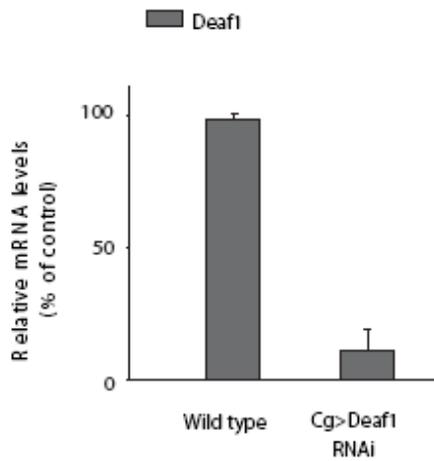
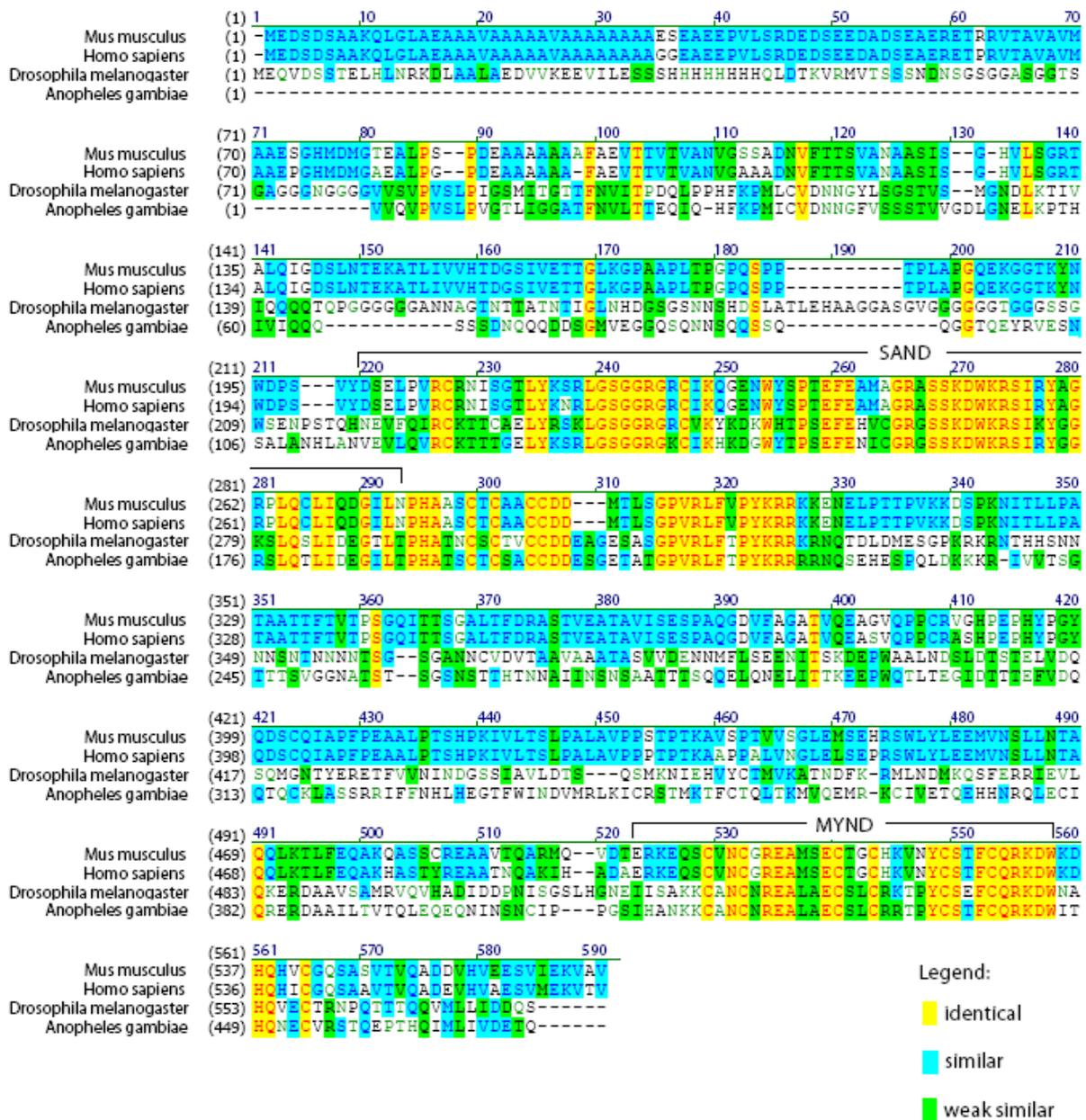


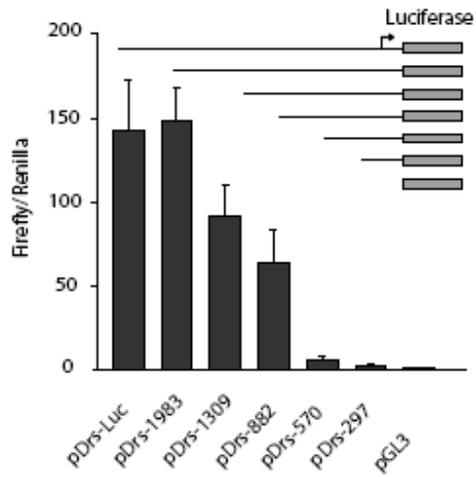
Online Supplementary Material



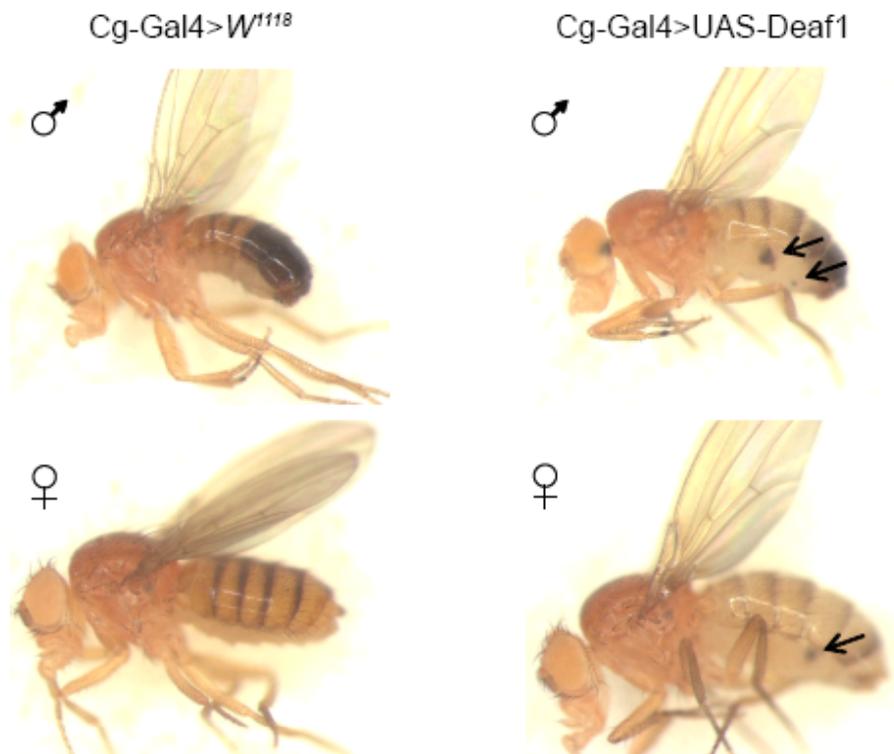
Online Supplementary Fig. 1. Analysis of UAS-Deaf1 RNAi efficiency. Adult fat bodies of 5 day old flies expressing the Deaf1 RNAi construct (Cg>Deaf1 RNAi) were dissected to assess Deaf1 mRNA levels. *Deaf1* mRNA levels in wild-types were set to 100%. Error bars indicate two technical replicates. Experiment was independently reproduced twice.



Online Supplementary Fig. 2. Alignment of Deaf1 protein sequences from different species. Deaf1 Protein sequences of *Drosophila melanogaster*, *Anopheles gambiae*, *Mus musculus* and *Homo sapiens* were aligned using AlignX algorithm implemented in the VectorNTI program (Invitrogen). Residues in the alignment are colored according to the following scheme: black font on white background: no conservation. Blue on cyan: consensus residue derived from a block of similar residues at a given position. Black on green: consensus residue derived from the occurrence of greater than 50% of a single residue at a given position. Red on yellow: consensus residue derived from a completely conserved residue at a given position. Green on white: residue weakly similar to consensus residue at given position. Location of SAND and MYND domains are indicated by a black bracket.



Online Supplementary Fig. 3. Identification of a minimal *Drs* enhancer sequence. Enhancer elements of the *Drs* promoter ranging from +50 to -1983, -1309, -882, -570 and -297 were cloned upstream of a luciferase gene in the pGL3 vector (Promega). All reporter constructs were transiently transfected with Toll^{ALLR} and *Renilla* coreporter to test for Luciferase expression. Experiment was independently reproduced twice. Error bars account for the standard deviation of quadruplicates.



Online Supplementary Fig. 4. Overexpression of Deaf1 in adult fatbody and hemocytes induces melanotic tumors. Flies carrying UAS-Deaf1 construct (7), were crossed with the Cg-Gal4 driver and kept at 29°C. Arrows indicate melanotic tumors. ^{w¹¹¹⁸} flies crossed with Cg-Gal4 individuals served as the negative control.

Supplemental Methods

Large scale RNAi screening

Hemocyte derived *Drosophila* SL2 cells were maintained in Schneider's medium (Invitrogen) supplemented with 10% fetal calf serum (PAA) and penicillin-streptomycin (Invitrogen). Cells were grown at 25°C at sub-confluent densities. All screening experiments were performed in 96-well tissue culture plates (Microtest Tissue Culture Plate 96 well Flat Bottom, Falcon). Screening plates were pre-loaded with an average of 1µg dsRNA per well in 10µl of 1mM Tris pH7 with a Biomek FX robot (Beckmann-Coulter). To transfect *EGFR-Toll*, *Drs-luc* and *pIz-RL* reporter constructs, 5 million SL2 cells were seeded per 35mm dish (Cellstar, Greiner) and cultured for 24h. Then 0.7µg of h*EGFR-Toll* (1), 0.7 µg *Drs-luc* plasmid DNA (2) and 0.7µg *pIz-Rluc* (*pIZ-RL*, Renilla luciferase expression plasmid, Invitrogen) were co-transfected using Effectene (Qiagen) according to manufacturer's instructions. Sixteen hours after transfection, cells were resuspended in serum free medium, adjusted to 10,000 cells per µl and 50µl were subsequently seeded per well of the 96-well tissue culture plates containing the dsRNA using a liquid dispensing unit (Multidrop, Thermo Labsystems). After 45 minutes of starvation, 50µl of complete medium was added to each well. Cells were incubated in sealed assay plates at 25°C. Ninety-six hours after RNAi treatment, cells were stimulated by adding recombinant human EGF (Biomol) to a final concentration of 0.2ng/µl. Sixteen hours after induction, cells were lysed and the lysate was split to read Firefly and *Renilla* luciferase activities in separate assay plates (white 96-well LIA Plates, Greiner) using a Mithras LB940 plate reader (Berthold Technologies). The screen was performed in duplicate.

Computational analysis

To identify candidate genes that significantly increase or decrease Toll pathway activity, the ratio of Firefly and *Renilla* raw luciferase results were normalized by median centering of each 96well plate. z-scores were calculated as the number of median-adjusted standard deviation (mad) that a particular well differed from the median of the 96well plate. All calculations were performed on the R-biostatistical platform using cellHTS package (3). To minimize false negatives, we applied a set of low-stringency criteria to generate a list of candidate genes. First, we filtered dsRNA treatments with z-scores > 2.5 for negative regulators or <-2.5 for positive regulators, respectively. Treatments that showed a high variability between duplicates were excluded (standard deviation higher than 50% of the replicates average). We also filtered against previously identified cell viability modifiers that show a phenotype in cultured *Drosophila* cells (4). We further excluded genes that showed phenotypes in other screens (M.B., unpublished observations). These filtering steps led to a final list of approximately 32 candidates (Supplementary table 1).

Cloning of Deaf1 full length and deletion constructs

cDNA from reverse transcribed total RNA served as template to amplify Deaf1 in a PCR reaction using AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen) following manufacturer's instructions. Fragments generated by combinations of forward primers EcoRI-CTATCGTTGGACCGCAGAATAAGTG, EcoRI-CTAGTATGCGTCTGCTGCGACG-ACGAAGC and reverse primers XbaI- GGAATGATCGTCGATCAACAGC, XbaI-GTCCGCGTGCACCTTGACAC were subcloned into EcoRI and XbaI sites of pAc5.1V5/His expression vector (Invitrogen).

***Drosomycin* promoter luciferase reporters**

All *Drs* promoter sequences were subcloned into NheI/NcoI sites of pGL3-basic luciferase reporter plasmid (Promega). To generate *Drs* reporters of different length we combined 3' primer NcoI-TGGAAAAGGTTCTCACGGAG with 5' primer NheI-GTTTCAGCGGAGAGTTCCAG (pDrs-1983), NheI-GGCAATCTTAGCAACAACGA (pDrs-1309), NheI-CGGTCGTTTCGAATTTTGTT (pDrs-882), NheI-CCGTGCATTC-TTAAAGCAA (pDrs-570) and NheI-CGAATCATTTTTGCTGGACA (pDrs-297).

Site directed mutagenesis

To introduce mutations in TTCG sites of the construct pDrs-882 we used the QuickChange® II Site-Directed Mutagenesis kit (Stratagene) according to manufacturer's instructions. Site location, mutated sequence and primers are given in Supplementary table 3.

Cloning of a *Deaf1* *in-vivo* hairpin RNAi construct

XbaI-GCCCTGTCCGTCTCTTACA and XbaI-TCCTTTTGC-AGAACCTCGAT primers were used to amplify a 517bp long fragment of exon three of *Deaf1* for *in vivo* RNAi. The PCR generated amplicon was subcloned into NheI and SpeI sites of the pWiz plasmid (5) to generate an inverted repeat separated by the white intron 2. The resulting plasmid, pWiz-*Deaf1*-RNAi, was first tested in cell culture for RNAi efficiency and then injected into *w*¹¹¹⁸ embryos to generate transgenic flies.

Supplementary Table 1 Screening results

GeneID	CG	z-score ^a	dsRNA ID	Primer 1	Primer 2	Specificity / Efficiency / % ^b
Rel	CG11992	-11.19	HFA16819	CAGAGCCCTCGTACAAA	TCCTGTTTAAATTTCGAATAA	100
Myd88	CG2078	-10.15	HFA06815	ACTCGTGGCCCATCTCC	CTGGATATCGTGGCAGAC	100
Trf2	CG18009	-9.86	HFA18727	CTAGCGTAAACCAAGTGTGA	GTAACGTGGACGCTATTCT	100
pil	CG5974	-8.22	HFA17026	GGCGTCAAGAACTCAA	CGAGGCTGATCTAAAC	100
tkk	CG1856	-7.68	HFA17091	ACAGCGATGGCGAAGGT	GACCCAGCAGCCTTGAAC	100
Deaf1	CG8567	-6.16	HFA11143	GATGTCATCCAGCAACAG	TGACTACCTGCTGCTC	100
TRIA-L	CG5930	-5.95	HFA16882	ATGCCAAGGCCAAGAAAG	ACAGGCATATGGATTGG	100
TRIA-S	CG5163	-5.46	HFA16883	TGAACTGTACCGCAACCC	AGAACTCGCGCTCTTG	79
M-2	CG8103	-5.33	HFA11222	CGGAGGCGAAGATGG	ACCCATTGGGAATGCCAG	100
bon	CG5206	-5.28	HFA16914	AGGTGCTGGTGGAAAAGAA	ACCCGTCACCCGAAAG	100
Eip93F	CG18389	-5.07	HFA16661	GGCATAATGAACGAGGGAC	CATCATCTCTGTATGCTAGAC	102
kay	CG15509	-4.99	HFA16977	CGGAAATACCTGAAATAAG	GCGCCGGCTTGAGA	100
CG1244	CG1244	-4.27	HFA08274	CAATTGAGACCAAGTATGGG	GCGCATTGATGGCCATT	100
Su(Hw)2-10	CG8068	-4.23	HFA07721	CATGCCATTTCCGCTCTTT	GTAAGAAAGGCACCTCTTGGG	100
Taf4	CG5444	-4.03	HFA11297	ATTGTGCAATACGTGGACC	GGCTGGGTTCTTTT	100
CG32180	CG32180	-3.96	HFA11160	CGAGTCAAAGTCTCTATAAT	TTGATCAAGCTCTCTTGTAG	100
hay	CG8019	-3.2	HFA11354	TACCAACAATCCAGACATTAAT	ATCCAGCTTCAATGAGA	100
Fer1	CG33323	-3.04	HFA12555	CTGTACCGCAAGGGTGA	ACTGCCGCTGCTGAAAA	100
CG3711	CG3711	-3.01	HFA18647	TCTTCGAGGCGCTAC	CCCAACCCGTAAGCCTTC	100
gce	CG6211	3.11	HFA19974	CAATAGTTCATATGGCGAGTC	GTTTTGGGTTACCATTGCC	100
CG30426	CG30426	3.14	HFA04119	CGGTACACCAAGGAGATGG	CAAAATTGCTACGTCAACAC	100
CG31441	CG31441	3.14	HFA15723	TGCGAAAAAGCGGATAGT	GCTACAGAACCGCAAGC	100
CG7015	CG7015	3.19	HFA10761	AGTGGCGATCCGACCAC	CITCAGTAAAGTGGAAAAATAAAC	91
stw	CG3836	3.32	HFA11400	TTCGAGCGACCGATG AAG	AATATCGGAGAAAGGATCTG	100
pb	CG31481	3.33	HFA12629	ATCAAAAAGGAGCGGCTC	GTTGGTCTGTTGTAGTTGT	100
CG33936	CG33188	3.56	HFA15147	CGCTTAGAAAAAGCAACAG	ACTCCTCTGCAAAACATCTCC	80
BhbVII	CG11494	3.73	HFA08151	TGCAATGGACAGTTGTTGATT	GCCGCTCAGGGGCT	100
CG33213	CG33213	4.07	HFA14487	CGCAACCGGCAAAAGGT	CCACTTGGTCTCCAGCG	100
CG32767	CG32767	4.14	HFA18386	CAGCAACATCATCTGCTAAC	TGTAGACAGGTGGTCTGTTG	100
Bk	CG6500	4.2	HFA19350	TGATGACTATGGACATACCCAAA	GCTTGCAGAGCATCAGGT	100
osa	CG7467	4.21	HFA17022	CGGTGGTGGGATGGTAG	CATTGAAAAGCGTGTCT	100
CG3726	CG3726	4.35	HFA18320	CCAGATGCGCCCAATC	CGTCTCGATCTCTCCAC	100
dalao	CG7055	4.83	HFA18419	CCTCCAGGAGGAGCTAAA	CTCGACTAGCGTGGACTG	100
brn	CG5942	4.87	HFA11330	GTTTGGCTGTACAATAACAATC	ATGTTGGAGCAGGACTTAAAG	100

^a negative scores correspond to significant impairment of pathway activity. Positive scores correspond with hyperactivation. Calculation of Z-scores were performed using BioConductor package cellHTS (Boutros et al., 2006)

^b percentage of all possible 21 nucleotide long siRNA originating from dsRNA sequence which match completely to target gene sequence. (Arizman et al., 2005)

^c percentage of matching siRNAs which are efficient according to Reynolds criteria (Reynolds et al., 2004; Arizman et al., 2005).

Supplementary Table 2 dsRNA template and qPCR primers

Gene	Primer 1	Primer 2	Probe library # ^a
T7 tagged primer for RNAi probe synthesis			
GFP	TAAATACGACTACTATAGGACCCCTGTGACCACCCCTGACCTAC	TAATACGACTCACTATAGGGGACCAATGTGATCGCGCTTCTCGT	
Myd88	TAATACGACTCACTATAGGGAACAACATCA.CAGTGGCCAAAAGTG	TAATACGACTCACTATAGGGGAGTAGATCGAAAAGGCATCCAGA	
Cactus	TAATACGACTCACTATAGGGAA.GGCAACAGCAACAAGTGA	TAATACGACTCACTATAGGGCCTGA.TCCTCTTCTCGTCTCG	
Dif	TAATACGACTCACTATAGGGCCCAAAATTGGACCAC	TAATACGACTCACTATAGGGTGGCACTCATTTCTGACTTA	
Dorsal	TAATACGACTCACTATAGGACTACA.ATCGTTTGCCAG	TAATACGACTCACTATAGGGTGATTTGTGGTCAGGTTCCA	
IMD	TAATACGACTCACTATAGGAGA.ATGTCAAAGCTCAGGAACCT	TAATACGACTCACTATAGGGAGAAATGCTGACCCGTTTTGCGCG	
Relish	TAATACGACTCACTATAGGTGGACACATGGATCGCTAA	TAATACGACTCACTATAGGCGGACTTGGGTTATTGATT	
Deaf1 1	TAATACGACTCACTATAGGTGATGAGGAAGAAGAGGAGGA	TAATACGACTCACTATAGGGTTCACATTATTCTGCGGTCCA	
Deaf1 2	TAATACGACTCACTATAGGGATCGTCA.TCCAGCAACAG	TAATACGACTCACTATAGGGTGACTCACCTGCTTCGTC	
Deaf1 3	TAATA.CGACTCACTATAGGGCCCTGTCGTCCTTTTACA	TAATACGACTCACTATA.GGGGCCATCGTTGATGTTCA.CCAC	
Deaf1 4	TAATACGACTCACTATAGGGCTAAAAGT.CCTTGCAGTTCGGTT	TAATACGACTCACTATAGGGGCTTGCCTACGTTCA.ATA	
qPCR primer sequence			
RP49	CGGATCGATATGCTAAGCTGT	GCGCTTGTTCGATCCGTA	105
Drosomylin	TACTTGTTGCCCCCTCTTCG	GTATCTTCCGGACAGGCAGT	131
Diptericin	CCGAGTACCACCTCAATCT	ACTGCAAAGCCAAAACCATC	70
Deaf1	CCGAGGATGTTGT.GAAAGAAG	ATGCGAACCTTGGGTGCC	19
			84

^a Primers for qPCR analysis were generated using the online tool at www.universalprobelibrary.com

Supplementary Table 3 Primers used to introduce point mutations.

Name	site location ^a	Wild Type sequence	mutated sequence	Primer 1	Primer 2
Drs-mutA	-23	CGAA	AGAG	GTGCACTATAAAGCTTCTCCTAGAGGTTCCCAAGCGACTTGTGGCTTGGGAACCTCTAGGAGAAGCTTCCACAAGTCCGATATAGTGCAC	ATATAGTGCAC
Drs-mutB	-88	TTCG	A GAG	GTATCATCATAAATTTTGTGATATACAGAGTTTATA GCCGATGGGTAGTCGGGTATAAACTCTGTATATCACCCGACTACGCATCGGC	ACAAAATTATGATGATAC
Drs-mutC	-140	CGAA	AGAG	CAAAGTAGTTCCCTACAGAGGGCCTATAAATGTG CATGTCCAGTCCACATTTATAGGCCCTCTGTAGGGGACTGCACATG	AACTACTTTCG
Drs-mutD	-169	TTCG	AGAG	GCAATGCTTTTTCGCTTAGAGATAAGCGTAAAGGAACTACTTGTACCGCTTATCTCTAAAGCGTAAGCCGCAAGTAGTTCC	AAAAGCATTTGC
Drs-mutE	-182	TTCG	AGAG	CAAATGAGGCTCTAAGCAATGCTTAGAGCTTACG CGCTTATCGAAAAGCGTAAGCTCTAAGCAATTGCTTCTTTTCGATAAGCG	AGAGCCCTCATTTG

^a location of TTCG motif relative to transcriptional start site of *Drosomycin*

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