Figures and figure supplements

The composition and organization of *Drosophila* heterochromatin are heterogeneous and dynamic

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Figure 1. Workflow to identify novel heterochromatin components and regulators. We devised an unbiased strategy to identify novel components of heterochromatin. First, we identified candidates by performing HP1a immunoprecipitation followed by mass spectrometry (IP-MS) and a genome-wide RNAi screen. Candidates that localized to heterochromatin were assayed for effects on PEV. Finally, we investigated their spatial and temporal localization with respect to heterochromatin.

DOI: 10.7554/eLife.16096.003
Figure 2. A genome-wide image-based RNAi screen identifies HP1a regulators. Drosophila Kc cells transfected with dsRNA were analyzed for HP1a localization by IF, and DNA was counterstained with DAPI. Cells were visualized using high-throughput fluorescent microscopy and imaging features were extracted using custom Matlab scripts. Wells were normalized and checked for replicate consistency using the Rank Product test and a p-value was calculated. Putative candidates involved in HP1a recruitment/maintenance were selected by identifying amplicons that lowered HP1a intensity, or clustered with HP1a depletions after hierarchical clustering or Support Vector Machine (SVM) analysis. (B) Genes that clustered using unsupervised hierarchical clustering with either HP1a or Su(var)3–9 positive control depletions are represented by the yellow circle. Supervised machine learning models (SVMs) were trained to identify genes that disrupt HP1α staining (blue circle) using HP1a depletion controls. HP1α intensity measures (mean, maximum, relative maximum and kurtosis) were used to identify another set of candidate genes (red circle). Genes identified by multiple methods are indicated by color below the Venn diagram. See Figure 2—source data 1 for a list of all genes identified in the RNAi screen and the method used to identify them.

DOI: 10.7554/eLife.16096.008
The following source data is available for figure 2:

Source data 1. 374 genes putatively regulate heterochromatin.
DOI: 10.7554/eLife.16096.009
Figure 2—figure supplement 1. The rank product test is more effective than the robust Z-Score at identifying HP1a knockdowns. HP1a mean intensity was normalized using the robust z-score (A) or the rank product test (B). The normalized value (or p-value) is plotted versus a ranked list of the amplicons, with a value of one indicating the strongest hit. HP1a RNAi (positive controls) are noted in red and the percentage for the highest ranked positive control is indicated with an arrow.

DOI: 10.7554/eLife.16096.010
Figure 3. Identification of candidates that co-localize with HP1a. Proteins were selected from the HP1a IP-MS (red circles) or the RNAi screen (blue circles), tagged with GFP (green), and analyzed for localization with respect to mCherry-tagged HP1a (red). GFP-tagged HP1a was used as a positive control (gray circles). The Pearson correlation coefficient (PCC) between mCherry-HP1a and GFP-tagged proteins left of the dashed line was significantly higher than the PCC between mCherry-HP1a and GFP-mod (green triangle), using the two-sided unpaired Mann-Whitney test (p-value < 0.05). Numbers on graph correspond to representative images (right panel). Scale bar is 5 μm. See Figure 3—source data 1 for the PCC of all proteins tested.

DOI: 10.7554/eLife.16096.012

The following source data is available for figure 3:

Source data 1. Identification of candidates that co-localize with HP1a.

DOI: 10.7554/eLife.16096.013
Figure 4. HPips and RNAi screen candidates are suppressors of variegation. (A) Color Inspector 3D in ImageJ was used to determine the RGB values of ‘red’ pixels (indicating loss of suppression). The percent of the eye composed of red pixels was then calculated. (B) Fly mutants and RNAi lines were tested for impact on white variegation in y, w, KV108 males, and are organized by p-value. Mutations were tested for dominant effects if they were recessive lethal, otherwise homozygotes were analyzed. CG7357[f00521] was scored for variegation using the yellow reporter gene, since the line harbors a mini-white reporter that precludes assessment of white variegation. The p-values were calculated using a 2-tailed, 2-sample unequal variance t-test for white variegation and a 2-sample Kolmogorov-Smirnov test for yellow variegation. Positive and negative controls were performed and are listed in the Figure 4—source data 1 along with the genotypes of all the fly lines used. CG2129, Ssrp and Ref1 could not be tested for effects on variegation using RNAi lines, due to lethality.

DOI: 10.7554/eLife.16096.014

The following source data is available for figure 4:

Source data 1. HPips and RNAi screen candidates are suppressors of variegation. DOI: 10.7554/eLife.16096.015

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a Indicates protein was not tested or did not colocalize with HP1a in the low-resolution colocalization screen.
b OE of gene lead to modification of variegation in Schneiderman, J. I. et al., 2010.
c Only found enriched in one HP1a IP-MS

****P < 0.0001; ***P < 0.001; **P < 0.005; *P < 0.05; N.S. = Not Significant
Figure 5. Heterochromatic proteins display diverse localization patterns. HP4 and HP5 broadly overlap with HP1a. SuUR and FK506-bp1 overlap with the interior of HP1a (narrow). crol and l(3)neo38 form a focus within the HP1a domain (focal). Focal proteins are presented as slices, broad and narrow proteins are projections. mCherry-tagged HP1a is in red, GFP-tagged ORF is in green. Scale bar is 5 μm.

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Figure 5—figure supplement 1. Heterochromatic proteins display diverse localization patterns. mCherry-tagged HP1a is in red, GFP-tagged ORF is in green. Localization patterns are grouped into 7 categories: ‘Broad’ - almost complete overlap with HP1a; ‘Narrow’ - only partial overlap with HP1a; ‘Focal’ - forms a foci or focus that overlap with HP1a; ‘Pan-nuclear’ - everywhere in the nucleus; ‘At HC boundary’ - enriched at the periphery of heterochromatin; ‘Foci outside of HC’ - forms foci outside of heterochromatin; and ‘Nucleolus’ - localizes to the nucleolus. Proteins labelled with + indicates that in a population of S2 cells they display patterns that fit in more than one category. * indicates that the localization pattern is dependent on cell type or location (N- or C-term) of the tag. Proteins labelled with ^ indicate a slice, otherwise images are projections. Prod was tagged with mCherry and image was false-colored; red indicates GFP-tagged HP1a and green indicates mCherry-tagged prod. & indicates experiment done in Kc cells. Hr83: narrow when N-terminal tagged in Kc cells (stable), pan-nuclear when N-term tagged in S2 cells (transient), nucleolar when C-term tagged in S2 cells (transient); CG2129: N-terminally GFP tagged construct (transient Kc and S2) shows nucleolar localization in lowly expressing cells and foci appear in highly expressing cells; blanks when N-term tagged in stable Kc cells broadly co-localizes with HP1a, N-term tagged in transient S2 has foci next to the HP1a domain, C-term tagged in transient S2 or Kc cells is pan-nuclear, antibody staining is also pan-nuclear with some structure throughout the nucleus. Su(var)3–9 is tagged with mClover. BioTAP tagged ADD1-RA (red) was visualized using a Peroxidase antibody followed by immunofluorescence.

DOI: 10.7554/eLife.16096.018
Figure 6. HP2 time-lapse imaging reveals dynamic regulation and overlap with PCNA throughout S-phase. HP2 partially overlaps and is enriched at the boundary of HP1a in G2, released from chromatin during mitosis and broadly colocalized with HP1a during G1. Mitosis is used to discriminate G1 from G2. Dotted lines indicate the cell periphery during mitosis. mCherry-tagged HP1a is in red, GFP-tagged HP2 is in green. Scale bar is 10 μm. (B) HP2 overlaps with PCNA foci in early, mid and late S-phase. Representative images of early, mid and late S-phase are shown. mCherry-tagged PCNA is in red, GFP-tagged HP2 is in green. Scale bar is 5 μm. DOI: 10.7554/eLife.16096.019
Figure 7. Time-lapse imaging reveals a variety of dynamic localization patterns within heterochromatin. A graphical representation of the localization patterns of heterochromatin proteins throughout the cell cycle is shown. HP1a is depicted in red, the heterochromatin protein (HPip) in green and overlap between the two in yellow. A dotted circle indicates that FK506-bp1 forms a ring around the nucleolus. * indicates foci overlap completely with PCNA during S-phase.

DOI: 10.7554/eLife.16096.027
Figure 7—figure supplement 1. Combined SuUR, HP1a and PCNA time-lapse imaging reveals dynamic regulation. SuUR colocalizes with HP1a during G2 and G1, and colocalizes with PCNA during S-phase. Dotted lines indicate the cell periphery during mitosis. Mitosis is used to discriminate G1 from G2, while PCNA foci indicates S-phase. Cerulean tagged HP1a is in blue, YFP tagged SuUR is in green, mCherry tagged PCNA is in red. Scale bar is 10 μm.

DOI: 10.7554/eLife.16096.028
Figure 7—figure supplement 2. ADD1-PB time-lapse imaging reveals dynamic regulation. ADD1-PB forms focal subdomains that abut and overlap HP1a, and does not overlap with the centromeric or telomeric markers CID and HOAP (data not shown), respectively. In G2 ADD1-PB is predominantly focal at the heterochromatin boundary. A small amount of discrete signal remains on chromatin during mitosis and persists at low levels into G1, before eventually increasing in intensity, which suggests loading at the end of G1 or during S-phase. Mitosis is used to discriminate G1 from G2. Dotted lines indicate the cell periphery during mitosis as it divides into two daughter cells (G1). mCherry tagged HP1a is in red, GFP tagged ADD1-PB is in green. Scale bar is 10 μm.

DOI: 10.7554/eLife.16096.029
Figure 7—figure supplement 3. Oddjob time-lapse imaging reveals dynamic regulation. Odj broadly co-localizes with HP1a at the end of G2 and disperses from chromosomes during mitosis. It reforms as a focal subdomain after mitosis that gradually increases in size, until it broadly overlaps HP1a again. Mitosis is used to discriminate G1 from G2. Dotted lines indicate the cell periphery during mitosis as it divides into two daughter cells (G1). mCherry tagged HP1a is in red, GFP tagged Odj is in green. Scale bar is 10 μm.
DOI: 10.7554/eLife.16096.030
Figure 7—figure supplement 4. FK506-bp1 time-lapse imaging reveals dynamic regulation. FK506-bp1 narrowly co-localizes with HP1a throughout much of the cell cycle and loses co-localization with HP1a 20 min to 1 hr before HP1a is released from chromosomes (prophase). After mitosis, the narrow co-localization pattern of FK506-bp1 is restored, with a weak ring around the nucleolus, which is located adjacent to the HP1a domain. FK506-bp1 foci then begin to accumulate outside of heterochromatin until just before prophase, when they disappear prior to HP1a removal. Foci do not track with PCNA (replication), CID (centromeres) or HOAP (telomeres) foci (data not shown). Mitosis is used to discriminate G1 from G2. Dotted lines indicate the cell periphery during mitosis as it divides into two daughter cells (G1). mCherry tagged HP1a is in red, GFP tagged FK506-bp1 is in green. Scale bar is 10 μm.

DOI: 10.7554/eLife.16096.031
Figure 7—figure supplement 5. Lhr time-lapse imaging reveals dynamic regulation. Lhr broadly co-localizes with HP1a and is released from chromatin during mitosis. Mitosis is used to discriminate G1 from G2. Dotted lines indicate the cell periphery during mitosis as it divides into two daughter cells (G1). mCherry tagged HP1a is in red, GFP tagged Lhr is in green. Scale bar is 10 μm. (B) Lhr partially overlaps centromeres. mCherry tagged CID is in red, GFP tagged Lhr is in green. Scale bar is 5 μm. (C) Some Lhr and XNP foci overlap, but others do not (arrows). mCherry tagged Lhr is in red, GFP tagged XNP is in green. Scale bar is 5 μm.
DOI: 10.7554/eLife.16096.032
**Figure 7—figure supplement 6.** XNP time-lapse imaging reveals dynamic regulation. XNP colocalizes with a portion of HP1a in G2. The majority of XNP is removed during mitosis, however 1–2 foci remain chromatin-bound. In G1 XNP is focal within the HP1a domain while gradually accumulating and colocalizing with more HP1a. Mitosis is used to discriminate G1 from G2. Dotted lines indicate the cell periphery during mitosis as it divides into two daughter cells (G1). mCherry-tagged HP1a is in red, GFP-tagged XNP is in green. Scale bar is 10 μm. (B) XNP and CID partially overlap. mCherry-tagged CID is in red, GFP-tagged XNP is in green. Scale bar is 5 μm.

DOI: 10.7554/eLife.16096.033
Figure 8. Models for subdomain formation within heterochromatin. We propose three non-mutually exclusive models for subdomain formation of HP1α interacting proteins (HPips) within the HP1α (teal) heterochromatin holodomain. (A) The HPip (red) may be recruited to a specific sequence and seeds the formation of a subdomain (as observed for D1 [Aulner et al., 2002] and GAGA [Raff et al., 1994] factor). (B) HP1α and its orthologs are extensively post-translationally modified by SUMOylation, acetylation, methylation, formylation, ubiquitination and poly(ADP-ribosyl)ation (Alekseyenko et al., 2014; Lomberk et al., 2006; LeRoy et al., 2009). An HPip could have an increased binding affinity for a specific HP1α PTM (yellow). Thus, HP1α PTMs may regulate HP1α complex formation and spatially restrict HPip recruitment. Consistent with the PTM model, HP2 and PIWI have been shown to have higher binding affinities for HP1α proteins containing phospho-mimic mutations in the HP1α chromo shadow domain (Mendez et al., 2011). (C) Subdomains could form by a cooperative binding mechanism (Bray and Duke, 2004; Bai et al., 2010). HP1α can oligomerize at least up to tetramers (Wang et al., 2000; Zhao et al., 2000; Canzio et al., 2011), forming a multivalent platform for HPip binding (i.e. more than one HPip binding site per HP1α oligomer). Thus, initial binding by an HPip could induce a higher binding affinity between a neighboring HP1α molecule and the HPip. The dotted arrow indicates potential self-interactions between HPips and solid arrows indicate hypothetical HPip on/off rates.

DOI: 10.7554/eLife.16096.034
Appendix 1—figure 1. HP1α staining is significantly decreased in dying cells. The negative log of the RP p-value for HP1α mean intensity and number of nuclei were plotted against each other. Green dots indicate thread depletions (dying cells) and red dots indicate HP1α depletions.

DOI: 10.7554/eLife.16096.041