Supplementary Table S1. Effect of *pont* and *rept* mutations on the dominant extra sex comb phenotype induced by *Pc* mutation on the second leg in males.

Genotype	Number of transformed flies*/total	% transformed flies	Expressivity§ (%)
+/+	0/120	0	0
$pont^{5.1}$/+	0/60	0	0
$rept^{35}$/+	0/70	0	0
$pont^{5.1}$,$rept^{35}$/+	0/90	0	0
Pc^{XT109}/+	91/185	49	10
Pc^{XT109}/$pont^{5.1}$	24/136	18	2.9
Pc^{XT109}/$pont^{5.1}$;*P(pont)*	90/195	46	10.7
Pc^{XT109}/$rept^{35}$	136/200	68	18.1
Pc^{XT109}/$rept^{35}$;*P(rept)*	100/185	54	14
Pc^{XT109}/$pont^{5.1}$,$rept^{35}$	58/106	55	14

* Number of males with extra sex comb teeth on the second leg.
§ Average number of ectopic sex comb teeth on the second leg, with reference to a number of 10 teeth on the first leg of wild type males (100%)

Supplementary Table S2. Genetic interactions of *pont* and *rept* mutations with the gain-of-function allele Scr^{Msc}.

Genotype	Average number of teeth on T2	Average number of teeth on T3	Number of flies examined
+/+	0	0	60
Scr^{Msc}/+	2.77	1.25	75
$pont^{5.1}$/+	0	0	100
$rept^{35}$/+	0	0	100
$pont^{5.1}$,$rept^{35}$/+	0	0	100
Scr^{Msc}/$pont^{5.1}$	1.93	0.23	87
Scr^{Msc}/$pont^{5.1}$;*P(pont)*	2.72	1.07	190
Scr^{Msc}/$rept^{35}$	3.33	1.74	89
Scr^{Msc}/$rept^{35}$;*P(rept)*	2.62	1.28	134
Scr^{Msc}/$pont^{5.1}$,$rept^{35}$	2.81	0.92	126

Supplementary Table S3. MuDPIT analysis of proteins copurifying with the Brm complex[a]

Protein	Accession	No. of peptides	% sequence coverage
Snr1	Q24090	27	70.5
Bap55	Q9V814	29	61.6
Moira	AAF55259	83	55.7
Bap60	Q9VYG2	35	55.1
Actin	P10987	15	50.3
Polybromo	Q9VC36	85	48.6
CG7154	Q9VLX2	27	44.4
Brahma	P25439	79	41.9
Pontin	Q9VH07	13	41.4
Bap111	Q9W384	28	38.2
Bap170	Q9V9D0	54	37.1
Dd4	Q9V9F1	14	33.8
Hsc3	Q9VYU3	18	30.8
Sin3A	Q8ML45	43	26.3
Osa	Q8IN94	51	17.9
e(y)3	AAF48990	29	16.9
lid	Q9VMJ7	20	16.2

[a]The Brm peak fraction from the final glycerol gradient purification step from two independent purifications was analysed by MuDPIT mass spectrometric analysis and proteins identified by at least five independent peptides on both analyses are indicated. The number of peptides along with the percentage of primary sequence covered by the identified peptides are shown for one four-step MuDPIT analysis.

Supplementary Methods

Antibody production

Anti-Pont (Pont67) and anti-Rept (Rept66) antibodies were raised in rabbit against synthetic peptides:

H_2N-CKVNGRNQISKDDIED-$CONH_2$ for Pont;

H_2N-FTRARDYDATGAQTC-$CONH_2$ for Rept.

The peptide sequences were chosen based on hydrophilicity and predicted surface exposure and showed no homology with the paralogous protein sequence. Peptide synthesis, coupling, immunisations, animal rearing, and antibody purification against immobilized peptides were performed by Eurogentec. Specificity was determined by western blot analyses of *Drosophila* nuclear extracts and non-cross-reactivity with the paralogous protein determined by the non-detection (western blot) of 5µg paralogous protein purified from baculovirus-infected Sf9 cells (data not shown, but see Figure 3D). A second anti-Pont antibody (Pont53) was used (Fig. 4E) and has been described elsewhere (Bauer et al., 2000).

Eye pigmentation quantitation assays

PEV assays were performed in *In(1)w*^{m4h} context according to Sass and Henikoff (1998). Analyses of effects of *pont* and *rept* on PRE-mediated regulation of *mw* were performed in

Eye pigment values were determined spectrophotometrically at OD_{485} according to Zink and Paro (1995).

Brm-C purification

Brm-C was purified using a multi-step FPLC purification strategy. Nuclear embryonic extracts, prepared according to (Shao *et al*, 1999), were applied to a Biorex 70 column (Bio-Rad) using 10 mg extract/ml of beads in BC buffer (20 mM Hepes,pH 7.9, 10% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) containing 280 mM KCl (BC280). Proteins were eluted by stepwise gradient with BC425, adjusted to 500 mM KCl and applied to a Butyl-S Sepharose 6 FF column equilibrated in BC500 supplemented with 10 µM ZnCl2 and 0.05% NP-40 (PCB500). The flow through was collected and 4 ml were fractionated on a 120 ml Sephacryl S-300 column in PCB500 (7 cm/h). Peak Brm/Pont fractions were pooled and dialysed overnight against TBZ buffer (20 mM Trix.Cl, pH 8.5, 10% glycerol, 0.2 mM EDTA, 10 µM $ZnCl_2$ and 0.05% NP-40, 0.5 mM DTT, 0.2 mM PMSF) containing 100 mM KCl (TBZ100) and applied (~500 µg) to a 1 ml Mono Q HR 5/5 column and bound proteins fractionated with a 20-column volume 0.1-1 M KCl linear gradient in TBZ (153 cm/h), collecting 0.5 ml fractions. 250 µl peak Brm/Pont fraction was loaded onto a 4 ml 10-45% glycerol gradient in TBZ150, run for 24 h at 84,000 *g* and 200 µl collected fractions were analysed by western. The fraction containing the most Brm and Pont was analysed by silver stain, western and MuDPIT. All protein manipulation techniques were carried out at 4^{o}C.

Purification of Flag-Pont

Recombinant baculovirus encoding a N-terminal Flag tag Pont fusion protein was used to infect Sf9 cells. 48 hours following infection, whole cell extracts were prepared by rapid freeze/thawing the PBS-washed cell pellet in BC280 buffer (2 ml/ml pellet), followed by sonication with 3x 10 second bursts using a microtip (50% duty cycle). Flag tagged Pont was

subsequently purified following the same protocol as for PCC purification, and analysed by western analyses using anti-Pont antibodies (Pont53 and Pont67), and anti-Flag (M2, Sigma)

Sample preparation for MuDPIT mass spectrometry analysis

While preserved in its buffer solution (50 µl of 20 mM HEPES.K+ pH 8.5, 150 mM KCl, 5 µM $ZnCl_2$, 0.05% NP-40, 25% glycerol), 50 µl of 16 M urea, 100 mM Tris, pH 8.5 were added to denature the proteins. The subsequent mixture was then reduced by adding 0.5 µl of 1 M TCEP (to a final concentration of 5 mM TCEP) and incubated at room temperature. To alkylate, 2.0 µl of iodoacetamide (10 mM final concentration) were added and the sample was subsequently incubated at room temperature while in the dark for 15 mins. 1 µl endoproteinase Lys-C (0.1 µg/µl) was then added and shaken for 4 h while incubated in the dark at 37ºC. The addition of 300 µL of 100 mM Tris pH 8.5 diluted the solution to 2 M urea. Calcium chloride (100 mM) was then added (5.0 µL) for a final concentration of 1 mM $CaCl_2$. Trypsin (0.5 µg/µL) was added in the amount of 4.0 µL. The resulting mixture was then shaken for 18 h and incubated in the dark at 37ºC. To neutralize the solution, 25.0 µL formic acid (90%) was added to a final concentration of 5% and the sample was centrifuged for 30 mins at 2ºC on a table top centrifuge.

Multidimensional protein identification technology (MuDPIT)

Upon completion of the digestion, the protein was pressure-loaded onto a fused silica capillary desalting column containing 3 cm of 5-µm strong cation exchange (SCX) followed by 3 cm of 5-µm C18 (reverse phase or RP material) packed into an undeactivated 250-µm i.d capillary. Using 1.5 mL of buffer A (95% water, 5% acetonitrile, and 0.1% formic acid) the

desalting column was washed overnight. Following the desalting process, a 100-µm i.d capillary consisting of a 10-µm laser pulled tip packed with 10 cm 3-µm Aqua C18 material (Phenomenex, Ventura, CA) was attached to the filter union (desalting column–filter union–analytical column). The resulting split-column was placed inline with a ThermoFinnigan Surveyor MS Pump (Version 2.3; Palo Alto, CA) and analyzed using a customized 4-step separation method (90, 110, 110, and 150 minutes respectively).

Step 1 utilized only buffer A (95% water, 5% acetonitrile, and 0.1% formic acid) and buffer B (80% acetonitrile, 20% water, and 0.1% formic acid). It began with 5 min of 100% Buffer A, followed by the following buffer B gradients: 5 min of 0-10%, 40 min of 10-45%, and 10 min of 45-100%. Twenty minutes of 100% buffer B ensued and the gradient program ended with 10 min of 100% buffer A. Steps 2-4 utilized Buffers A, B, and C (500 mM ammonium acetate, 5% acetonitrile, and 0.1% formic acid). Steps 2 and 3 each began with: 3 min of 100% buffer A, 7 min of X% buffer C, a 5 min gradient from 0 – 10% buffer B, a 75 min gradient from 10-45% buffer B, and then 5 min of a 45-100% buffer B gradient. Five minutes of 100% buffer B followed and then the sequence ended with 10 min of 100% buffer A. The buffer C portions consisted of 20% for step 2 and 50% for step 3. Step 4 began in a similar fashion (3 min of 100% buffer A, 7 min of 100% buffer C, and a 5 min gradient from 0–10% buffer B) yet its 10-45% buffer B gradient lasted for 85 minutes and the 45-100% buffer B gradient was for 10 min. Ten minutes of 100% and then a gradient of 0-100% buffer B ensued with the run ending with 10 min of 100% buffer A.

By increasing the salt concentration (buffer C) peptides “bump” off the SCX and then with a gradient of increasing hydrophobicity (buffer B) the peptides can elute from the RP into the ion source. To elute the peptides from the micro capillary column, a distal 2.5 kV spray

voltage was applied. The applied voltage caused the peptides to directly electro spray into an LTQ 2-dimensional ion trap mass spectrometer (ThermoFinnigan, Palo Alto, CA). First a cycle of one full-scan mass spectrum (300-2000 m/z) and then 5 data-dependent MS/MS spectra at a 35% normalized collision energy was performed throughout each step of the multidimensional separation. The aforementioned HPLC solvent gradients and MS functions were all controlled by the Xcalibur data system (Version 1.4).

Ion Trap mass spectrometry of gel-excised bands

Flag-tagged Pontin was overexpressed and purified by anti-Flag affinity chromatography (M2 beads: Sigma). Purified protein was resolved by 8% SDS-PAGE and stained by colloidal Coomassie Blue stain (Sigma). Protein bands were excised and trypsinised (10 ng/μl in 25mM NH_4CO_3) for 6 h at 37^{o}C. The resulting peptides were applied to an ion trap mass spectrometer (LCQ-Deca XP, ThermoFinnigan) in nano-ESI-IT configuration coupled to two-dimensional HPLC (BioBasic SCX cationic exchange followed by reverse-phase C 18 PicoFrit chromatography).

Analysis of tandem mass spectra

As each step was executed, its spectra were recorded to a RAW file. This data was then converted into .ms2 format through the use of RawXtract (Version 1.8). From the .ms2 files, poor quality tandem mass spectra were removed using an automated spectral quality assessment algorithm known as a "PARC" filter (Bern et al. 2004). The remaining MS/MS spectra were searched with the SEQUEST™ algorithm (Eng et al. 1994) against the NCBI RefSeq *Drosophila* (vers. 10-31-06) protein sequence database. Protein sequences from the database were reversed

(decoy database) to assess the false positive rate (Peng et al. 2003). A computer cluster consisting of 100 1.2 GHz Athlon CPUs was used to perform the search (Sadygov et al. 2002). The SEQUEST search did not employ any enzyme specificity and the final data set was filtered using the DTASelect™ (version 2.0) program (Tabb et al. 2002). The type of digestion method used was specified (--trypstat for tryptic digests and –modstat for modified peptides) so as to specifically filter for peptides with trypsin specificity. Modifications for ubiquitin (+114 on K) were searched for from the MS/MS spectra. A 7.8% false positive rate was used to dynamically set XCorr and DeltaCN thresholds through quadratic discriminant analysis. This dataset was then further filtered to remove contaminants (i.e. keratin) through the use of Contrast (version 2.0). A minimum of 5 peptides and half tryptic status (-p 5 –y 1) were set in the Contrast.params file.

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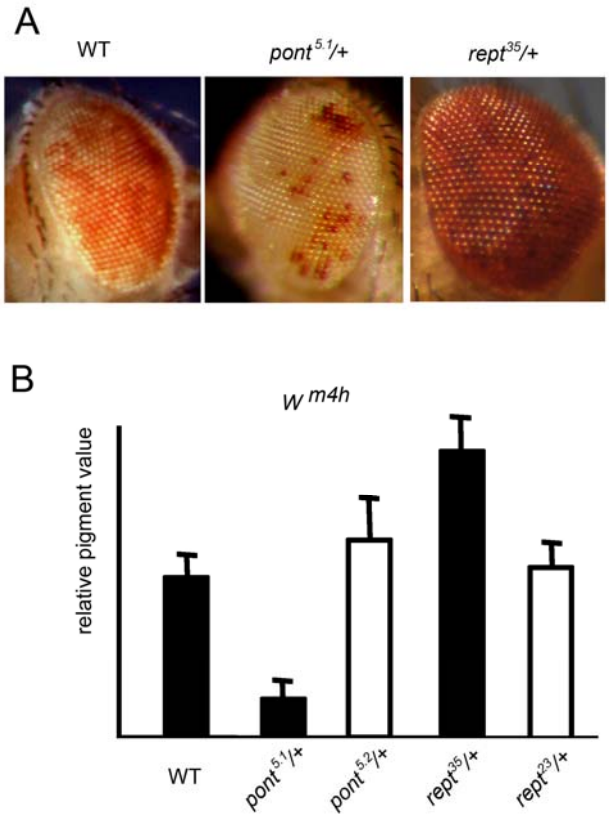
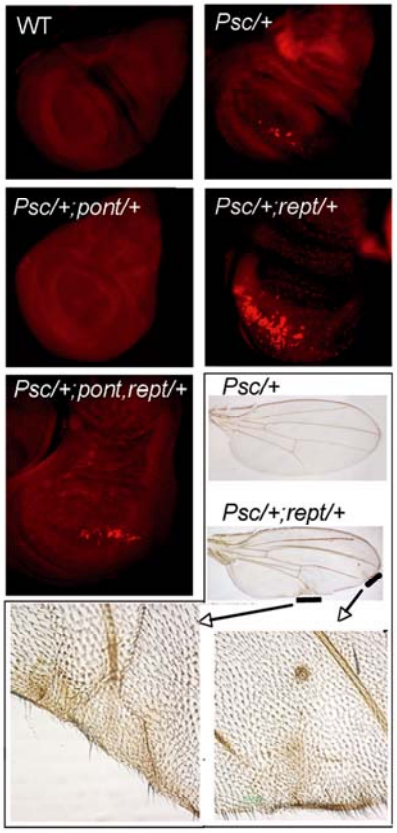
Supplementary Figures



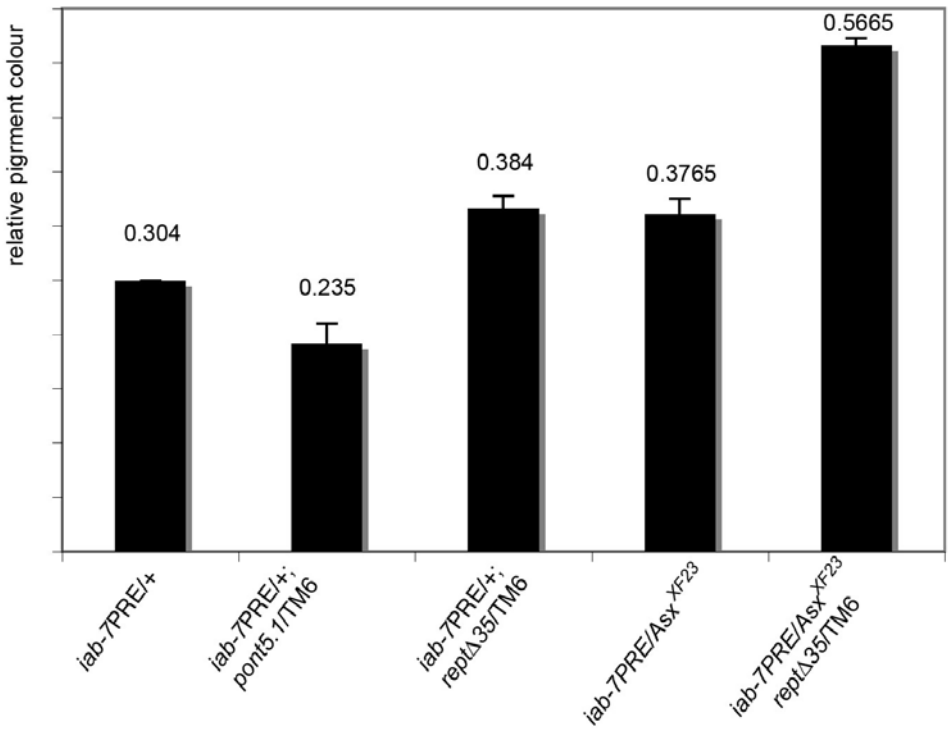
Supplementary Figure S1. Pont and Rept function antagonistically in gene silencing mediated by pericentric heterochromatin

PEV enhancement of *w* by *pont* and suppression by *rept*, as illustrated by the inactivation of *w* in most eye cells of a male carrying the w^{m4h} chromosome and heterozygous for $pont^{5.1}$, the increased expression of *w* caused by w^{m4h} male heterozygous for $rept^{35}$ (A), and by eye pigment quantification (arbitrary units) (B). $pont^{5.2}$ and $rept^{23}$ chromosomes correspond to perfect excision events of P-elements inserted in *pont* and *rept* genes (Bauer et al. 2000). Error bars indicate standard deviations.



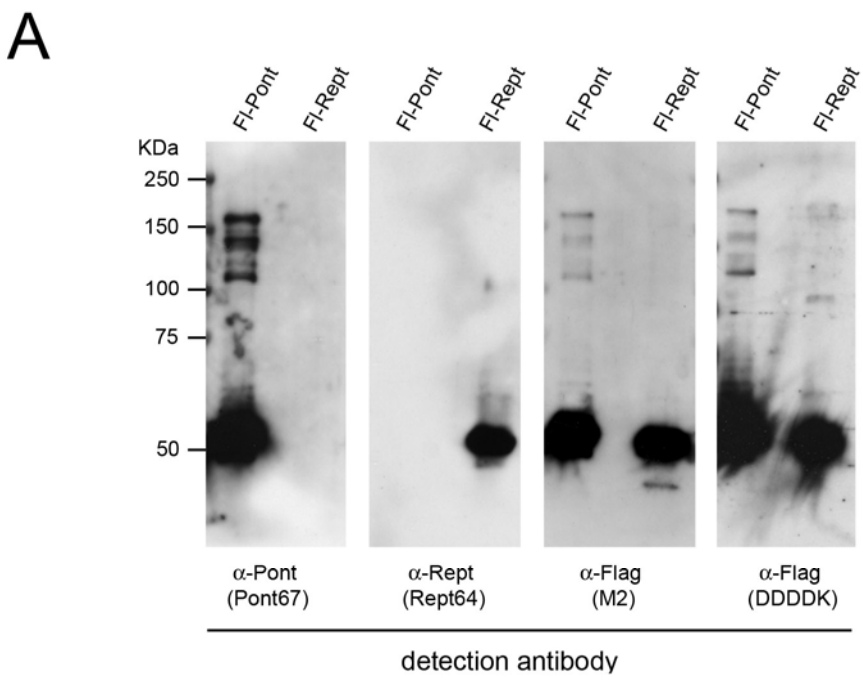
Supplementary Figure S2. *pont* and *rept* interfere in an opposite manner with *Psc* in the control of Ubx expression in the wing imaginal disc

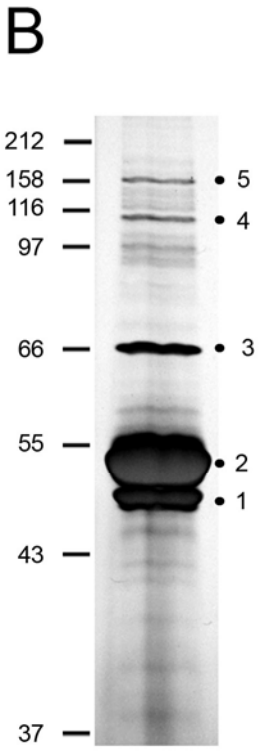
Ubx protein is not expressed in the epithelium of wild type wing imaginal discs (top left panel) and becomes derepressed in few cells from disc epithelium heterozygous for *Psc* (top right panel). This derepression is abolished when one copy of *pont* is simultaneously removed (middle top left panel) and significantly enhanced by a dosage reduction of *rept* (middle top right panel), whereas an expression level comparable to expression in *Psc/+* discs is restored in wing discs from *Psc*, *pont* and *rept* triple heterozygous larvae (middle bottom left panel). Ubx derepression in *Psc/+;rept/+* imaginal wing disc results in a transformation of the wing structure (magnified views in bottom panels).



Supplementary Figure S3. *pont* and *rept* regulate PRE-controlled gene expression

Expression of *mw* under the control of the *iab-7* PRE was measured by eye pigment quantitation in flies containing one copy of the *iab-7* PRE. *mw* expression was then determined as a function of this reference value in flies carrying the *iab-7 PRE* and mutated for either *pont*, *rept*, *Asx*, or *Asx* and *rept* showing an increase in silencing of *mw* by the PRE upon mutation of *pont* or a loss of silencing upon mutation of *rept*, *Asx*, or *Asx* and *rept*. Average mean OD_{485} values are shown above the bars.





C

Band	ID	Accession	% Coverage	*p*
1	Reptin	75026227	12.06	1.37×10^{-8}
2	Pontin	75026935	74.34	6.96×10^{-7}
3	Hsc4	28381304	12.29	2.10×10^{-6}
4	Pontin	75026935	42.30	4.20×10^{-6}
5	Pontin	75026935	25.20	1.04×10^{-5}

Supplementary Figure S4. Purification and identification of high molecular weight forms of Pont

(A) 2.5 μg flag-tagged Pont or Rept, overexpressed in Sf9 cells and purified by M2 affinity chromatography, were resolved by SDS-PAGE and detected by immunoblotting using anti-peptide antibodies against either Pont (Pont67), Rept (Rept64) or Flag (DDDDK; Abcam and M2; Sigma). Of note is the non-cross reaction of anti-Pont and anti-Rept antibodies with their paralogous protein counterpart, and the detection of identical high molecular weight forms of Pont by Pont67 and anti-Flag antibodies. (B) Silver stain analysis of Fl-Pont purified from Sf9 cells. Numbered bands were excised and the protein identified by ion trap nano-ESI-IT mass spectrometry (C). Note: the identification of *Drosophila* Hsc4 and Reptin correspond to their *Spodoptera frugiperda* orthologues.