

Supplementary Information for

Waves of *hedgehog* gene expression undergo splitting during Spider Head Segmentation

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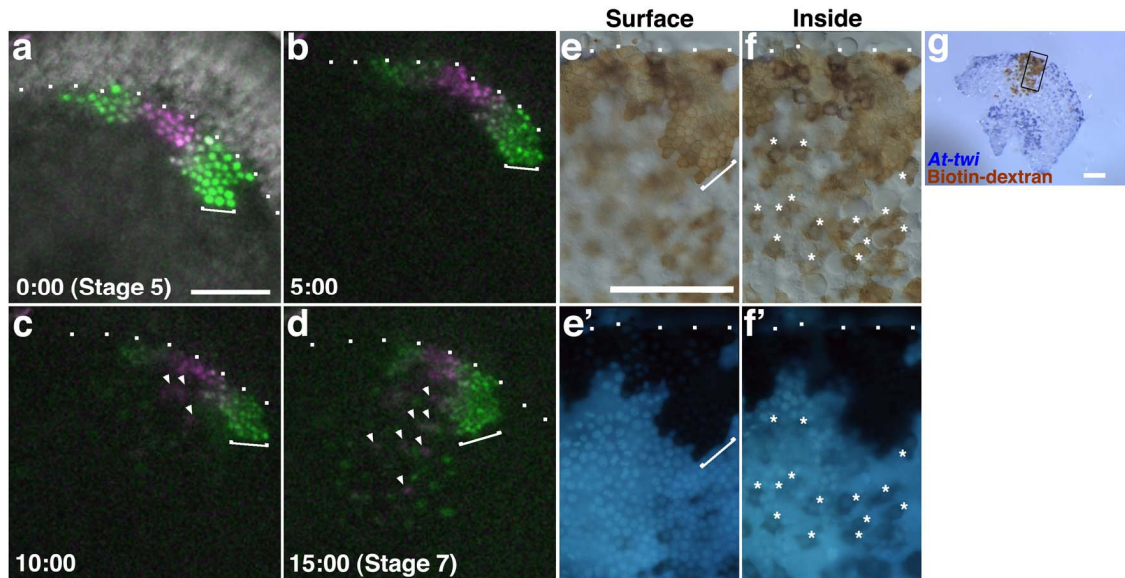
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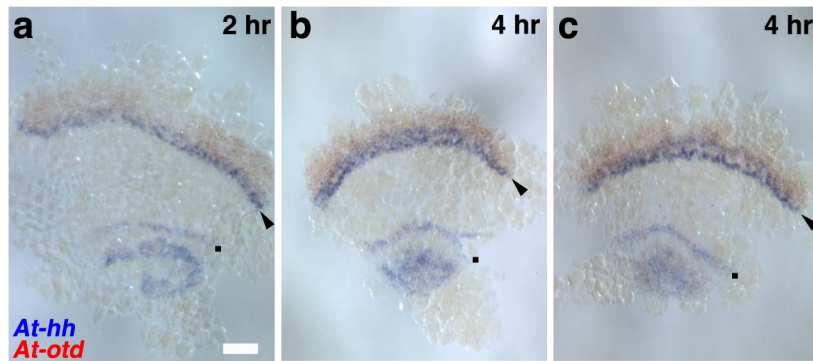
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Supplementary Figures S1 to S8

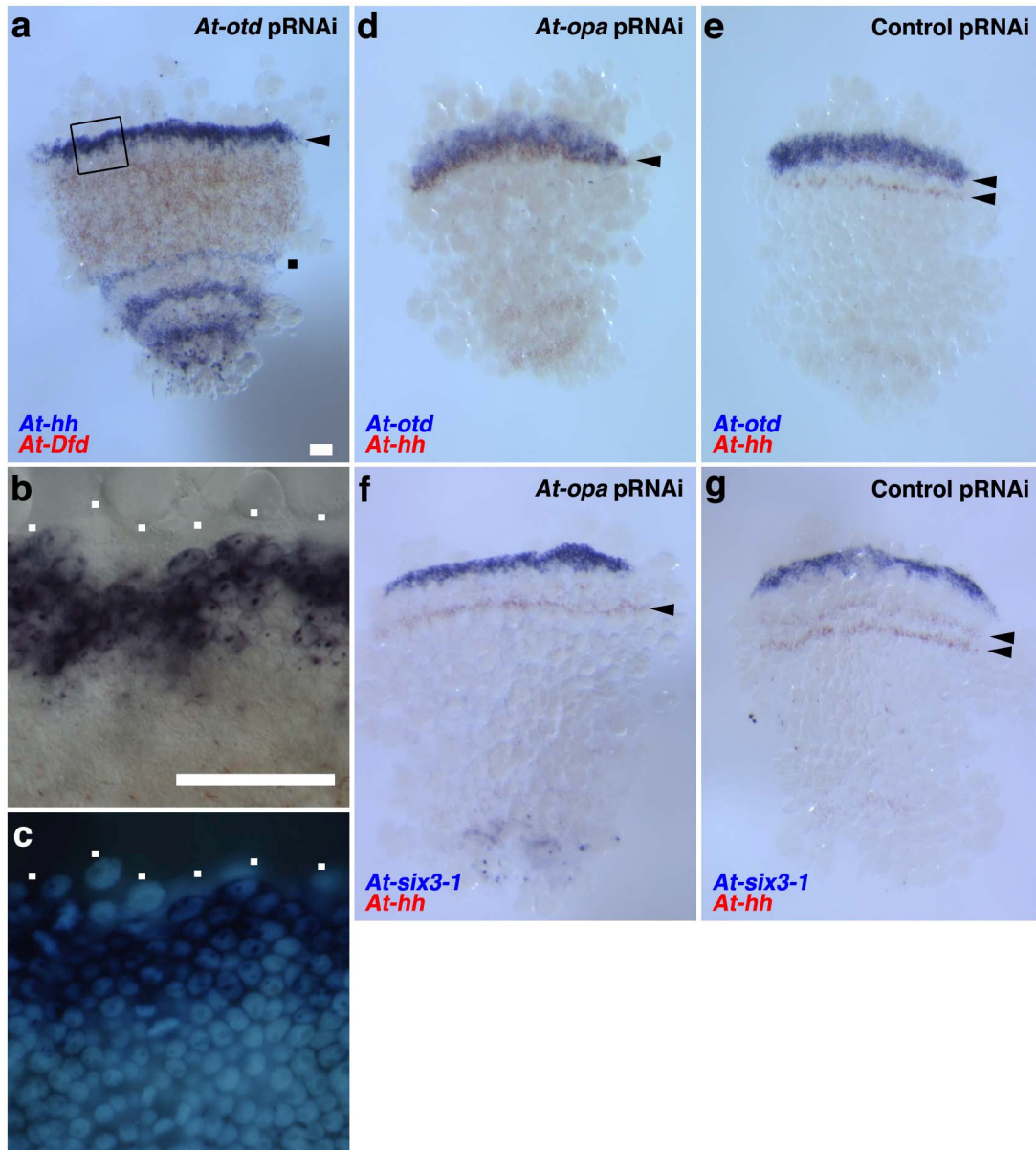
Supplementary Tables S1 and S2



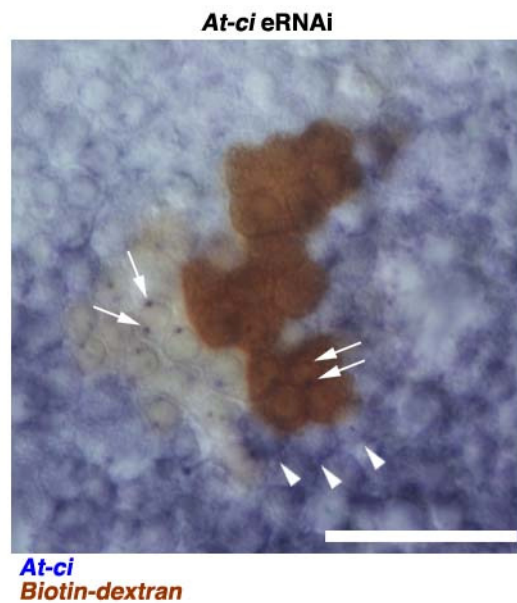
Supplementary Figure S1: Internalization of cells from the rim of the germ disc. (a-d) Projections of confocal images of a cell clone expressing NLS-tdEosFP at the rim of the germ disc (dotted) observed by time-lapse confocal microscopy. Images were collected at the time indicated (hr:min). The tdEosFP was photoconverted from the green (shown in green) to the red state (shown in purple) in a part of the cell clone before recording, which began at early stage 5. In a, a transmission image is merged to show the outline of the germ disc. Some of the cells labelled in purple were internalized (c, d, arrowheads). (e-g, e', f') A flat preparation of the observed embryo, which was fixed approximately 15 min after the images shown in d were collected and was stained for *At-twi* transcripts (purple), the lineage tracer biotin-dextran (brown), and DNA. e and f are differential interference contrast images of the boxed area in g; e' and f' are fluorescence images of e and f for DNA staining. The focal plane was adjusted to the surface cell layer in e and e' and to an inner cell layer in f and f'. Asterisks indicate labelled cells in which the *At-twi* transcripts were detectable. Cells indicated by brackets in a-e and e' are the same cell population that contributed to the surface ectoderm. Scale bars, 100 μ m.



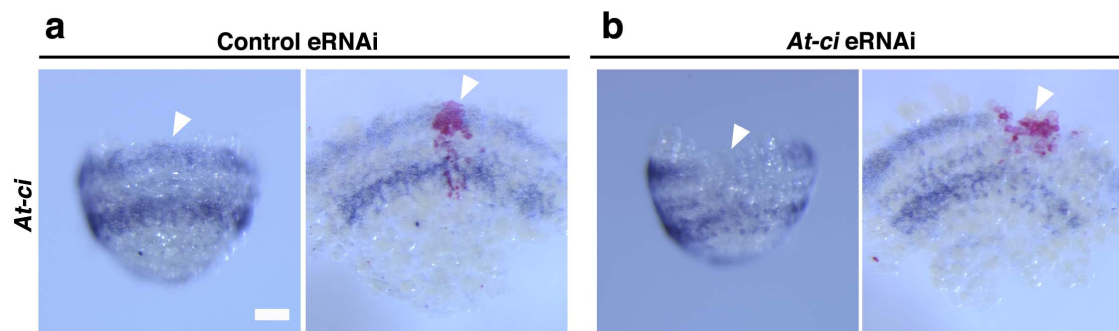
Supplementary Figure S2: Examples of splitting *At-hh* stripes in the presumptive head ectoderm. (a-c) Flat preparations of embryos double-stained for *At-hh* (purple) and *At-otd* (red) transcripts. These additional samples came from the experiments shown in Fig. 3. The embryo shown in **a** was fixed at the 2-hr time point, and those shown in **b** and **c** were fixed at the 4-hr time point. Arrowheads indicate the splitting of the *At-hh* stripe in the presumptive head ectoderm. Dots indicate the *At-hh* stripe corresponding to the L4 segment. Anterior is to the top. Scale bar, 100 μ m.



Supplementary Figure S3: *At-opa* pRNAi does not disrupt anterior terminal patterning. (a-c) Flat preparation of an *At-otd* pRNAi embryo at germ band-forming stage stained for *At-hh* (a, b, purple) and *At-Dfd* (a, b, red) transcripts and DNA (c). The area boxed in a is magnified in b and c. The *At-hh* stripe (arrowhead) persisted at the anterior margin of the nascent germ band (white dots). This embryo is approximately six hr older than the control embryo shown in Fig. 6c. The black dot indicates the *At-hh* stripe corresponding to the L4 segment. (d-g) Flat preparations of *At-opa* and control pRNAi embryos at germ band-forming stages stained for *At-hh* (red) and *At-otd* (d, e, purple) or *At-six3-1* (f, g, purple) transcripts. The *At-hh* stripes in the presumptive head ectoderm are indicated by arrowheads. Scale bars, 50 μ m.



Supplementary Figure S4: Cell-autonomous effects of eRNAi. Part of a stage 5 embryo microinjected with *At-cil* dsRNA and stained for *At-ci* transcripts (purple) and the lineage tracer biotin-dextran (MW 10,000, brown). Note that cells in the labelled area can be grouped into two sub-populations based on the level of the brown signal. These two groups appear to reflect two descendant cell populations of sister cells of the first cell division after microinjection. Although the lineage tracer is inherited asymmetrically, *At-ci* transcripts are depleted from the cytoplasm in both cell populations but not in adjacent cells (arrowheads). Arrows point at paired dots of nuclear signals implying that the transcription is active. Scale bar, 50 μ m.



Supplementary Figure S5: Validation of eRNAi against *At-ci*. (a, b) Detection of *At-ci* transcripts in embryos microinjected with control *gfp* dsRNA (a) and *At-ci1* dsRNA (b). Each embryo was photographed before (left) and after (right) staining for the lineage tracer FITC-dextran (pink). Arrowheads indicate areas where the lineage tracer and presumably the dsRNAs were introduced. Specific reduction in the level of the signal for *At-ci* transcripts was observed in b, but not in a. Scale bar, 100 μ m.

a

ZF1 **ZF2**

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* * * * *
At-Opa CLWIDPDQ----MPPKKPCNKI FQTMHEIVSHIAVEHVGGPECTN-HTCCWQECPRNGRPFKAKYKLVNHIRVHTGKPPFPCPF
Dm-Opa .....PGLVPPGGR.T...V.HS.....T.LT.....T-..A.F.VG.S.....A..H
Bf-Zic .....PE.....S.S.....T.VT.....A.F.KD..D..A.....
Hr-ZicN .K..VSGKS---GEENAIT.DRE.YS.NQL.D.VT.D....HDQAD...Y.KD.T.E-KS.Q.....I..L.
Mm-Zic1 .K..E.E.E---LAN...S...T.S...L.T.VT.....QS...I.F.E...E.K.....
Xl-Zic1 .K..E.E.E---LAN...S...T.S...L.T.VT.....QS...I.V.E...E.K.....I.....
Dr-Zic1 .K..E.E.E---LTN...S...T.S...L.T.LT.....QS...I.F.E.G.E.K.....
At-Ci .H.....S.G.E.M.QE.L.Q..NKD.IQ.SRK.S-FV.R.KD.S.EEK...Q.M..V.M.R.....NK.T.
Dm-Ci .H.....RS.R.IE.I.QD.L.K..NND.IQNKKA--FV.R.ED.T.GEK...Q.M..V.M.R.....HK.T.
Tc-Ci .H.....D.GTE...QD.L.K..NND.IHANKKS--FV.R.DG.S.AEK...Q.M..V.M.R.....HK.T.
Bf-Gli .H.DG-----S.E.D.QDQL.H..NND.IH.EKKE--FV.R.S..T.EQK...Q.M..V.M.R.....HK.T.
Mm-Gli .R.DG-----SQE.DSQEQL.H..NS..IH.ERKE--SV.H.GG.S.EL...Q.M..V.M.R.....HK.T.
Xl-Gli2 .H.DG-----S.E.D.QDQL.H..NND.IH.EKKE--FV.R...S.EQK...Q.M..V.M.R.....HK.T.
Dr-Gli1 .H.ES-----S.E.D.QEQL.H..NN..IH.EKKE--FV.H.KD.S.EQ...Q.M..V.M.R.....HK.T.
Dm-Lmd .R.TG-----DEE.PHQQAF.E..EKC.DVRKGED-FS.F.LD..RYK..N.R...LI.M..S...NK..M.
Dm-Sug .N.T-----DRV.D.LDALAQ.VTQR.AIASLTDGLYY.R.R.G.Q.SE.G.N.R..MLV.T.T..K...HR..
Mm-GLIS1 .R.V-----CAAYEQQE.L.R..EKS.IDQRKGED-F..F.AG.V.RYK..N.R...LI.M..S...NK.M.
Dr-GLIS3 .C.LH-----GA.YSHRDAL.K..EQI..DQRKGED-F..F.AG.A.KHK..N.R...LI.M..S...NK.S.
Dm-Snail .....DE.Q.MYS.SMGLSK.-RQF.CPAA..N--QEKKTHS.EEC.KLYTTIGA.KM...T.....L..KC
At-Snail .....SA.S.NYA.YSGLSR.-KQF.CITL.K-----KAFN.KYCDKIYVSLGA.KM...T.....L..KC

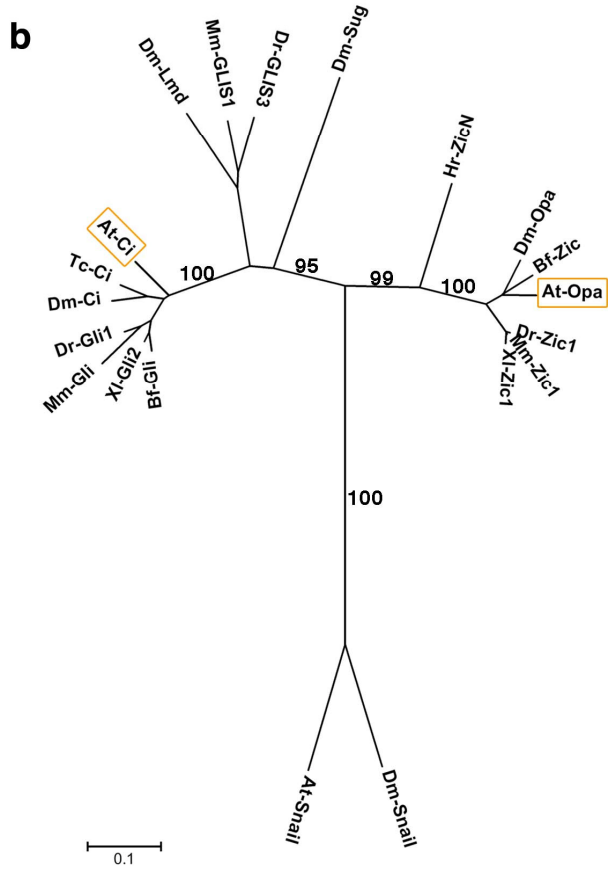
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ZF3 **ZF4** **ZF5**

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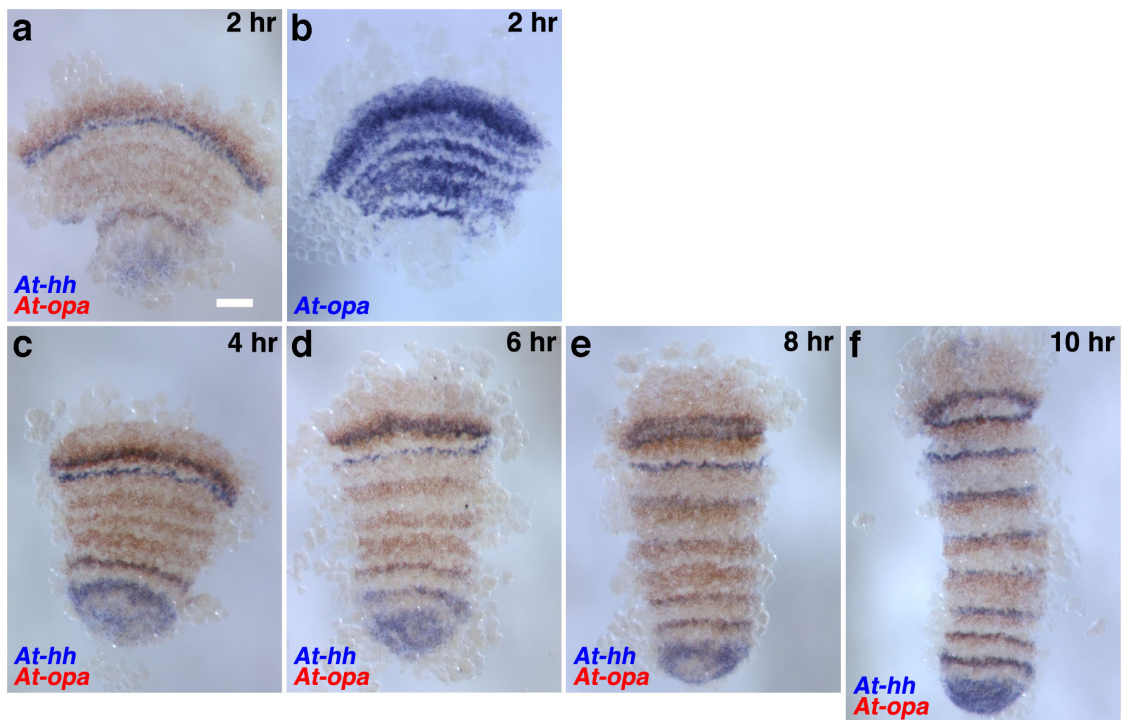
* * * * *
At-Opa PGCCKVFARSENLIKHKRTHTGKPPFKCEYAGCDRKFANS.SDRKKHS-HVHTSDKPYNCKIRGCDKSYTHPSLKRKHMK--VHG
Dm-Opa .....HE.....R.....R.N.....
Bf-Zic .....L.....FE.....R.....V.....
Hr-ZicN .N.....V.PFD...R.....T-YT.STS..A..VQ..K.....L--M.E
Mm-Zic1 .....FE.....R.....M.....L.M--.....E
Xl-Zic1 .....FE.....R.....M.....L.M--.....E
Dr-Zic1 .....FE.....R.....M.....L.M--.....E
At-Ci .E.S.AYS.L...T.L.S...YT.FP.SKA.S.A..A..QNRT.SNT..A..AP.T.R.D...V.T...
Dm-Ci .E.F.AYS.L...T.L.S...YT.P.SKA.S.A..A..QNRT.SNE..I..AP.T.R.D...V.T...
Tc-Ci .E.V.AYS.L...T.L.S...YT.P.SKA.S.A..A..QNRT.SNE..V..AP.T.R.D...V.T...
Bf-Gli .E.N.AYS.L...T.L.S...YV.HE.NKA.S.A..A..QNRT.SNA..V..P.T.R.D...V.T...
Mm-Gli .E.R.SYS.L...T.L.S...YM.QE.SKA.S.A..A..QNRT.SNE..V..LP.T.R.D...V.T...
Xl-Gli2 .E.F.AYS.L...T.L.S...YV.HE.NKA.S.A..A..QNRT.SNE..I..P.T.R.D...V.T...
Dr-Gli1 .E.N.AYS.L...T.L.S...YV.HE.NKA.S.A..A..QNRT.SNE..I..P.T.R.D...V.T...
Dm-Lmd .N.A.S.L...Q.S...R.YG.Q.K.LKA.S...A..Q-RT.YDT..A.QLP.T.R.D...V.N--A
Dm-Sug .H.L.E.S.S.A...I.S.S...Y.SFE.QKAYS...F..T-RT.SME..M.VA.Q.R.D...V.TFK.S
Mm-GLIS1 .E.S.A.S.L...L.S...YL.QHP.QKA.S...A..Q-RT.LDT..A.Q.P.S.R.D...V--A.S
Dr-GLIS3 .E.Q.A.S.L...L.S...YV.PHP.LKA.S...A..Q-RT.VDT..A.Q.Q.G.R.D...V.S--S
Dm-Snail .I...A.S.PWL.QG.I...Q.P--D.P.S..DR.NLRA.Q-QT.VDV.K.A.QV--H..FSRM.L.N..SSSNCTI
At-Snail .NL...A.S.PWL.QG.I...A.P--H.S.A..DR.NLRA.L-QT.SDV.K.S.NA--S.TFSRM.L.L..NDGGCGA

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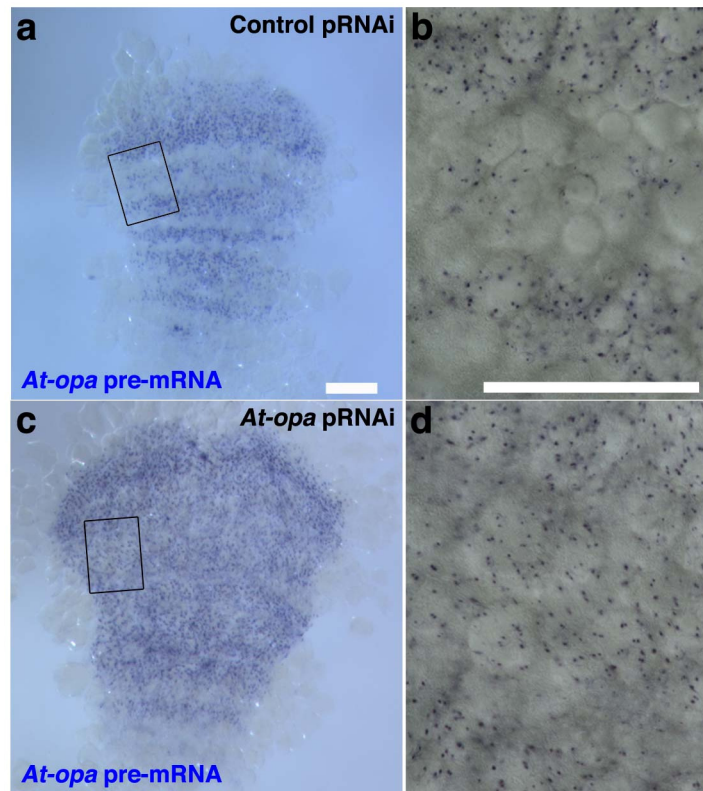


Supplementary Figure S6. Molecular characterization of At-Opa and At-Ci. (a)

Alignment of the amino acid sequences of five zinc finger domains, ZF1-5, of the Opa/Zic, Ci/Gli, Glis and Snail family proteins. Residues that are conserved with the At-Opa protein are shown in dots. Gaps that were introduced to optimize the alignment are indicated by dashes. Asterisks indicate the conserved Cys and His residues among the proteins. **(b)** Neighbour-joining tree constructed based on the alignment shown in **a**. The numbers at the internal branches are bootstrap values (%). The accession numbers of the proteins are as follows: At-Opa, AB605264; Dm-Opa, NP_524228.2; Bf-Zic, AB231866.1; Hr-ZicN BAC23063.1; Mm-Zic1, AAH60247.1; Xl-Zic1, BAA33406.1; Dr-Zic1, NP_571008.1; At-Ci, AB605263; Dm-Ci, AAF59373.2; Tc-Ci, EFA01291.1; Bf-Gli, CAB96572.1; Mm-Gli, AAC09169.1; Xl-Gli2, AAD28180.1; Dr-Gli1, NP_840081.1; Dm-Lmd, AAN13923.1; Dm-Sug, AAF58441.1; Mm-GLIS1, CAM23304.1; Dr-GLIS3, AAI29174.1; Dm-Snail, AAF53463.1; At-Snail, BAD44735.1. Species abbreviations are as follows: At, *Achaearanea tepidariorum*; Bf, *Branchiostoma floridae*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Hr, *Halocynthia roretzi*; Mm, *Mus musculus*; Tc, *Tribolium castaneum*; Xl, *Xenopus laevis*.



Supplementary Figure S7: Expression patterns of *At-opa* transcripts. (a-f) Flat preparations of embryos double-stained for *At-hh* (purple) and *At-opa* (red) transcripts (a, c-f) or single-stained for *At-opa* (b). These samples came from the pools of fixed embryos used in Fig. 3. The time points at which embryos were fixed are indicated. Scale bar, 100 μm .



Supplementary Figure S8: *At-opa* pRNAi disrupts the striped pattern of *At-opa* transcription. (a-d) In situ hybridization of *At-opa* pre-mRNA in control (a, b) and *At-opa* pRNAi (c, d) embryos with an intron probe. The areas boxed in a and c are magnified in b and d, respectively. Dots of nuclear signals were detected in most (but not all) cells of the surface ectoderm in the *At-opa* pRNAi embryo, with no clear striped pattern being evident. Scale bars, 100 μ m.

Supplementary Table S1

dsRNAs and methods to validate reduction of target transcripts

dsRNA	region in cDNA	experiment	method for validation*
<i>At-otd1</i>	nt 1-1190	pRNAi	1
<i>At-otdXSE</i>	nt 1-480	eRNAi	2
<i>At-otdXE</i>	nt 481-1190	pRNAi, eRNAi	1, 2, 3
<i>At-opa1</i>	nt 83-1898	pRNAi	1
<i>At-opaF</i>	nt 83-547	pRNAi, eRNAi	1, 2, 3
<i>At-opaH</i>	nt 1020-1898	pRNAi, eRNAi	1, 2, 3
<i>At-ci1</i>	nt 580-1346	eRNAi	2
<i>At-ci2</i>	nt 1343-2480	eRNAi	2
<i>At-hh1</i>	nt 1-1020	pRNAi	1, 3**
<i>At-hh2</i>	nt 1017-1940	pRNAi	1**
<i>gfp***</i>	nt 979-1698 (pQBI25)	pRNAi, eRNAi	1, 2, 3

*1, in situ hybridization for pRNAi; 2, in situ hybridization for eRNAi; 3, Quantitative RT-PCR for pRNAi. **For detail, see ref. 27. ***Used as control.

Supplementary Table S2

Summary of a microarray-based screen for genes down-regulated in *At-hh* pRNAi embryos

Array No.	REF_ ID	[<i>At-hh</i> pRNAi] / [normal]	EST clone ID or gene name	Accession No.	Top hit in BLASTX search with <i>Drosophila melanogaster</i> proteins (comments)
Spots in which the signal intensity ratio was lower than 0.5					
1	6820	0.417279	At_eW_008_H09	FY219229	reflNP_001104348.1 rolled, isoform F
1	11491	0.47383	At_eW_012_M09	FY220493	reflNP_524071.2 dally-like, isoform A
1	10627	0.419288	At_eW_018_C03	FY221871	(3' untranslated region of <i>At-patched</i> mRNA[AB433900])
1	11579	0.416804	At_eW_019_D19	FY222272	reflNP_511057.1 spaghetti squash
1	6991	0.403571	At_eW_021_C06	FY222963	
1	5807	0.48493	At_eW_022_E14	FY223390	gblADK20123.1 eater
1	10269	0.39045	At_eW_022_J19	FY223508	
1	140	0.491324	At_eW_024_O04	FY224339	
1	9011	0.450229	At_eW_025_F13	FY224510	
1	8275	0.494544	At_eW_025_G01	FY224522	
1	5244	0.401155	eS6_d1_08_C03	FY372343	
1	10904	0.477473	eS6_d1_08_C03	FY372343	
1	12457	0.36501	eS6_d1_23_H04	FY373674	
2	8324	0.346165	eS6_d1_33_F05	FY374464	
2	11332	0.476639	eS6_d1_33_F05	FY374464	
2	9755	0.499719	eS6_d1_46_B01	FY375479	
2	5663	0.327032	eS6_d1_54_H12	FY376203	reflNP_524228.2 odd paired (clustered with At_eW_022_A24)
2	3615	0.482977	eS7_003_H01	FY376814	
2	1644	0.176031	eS7_SB_031_C08	FY380182	reflNP_524228.2 odd paired (clustered with At_eW_022_A24)
2	8681	0.201762	eS7_SB_031_C08	FY380182	reflNP_524228.2 odd paired (clustered with At_eW_022_A24)
2	501	0.335239	eS7_SB_042_E09	FY381074	
2	10252	0.406792	eS7_SB_042_E09	FY381074	
2	11471	0.351495	eS7_SB_042_E12	FY381077	
2	7029	0.435581	S7_d1_02_D06	FY368355	reflNP_524071.2 dally-like, isoform A
Positive controls					
1	3800	0.155415	<i>At-hedgehog</i>	AB125742	reflNP_524459.2 hedgehog, isoform A
1	11894	0.452442	<i>At-hedgehog</i>	AB125742	reflNP_524459.2 hedgehog, isoform A
2	6843	0.134035	<i>At-hedgehog</i>	AB125742	reflNP_524459.2 hedgehog, isoform A
1	1079	0.179233	<i>At-orthodenticle</i>	AB096074	emblCAA41732.1 orthodenticle
1	6105	0.26431	<i>At-orthodenticle</i>	AB096074	emblCAA41732.1 orthodenticle
2	3062	0.217181	<i>At-orthodenticle</i>	AB096074	emblCAA41732.1 orthodenticle
Negative controls					
1	6978	0.851721	At_eW_003_D02	FY217447	reflNP_524219.1 alpha catenin
1	7997	0.766973	At_eW_003_D02	FY217447	reflNP_524219.1 alpha catenin
1	9278	0.943404	At_eW_003_D02	FY217447	reflNP_524219.1 alpha catenin
1	11582	0.896976	At_eW_003_D02	FY217447	reflNP_524219.1 alpha catenin
2	6342	0.79843	At_eW_003_D02	FY217447	reflNP_524219.1 alpha catenin
2	11607	1.031567	At_eW_003_D02	FY217447	reflNP_524219.1 alpha catenin
1	4354	1.131876	eS7_003_G08	FY376809	reflNP_477375.1 elongation factor 1alpha48D, isoform A
1	10730	1.34441	eS7_003_G08	FY376809	reflNP_477375.1 elongation factor 1alpha48D, isoform A
2	430	1.512315	eS7_003_G08	FY376809	reflNP_477375.1 elongation factor 1alpha48D, isoform A
1	3623	0.935976	eS7_SB_037_C01	FY380578	reflNP_511095.1 histone H3.3B, isoform A
1	9723	0.925602	eS7_SB_037_C01	FY380578	reflNP_511095.1 histone H3.3B, isoform A
2	6417	0.991825	eS7_SB_037_C01	FY380578	reflNP_511095.1 histone H3.3B, isoform A