP40 nobo*^{KO}*/CyO*	0	117

Supplementary table S2. Fly strains used for transgenic rescue of *nobo* RNAi

The lethality of *nobo* RNAi animals with the expression of *nobo* and other GST genes. Numerical data are the same as shown in Table 1. Each cross of parental males with parental females is indicated. Genetic markers of the *CyO*, *TM6B* and *TM3* balancer chromosomes are Cy^-, Hu^- and Sb^-, respectively. $nobo^{KO}$ RNAi and viable non-RNAi adults were Cy^+ Hu^+ Sb^+ and Hu^-, respectively.

Back-ground	Transgene	Parents (males)	Parents (females)	CyO^+, Hu^+, Sb^+	Hu^-
nobo RNAi	*nobo-Bm#1*	*w; UAS-dicer2; phm-GAL4#22/TM6*	*w; P{UAS-nobo-Bm-HA}#1/CyO; P{UAS-nobo-IR}#40316/TM6B*	120	498
	nobo-Bm#2	*w; UAS-dicer2; phm-GAL4#22/TM6*	*w; P{UAS-nobo-Bm-HA}#2/CyO; P{UAS-nobo-IR}#40316/TM6B*	105	350
	GSTe4	*w; UAS-dicer2; phm-GAL4#22/TM6*	*w; P{UAS-GSTe4}attP40/CyO; P{UAS-nobo-IR}#40316/TM6B*	0	92
	GSTe12	*w; UAS-dicer2; phm-GAL4#22/TM6*	*w; P{UAS-GSTe12}attP40/CyO; P{UAS-nobo-IR}#40316/TM6B*	0	105
	sepia	*w; UAS-dicer2; phm-GAL4#22/TM6*	*w; P{UAS-sepia}; P{UAS-nobo-IR}#40316/TM6B*	0	249
	CG6673A	*w; UAS-dicer2; phm-GAL4#22/TM6*	*w; P{UAS-CG6673A}; P{UAS-nobo-IR}#40316/TM6B*	0	120
	CG6673B	*w; UAS-dicer2; phm-GAL4#22/TM6*	*w; P{UAS-nobo-IR}#101884; P{UAS-CG6673B}/TM6B*	0	278
	CG6662	*w; UAS-dicer2; phm-GAL4#22/TM6*	*w; P{UAS-CG6662}; P{UAS-nobo-IR}#40316/TM3 Sb*	0	107

Supplementary table S3. List of primers used in this study.

Each gene specific sequence is indicated in upper case letters. The regions that are recognised by restriction enzymes are underlined.

Purpose	Name of primers	Sequence (5´> 3´)	Remarks
qRT-PCR primers for quantifying *nobo*	nobo-qRT-PCR-F2	CGGTCCGCAGTTGCCTTATGC	
	nobo-qRT-PCR-R2	GGACTAGGGTGGGAACACTGTGCTG	
Vector construction to overexpress *nobo*	UAS-nobo-HA-F	gtcagatctATGTCTCAGCCCAAGCCGATTTTG	
	UAS-nobo-HA-R	actgcggccgccCTCCACCTTCTCGGTGACTACCGCTG	
Vector construction to overexpress *nobo-Bm*	UAS-BmGSTe7-HA-F	gtcagatctATGTCCATTGTTCGGTGTAATATG	
	UAS-BmGSTe7-HA-R	actgcggccgccGTTTGGCTTGTAAAGACTCATAAAATA	
Vector construction to overexpress *GSTe4*	GstE4-CDS-F	agtcagatctATGGGTAAGATATCGCTATAC	
	GstE4-CDS-R	tcagtctagaTTACGAAACTATGGTGAAG	
Vector construction to overexpress *GSTe12*	CG16936-CDS-F	agtcagatctATGTCAAAGCCAGCTCTGTATT	
	CG16936-CDS-R	tcagtctagaCTACTTGCCACGGTTTTCTG	
Vector construction to knock-out *nobo*	noboKO5F-*Xho*I	CTCGAGTAGCCTGATGCTGTCTCCAAGC	
	noboKO5R-loxP-*Not*I	GCGGCCGCATAACTTCGTATAGCATACATTATACGAAGTTATTAGCCACAGTACTGATTGATGGTGG	
	noboKO3F-loxP-*Hind*III	AAGCTTATAACTTCGTATAATGTATGCTATACGAAGTTATCACCGAGAAGGTGGAGTAGCACTAG	
	noboKO3R-*Sph*I	GCATGCCCAAACGTAAAATCCTGAGACGTAAGC	
Genotyping for *nobo*^{KO} allele	noboKO-genotype	GGCGCGAGAGGTACATTGTTTAGC	‘F’ in Fig. 2a
	noboKO-genotype-R	CACTTGGCAGCTGGAAAGTCAGAG	‘R’ in Fig. 2a

Supplementary table 4. GST proteins used for generating the phylogenic tree in Fig. 1h and Supplementary fig. S2.

Red characters indicate Nobo sub-clade.

Species	Gene ID	Cluster
Aedes aegypti	AAEL000092	GSTx1
	AAEL004229	GSTt4
	AAEL001054	GSTd4
	AAEL001059	GSTd3
	AAEL001071	GSTd5
	AAEL001078	GSTd2
	AAEL001061	GSTd1
	AAEL001090	GSTd7
	AAEL006764	Delta-GST
	AAEL007955	GSTe8
	AAEL007954	GSTe1
	AAEL007962	GSTe4
	AAEL007951	GSTe2
	AAEL007964	GSTe5
	AAEL007948	GSTe7
	AAEL007946	GSTe6
	AAEL007947	GSTe3
	AAEL009020	GSTt3
	AAEL009017	GSTt1
	AAEL009016	GSTt2
	AAEL009602	
	AAEL010500	GSTx2
	AAEL010582	GSTd11
	AAEL010591	GSTd6
	AAEL011741	GSTs1
	AAEL011752	GSTi1
	AAEL011934	GSTz1
	AAEL015336	GST
	AAEL017085	GSTo1
Anopheles gambiae	AGAP005749	GSTO1
	AGAP006132	GST
	AGAP002898	GSTZ1
	AGAP003257	GSTU2
	AGAP004163-PB	GSTD7
	AGAP004164-PC	GSTD1
	AGAP004165	GSTD2
	AGAP004171	GSTD8
	AGAP004172	Delta-GST

	AGAP004173	GSTD5
	AGAP004378	GSTD11
	AGAP004379	GSTD6
	AGAP004380	GSTD12
	AGAP004381	GSTD4
	AGAP004382	GSTD3
	AGAP004383	GSTD10
	AGAP010404	GSTS1
	AGAP009190	GSTE8/U4
	AGAP009191	GSTE6
	AGAP009192	GSTE5
	AGAP009193	GSTE4
	AGAP009194	GSTE2
	AGAP009195	GSTE1
	AGAP009196	GSTE7
	AGAP009197	GSTE3
	AGAP009342	GSTU3
	AGAP000761	GSTT1
	AGAP000888	GSTT2
	AGAP000947	GSTU1
	AGAP012702	Delta-GST
	AGAP012838	Delta-GST
	AGAP012839	Partial;Delta-GST
Tribolium castaneum	Tc_04450	Epsilon-GST
	Tc_04449	Epsilon-GST
	Tc_04448	Epsilon-GST
	Tc_04447	Epsilon-GST
	Tc_04940	Epsilon-GST
	Tc_04941	Epsilon-GST
	Tc_04942	Epsilon-GST
	Tc_04446	Epsilon-GST
	Tc_04445	Epsilon-GST
	Tc_04444	Epsilon-GST
	Tc_04443	Epsilon-GST
	Tc_04442	Epsilon-GST
	Tc_00522	Theta-GST excluded
	Tc_03231	Sigma-GST
	Tc_03232	Sigma-GST
	Tc_03233	Sigma-GST
	Tc_03336	N-terminal domain only
	Tc_03104	Partial; Epsilon-GST
	Tc_03345	Epsilon-GST
	Tc_03346	Epsilon-GST

	Tc_03347	Partial; Epsilon-GST
	Tc_03348	Epsilon-GST
	Tc_03103	Epsilon-GST
	Tc_03496	Sigma-GST
	Tc_02878	Sigma-GST
	Tc_00067	Sigma-GST
	Tc_00055	Omega-GST
	Tc_00054	Omega-GST
	Tc_03873	Omega-GST
	Tc_07571	Delta-GST
	Tc_09482	Epsilon-GST
	Tc_09842	Zeta-GST
	Tc_06215	Theta-GST
Culex quinquefasciatus	CPIJ000031	Omega-GST
	CPIJ006159	Sigma-GST
	CPIJ006160	Sigma-GST
	CPIJ018624	Epsilon-GST
	CPIJ018625	Epsilon-GST
	CPIJ018626	Epsilon-GST
	CPIJ018627	Epsilon-GST
	CPIJ018628	Partial; Epsilon-GST
	CPIJ018629	Epsilon-GST
	CPIJ018630	Epsilon-GST
	CPIJ018631	Epsilon-GST
	CPIJ018632	Epsilon-GST
	CPIJ018633	Epsilon-GST
	CPIJ019572	Theta-GST
	CPIJ000304	Delta-GST
	CPIJ009434	Delta-GST
	CPIJ009240	GST
	CPIJ020053	Theta-GST
	CPIJ010814	Partial; Delta-GST clusters with CPIJ002680
	CPIJ002660	Delta-GST
	CPIJ002661	Delta-GST
	CPIJ002663	Delta-GST
	CPIJ002674	Delta-GST
	CPIJ002675	Delta-GST
	CPIJ002676	Delta-GST
	CPIJ002677	C-terminal domain only
	CPIJ002678	Delta-GST
	CPIJ002679	Delta-GST
	CPIJ002680	Delta-GST
	CPIJ002681	Delta-GST

	CPIJ002682	Partial; Delta-GST
	CPIJ002683	Delta-GST
	CPIJ014051	Theta-GST
	CPIJ014052	Theta-GST
	CPIJ014053	Theta-GST
	CPIJ014054	Theta-GST
	CPIJ014694	Delta-GST
	CPIJ016212	Delta-GST
	TBLASTN	Cp_GSTZ; overlaps with CPIJ009709
Drosophila melanogaster	FBpp0087660	Epsilon-GST
	FBpp0087548	Theta-GST
	FBpp0087549	Theta-GST
	FBpp0086857	Epsilon-GST
	FBpp0086157	GSTS1
	FBpp0085905	GSTE10
	FBpp0085850	GSTE1
	FBpp0085851	GSTE2
	FBpp0085852	GSTE3
	FBpp0085853	GSTE4
	FBpp0085854	GSTE5
	FBpp0085855	GSTE6
	FBpp0085856	GSTE7
	FBpp0085857	GSTE8
	FBpp0085858	GSTE9
	FBpp0085876	Epsilon-GST
	FBpp0072341	Epsilon-GST
	FBpp0076348	Omega-GST
	FBpp0076349	se;Omega
	FBpp0076378	Omega
	FBpp0076376	Omega-GST
	FBpp0113023	gfzf;Delta/Epsilon superclass2
	FBpp0081522	Zeta-GST
	FBpp0081520	Zeta-GST
	FBpp0082079	GSTD10
	FBpp0082078	GSTD9
	FBpp0082077	GSTD1
	FBpp0082041	GSTD2
	FBpp0082042	GSTD3
	FBpp0082043	GSTD4
	FBpp0082044	GSTD5
	FBpp0082045	GSTD6
	FBpp0082046	GSTD7
	FBpp0082047	GSTD8

	FBpp0113057	Delta-GST
	FBpp0073609	Theta-GST
	FBpp0077002	Theta-GST
Bombyx mori	BGIBMGA002222	GSTd2
	BGIBMGA002211	GSTd3
	BGIBMGA002279	GSTe2
	BGIBMGA005064	GSTz1
	BGIBMGA006537	GSTd1
	BGIBMGA006538	Delta-GST
	BGIBMGA006639	Partial(C-terminal domain only)
	BGIBMGA007860	Partial;divergent;GSTz2
	BGIBMGA009106	GSTs1
	BGIBMGA009107	GSTs2
	BGIBMGA009607	Partial;GSTo3;condirmed by SilkDB
	BGIBMGA009935	GSTt1
	BGIBMGA009951	GSTe3
	BGIBMGA010094	GSTe4
	BGIBMGA010093	GSTe5
	BGIBMGA011658	GSTo2
	BGIBMGA011819	GSTo1
	BGIBMGA011820	GSTo4
	BmGSTe7	BmGSTe7
Pediculus humanus	PHUM009630	Sigma-GST
	PHUM097960	Partial;Delta-GST
	PHUM189430	Delta-GST
	PHUM189440	Delta-GST
	PHUM236630	
	PHUM284550	Sigma-GST
	PHUM284560	Partial;Sigma-GST
	PHUM284770	Sigma-GST
	PHUM333090	Delta-GST
	PHUM454040	Theta-GST
	PHUM530530	Omega-GST
Nasonia vitripennis	XP_001605303	GSTT2
	XP_001608225	GSTS1
	XP_001607824	Partial;GSTS22;Clusters;with S3,S8,S1
	XP_001608207	GSTD5
	XP_001599411	GSTS3
	XP_001603942	GSTS4
	XP_001605098	Partial;GSTS61
	XP_001605456	GSTS5
	XP_001600473	GSTS7
	XP_001600977	GSTS8

	XP_001600187	GSTD4
	XP_001600762	GSTO1
	XP_001601032	GSTO2
	XP_001606174	GSTD3
	XP_001607855	GSTD1
	XP_001603686	GSTT1
	XP_001603714	GSTT3
	TBLASTN	GSTZ1
Apis mellifera	GB11466	GSTO1
	GB14372	GSTS4
	GB17672	GSTZ1
	GB18045	GSTD1
	GB30268	Partial;GSTS21
	GB16959	GSTS1
	GB10031	GST
	GB19678	Partial
	GB12047	GSTT1
Acyrthosiphon pisum	ACYPI002127	Sigma-GST
	ACYPI002679	Sigma-GST
	ACYPI000794	Sigma-GST
	ACYPI009586	Delta-GST
	ACYPI009122	Theta-GST
	ACYPI007233	Theta-GST
	ACYPI008340	Omega-GST
	ACYPI008042	Delta-GST
	ACYPI009519	Sigma-GST
	ACYPI008550	Delta-GST
	ACYPI009326	Sigma-GST
	ACYPI52132	Partial
	ACYPI001068	Delta-GST
	ACYPI008657	Delta-GST
	ACYPI006899	Delta-GST
	ACYPI52302	Delta-GST
	ACYPI005620	Delta-GST
Danaus plexippus	DPGLEAN14383	GSTE3
	DPGLEAN07422	GST-containing FLYWCH zinc-finger protein, isoform B
	DPGLEAN00802	GSTD1
	DPGLEAN00407	CG16936
	DPGLEAN04701	CG9363-A
	DPGLEAN15774	CG6776
	DPGLEAN17330	GSTE4
	DPGLEAN10649	GSTE7
	DPGLEAN10896	suppressor of ref(2)P sterility

	DPGLEAN09486	GSTE7
	DPGLEAN12507	GSTD1-A
	DPGLEAN06785	GSTS1-C
	DPGLEAN06787	GSTS1-C
	DPGLEAN13104	GSTE7
	DPGLEAN21737	failed axon connections, isoform C
	DPGLEAN03237	CG6776
	DPGLEAN03240	CG6776
	DPGLEAN19980	chloride intracellular channel
	DPGLEAN02728	CG4623
	DPGLEAN04969	CG9362
	DPGLEAN02611	GSTS1-C
	DPGLEAN04127	CG9393
	DPGLEAN02423	CG1702-B
	DPGLEAN01745	GSTS1-C
	DPGLEAN01768	CG17639-A
	DPGLEAN14398	GSTE1
Caenorhabditis elegans	CeGST43	
	CeGST42	
	CeY53G8B1	
	CeY53F4B33	
	CeY53F4B29	
Homo sapience	HsGSTA3	
	HsGSTA2	
	HsGSTA1	