Materials and Methods

HP1B and *Umbrea* presence/absence

Genomic DNA was prepared from 32 species of Drosophila (Fig. 2A), using previously described methods (*26*). Primers HSM208: CAACCAGTTCGCATGAAAATGCATAATCAATC and HSM215: AGATGAAGATGCACCAATGATACGCTCAATGGG were designed to sequences flanking *Umbrea* in the first intron of *dumpy.* These sequences are conserved from *D. melanogaster* to *D. pseudoobscura* and therefore unambiguously amplified the *Umbrea* syntenic locus in all species tested. The presence or absence of *Umbrea* was identified by size differences on an agarose gel, and confirmed by sequencing (Figure S6). *Umbrea* sequences have been uploaded to GenBank, with Accession numbers KC660086- KC660100.

Evolutionary analysis

Umbrea and HP1B protein sequences from different species were aligned using ClustalX (*27*) and manually adjusted for gaps. Nucleotide alignments that honored codon positions were made using Pal2Nal (*28*). Phylogenies were created using DNA sequences and compared to published phylogenies (*25*). Maximum-likelihood analysis of *Umbrea* and *HP1B* gene sequences was performed with codeml of the PAML software package (*29*), and web implementation of the HyPhy analysis package (www.datamonkey.com) (*30*). dN/dS ratios between lineages were calculated in PAML using a free ratio model, allowing dN/dS variation along the branches of the phylogeny. To detect amino acids under selection in Umbrea and HP1B, we fit the multiple-alignment of amino acid sequences to either the F3x4 or the F61 codon frequency models. Likelihood ratio tests were performed by comparing *NS* sites models M1 (neutral) and M2 (selection), M7 (neutral, beta-distribution of omega <1) and M8 (selection, beta distribution, omega >1 allowed), or M8a (selection, beta distribution, omega >1 allowed) and M8. PAML analysis identified sets of amino acids in the *Umbrea* phylogeny with high posterior probabilities for positive selection by using Bayesian methods.

Cultured cell cytology

All constructs used for cytology were generated by amplifying genomic DNA (*Umbrea* lacks introns) and directionally cloning into pENTR-D-TOPO (Invitrogen). Upon sequence verification, these entry clones were recombined via LR clonase (Invitrogen) reactions into Gateway Destination vector pHGW 1073 acquired from the Drosophila Genomics Resource Center (DGRC, Bloomington, IN). *Kc* cells were seeded onto coverslips and transfected with FuGene (Roche) overnight with 2ug of plasmid DNA. Expression of fusion proteins was transiently induced by heat-shock at 37^{o}C for 45 minutes and cells were allowed to recover for 2 hours at room temperature. Nuclei were prepared by incubating cells in 0.5% sodium citrate and spinning away cytoplasm using a Shandon Cytospin3 at 1900 rpm. Nuclei were fixed for 15 minutes in 1X phosphate buffered saline plus 0.3% Tween-20 (PBST) and 4% paraformaldehyde. Following fixation, nuclei were washed with PBST and then blocked for 45 minutes at room temperature with either PBG (PBST plus 0.2% cold water fish gelatin (Sigma) and 0.5% BSA (Sigma) or PBST plus 5% BSA. Primary antibodies diluted in PBG (see below) were incubated with nuclei for 1 hour at room temperature or overnight at 4^{o}C. Following primary antibody staining, nuclei were washed in block and incubated with Alexa Fluor secondary antibodies diluted in block. *D. melanogaster* Kc cells and *D. teisseiri* cells were acquired from the DGRC.

Cytology of *Drosophila* testes and imaginal discs
To assay Umbrea localization in the male germline, virgin adult male Canton S flies were isolated and aged for 2-5 days before testes were dissected and prepared for imaging as previously described(*31*). To assay Umbrea localization in developing larval imaginal discs, wandering L3 larvae were isolated and inverted in cold 1X PBS. Whole carcasses were subsequently fixed in 1X PBS plus 4% paraformaldehyde, washed in 1X PBS plus .3% Triton-X 100 (PBST), and blocked in PBST plus 3% BSA. Primary antibody staining was performed in block at 4 degrees C overnight. Testes and discs were imaged using a Leica TCS SP5 II confocal microscope and images were acquired with LASAF software. Lif files were converted to maximally projected tifs using MetaMorph software. For each image, data from the red channel was merged into the blue channel to create magenta (*32*).

Western blot analysis
Nuclear extracts from Kc and S2 cells transfected with heat-shock inducible Umbrea-GFP or untransfected cells were prepared as below (see Purification and Mass Spectroscopy), boiled in SDS loading buffer plus beta-mercaptoethanol and run on 4-12% NuPAGE Bis-Tris gels. After transfer, membranes were blocked in milk, probed with anti-Umbrea or anti-beta tubulin primary antibodies and Li-Cor IRDye secondary antibodies. Blots were imaged using the Li-Cor Odyssey system.

Antibodies used
Anti-Umbrea (rabbit polyclonal, developed in this study), 1:5000. Umbrea antibody (Covance) was raised against peptides KLTSSTALPVKQRNGFDL (aa6-aa22) and LESDNGYETTPSPRKKRS (aa86-aa103). Both peptides were injected into single rabbits, and immunized over ~two months.
Anti-cid (rabbit polyclonal, Abcam) 1:500
Anti-cid (chicken polyclonal, gift from Karpen lab(*33*)), 1:1000
Anti-CENP-C (rabbit polyclonal, gift from Lehner lab(*34*)), 1:5000
Anti-HOAP: (rabbit polyclonal, gift from Theurkauf lab(*35*)), 1:1000
Anti-HipHop: (rabbit polyclonal, gift from Rong lab(*36*)), 1:500
Anti-beta tubulin: (E7 mouse monoclonal, Developmental Studies Hybridoma Bank),1:1000
Goat anti-rabbit 488 (Invitrogen), 1:5000
Donkey anti-Chicken 568 (Invitrogen), 1:5000
Goat anti-mouse 568 (Invitrogen), 1:5000
IRDye680 anti-mouse, 1:10000
IRDye800 anti-rabbit, 1:10000

***Umbrea* transgenesis for RNAi rescue**
Umbrea genes were synthesized by GenScript that were re-encoded such that all synonymous sites were changed, leaving the amino acid sequence intact. Transgenes were cloned into pENTR D-TOPO (Invitrogen), then recombined using Gateway technology into expresion vector pUASp-GFP, with miniwhite rescue. Transgenesis by standard embryo injection was performed by The Best Gene Inc. (Chino Hills, CA, US).

***Drosophila* maintenance and strains**
All *Drosophila* strains were maintained on standard molasses-cornmeal medium in uncrowded conditions.

The following strains were obtained from the Bloomington Drosophila Stock Center for use in complementation analysis of *dumpy (dp)* and *Umbrea* :
dp^{lv1} b^{1}/SM5 (Stock number: 278)
dp^{olvR}/SM5 (Stock number: 280)
w^{1118}; net^{1} P{$w^{+mGT=GT1}$}$HP6^{BG0142}$] $dp^{BG01429}$/In(2LR)Gla, wg^{Gla-1} Bc^{1} (Stock number: 12747

The following Vienna Drosophila RNAi Center (VDRC) line was used in *in vivo* RNAi knockdown experiments:
w^{1118}; P{GD4434}v13074/CyO (Stock number: v13074)

The following GAL4 driver lines were obtained from the Bloomington Drosophila Stock Center:
y^{1} w*; P{Act5C-GAL4-w}E1/CyO (Stock number: 25374)

RNAi knockdown of Umbrea in S2 cells
dsRNA was prepared using the MegaSCRIPT® T7 kit (Applied Biosystems) following the manufacturer's instructions. Templates were generated by PCR from genomic DNA (for Umbrea) and from the pCopia-LAP-CID plasmid (for the control dsRNA), using the following primers:
Umbrea forward, TAATACGACTCACTATAGGGCGCCCAGCTCCACTTTGAC,
Umbrea reverse, TAATACGACTCACTATAGGGCGCATTTCGTGATCGTTTCTT,
scrambled forward, TAATACGACTCACTATAGGGCAAGAGCTTGGCGGCGAAT,
scrambled reverse, TAATACGACTCACTATAGGGCCGCGGGTTCCTTCCGGTA.
The dsRNA was transfected into the cells using DOTAP liposomal transfection reagent (Roche). 10^{6} logarithmically growing S2 cells were plated in 1 ml of serum medium in a 6-well plate.10µg dsRNA were transfected with DOTAP (Roche), following the manufacturer's instructions. After 24hr, the DOTAP containing medium was replaced with new serum containing medium, and cells were incubated for 4 additional days. Samples were taken on day 5 and were subjected to indirect IF and time-lapse analysis.

Immunofluorescence (IF) on fixed S2 cells
S2 cells were settled on positively-charged glass slides in PBS for 15min followed by fixation with 3.7% formaldehyde for 10 min. After three washes with PBST (PBS with 0.1% Triton X-100) for 5 min each, cells were blocked in 5% milk in PBST for 20 min before primary antibody incubation overnight at 4°C. This was followed by three washes of 5 min in PBST. The primary antibodies used were 1:1000 chicken anti-CID (*33*), mouse anti-α-tubulin (Sigma-Aldrich), and rabbit anti-phospho-H3S10 (PH3; Millipore). All of the secondary antibodies (Cy5 anti-chicken, 546 anti-mouse and 488 anti-rabbit, Invitrogen) were diluted 1:500 in 5% milk in PBST and incubated for 45 min at room temperature. After three 5-min washes in PBST, cells were mounted in SlowFade® Gold anti-fade reagent (Invitrogen) containing DAPI. All images were taken on a Personal DeltaVision (DV) microscope (Applied Precision, LLC) and deconvolved using softWoRx (with iteration set to conservative, 5 cycles; Applied Precision, LLC). Images were taken as z stacks of 0.4- or 0.5-µm increments using 60× and 100× oil-immersion objectives. For each image, data from the red channel was merged into the blue channel to create magenta(*32*). Mitotic cells were scored as defective or normal based on chromosome

and spindle morphology compared with control cells, and the p-value was calculated using the Fisher's Exact Test.

Time-lapse analysis of mitosis
Time-lapse videos were performed using a Personal DV microscope using a 60× oil-immersion objective. S2 cells expressing mCherry-tubulin and H2B-GFP (gift of G. Goshima) were mounted using the hanging drop method (*37*) 5 days after RNAi treatment. Images of cells in prophase/prometaphase were taken every 2 to 4 min until cytokinesis or for up to 90 min in cases where cells were experiencing delays in mitosis. 14 control and 22 Umbrea RNAi videos of randomly selected cells in prophase were made. Videos were deconvolved and quick-projected, and the time (in minutes) in the still images reflects the actual elapsed time during image acquisition.

Quantitative Real-time PCR
For reverse transcription, total RNA was extracted from the S2 cells 5 days after RNAi using the RNeasy kit (QIAGEN). Genomic DNA was removed from the sample with RQI RNase Free DNase (Promega). 2 μg RNA was used as the template for cDNA synthesis, which was performed using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). The cDNA was then used as a template for quantitative real-time PCR, which was run using a BioRad iCycler iQ™. Briefly, the program was run in reaction volumes of 15 μl containing the following reagents: 7.5 μl EXPRESS SYBR® GREEN**ER**™ SuperMix with Premixed ROX (Invitogen), 6.0 μl molecular grade water, 0.5 μl cDNA template, and 1 μl forward and reverse primer mix. The following primers were used for qPCR:
Umbrea forward: CTTCCGTTTGGTTTTAGATATCGT
Umbrea reverse: AAACACTTGACAAAACGTGACAAT
Actin5C forward: GATCTGTATGCCAACACCGT
Actin5C reverse: ATGGCCGAATTCTCAGTGGA

Each cDNA/primer set combination was run in triplicate. Cycle threshold (CT) values for Actin5C and Umbrea in the scrambled RNAi sample and Umbrea RNAi sample were used to calculate the ΔΔCT values for Umbrea expression.

Purification and Mass Spectrometry
Ammonium sulfate nuclear extracts were prepared from large scale cultures of S2 cells expressing Flag-tagged proteins. Nuclei were prepared by Dounce homogenization of cells swollen in hypotonic buffer (15 mM KCl, 10 mM HEPES pH7.6, 2 mM $MgCl_2$, 0.1 mM EDTA). Nuclei were pelleted, resuspended in Buffer B (114 mM KCl, 14 mM HEPES pH7.6, 4.8 mM $MgCl_2$, 0.1 mM EDTA), following which 0.1 volumes of 4M ammonium sulfate was added and chromatin bound proteins were extracted for 30 minutes. The solubilized proteins were recovered after centrifugation and precipitated under non-denaturing conditions by the addition of 0.3 g/mL fine ammonium sulfate powder. The resulting pellet was dialysed against Buffer C (150 mM KCl, 25 mM HEPES pH7.6, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 10% Glycerol), cleared of non-solubilized material by centrifugation, and used for affinity purification. All buffers were supplemented with DTT and protease inhibitors.

For co-immunoprecipitations (CoIP) studies, small scale extracts were prepared as for the ammonium sulfate extracts, except that nuclei were prepared in hypotonic buffer by the addition of Triton-X100 to a final concentration of 0.02% and directly dialysed against Buffer C after the 30 minute ammonium sulfate extraction step. Affinity purification of

interaction partners was performed using anti-FLAG M2 Sepharose (Roche): 60 µl of 50% slurry beads and 5 mg of nuclear extract were incubated for 2 hours at 4°C, washed three times 10 minutes in buffer C and eluted with 0.5 mg/mL FLAG peptide dissolved in Buffer C.

50% of the eluate was boiled with Laemmli buffer and separated on a 15% PAA gel until the running front migrated three cm into the separation gel. Proteins were stained with Coomassie Brilliant blue and the whole lane was excised from the gel with a disposable gridcutter (Gel Company) and split into 8 vials. After in-gel tryptic digestion, 50% of the samples were injected in an Ultimate 3000 HPLC system (LC Packings Dionex). Samples were desalted on-line by a C18 micro column (300 mm i.d. 5 mm, packed with C18 PepMapTM, 5 mm, 100A˚ by LC Packings), and peptides were separated with a gradient from 5% to 60% acetonitrile in 0.1% formic acid over 40 min at 300 nL/min on a C18 analytical column (75 mm i.d. 10 cm, packed with C18 PepMapTM, 3 mm, 100A˚ by LC Packings). The effluent from the HPLC was directly electrosprayed into the LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific). The MS instrument was operated in the data-dependent mode to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra (*m/z* 580-830) were acquired in the Orbitrap with resolution 60 000 at *m/z* 400. The six most intense peptide ions with charge states between two and five were sequentially isolated (window = 350-2000 *m/z*) to a target value of 10000 and fragmented in the linear ion trap by collision-induced dissociation (CID). Fragment ion spectra were recorded in the linear trap of the instrument. For all measurements with the Orbitrap detector, three lock-mass ions from ambient air (m/z=371.10123, 445.12002, 519.13882) were used for internal calibration as described. Typical mass spectrometric conditions were: spray voltage, 1.4 kV; no sheath and auxiliary gas flow; heated capillary temperature, 200°C; normalized collision energy, 35% for CID in linear ion trap. The ion selection threshold was 10 000 counts for MS2. An activation q=0.25 and activation time of 30 ms were used.

Proteins were identified and quantified using the Andromeda algorithm of the MaxQuant 1.2.2.5 software package (9). Identified proteins were considered as interaction partners if their MaxQuant intensities displayed a greater than 8-fold enrichment compared to control anti-FLAG purifications from L2-4 nuclear extracts not expressing any FLAG-tagged protein. Proteins are ranked according to their fold enrichment in the Umbrea immunoprecipitation relative to a mock purification, with the top 50 listed. To estimate protein levels in the Umbrea pulldown, we used the intensity based absolute quantification values (iBAQ)(10), which corresponds to the sum of peak intensities of all peptides matching to a specific protein divided by the number of theoretically observable peptides. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD000163(*38*).

Supplemental Figure Legends

Fig. S1. Umbrea is an essential gene and Umbrea-GFP is functional *in vivo.* (**A**) dsRNA homologous to *Umbrea* causes penetrant lethality in *D. melanogaster,* partially rescued by coexpression of RNAi-resistant Umbrea-GFP (recoded at synonymous sites)**.** Overexpression of Umbrea-GFP does not induce lethality. Rescue was calculated by comparing observed progeny from a single cross to predicted Mendelian ratios based on total progeny counts. **(B)** Schematic of the *Umbrea* locus. P-element insertion (red triangle) and Umbrea^{35} and Umbrea^{366} imprecise deletion mutations are indicated. Umbrea^{35} encodes a protein lacking a nuclear localization signal (NLS) and fails to enter the nucleus when expressed in Kc cells (data not shown) whereas Umbrea^{366} represents an almost complete deletion of the Umbrea locus. **(C)** Genetic complementation analysis. We investigated the effects of P-element insertion into *Umbrea, P(GT1)HP6$^{[BG01429]}$*, which causes early embryonic lethality. We found that this P-element insertion affected both *Umbrea* and *dumpy* (*dp*) expression, since it was not able to complement *dp* mutations. Imprecise excision of this P-element yielded two alleles that only affected *Umbrea* (fully complemented *dp* mutations) and also resulted in late larval-pupal lethality, consistent with the highly penetrant late-stage lethality seen in the RNAi knockdown experiments (Fig. S1A). Parental genotypes in each cross are shown, along with total progeny counts for control and trans-heterozygote F1s. In each case, the balancer chromosome was Curly of Oster (CyO).

Fig. S2. HP1B^{ana} localizes to heterochromatin and euchromatin. HP1B^{ana} fused to GFP (green), expressed in *D. melanogaster* Kc cells. Centromeres are stained with anti-Cid (magenta), with DAPI (blue), bar = 5 microns.

Fig. S3. Umbrea^{mel}-GFP does not colocalize with telomere markers. Umbrea^{mel}-GFP (green) expressed in *D. melanogaster* Kc cells along with either anti-HOAP (magenta, **A**), which stains telomeres and some centromeres (arrow), and anti-HipHop (magenta, **B**), which only localizes to telomeres (bar = 5 microns).

Fig. S4. Umbrea-specific antibody staining reveals endogenous Umbrea localizes to centromeres *in vivo*. **(A)** Western blot of Kc and S2 control cells or cells expressing GFP-Umbrea shows that anti-Umbrea serum (green, 1:5000 dilution) is specific for Umbrea. Beta-tubulin loading control is shown in red, as is JL8 anti-GFP. **(B)** Anti-Umbrea serum (green) from immunized rabbits reveals centromere co-localization with anti-Cid (magenta), bar = 5 microns. **(C)** Umbrea (green) localization to centromeres (Cid, magenta) in cells from a larval haltere imaginal disc (DAPI in blue).

Fig. S5. (A) Effective RNAi knockdown of *Umbrea* transcript in S2 cells. *Umbrea*-specific qRT-PCR results are presented from control dsRNA cells and cells treated with dsRNA homologous to *Umbrea.* **(B)** Time-lapse live imaging of Umbrea-depleted cells expressing mCherry-tubulin (in magenta) and H2B-GFP (in green) revealed specific mitotic defects.

Fig. S6. (A) Amplification of *Umbrea* locus (within *dumpy* first intron) from representative species of *Drosophila*, using primers recognizing conserved sequence in the shared syntenic sites of the *dumpy* first intron . **(B)** Bands were subsequently excised and DNA sequenced to determine the absence of *Umbrea,* or presence of either full-length or CSD-only (+) genes.

Fig. S7. Molecular evolution of *Umbrea* and *HP1B* across 15 million years of *Drosophila* evolution. (**A**) *Umbrea* sequences were used to calculate dN/dS values for each branch, calculated using the free-ratio model in PAML. Branches with dN/dS ratios greater than 1 are in bold, with the number of nonsynonymous to synonymous changes shown in parentheses. (**B**) Codon-specific tests for positive selection. Model M7, a null model where dN/dS>1 is not permitted, is compared versus M8, which allows sites to have dN/dS>1 in likelihood ratio tests. A significantly better fit to M8 versus M7 (p-values indicated) suggests that Umbrea has recurrently evolved under positive selection. (**C**) Codons in *Umbrea* identified as evolving under recurrent positive selection by PAML (Naïve Empirical Bayes test), or FUBAR (www.datamonkey.com), with posterior probabilities greater than 0.9 in either test are presented. 2 of the 4 codons are identified with high probability in both tests. (**D**) Gapped amino acid alignment of all Umbrea proteins. Chromodomains in four proteins and chromoshadow domains in all proteins are highlighted in yellow and blue respectively. We could not reliably align the tail regions outside the dotted lines, and these were excluded from all molecular evolution analyses to detect selection signatures, even though these regions harbor the highest divergence of all domains (and we would therefore underestimate the degree of positive selection). Residues identified as having evolved under recurrent positive selection (**C**) are indicated by thick bars.

Fig. S8. Amino acid alignment of chromodomains from HP1A^{mel}, HP1Bs, and "full-length" chromodomain-containing Umbreas. Positions critical for histone H3K9me-binding are highlighted in red.

Fig. S9: (A) PxVxL-recognition residues altered along the branch leading to the *melanogaster* subgroup highlighted (yellow) on a crystal structure of a CSD dimer from mouse HP1beta (green and teal) bound to CAF-1 peptide (black) (ProteinDataBank 1S4Z).

Fig. S10. (**A**) Umbrea^{mel}-GFP (green) colocalizes with Cid (magenta) in *D. melanogaster* Kc cells, unlike HP1B^{mel}-CSD-GFP (**B**), which shows no discrete localization (bar = 5 microns). (**C)** Umbrea^{ptak}-GFP (green) localizes to heterochromatin surrounding centromeres.

Table S1. Identification of Umbrea-interacting proteins. Proteins were identified and quantified using the Andromeda algorithm of the MaxQuant software package (*30*). Proteins are ranked according to their fold enrichment in the Umbrea immunoprecipitation relative to a mock purification, with the top 50 listed. To estimate protein levels in the Umbrea pulldown we used the intensity based absolute quantification values (iBAQ, see Methods), which corresponds to the sum of peak intensities of all peptides matching to a specific protein divided by the number of theoretically observable peptides. Missing values in the background purification were set to an arbitrary value of 1 to facilitate the calculation of enrichment values. Previously known heterochromatin or centromeric proteins are highlighted in bold.

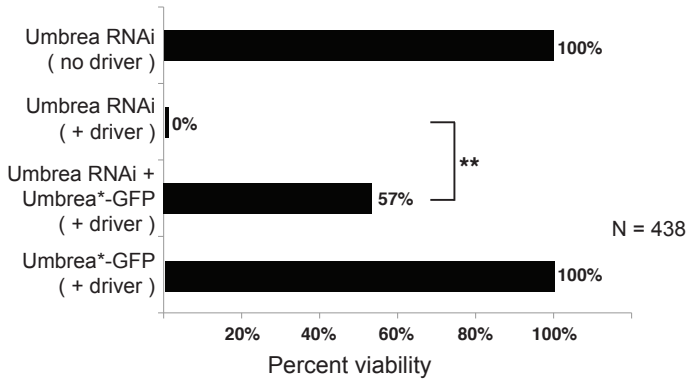
Movie S1. Time-lapse live imaging of Umbrea-depleted treated with dsRNA against Umbrea expressing mCherry-tubulin (in red) and H2B-GFP (in green) followed through mitosis.

Movie S2. A second example of time-lapse live imaging of Umbrea-depleted cells treated with dsRNA against Umbrea expressing mCherry-tubulin (in red) and H2B-GFP (in green) followed through mitosis.

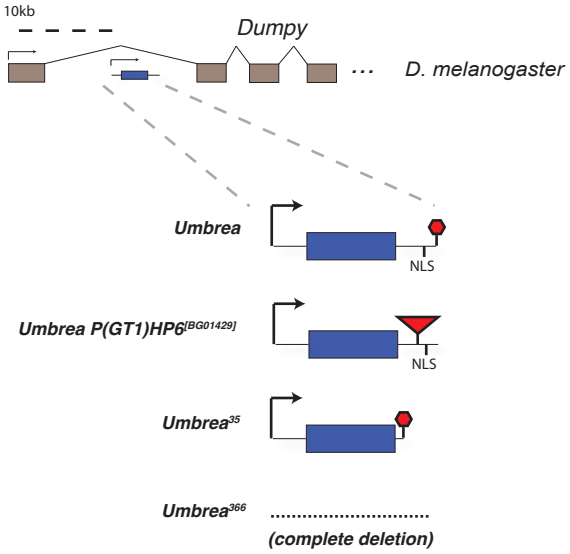
Movie S3. Time-lapse live imaging of control cells treated with scrambled dsRNA expressing mCherry-tubulin (in red) and H2B-GFP (in green) followed through mitosis.

Figure S1

A



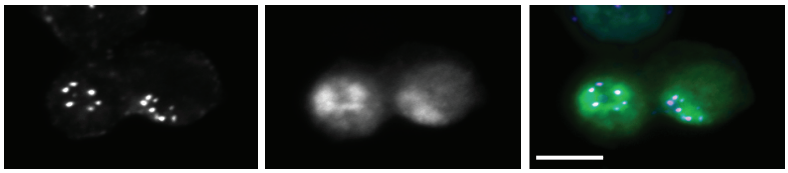
B



C

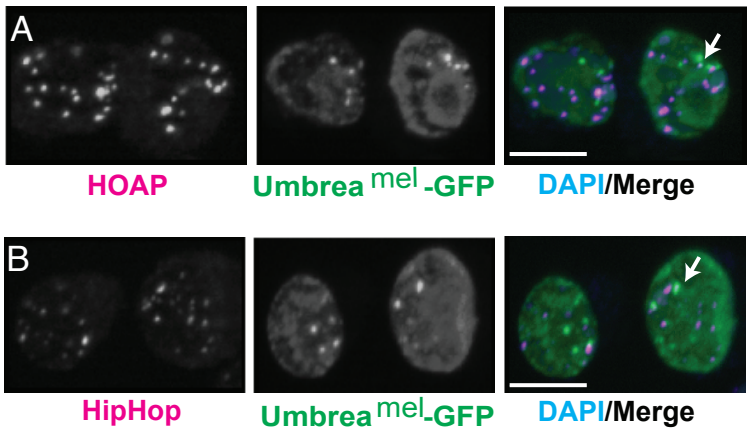
Complementation cross			F1 progeny Control	Trans-het.	N =	Complements?
P{GT1}HP6[BG01429] / Bal.	X	*Umbrea35 / Bal.*	83	89	172	Yes
P{GT1}HP6[BG01429] / Bal.	X	*Umbrea366 / Bal.*	80	94	174	Yes
P{GT1}HP6[BG01429] / Bal.	X	*dumpylvl / Bal.*	226	0	226	No
P{GT1}HP6[BG01429] / Bal.	X	*dumpyolvr / Bal.*	61	0	61	No
Umbrea35 / Bal.	X	*dumpylvl / Bal.*	167	157	324	Yes
Umbrea366 / Bal.	X	*dumpylvl / Bal.*	105	131	236	Yes
Umbrea35 / Bal.	X	*dumpyolvr / Bal.*	290	327	617	Yes
Umbrea366 / Bal.	X	*dumpyolvr / Bal.*	94	82	176	Yes
Umbrea35 / Bal.	X	*Umbrea366 / Bal.*	234	2	236	No

Figure S2



Cid HP1B^{ana}GFP DAPI/Merge

Figure S3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HOAP
Umbrea mel -GFP
DAPI/Merge
B
HipHop
Umbrea mel -GFP
DAPI/Merge

Figure S4

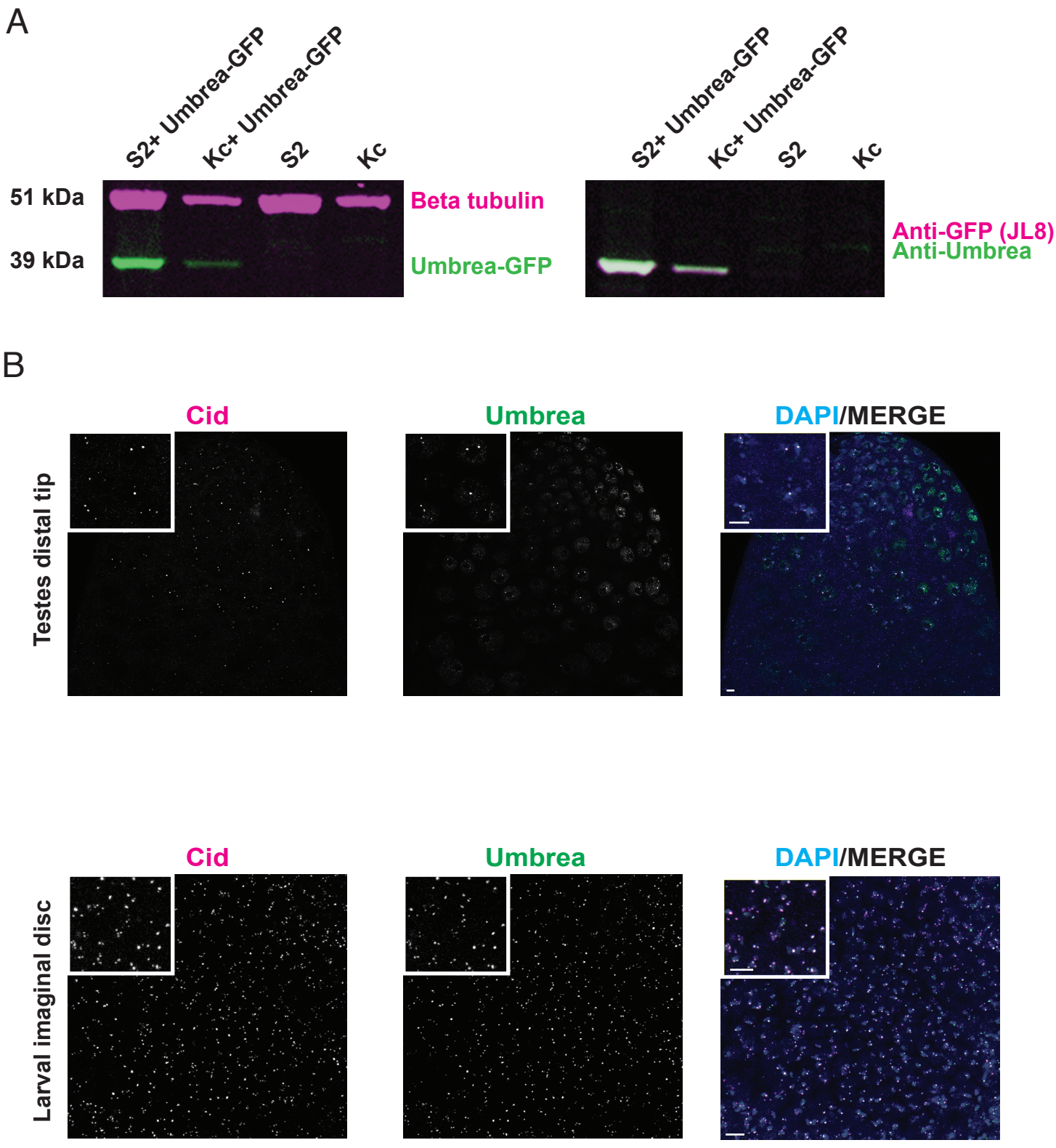

A
S2+ Umbrea-GFP
Kc+ Umbrea-GFP
S2
Kc
51 kDa
39 kDa
Beta tubulin
Umbrea-GFP
S2+ Umbrea-GFP
Kc+ Umbrea-GFP
S2
Kc
Anti-GFP (JL8)
Anti-Umbrea
B
Cid
Umbrea
DAPI/MERGE
Testes distal tip
C
Cid
Umbrea
DAPI/MERGE
Larval imaginal disc

Figure S5

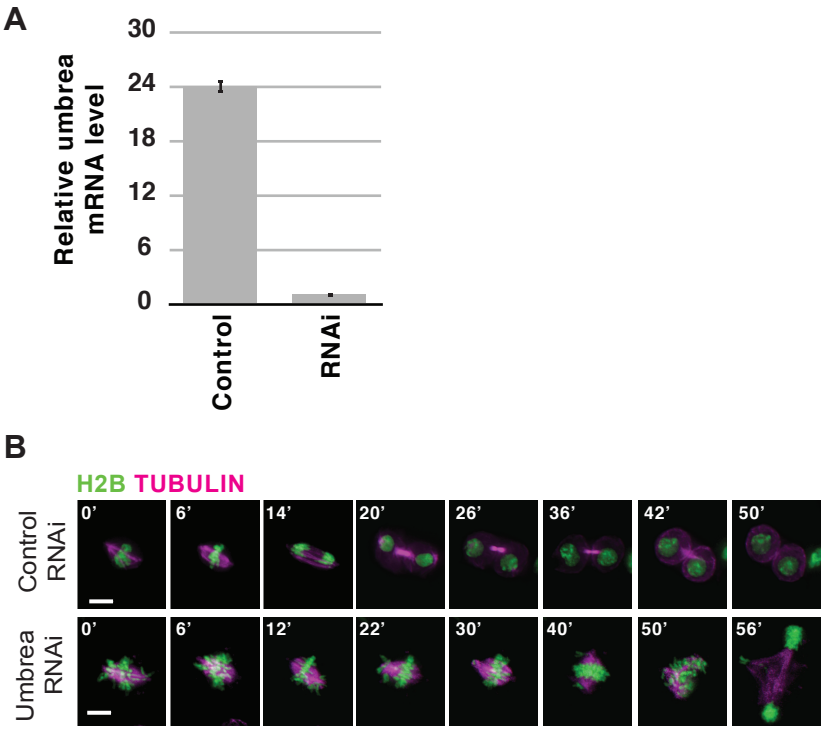

A
Relative umbrea mRNA level
30
24
18
12
6
0
Control
RNAi
B
H2B TUBULIN
Control RNAi
0'
6'
14'
20'
26'
36'
42'
50'
Umbrea RNAi
0'
6'
12'
22'
30'
40'
50'
56'

Figure S6

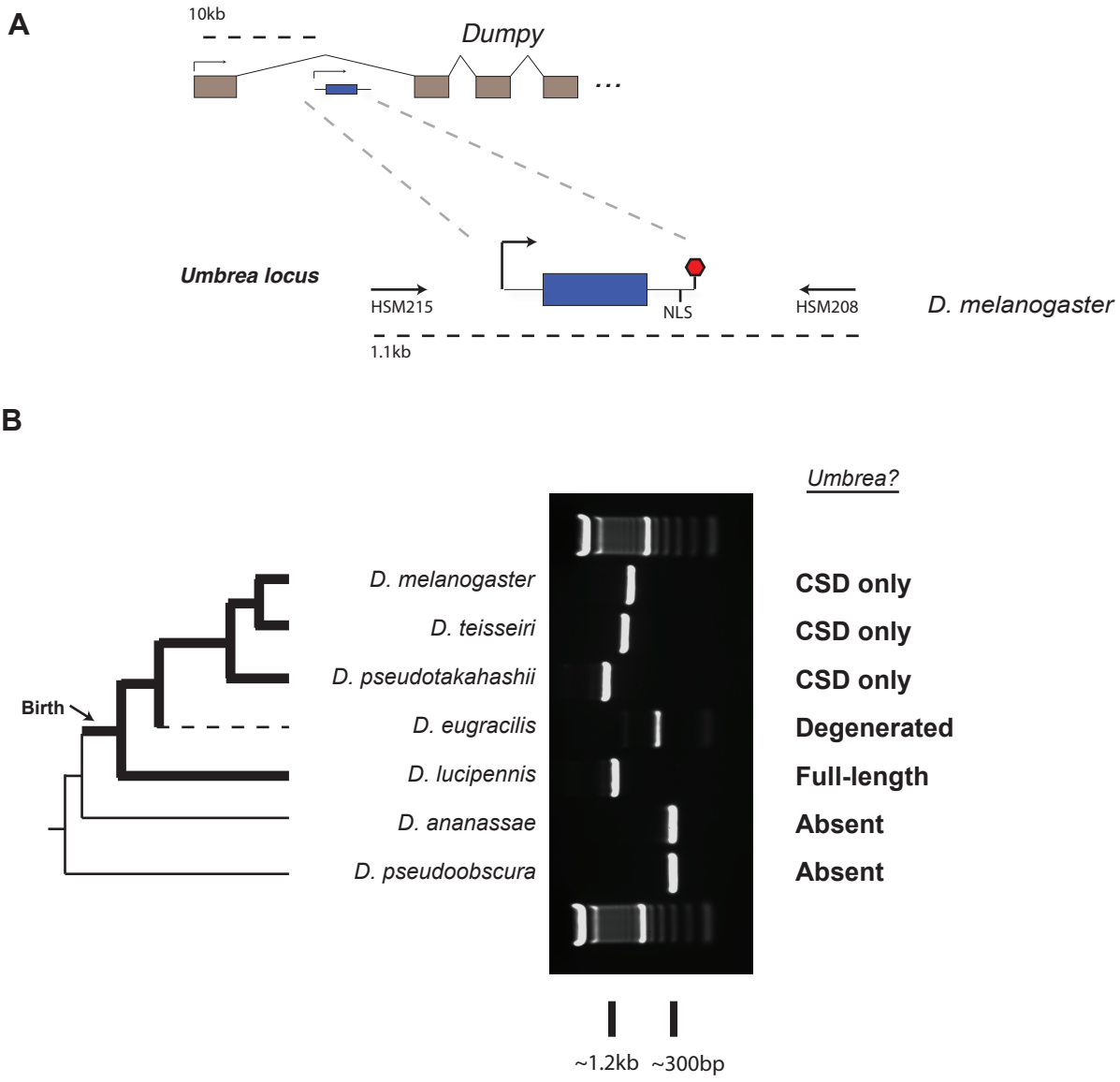
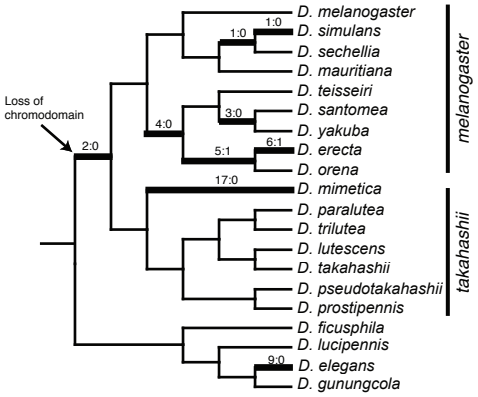

A
10kb
Dumpy
...
Umbrea locus
HSM215
NLS
HSM208
D. melanogaster
1.1kb
B
Umbrea?
Birth
D. melanogaster
D. teisseiri
D. pseudotakahashii
D. eugracilis
D. lucipennis
D. ananassae
D. pseudoobscura
CSD only
CSD only
CSD only
Degenerated
Full-length
Absent
Absent
~1.2kb
~300bp

Figure S7

A



B

Comparison of NsSites models M7 vs M8

Codon Frequency Model	Degrees of Freedom	2 lnL λ	**p-value**	% sites with dN/dS >1 (Average dN/dS)
F3X4	2	10.3	**0.006**	14(1.7)
F61	2	9.5	**0.008**	13 (1.7)

C

Posterior probability that dN/dS > 1

Umbrea Residue	Naive Empirical Bayes (PAML)	FUBAR (HyPhy)
R12	**0.99**	**0.99**
N13	**0.92**	0.79
V56	**0.92**	**0.94**
E75	**0.96**	0.77

D

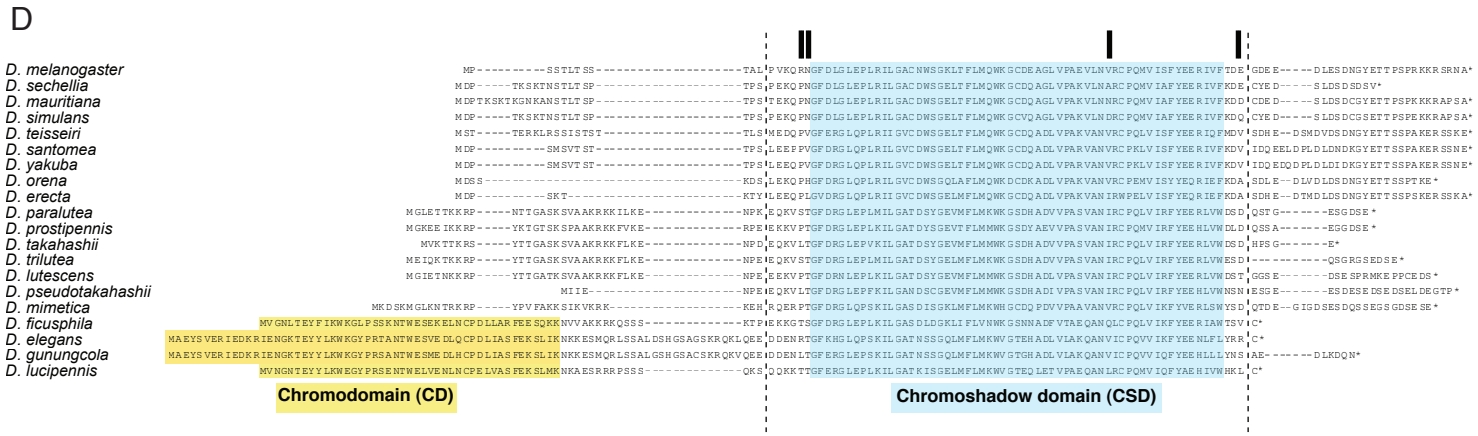


Figure S8

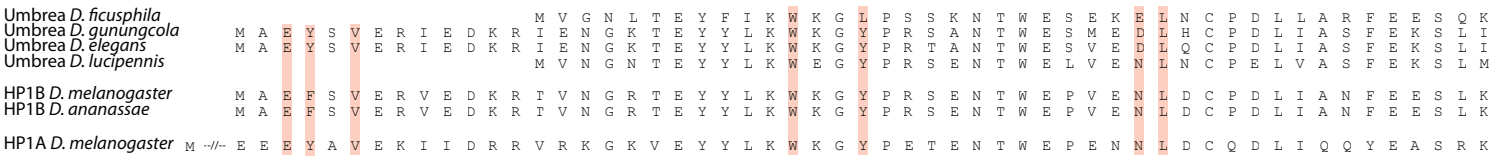

Umbrea D. ficusphila
Umbrea D. gunungcola
Umbrea D. elegans
Umbrea D. lucipennis
HP1B D. melanogaster
HP1B D. ananassae
HP1A D. melanogaster

Figure S9

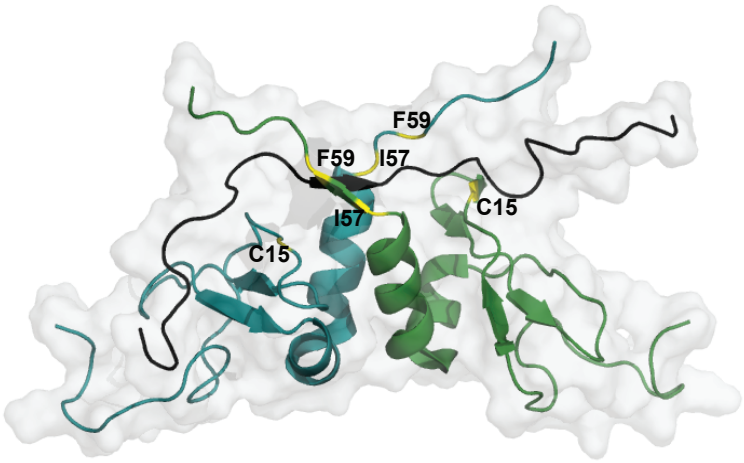


Figure S10

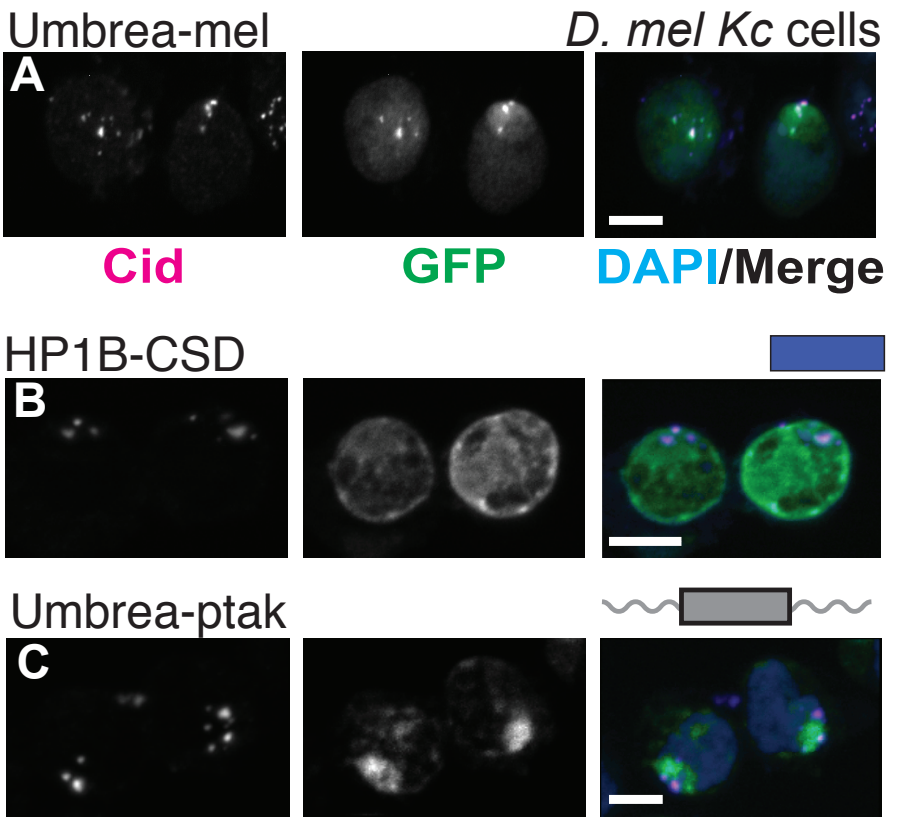

Umbrea-mel
D. mel Kc cells
A
Cid
GFP
DAPI/Merge
HP1B-CSD
B
Umbrea-ptak
C

Table S1

Name	FBgn	Peptides	iBAQ Umbrea	iBAQ Ctrl	Enrichment
Umbrea	**FBgn003161**	**7**	**2.10E+08**	**0.00E+00**	**2.10E+08**
CG3680	FBgn0037027	52	4.96E+07	0.00E+00	4.96E+07
Nlp	FBgn0016685	6	2.36E+07	0.00E+00	2.36E+07
bel	FBgn0000171	47	2.26E+07	0.00E+00	2.26E+07
HP4	**FBgn003582**	**10**	**1.42E+07**	**0.00E+00**	**1.42E+07**
glo	FBgn0259139	29	9.81E+06	0.00E+00	9.81E+06
Hmr	**FBgn000120**	**92**	**9.36E+06**	**0.00E+00**	**9.36E+06**
Lhr	**FBgn003421**	**24**	**9.08E+06**	**0.00E+00**	**9.08E+06**
HP5	**FBgn003030**	**49**	**8.51E+06**	**0.00E+00**	**8.51E+06**
Nipped-B	FBgn0026401	56	8.36E+06	0.00E+00	8.36E+06
CG1647	FBgn0039602	30	8.21E+06	0.00E+00	8.21E+06
CG4788	FBgn0032354	9	5.82E+06	0.00E+00	5.82E+06
Pen	FBgn0011823	17	5.36E+06	0.00E+00	5.36E+06
Cenp-C	**FBgn008669**	**41**	**4.96E+06**	**0.00E+00**	**4.96E+06**
mu2	FBgn0002872	34	3.63E+06	0.00E+00	3.63E+06
Su(var)2-HP2	**FBgn002642**	**72**	**2.90E+06**	**0.00E+00**	**2.90E+06**
nonA	FBgn0004227	22	2.87E+06	0.00E+00	2.87E+06
CG14438	FBgn0029899	54	2.18E+06	0.00E+00	2.18E+06
MRG15	FBgn0027378	11	1.95E+06	0.00E+00	1.95E+06
CG7236	FBgn0031730	2	1.95E+06	0.00E+00	1.95E+06
XNP	FBgn0039338	84	1.78E+06	0.00E+00	1.78E+06
tral	FBgn0041775	16	1.65E+06	0.00E+00	1.65E+06
Rpd3	FBgn0015805	17	1.49E+06	0.00E+00	1.49E+06
ash1	FBgn0005386	33	1.49E+06	0.00E+00	1.49E+06
CG8290	FBgn0026573	80	1.38E+06	0.00E+00	1.38E+06
CG7239	FBgn0031740	11	1.34E+06	0.00E+00	1.34E+06
porin	FBgn0004363	17	1.33E+06	0.00E+00	1.33E+06
His3.3B	FBgn0004828	10	1.28E+06	0.00E+00	1.28E+06
mof	FBgn0014340	10	9.77E+05	0.00E+00	9.77E+05
CoRest	FBgn0261573	9	9.71E+05	0.00E+00	9.71E+05
Su(var)3-3	**FBgn026039**	**14**	**9.37E+05**	**0.00E+00**	**9.37E+05**
CG33107	FBgn0053107	8	8.15E+05	0.00E+00	8.15E+05
Su(var)3-9	**FBgn000360**	**13**	**7.28E+05**	**0.00E+00**	**7.28E+05**
SuUR	**FBgn002535**	**10**	**5.74E+05**	**0.00E+00**	**5.74E+05**
RpL26	FBgn0036825	11	5.67E+05	0.00E+00	5.67E+05
E(var)3-9	FBgn0260243	10	5.63E+05	0.00E+00	5.63E+05
msl-3	FBgn0002775	7	5.62E+05	0.00E+00	5.62E+05
torp4a	FBgn0025615	8	5.52E+05	0.00E+00	5.52E+05
rin	FBgn0015778	13	5.50E+05	0.00E+00	5.50E+05
CG16972	FBgn0032481	96	5.06E+05	0.00E+00	5.06E+05
AnnIX	FBgn0000083	27	4.14E+05	0.00E+00	4.14E+05
betaTub85D	FBgn0003889	20	4.07E+05	0.00E+00	4.07E+05
CG6523	FBgn0032509	2	3.92E+05	0.00E+00	3.92E+05
CG5664	FBgn0037082	8	3.57E+05	0.00E+00	3.57E+05
msl-2	FBgn0005616	5	3.42E+05	0.00E+00	3.42E+05
CG4203	FBgn0038300	10	3.03E+05	0.00E+00	3.03E+05
CG33213	FBgn0053213	6	3.00E+05	0.00E+00	3.00E+05
CG7172	FBgn0037102	1	2.80E+05	0.00E+00	2.80E+05
CG14005	FBgn0031739	10	2.76E+05	0.00E+00	2.76E+05