Investigating repeat-induced silencing in *Drosophila melanogaster*

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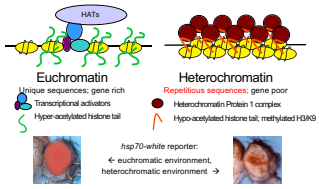
Abstract: We investigated the role that repetitious DNA sequences play in the formation and spreading of heterochromatin by creating transgenic constructs containing a repeat fragment (transposable element *1360*, *lacO*$_{256}$ tandem repeats, or a GAA$_{310}$ triplet repeat) upstream of an *hsp70-white* reporter. These constructs were placed in a euchromatic region in a site within an actively transcribed gene, *nesd*, but close to a block of repetitious DNA packaged as heterochromatin. The resulting PEV eye phenotypes suggest that ectopic heterochromatin formation has occurred in all three cases. However, the PEV phenotype is much stronger in *lacO*$_{256}$*-hsp70-white* and in *GAA*$_{310}$*-hsp70-white* than in *1360-hsp70-white* transgenic flies. Excision of the repeat fragment reverses the PEV phenotype to a full red eye in all cases, demonstrating that the stochastic silencing is dependent on the presence of repetitious elements. In all cases the PEV reporters are sensitive to HP1a depletion, confirming that the silencing is due to heterochromatin formation. Tests for sensitivity to mutations in *piwi* and *AGO3* indicate that the *1360-hsp70-white* reporter is dependent on the RNAi mechanism. Silencing in the *lacO*$_{256}$ repeat-containing case shows little dependence on H3K9 histone methyl transferases; dependence on *Su(var)2-1* for silencing, and loss of silencing on over-expression of *Gcn5* suggest reliance on the histone deacetylation process. *lacO*$_{256}$*-hsp70-white* transgenic flies show a surprising loss of silencing at 18° C. The GAA repeat-containing reporters are sensitive to both HMTs and HDAC pathway mutations. Overall, our results indicate distinct mechanisms for targeting and maintenance of local heterochromatin domains induced by various types of repeats.

Heterochromatin formation results in greater nucleosome stability, driving gene silencing

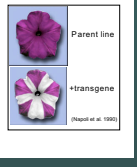


What triggers heterochromatin formation?

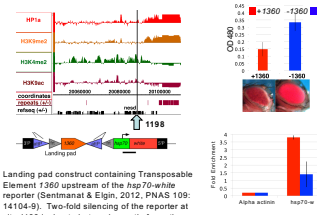
Repetitious elements are recognized as such and silenced, whether tandem arrays of genes, transposable elements, or tri-nucleotide arrays.

- Rich Jorgenson – a more purple petunia?
- Extra copy of pigment gene leads to silencing!
- Humans: triplet repeat expansion leads to silencing of gene, genetic disability.
- Examples:
  - Fragile X mutation: CGG
  - Friedreich's ataxia: GAA
  - myotonic dystrophy: CTG

Fragile X Foundation
Zoghbi and Beaudet 2007



1360 can induce ectopic assembly of heterochromatin & accumulation of HP1a with concomitant silencing

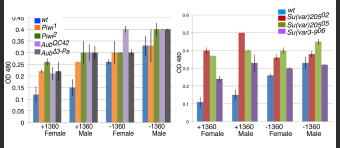


Landing pad construct containing Transposable Element *1360* upstream of the *hsp70-white* reporter (Sentmanat & Elgin, 2012, PNAS 109: 14104-9). Two-fold silencing of the reporter at site 1198 is due to heterochromatin formation, which is dependent on *1360* (see *frt/Flp* excision results, right).

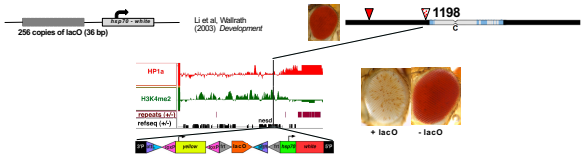
HP1a ChIP

1360-dependent PEV is sensitive to mutations in piRNA pathway components, as well as mutations in *Su(var)205* (HP1a) and *Su(var)3-9* (HMT)

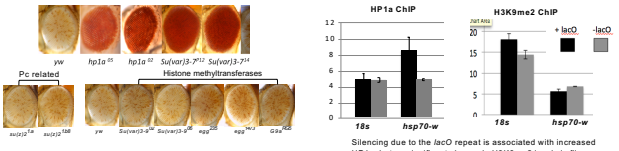
(eye pigment measurements)



lacO repeats – a tandem array of a foreign DNA sequence - initiate silencing when inserted at site 1198



Silencing of 1198 *lacOhsp70-white* is sensitive to mutations in the HP1a complex, but not to mutations in the genes for individual histone H3K9 methyltransferases. ChIP assays indicate no change in H3K9me2 on insertion of repeats, suggesting a different mechanism for stabilizing heterochromatin and maintaining silencing.



Silencing due to the *lacO* repeat is associated with increased HP1a, but no significant change in H3K9me2 levels in flies maintained at 25° C. This suggests that histone deacetylation, an alternative mechanism for silencing, may play a role.

Results of a classic genetic screen and RNAi knockdowns suggest the importance of some HDACs but not others for this heterochromatin formation.

Allele	System	Dominant Impact on Reporter Expression?
AGO2^{414}	siRNA	No
hs^{mutator4}	piRNA	No
mael^{28}	piRNA	Yes
Sin3A^{hm}	HDAC	Yes
Sin3A^{MI02109}	HDAC	Yes
Su(z)^{2}	HDAC	No
Su(z)$^{2D-11}$	HDAC	No
Rpd3^{hm}	HDAC	No
Rpd3^{hm}	HDAC	No
Su(var)205^{mut}	HP1a	Yes
Su(var)205^{mut}	HP1a	Yes
E(z)^{731}	Polycomb	No

TRIP Alleles	Impact on Reporter Expression?
HDAC4^{mutator}	Suppression of variegation
HDAC4^{mutator}	None
HDAC6^{mutator}	Suppression of variegation
HDAC11^{MI03018}	None
Sir2^{mutator}	Suppression of variegation
Sir2^{mutator}	Lethal
Sirt5^{MI01383}	None
Sirt6^{mutator}	None
Sirt7^{mutator}	None
Sirt2^{MI03209}	Enhancement of variegation

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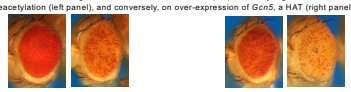
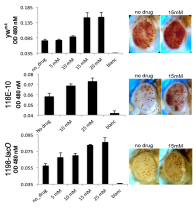
lacO-induced silencing is also lost with mutations in *Su(var)2-1*, a key regulator of histone deacetylation (left panel), and conversely, on over-expression of *Gcn5*, a HAT (right panel).

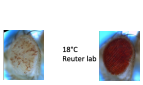


Nicotinamide (a non-specific HDAC inhibitor) results in loss of PEV silencing

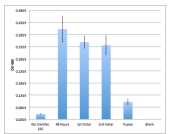


Dietary application of a known HDAC inhibitor, nicotinamide, resulted in a dose-dependent loss of silencing measured by eye pigmentation levels. This effect implies a role for HDACs in the 1198-*lacO* silencing, as well as in other cases exhibiting PEV (*w*^{m4}, X-chromosome rearrangement; 118E-10, transposon insertion into a pericentric fourth chromosome site).

lacO-hsp70-white trangenic flies show a surprising loss of silencing at 18° C



Flies kept at 25°C maintain a PEV phenotype, which differs dramatically from flies transferred to 18°C.



Flies transferred to 18°C from 48 hours after the start of egg-laying through larval stages show a prominent shift to red-eye phenotype.

Thus the temperature sensitive component is required for heterochromatin maintenance.

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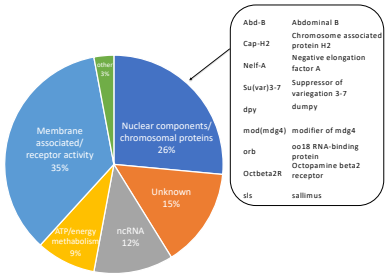
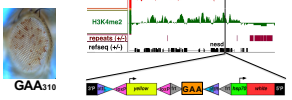
A genetic screen of EMS mutagenized flies for novel PEV suppressors of *lacO-hsp70-white* identified several candidates.

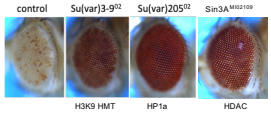


Suppressors of *lacO* mediated silencing were identified by crossing mutagenized males to the *lacO-hsp70-white* reporter line. To exclude known *Su(var)*s we conducted a secondary screen with a 'classic' *hsp70-white* PEV reporter (118E-10, P element located in the pericentric heterochromatin of the 4th chromosome), discarding these suppressors. Only a portion of the screen hits are nuclear components; a significant group (35%) represents genes associated with membranes and/or have receptor activity.

Friedrich's Ataxia Model in *Drosophila* (GAA$_{310}$ swapped into site 1198)

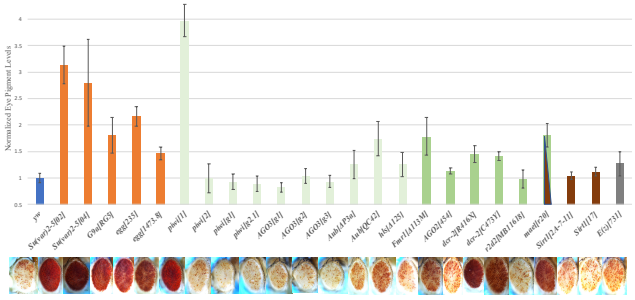


Transgenic construct with a DNA fragment of 310 copies of the triplet GAA, originating from a Friedrich's Ataxia patient, inserted upstream of an *hsp70-white* reporter at site 1198 results in strong PEV. (GAA fragment provided by Richard Festenstein, Imperial College, London.)



GAA$_{310}$*-hsp70-white* silencing is suppressed by mutations in genes coding for HP1, Su(var)3-9, and Sin3A.

GAA$_{310}$ mediated silencing is suppressed by mutations in genes coding for histone H3K9 methyltransferases (*Su(var)3-9*, *G9a*, *egg*). The piRNA system does not play a major role based on the absence of dominant effects from *AGO3*, *piwi* (with the exception of [1]), and *hls* mutations.



Summary

- Foreign repeats can induce robust silencing at position 1198 (base of 2L).
- A triplet repeat (GAA$_{310}$) from a Friedreich's Ataxia patient drives typical heterochromatin formation, dependent on H3K9me2/3 and HDACs; it apparently does not use the RNAi system for recognition, whereas TE's appear to do so.
- Silencing of *lacO* repeats shows greater dependence on HDACs than on HMTs; the *lacO* system shows unusual temperature dependence, a unique feature.

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