Supplemental Information for:

"*Drosophila* dosage compensation loci associate with a boundary forming insulator complex."

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Experimental Procedures:

EMSA. Purified DNA probes (1 picomole) were 5' end labeled with [γ-^{32}P]ATP (MP Biomedicals/ Perkin Elmer) using T4 polynucleotide kinase (New England Bio-Labs) in a 50 ul total reaction volume at 37°C for 1 h. Samples were run through columns packed with Sephadex G-50 fine gel (Amersham Biosciences) to separate free ATP from the labeled probes. The volume of the sample eluted from the column was adjusted to 100 ul using deionized water so that the final concentration of the probe was 10 fmol/ul.

Binding reactions were performed in a 20 ul volume using the conditions described previously (Wolle et al., 2015) except for the concentration of the non-specific competitor poly(dA-dT):poly(dA-dT) in the binding reaction. The final concentration of poly(dA-dT):poly(dA-dT) was varied between 0.1 and 0.25 mg/ml depending on the DNA probe used. 1 ul of nuclear extract (corresponding to about 20 g of protein) or an equal volume of 360 mM nuclear extraction buffer (for negative control) was used. For the experiments with fractionated nuclear extracts, 10 ul of the indicated gel filtration fraction was used instead of the nuclear extract. In such cases, the addition of KCl into the reaction mixture was omitted. In some reactions, unlabeled competitor DNA was included so that the final concentration of the competitor would be in 5- to 100-fold excess. The reaction mixtures containing the ^{32}P- labeled DNA probes were incubated for 30 min at room temperature.

For supershift experiments, pre-immune rat or rabbit serum or antibodies against different proteins were pre-incubated in the reaction mixtures described above with the nuclear extract or gel column fractions for 30 min at room temperature to allow the protein-antibody association, followed by an incubation with ^{32}P- labeled DNA probes for 30 min at room temperature. Either 1 ul of rabbit polyclonal anti-CLAMP antibody A (SDIX, Larschan et al 2012), 4 ul of rabbit polyclonal anti-CLAMP antibody B (Abcam), 1 ul of rabbit polyclonal antibodies against GAF and E(y)2 or 1 ul of rat polyclonal against Mod(mdg4) (generous gift from Anton Golovnin and Pavel Georgiev, Elissa Lei [Mod(mdg4)], Carl Wu (GAF), and David Gilmour (GAF)) was used.

Binding reactions were electrophoresed using the conditions described previously (Wolle et al., 2015). The gels were run at 180 V for 3 to 4 h at 4°C, dried, and imaged using a Typhoon 9410 scanner and Image Gauge software or X-ray film.

Chromatin Preparation. Cells were crosslinked with 1% formaldehyde in T150 flasks. Samples were mixed (110 rpm) for 10 min at room temperature (~23°C) followed by quenching with

0.960 mLs 2.5 M glycine for 5 min at room temperature (110 rpm). The cells were then transferred to a 50 mL centrifuge tube and centrifuged for 5 min at 1500 g at 4°C. The pellet was resuspended in 30 mLs PBS containing 1x Protease Inhibitor cocktail (pancrease-extract 0.002 mg/ml, thermolysin 0.0005 mg/ml, chymotrypsin 0.002 mg/ml, trypsin 0.02 mg/ml, papain 0.33 mg/ml) (Roche) and 0.2 mM PMSF followed by centrifugation for 5 minutes at 1500 g at 4°C. Centrifugation and resuspension were repeated as described above with the following buffers: 1) 6 mLs Wash A (10 mM HEPES pH 7.6, 10 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 0.25% Triton-X 100, 1x PI, and 0.2 mM PMSF) and 2) 6 mLs Wash B (10 mM HEPES pH 7.6, 10 mM NaCl, 10 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 0.25% Triton-X 100, 1x PI, 0.2 mM PMSF). In between each wash, cells were rotated at 4C for 5 min. After Wash B, cells were pelleted and resuspended in Lysis buffer (0.1% SDS, 10 mM EDTA pH 8.0, and 50 mM Tris-HCL pH 8.0) at a concentration of 1.0 x 10^8 cells/mL.

Chromatin samples were sonicated on ice using a Bioruptor water bath sonicator for six 5 min cycles with 30 sec on 30 sec off on the high setting. A one min incubation on ice in between 5 min cycles was used to prevent the samples from overheating. Chromatin was diluted with 9 volumes Dilution Buffer (0.01% SDS, 1.2 mM EDTA pH 8.0, 16.7 mM Tris-HCL pH 8.0, 1.1% Triton X-100, and 167 mM NaCl) and filtered on a PolyPrep column (BioRad). Next, 200 µL was taken as 20% “Input” DNA sample and the remaining chromatin was aliquoted into 1 mL volumes for immunoprecipitation and flash frozen in liquid nitrogen and stored at -80°C until used.

For clean-up of input DNA samples, crosslinks were reversed overnight at 65˚C. 1 µL RNase A was added to the inputs and incubated at 37˚C for 30 min. 3 µL Proteinase K was added per tube and samples were incubated at 42˚C for 2.5 h. DNA was extracted using a Phenol-Chloroform-Isoamyl Alcohol (25:24:1) extraction using phase-lock tubes. The DNA was then precipitated with 5M NaCl, glycogen and ethanol at -80 for 1 h and resuspended in 50 µL UltraPure water. DNA was run on a 2% gel at 130 V for 1 h and post-stained with Gel Red for 20 min.

Immunoprecipitation (IP). Incubations of the chromatin with antibodies and magnetic beads were performed as described in the Experimental procedures. The following washes were performed with 1 mL buffer, with 3 min rotating at 4˚C followed by collection on a magnetic bead rack: 2X 1 mL Wash Buffer I (20 mM Tris-HCL pH 8.0, 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0, and 150 mM NaCl), 2X x 1 mL Wash Buffer II (20 mM Tris-HCL pH 8.0, 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0, and 500 mM NaCl), 1 mL TE wash buffer (10 mM Tris-HCL pH 8.0, 1 mM EDTA pH 8.0), and then eluted in Elution buffer (50 mM Tris-HCL pH 8.0, 10 mM EDTA pH 8.0, and 1% SDS) for 10 min in a 65˚C with vortexing every 2 min. Samples were centrifuged at max speed for 1 min, beads collected with a magnetic rack, and samples transferred to new tubes for DNA clean-up as described above (chromatin preparation supplemental experimental procedures) with a final resuspension in 50 µL UltraPure water.

Supplemental Table S1. DNA sequences used for the EMSA experiments.

Probe	Sequence	Primer sequences (5' to 3')	
Fab-7 GAGA3+4	CAAAGAGCGACACGTGAACAGGTGCAGTAGTAA ATATAAGCAAAGAGAGTTGGAAAGAGTATTGGCT AAGAGCGTCCGCTCACTAACACATAGATAAATTA AGAGAGACGTGATAAGAGAACCGCACGCACACC	F	CAAAGAGCGACACGTGAACAG
		R	GGTGTGCGTGCGGTTCTC
Fab-7 GAGA5+6	CGTGATAAGAGAACCGCACGCACACCACCGCAA AATCCAATTGGAAGAGAGCGACTGCTTGAGTGT ATTGGTTAGCAAGAGAGCGGCTAAGGTTTGATG GTTTGATTGGAATTCAGTTGCCGTTCG	F	CGTGATAAGAGAACCGCACGC
		R	CGAACGGCAACTGAATTCCAATC
roX1	TTATAGGGGTTATATGGCTTTACCGCTCTCTTTC GGGACTTGCAGTCCGCCCTATGGCCGTCTCACT CACCCGCTTGCGATGCAAACGCCTGGCCAGCCC GATAGAGAGTGATGGAGAGAGGGAGCCGTCTCT CCTTGTCTACAGTATCATTGTCTCGCTCGCATCC GCGTACCGCCACATGCATCCGATAGAGGTATGC	F	TTATAGGGGTTATATGGCTTTACCG
		R	GCATACCTCTATCGGATGC
roX1 mut	TTATAGGGGTTATATGGCTTTACCTTTTTTTTTCG GGACTTGCAGTCCGCCCTATGGCCGTCTCACTC ACCCGCTTGCGATGCAAACGCCTGGCCAGCCCG ATAAAAATTTTTTTTTTTTTGGAGCCGTCTCTCCT TGTCTACAGTATCATTGTTTTTTTTTCATCCGCGT ACCGCCACATGCATCCGATAGAGGTATGC		
roX1A	TTATAGGGGTTATATGGCTTTACCGCTCTCTTTC GGGACTTGCAGTCCGCCCTATGGCCGTCTCACT CACCCGCTTGCGATGCAAACGCCTGGCCAGCCC	F	TTATAGGGGTTATATGGCTTTACCG
		R	GGGCTGGCCAGGCGT
roX1B	GCTTGCGATGCAAACGCCTGGCCAGCCCGATAG AGAGTGATGGAGAGAGGGAGCCGTCTCTCCTTG TCTACAGTATCATTGTCTCGCTCGC	F	GCTTGCGATGCAAACGCC
		R	GCGAGCGAGACAATGATACTGTAG
roX1C	GATAGAGAGTGATGGAGAGAGGGAGCCGTCTCT CCTTGTCTACAGTATCATTGTCTCGCTCGCATCC GCGTACCGCCACATGCATCCGATAGAGGTATGC	F	GATGGAGAGAGGGAGCC
		R	GCATACCTCTATCGGATGC
roX2	ACAATTGCCAAATAATACAGATCGATTTAGAGCG AGATGACAATAGAGAGGCGATCTCTCTCGTATAC GAGTCTTGAAAAGAAAGAGAAGGCGAACGGTGC TGGCTTAGAGAGAGATGGCAATACTAATTAACTG CAAATACATTTCCGCCATTTTGTTGGCGCTAAAA GTAACGGAAATTCGAGATGCTTTTAGGGCTG	F	ACAATTGCCAAATAATACAGATCG
		R	CAGCCCTAAAAGCATCTCG
roX2 mut	ACAATTGCCAAATAATACAGATCGATTTATTTTTT TTTGACAATAGAGAGATTTATTTTTTTTTGACAAA GTCTTGAAAATTTTTTTTAGGCGAACGGTGCTGG CTTATTTTTTTTTGGCAATACTAATTAACTGCAAA TACATTTCCGCCATTTTGTTGGCGCTAAAAGTAA		

	CGGAAATTCGAGATGCTTTTAGGGCTG		
roX2A	ACAATTGCCAAATAATACAGATCGATTTAGAGCG AGATGACAATAGAGAGGCGATCTCTCTCGTATAC GAGTCTTGAAAAGAAAGAGAAGGCGAACGGTG	F	ACAATTGCCAAATAATACAGATCG
		R	CACCGTTCGCCTTCTCTTT
roX2B	CGAGTCTTGAAAAGAAAGAGAAGGCGAACGGTG CTGGCTTAGAGAGAGATGGCAATACTAATTAACT GCAAATACATTTCCGCCATTTTGTTGGCGCTAA	F	CGAGTCTTGAAAAGAAAGAGAAGG
		R	TTAGCGCCAACAAAATGG
roX2C	CTGGCTTAGAGAGAGATGGCAATACTAATTAACT GCAAATACATTTCCGCCATTTTGTTGGCGCTAAA AGTAACGGAAATTCGAGATGCTTTTAGGGCTG	F	CTGGCTTAGAGAGAGATGGCAATA
		R	CAGCCCTAAAAGCATCTCG
CG14446	GTGTGTGCGTGCGCCAGAGAAAGTGAGATAGAG AGAGAGAGAGAGCGAGCGAGAGCGAAAGTGAG GGAGACAGCGACAGAGAGAAGTTAAGAGAAAGA GG	F	GTGTGTGCGTGCGCC
		R	CCTCTTTCTCTTAACTTCTCTCTG
5C2	TTAGAGCAGAATGTATTTTAAATATCAATGTTTCG ATGTAGAAATTGAATGGTTTAAATCACGTTCACA CAACTTAGAAAGAGATAGCGATGGCGGTGTGAA AGAGAGCGAGATAGTTGGAAGCTTCATGGAAAT GAAAGAGAGGTAGTTTTTGGAAATGAAAGTTGTA CTAGAAATAAGTATTTTATGTATATAGAATA	F	TTAGAGCAGAATGTATTTTAAATATCAATG
		R	TATTCTATATACATAAAATACTTATTTCTAGTAC
5C2 mut1	TTAGAGCAGAATGTATTTTAAATATCAATGTTTCG ATGTAGAAATTGAATGGTTTAAATCACGTTCACA CAACTTATTTTTTTTTAGCGATGGCGGTGTGAAA GAGAGCGAGATAGTTGGAAGCTTCATGGAAATG AAAGAGAGGTAGTTTTTGGAAATGAAAGTTGTAC TAGAAATAAGTATTTTATGTATATAGAATA		
5C2 mut2	TTAGAGCAGAATGTATTTTAAATATCAATGTTTCG ATGTAGAAATTGAATGGTTTAAATCACGTTCACA CAACTTAGAAAGAGATAGCGATGGCGGTGTGAA AGATTTTTTTTTAGTTGGAAGCTTCATGGAAATGA AAGAGAGGTAGTTTTTGGAAATGAAAGTTGTACT AGAAATAAGTATTTTATGTATATAGAATA		
5C2 mut3	TTAGAGCAGAATGTATTTTAAATATCAATGTTTCG ATGTAGAAATTGAATGGTTTAAATCACGTTCACA CAACTTAGAAAGAGATAGCGATGGCGGTGTGAA AGAGAGCGAGATAGTTGGAAGCTTCATGGAAAT GATTTTTTTTTAGTTTTTGGAAATGAAAGTTGTAC TAGAAATAAGTATTTTATGTATATAGAATA		
5C2 mut1+2	TTAGAGCAGAATGTATTTTAAATATCAATGTTTCG ATGTAGAAATTGAATGGTTTAAATCACGTTCACA CAACTTATTTTTTTTTAGCGATGGCGGTGTGAAA GATTTTTTTTTAGTTGGAAGCTTCATGGAAATGAA AGAGAGGTAGTTTTTGGAAATGAAAGTTGTACTA GAAATAAGTATTTTATGTATATAGAATA		
5C2	TTAGAGCAGAATGTATTTTAAATATCAATGTTTCG		

mut1+2+3	ATGTAGAAATTGAATGGTTTAAATCACGTTCACA CAACTTATTTTTTTTTAGCGATGGCGGTGTGAAA GATTTTTTTTTAGTTGGAAGCTTCATGGAAATGAT TTTTTTTTAGTTTTTGGAAATGAAAGTTGTACTAG AAATAAGTATTTTATGTATATAGAATA		
bif	CGGTTGAGTTTTCCTCTTGCCTTCTTCTCGTTGG CAAACCCGTTTTGTTTGTGCTTTGTGCCGTTCTC CTCTCCCACTCCCTCTCCCTCTCTCTCTCTCTCT CTCTCTCTCCCTCTCAGCTGCCATGTTTCTCTCT ATGTCCATATCCATATCTCCATATCTCAATGGCT CCATGGCTCCATGG CTCTCTTCGCCCTCTT	F R	CGGTTGAGTTTTCCTCTTGCC AAGAGGGCGAAGAGAGCC
phk gamma	CGTGTGCACCTCTAACCCAAACACGAAGCCAAA AGAAAGAGATCGATCCGCATTGCCGAGTATCCA GATGGTTTAATCCGTATCCTTTGCCCTCTCTCTC TCTCCCTCTCTCTCTCTCTGTCTCTCTCTTTCTGA TGTTTAGCTGCGCAAACAAAGTCGCTTCAACGC GCGCAAAAAGTTCCAATTCGCCATACTCGTGA	F R	CGTGTGCACCTCTAACCCA TCACGAGTATGGCGAATTGGA
sw	GTGTAAGGGGGAAAGCGAGAGAGAGGGAGAGA AGGAGCATTGCAGCACATTGGTTCCTCTCGCCT GTCCTCGTCTTCTTTCGAGTGTGGCTGTGTGCGT GCGTGCATCACATCGGGGGAAAGGGATAGACAG AGGCAGAGAAAGTGAGTGAGAGGGAGAGAGAG AGAGAGAGAGAAAGGAAAGATGTGGCGGCAGG TGCA	F R	GTGTAAGGGGGAAAGCGAGA TGCACCTGCCGCCAC
rab35	TGTTTTCTTTTTTTCTGCTGCCTGCTCCCACACTC ACACACACAGGAGCACACTCCCTGCGCTTACTC TCTCTCTCTCTCTCTTTCGCACTCTCTCGGAAGC GCCCTCTCTTTCGCCCCAAGCTACGACCCTCTC GCTCGCACTGGTGCAGCCGGCTGAATATCTGAG ATAAGCTGTTGTTCTTTCTGGCTGCTGGTCTG	F R	TGTTTTCTTTTTTTCTGCTGCCT CAGACCAGCAGCCAGAAA
CES 11D1	AGCCTGAAGCCTGTGGACTCCGCTGATGCCGTG GAAACGGACAGCGTAAGTTGCATTGGCATTCCC ATCCATCCATCCATCTCGCTCATATTCGGGTTTT TTATTGCAGGCCAGCTACACGCCGAGCCTCTTG TCGCTGATGTGCATCTATGGATA	F R	AGCCTGAAGCCTGTGG TATCCATAGATGCACATCAGCG

Supplemental Table S2. Sequences used to generate dsRNA.

Target		Primer Sequence (5' to 3')
GFP	F	TAATACGACTCACTATAGGGAGAGGTGAGCAAGGGCGAGGAGCT
	R	TAATACGACTCACTATAGGGAGATCTTGAAGTTCACCTTGATGCCG
CLAMP	F	TAATACGACTCACTATAGGGGGGCGGCGATTCTATTG
	R	TAATACGACTCACTATAGGGCATGGGAGTGCTGCCCC
GAF	F	GAATTAATACGACTCACTATAGGGATGGTTATGTTGGCTGGCGTCAA
	R	GAATTAATACGACTCACTATAGGGATCTTTACGCGTGGTTTGCGT

Supplemental Table S3. Sequences used for ChIP-qPCR targets.

Target		Primer Sequence (5' to 3')
Fab-7	F	TAAGCCAACTGGTTTCCAACTCT
	R	TTGCCCAGGGTAAGTAACGGTAT
roX1	F	ATGCGAGCGAGACAATGATACT
	R	GACTTGCAGTCCGCCCTATG
roX2	F	ACGGTGCTGGCTTAGAGAGA
	R	AGTTCTGGTCACCCTGGAAA
CES 5C2	F	ATCAATGTTTCGATGTAGA
	R	CTTCCAACTATCTCGCTCT
CES 11D1	F	GTGGAAACGGACAGCGTAA
	R	CACATCAGCGACAAGAGGC

Additional Reference

Aoki T, Schweinsberg S, Manasson J, Schedl P. 2008. A stage-specific factor confers *Fab-7* boundary activity during early embryogenesis in *Drosophila*. Mol Cell Biol **28:**1047–1060.

Supplemental Figures

Fig. S1. GAF and CLAMP bind to the *Fab-7* boundary *in vivo*. Profiles for a 10 kb region spanning *Fab-7* are shown from genome-wide experiments on the localization of CLAMP (ChIP-seq: 31), GAF (53).

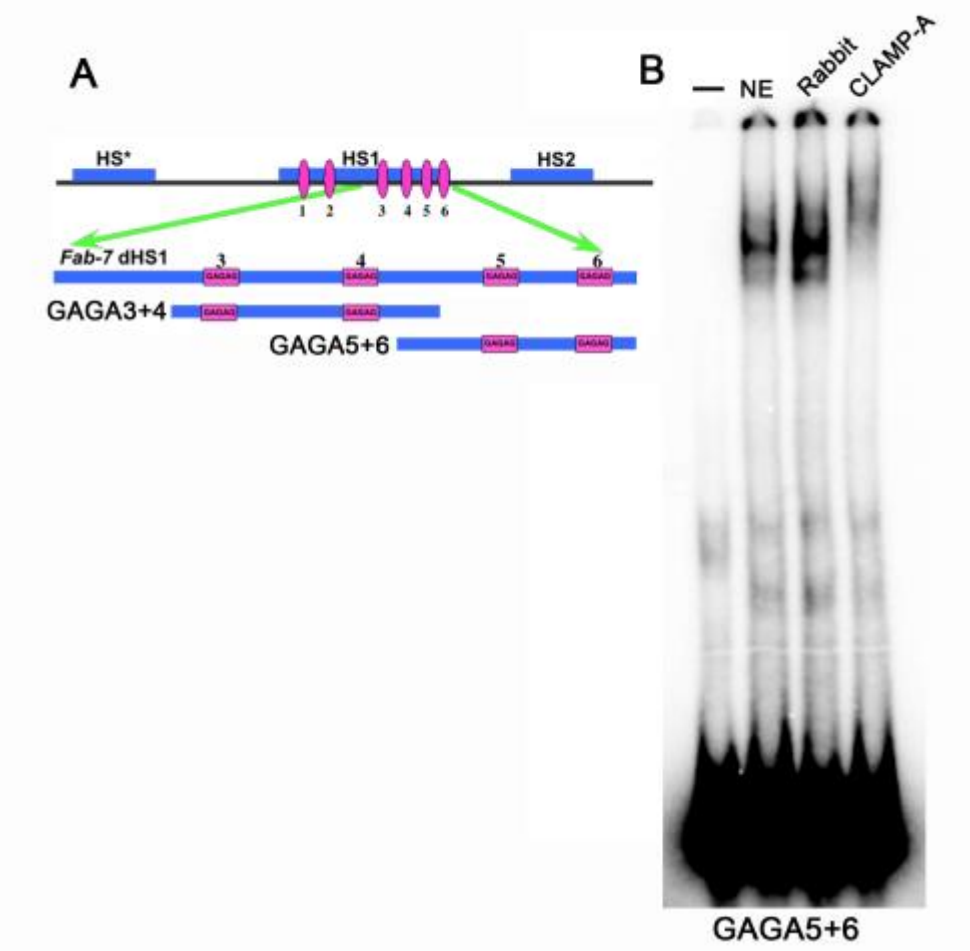

Fig. S2. CLAMP is part of LBC binding to Fab-7 GAGA5+6 sequence. (A) A schematic drawing of the *Fab-7* boundary with six GAGAG sites (pink) and probes (GAGA3+4 and GAGA5+6) used for EMSAs. (B) Probe GAGA5+6 was incubated with late nuclear extracts, without and with either a control preimmune rabbit serum (Rabbit) (lane 3) or the rabbit polyclonal CLAMP A antibody (lane 4).

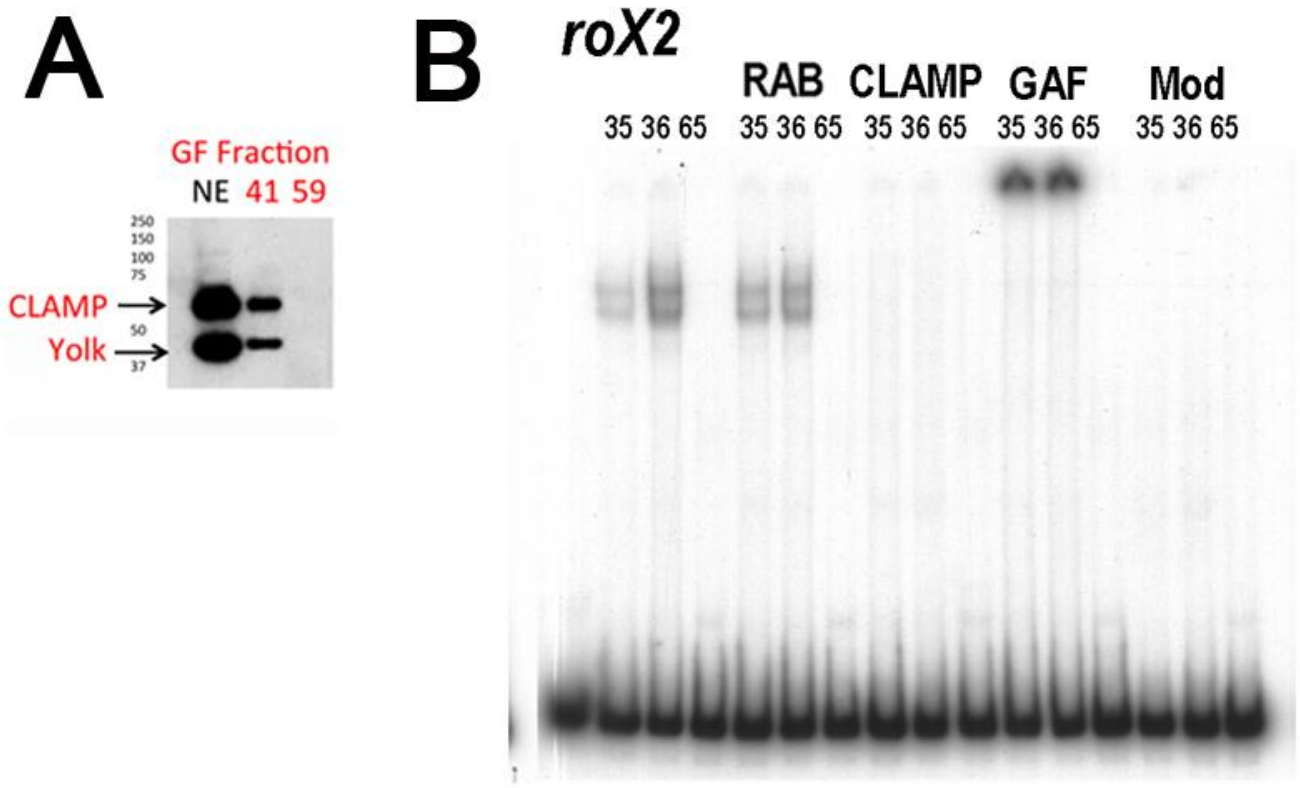

Fig. S3. CLAMP, GAF and Mod(mdg4) co-fractionate with the LBC. (A) Western blotting shows that CLAMP protein is present in a peak gel filtration fractions for LBC activity but not in a fraction that did not have LBC activity (as assayed by EMSAs). A non-specific band at 45kDa corresponds to yolk protein present in nuclear extract in excessive amounts. (B) CLAMP, GAF and Mod(mdg4) are integral components of the LBC. 10 ul of Superose 6 fractions 33, 36 and 65 were preincubated with no antibody, rabbit serum, CLAMP-B, GAF or Mod(mdg4) antibodies for 30 min. The labeled *roX2* probe was then added, and the incubation continued for 30 min prior to gel electrophoresis.

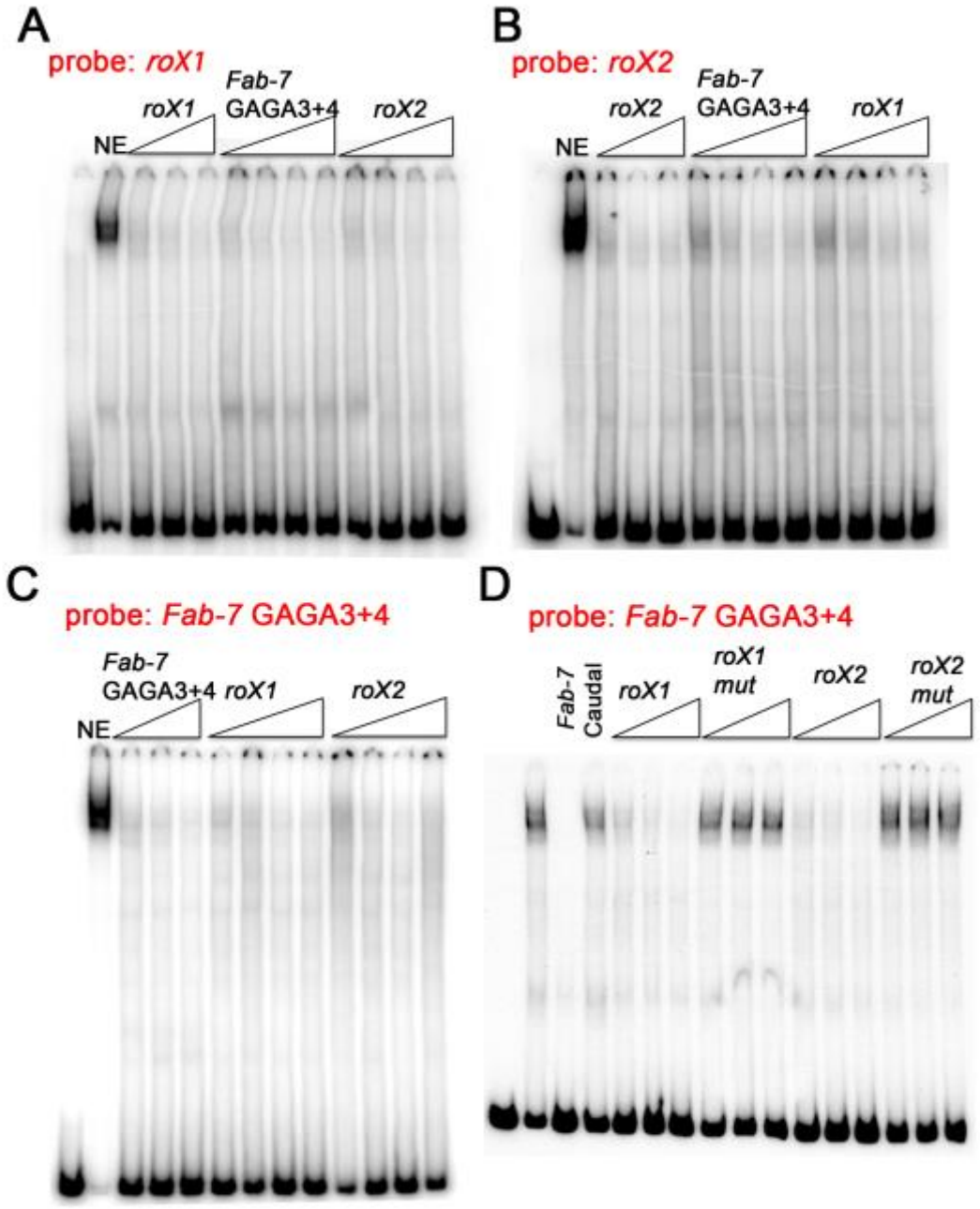

Fig. S4. Competition experiments with wild type and mutant *roX1* and *roX2* DNAs. Cross-competition experiments with labeled (A) *roX1*, (B) *roX2*, (C and D) *Fab-7* GAGA3+4 probes. Labeled probes (as indicated in red) were incubated with late nuclear extracts in the absence (NE) or presence of increasing amounts of unlabeled cold competitor as indicated above the lanes. For self-competition (first set of 3 in A, B and C) a 25x, 50x or 100x excess of cold competitor was added to the reaction mixtures. For cross competition (next two sets of four) 25x, 50x, 75x and 100x excess cold competitor as indicated was added to the reaction mix. (D) Mutated *roX1* and *roX2* probes fail to compete for LBC binding to the *Fab-7* GAGA3+4 probe. A 100 bp Caudal DNA competitor fails to compete for LBC binding and is used as a negative control. In each set of three 25x, 50x and 100x excess of cold competitor as indicated was added to the reaction mix.

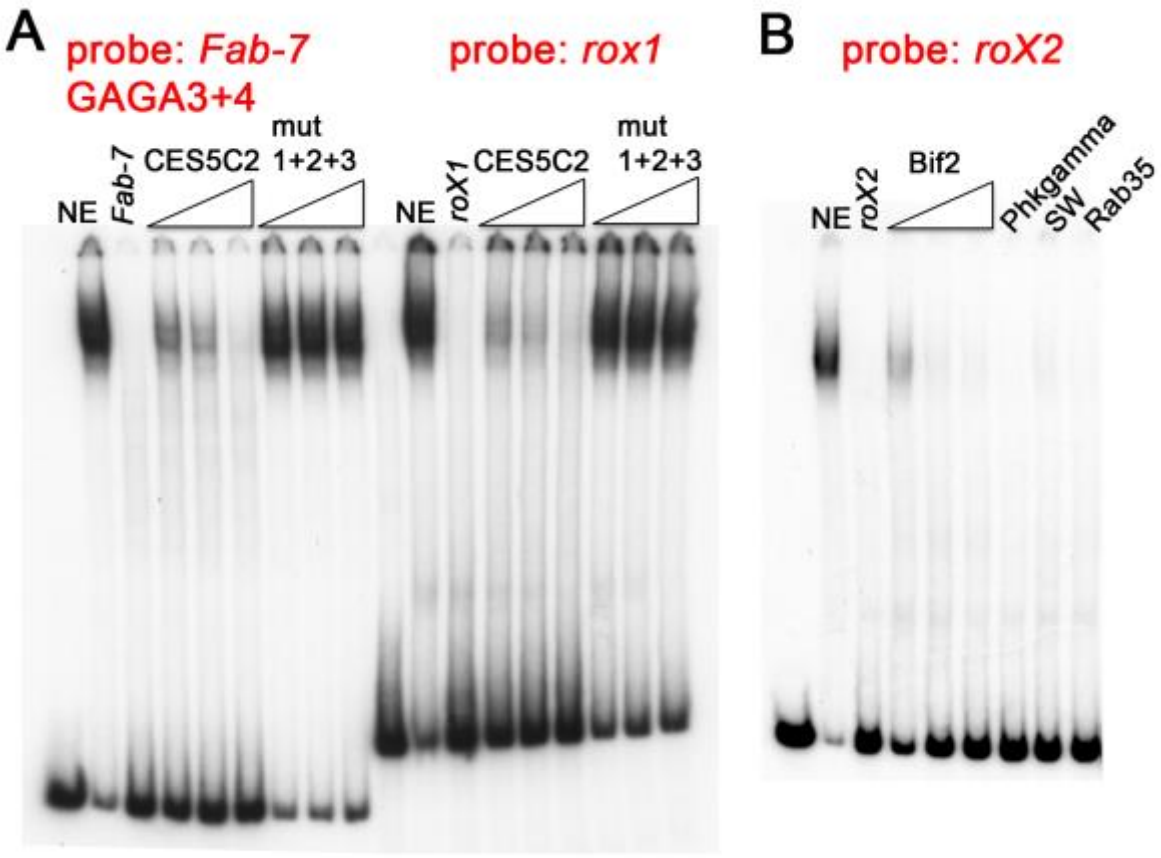

Fig. S5. Cross competition of CES 5C2, CES 5C2 mut 1+2+3 and interacting CES. (A) For the cross competition experiments the labeled probes were either *Fab-7* GAGA3+4 or CES 5C2 as indicated above in red. A 12.5x, 25x or 100x excess of the wild type or mutant CES 5C2 cold competitor was added. (B) Labeled *roX2* probe was competed with excess unlabeled DNA corresponding to the four interacting CES as indicated. For Bif2, a 12.5x, 25x, or 100x excess of the unlabeled DNA was added to the reaction mix. For the other unlabeled DNAs (*roX2*, *phkgamma*, *sw* and *Rab35* 100x excess was added.

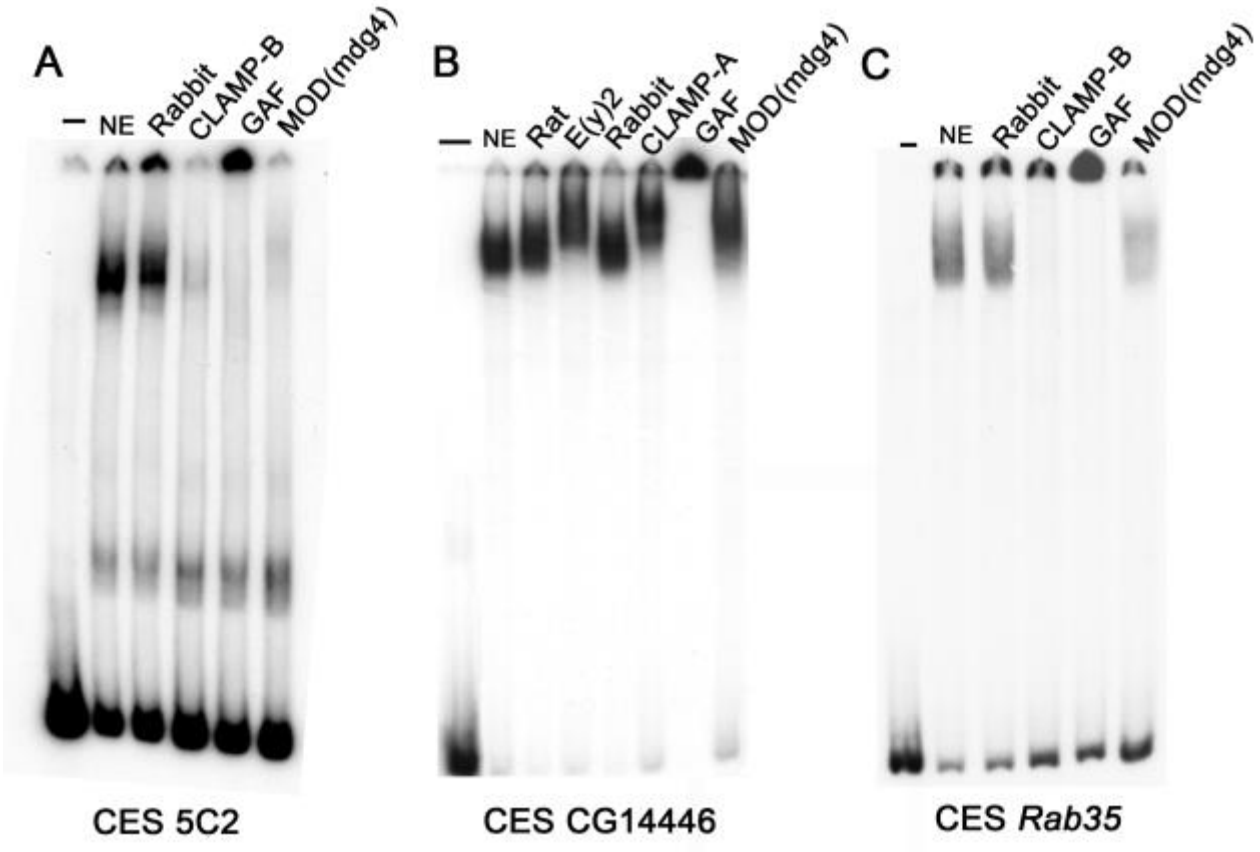

Fig. S6. Supershift experiments demonstrating that the LBC binds to (A) CES 5C2, (B) CG14446 and (C) Rab35. Probes were incubated with late nuclear extracts, without and with either a control preimmune rat or rabbit serum, a rat polyclonal E(y)2 antibody, two different rabbit polyclonal CLAMP antibodies (A and B), rabbit polyclonal GAF antibody or a rabbit polyclonal Mod(mdg4) antibody. A) CES 5C2, B) CES CG14446 and C) CES *Rab35*. For the experiments in panels A and C, the nuclear extract was preincubated with the indicated antibody for 30 min prior to the addition of the labeled probe, and then the reaction mix was incubated for an additional 30 min prior to gel electrophoresis. For panel B, the nuclear extract, antibody and labeled probe were added together and the reaction mix was then incubated for 30 min prior to gel electrophoresis.

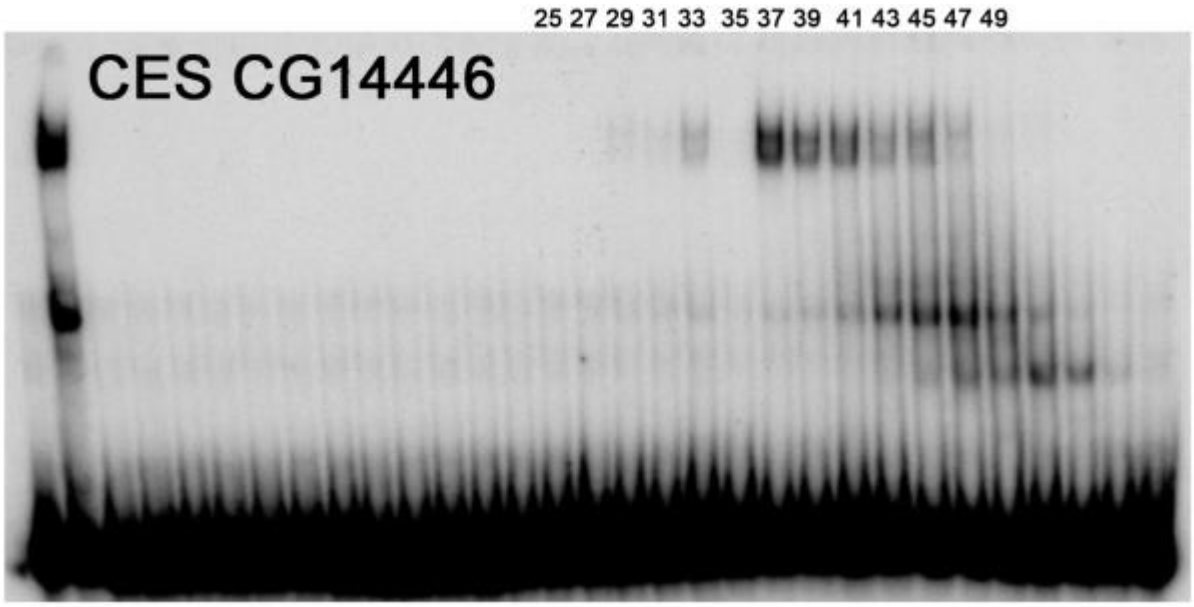

Fig. S7. LBC shift of CES CG14446 by Superose 6 column fractions. Nuclear extracts were size separated on a Superose 6 column and then the fractions used for EMSA of the CES CG14446 probe.. 10 ul of odd numbered column fractions (1-57) were incubated with the CES CG14446 probe. Note that the peak fractions are the same as observed with the *Fab-7* and *roX1* probes. Note: as in experiments with GAGA3+4 and *roX1*, the activity of fraction 35 is reduced compared to its neighbors. The reduction is, however, more pronounced for CG14446, probably because the fractions had been stored for a longer period of time when they were used for this EMSA experiment.