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Messenger RNA is a functional component of a chromatin insulator complex

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Chromatin insulators are DNA protein complexes situated throughout the genome capable of demarcating independent transcriptional domains. Previous studies point to an important role for RNA in *gypsy* chromatin insulator function in *Drosophila*; however, the identity of these putative insulator-associated RNAs is not currently known. Here we utilize RNA-immunoprecipitation and high throughput sequencing (RIP-seq) to isolate RNAs stably associated with *gypsy* insulator complexes. Strikingly, these RNAs correspond to specific sense-strand, spliced and polyadenylated mRNAs, including two insulator protein transcripts. In order to assess the functional significance of these associated mRNAs independent of their coding function, we expressed untranslatable versions of these transcripts in developing flies and observed both alteration of insulator complex nuclear localization as well as improvement of enhancer-blocking activity. Together, these data suggest a novel, noncoding mechanism by which certain mRNAs contribute to chromatin insulator function.

Keywords: RNA; chromatin; insulator; *Drosophila*; nuclear organization

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INTRODUCTION

Chromatin insulator complexes define transcriptionally independent chromatin domains throughout the genome likely through alteration of higher order chromatin interactions. Consistent with the enhancer-blocking and barrier activities by which chromatin insulators are functionally defined, genome-wide chromatin conformation studies have demonstrated that chromatin domain boundaries preferentially correspond to certain insulator sites as reviewed in van Bortle and Corces [1]. The well-characterized *Drosophila gypsy* insulator complex comprises three core protein components, which interact directly. Its binding specificity is defined by the zinc-finger DNA binding protein Suppressor of Hairy-wing (Su(Hw)), whose partners include the 2.2 kb isoform of Modifier of *mdg4* (Mod(mdg4)2.2) and Centrosomal protein 190 (CP190). Genome-wide mapping revealed thousands of Su(Hw)-binding sites, occurring most frequently in intergenic and intronic regions [2,3]. Moreover, *gypsy* insulator complexes coalesce at a small number of large nuclear structures termed insulator bodies, which have been proposed to correspond to higher-order chromatin domains likely mediated by CP190 and Mod(mdg4)2.2 BTB-domain interactions [4,5]. An alternative view is that insulator bodies are storage sites of reserve proteins not engaged in insulator activity [6]. Importantly, proper localization of insulator bodies are tightly correlated with *gypsy* insulator function and serves as a useful phenotypic readout [4,7,8].

A direct role for RNA in *gypsy* insulator function has been suggested by genetic and biochemical evidence implicating various RNA-binding proteins in the regulation of insulator activity. The putative RNA helicase Rm62 associates physically with CP190 complexes in an RNA-dependent manner [9]. In humans, the homologue of Rm62, p68, along with its associated noncoding RNA, SRA, interacts with the CTCF insulator protein and promotes its activity [10]. Recent work shows that the RNA-binding protein, Shep, interacts directly with *gypsy* insulator complexes and acts as a CNS-specific antagonist of insulator activity [11]. Therefore, utilization of RNA might be a generally conserved mechanism used to modulate the activity of chromatin insulators.

Here we report the unanticipated finding that certain messenger RNAs (mRNAs), and not other classes of RNA, specifically associate with *gypsy* insulator complexes. By native immunoaffinity purification and RNA sequencing (RIP-seq), we find that two of the most highly enriched transcripts are the *su(Hw)* and *Cp190* mRNAs themselves. In order to study the functional significance of these transcripts outside their coding capacity, we ectopically expressed untranslatable versions in a subset of tissues within the developing fly. Remarkably, we found that expression of these untranslatable RNAs both alter insulator body localization and improve *gypsy*-dependent enhancer-blocking activity in certain tissues. These findings suggest a novel, noncoding capacity for some mRNA transcripts in chromatin regulation.

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RESULTS AND DISCUSSION

Specific mRNAs copurify with *gypsy* insulator complexes

In order to identify RNAs stably associated with insulator complexes, we performed native sequential immunopurification of insulator complexes and high throughput sequencing. Nuclei from 40 g of mixed stage embryos were isolated by manual disruption and centrifugation through sucrose cushions. To achieve high specificity, nuclear extracts were first bound to an α-Su(Hw) column followed by mild salt elution, dilution and subsequent binding of the eluate to an α-CP190 column followed by high salt elution. Associated RNAs were 5′ end labelled and separated on a high percentage polyacrylamide gel. As certain RNA silencing mutants affect *gypsy* insulator activity [9], we were particularly interested to determine whether small RNAs associate with the *gypsy* insulator. However, we consistently observed 35–55 nt bands, considerably larger than known small RNA, enriched in IPs over unbound total nuclear extract (unpublished data). These products were gel extracted, directionally cloned and subjected to sequencing (Fig 1A). No products were obtained with α-Su(Hw) or α-CP190 preimmune sera used to preclear the nuclear extract, indicating specificity of the purification procedure.

A rigorous statistical approach was used to identify transcripts that specifically associate with *gypsy* insulator complexes. After clipping of adapter sequences, filtering of ribosomal RNA, mapping with TopHat [12], filtering of repetitive elements and removal of duplicate reads, we obtained ~1.1 M unique reads in the IP and ~2.3 M in the unbound total RNA samples. Together these libraries correspond to 11,523 annotated transcripts, including mRNA and other classes of noncoding RNA such as small nuclear RNA, small nucleolar RNA, transfer RNA and microRNA. We also considered reads that map to a set of ~1,800 *cis*-regulatory modules such as enhancers and silencers annotated in the REDFly database [13], thereby also sampling putative unannotated noncoding RNA. To distinguish transcripts specifically associated with Su(Hw)/CP190 from nonspecific contaminants, we applied the DESeq algorithm [14] to results from two independent purifications. DESeq calculates the statistical probability of enrichment on the basis of fold-change as well as variance of read counts corresponding to transcripts across biological replicates, providing a stringent measure of reproducibility. Using this method, only two transcripts, *su(Hw)* and *Cp190* mRNAs, emerge as significantly enriched ($p_{adj} < 2.0 \times 10^{-6}$ and $< 1.9 \times 10^{-2}$, respectively) compared with all other mRNA and annotated or putative noncoding RNA ($p_{adj} = 1$) (Fig 1B; supplementary Table S1 online). Reads map across the entirety of *su(Hw)* and *Cp190* transcripts, span known splice junctions and are primarily sense strand, suggesting that isolated RNAs are degradation products of full-length mRNA (Fig 1C,D, supplementary Fig S1 online), which were not directly sampled in this analysis. We examined the exon:intron ratio of all transcripts identified in these libraries and found that the Su(Hw)/CP190 RIP is enriched for exons compared with the unbound total RNA control ($P = 1.3 \times 10^{-8}$, Fisher's exact test). Due to the unavailability of more CP190 antibody, we validated the enrichment of spliced but not unspliced *su(Hw)* and *Cp190* mRNAs by Su(Hw) RIP compared with preimmune serum followed by quantitative reverse transcription polymerase chain reaction (RT–PCR) (supplementary Fig S1 online). These results indicate that RNA present in the Su(Hw)/CP190 RIP is mainly spliced and likely polyadenylated, and enrichment of both transcripts is not an artifact caused by the use of two antibodies.

We confirmed that Su(Hw)/CP190 RIP transcripts are spliced and polyadenylated by performing sequential Su(Hw)/CP190 RIP followed by oligo-dT selection instead of gel purification, followed by non-directional cloning. We found that *su(Hw)*, *Cp190* as well as seven additional mRNA transcripts are highly enriched by this more efficient method ($p_{adj} < 0.02$, Fig 2A, supplementary Table S2 and supplementary Fig S2 online). Only 2 of 9 transcripts are encoded by genes that coincide with a peak of Su(Hw) chromatin association defined by a genome-wide ChIP-chip study in embryos [3], similar to the frequency for all other transcripts ($P = 0.29$, Fisher's exact test). This result suggests that these mRNAs associate with insulator complexes *in trans*. We performed gene ontology analysis and found that 7 of 9 transcripts encode chromatin-associated proteins (Fig 2B). These transcripts could perhaps serve dual roles by also influencing insulator activity. In fact, mutation of *brm*, which encodes a Swi/Snf homologue involved in transcriptional activation, was shown to reduce *gypsy* insulator function [7], but given our results, this finding could reflect a requirement for either mRNA or protein. Enrichment of *su(Hw)* and *Cp190* mRNAs in Su(Hw) RIP but not with preimmune serum was confirmed by quantitative RT–PCR using the abundant transcript *RpL32* as a negative control, which is not enriched in the DESeq analysis (Fig 2A–C). These data demonstrate that *gypsy* insulator complexes associate with specific mRNAs mainly produced *in trans*.

RIP-seq of Dorsal as a negative control

Given the unexpected result of *su(Hw)* and *Cp190* transcript purification with insulator complexes, we considered the possibility that any chromatin-associated protein would copurify its own coding transcript using our protocol. We therefore performed RIP-seq analysis on the Dorsal (Dl) transcription factor, which is highly expressed in the embryo, is not known to be involved in insulator activity or to bind RNA directly, and has a well-defined embryonic chromatin association profile [15,16]. We utilized antisera used previously for ChIP-chip analysis [16], ensuring access to a chromatin-associated pool. Although Dl was efficiently immunoprecipitated from nuclear extracts using α-Dl but not control serum, the resulting amounts of RNA isolated in the IP versus control fractions were almost equal, suggesting that RNA is not abundantly associated with Dl. Owing to the extremely low RNA yield, we were unable to perform oligo-dT selection and instead depleted ribosomal RNA, subjecting the remainder to sequencing. DESeq analysis of two biological replicates identified 161 transcripts as significantly enriched ($p_{adj} < 0.02$) in Dl RIP compared with input (Fig 3A, supplementary Table S3 online). We suspect that the larger number of statistically significant enriched transcripts in the Dl RIP compared with Su(Hw)/CP190 RIP is mainly due to the higher consistency between biological replicates, and therefore higher sensitivity of detection, due to the simpler, single antibody purification. Importantly, the *dl* transcript is not significantly enriched in the Dl RIP ($p_{adj} = 0.37$), arguing against our purifications being contaminated with translating ribosomes. Consistent with these results, mass spec of similarly purified insulator complexes did not detect ribosomal proteins [9].

Unlike Su(Hw)/CP190-associated transcripts, those copurified with Dl appear to be generated *in cis* from transcription

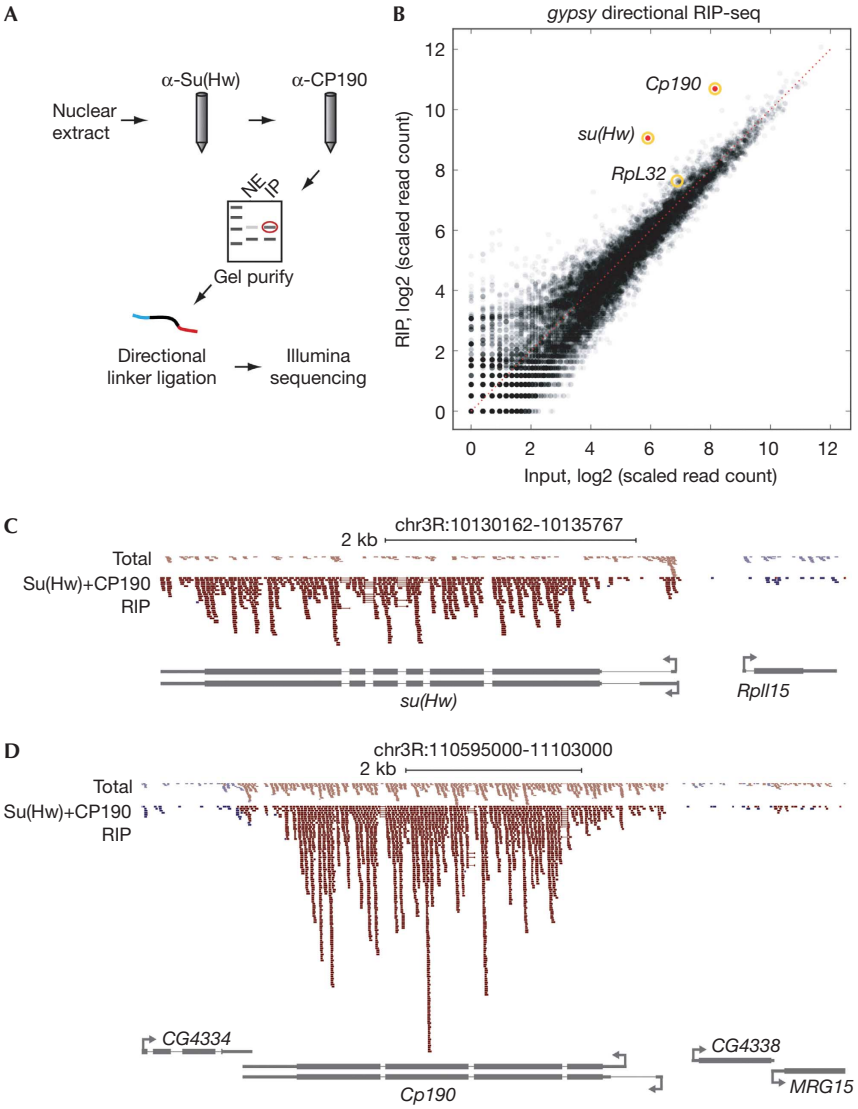

Fig 1 | Messenger RNA associates with *gypsy* insulator complexes. (**A**) Purification scheme for *gypsy* insulator-associated RNA. Embryo nuclear extracts were passed sequentially over α-Su(Hw) and α-CP190 immunoaffinity columns. Purified RNA was 5′ end labelled and purified by electrophoresis. 35- and 55-nt bands were excised from NE and IP lanes, directionally cloned and sequenced. (**B**) Scatterplot of annotated genes corresponding to transcripts identified in total unbound and Su(Hw)/CP190 RIP. Mean scaled tag count across replicates of total unbound input (x-axis) and Su(Hw)/CP190 RIP (y-axis) for each gene is calculated as the mean number of tags across replicates mapping to the entire gene body, scaled by library size. Transcripts corresponding to *su(Hw)* and *Cp190* ($p_{adj} < 0.02$, red) are indicated. All other transcripts (black) correspond to $p_{adj} = 1$. Note that genes displaying similar average fold-enrichment to *su(Hw)* and *Cp190* show high variance across biological replicates, resulting in lack of statistical significance. The *su(Hw)*, *Cp190* and *RpL32* transcripts are circled. (**C**) Reads mapping to the *su(Hw)* or neighbouring *RpII15* locus from total unbound (2.4 M read alignments) or Su(Hw)/CP190 RIP (1.3 M read alignments) libraries. The height of each Watson (blue) and Crick (red) read is scaled to respective library size. Thin bars interrupting reads span splice junctions. (**D**) Reads mapping to the *Cp190* locus as in (**C**). CP190, Centrosomal protein 190; IP, immunopurified; NE, nuclear extract; RIP, RNA immunoprecipitation; Su(Hw), Suppressor of Hairy wing.

factor binding sites. First, 142 or 88% of Dl-associated transcripts correspond to Dl ChIP peaks in the corresponding gene body, significantly more than expected compared with all other transcripts ($P = 1.2 \times 10^{-10}$, Fisher's exact test). Second, substantial numbers of Dl RIP reads map to introns or regions extending immediately 3′ beyond the annotated polyadenylation site (Fig 3B–D), consistent with some fraction of Dl-associated transcripts being nascent. The transcript association profile obtained for Dl contrasts starkly with that of the *gypsy* insulator, which mainly consists of processed mRNAs produced *in trans* to its binding sites. This important negative control also demonstrates that not all chromatin-associated proteins can associate with their own coding transcript.

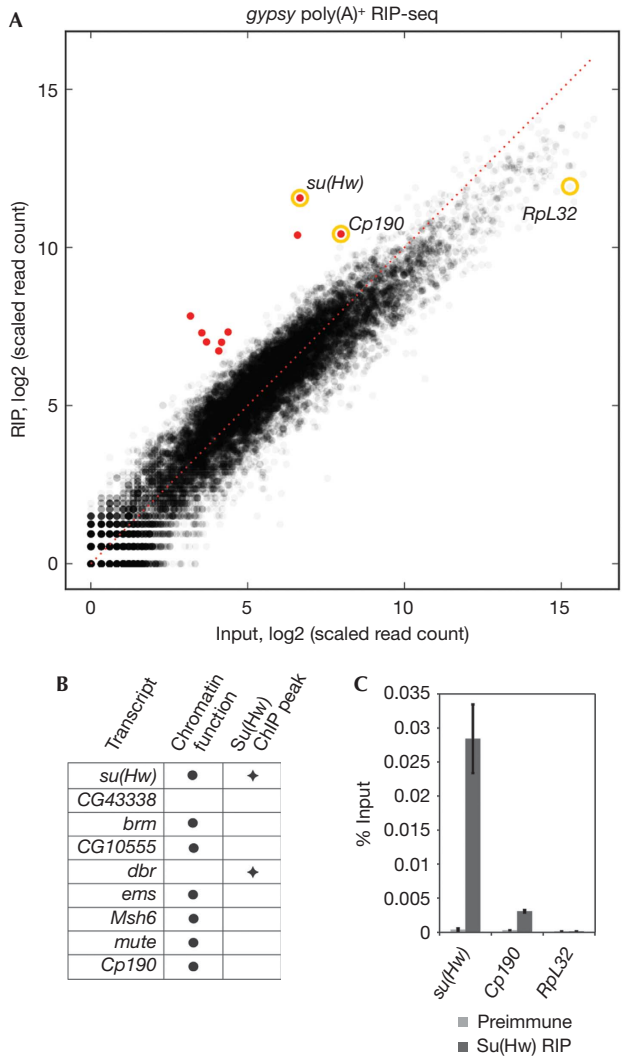

Fig 2 | Oligo-dT selected RNA associated with *gypsy* insulator complexes. (**A**) Scatterplot of annotated genes corresponding to transcripts identified in total nuclear extracts and Su(Hw)/CP190 RIP. Transcripts corresponding to $p_{adj} < 0.02$ are red. (**B**) Functional annotation of genes corresponding to significantly enriched transcripts ($p_{adj} < 0.02$) using FlyBase annotations and correspondence of gene locus with Su(Hw) ChIP-chip peaks [3]. (**C**) Enrichment of transcripts in Su(Hw) RIP determined by quantitative RT–PCR. Amount of *su(Hw)*, *Cp190* and *RpL32* in Su(Hw) IP or pre-immune control as a percentage of total. Parallel minus RT controls resulted in immeasurably low values. Error bars indicate standard deviation of quadruplicate measurements. ChIP, chromatin immunoprecipitation; IP, immunopurified; RIP, RNA immunoprecipitation; RT, reverse transcription; RT–PCR, reverse transcription polymerase chain reaction; Su(Hw), Suppressor of Hairy wing.

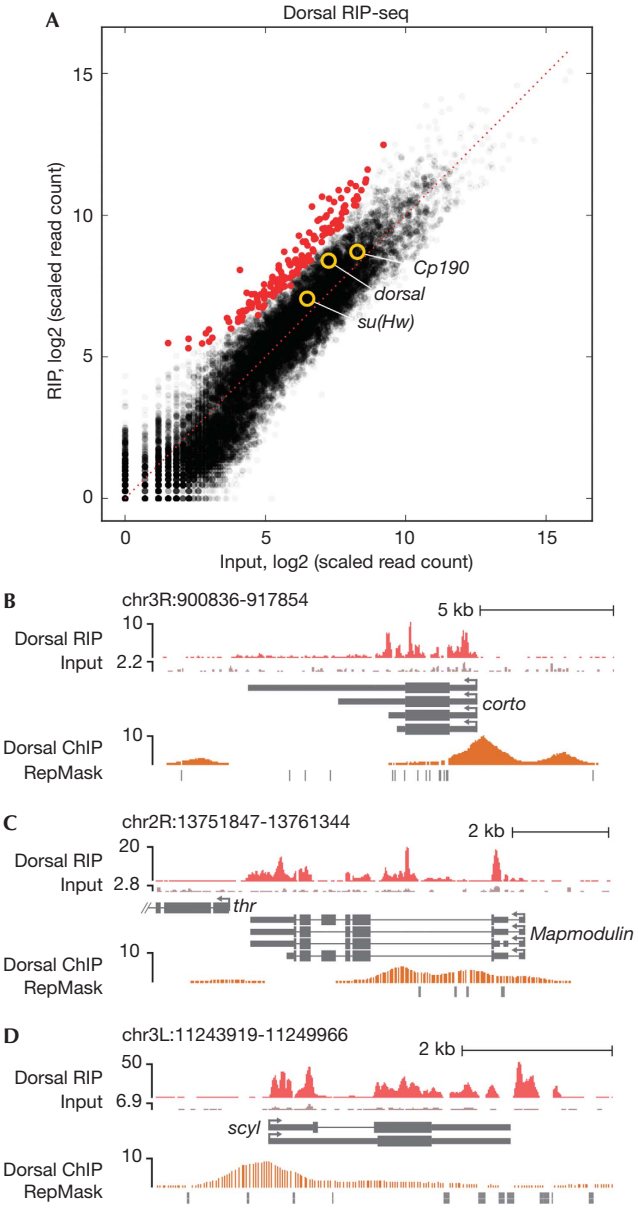

Fig 3 | RIP-seq of Dorsal-associated RNA. (**A**) Scatterplot of annotated genes corresponding to transcripts identified in total nuclear extracts and Dl RIP. Transcripts corresponding to $p_{adj} < 0.02$ are red. The *su(Hw)*, *Cp190* and *dl* transcripts are circled. (**B–D**) Reads mapping to the (**B**) *corto*, (**C**) *Mapmodulin* and (**D**) *scylla* loci from input (4.9 M read alignments) or Dl RIP (14.3 M read alignments) libraries. Input samples are shown on the same scale relative to respective IP, and the bottom of each scale bar corresponds to 0. Dl ChIP-chip signal at 1% FDR [15] is also shown. ChIP, chromatin immunoprecipitation; FDR, false discovery rate; IP, immunopurified; RIP, RNA immunoprecipitation; Su(Hw), Suppressor of Hairy wing.

In vivo expression of untranslatable transcripts using T7

In order to address whether *su(Hw)* and *Cp190* transcripts affect insulator activity, we expressed these transcripts uncoupled from RNA polymerase II transcription, processing, export and translation. This strategy avoids perturbing normal levels of Su(Hw) and CP190 proteins, which would confound subsequent analyses. The *su(Hw)* and *Cp190* cDNAs along with a negative control were expressed using UAS-Gal4 inducible T7 polymerase [17,18] (Fig 4A). The *mod(mdg4)2.2* transcript serves as an ideal negative control as the endogenous transcript is expressed at a

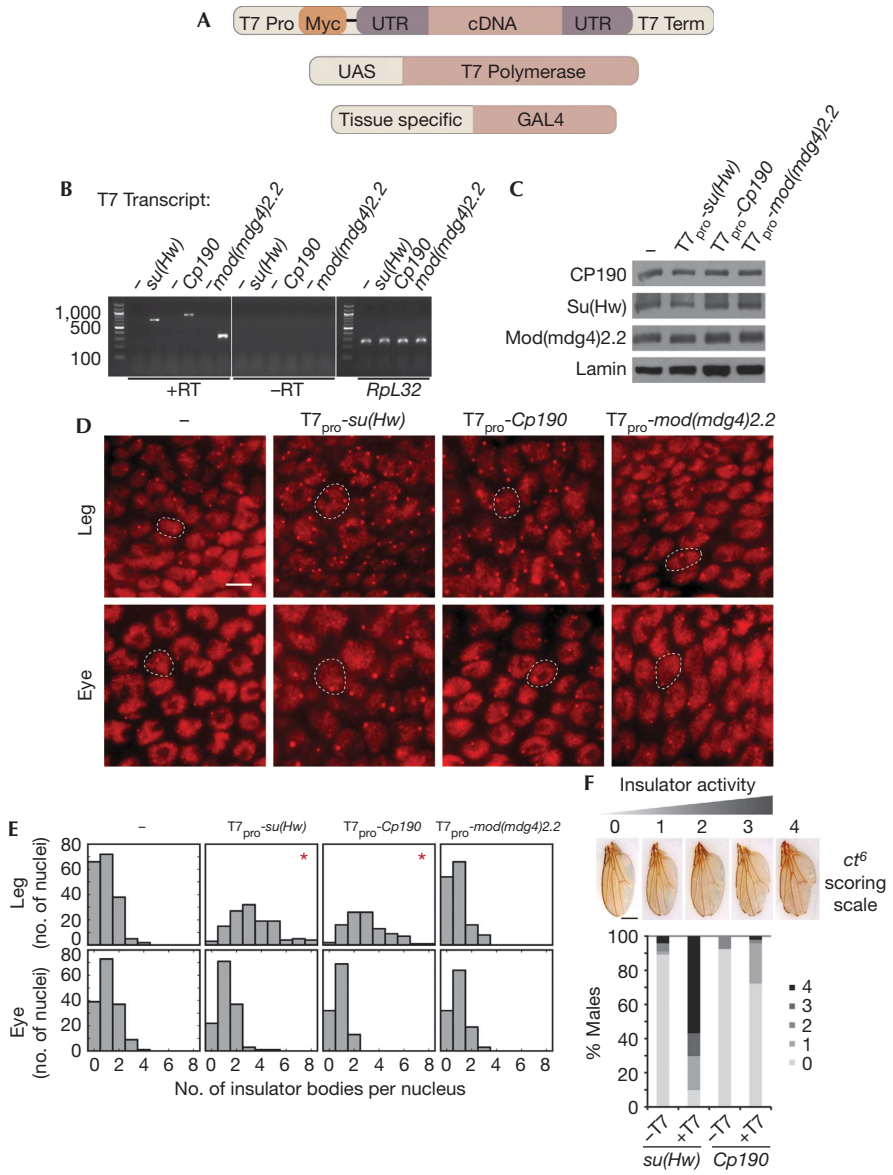

Fig 4 | Untranslatable versions of mRNA associated with the *gypsy* insulator affect insulator body localization and improve enhancer-blocking activity. (**A**) Schematic representation of three transgene system to produce untranslatable transcripts. Transcripts were cloned in their entirety including 5′ and 3′ UTRs; constructs contain a *myc* tag and are flanked by T7 promoter and T7 terminator hairpin sequence. Transcripts are induced by T7 polymerase under tissue-specific GAL4 control. (**B**) RT–PCR using *myc* and gene-specific primers to detect T7-*su(Hw)*, T7-*Cp190* and T7-*mod(mdg4)2.2* transcripts from larvae expressing T7 polymerase with (+RT) or without (−RT) reverse transcription. Endogenous *RpL32* transcript with RT serves as a positive control (right). (**C**) Western blotting of CP190, Su(Hw), Mod(mdg4)2.2 and Lamin in anterior larval extracts expressing T7 polymerase alone (−) or indicated T7 driven transcripts. (**D**) Indirect immunofluorescence of CP190 to detect insulator bodies in peripheral leg imaginal disc tissues expressing T7 polymerase alone or T7-*su(Hw)*, T7-*Cp190* or T7-*mod(mdg4)2.2* using *Ser*::Gal4 (top row). Insulator bodies in eye imaginal discs, with no T7 expression, from corresponding samples (bottom row). Rabbit α-CP190 is detected by α-rabbit Alexa-594. White dotted lines outline an example nucleus in each field. Scale bar equals 5 μm. (**E**) Histograms of number of nuclei (y-axis, n > 101) containing indicated number of insulator bodies per nucleus (x-axis) for samples shown in (**D**). Asterisks denote genotypes with distributions that show a statistically significant difference from the control ($P < 1.0 \times 10^{-10}$, Kruskal–Wallace test). (**F**) Effects of T7-*su(Hw)* and T7-*Cp190* on ct^6 phenotype in males without T7 (−T7) or with (+T7) polymerase driven by *Ser*::Gal4. All flies are homozygous for $mod(mdg4)^{u1}$. Percent of population scored on a 0–4 scale. 0, absence of notching; 1, light notch; 2, medium notch; 3, heavy notching; and 4, a single large notch in the distal wing margin, perhaps with notches in the wing tip. Scale bar is ~0.5 mm. Example wings are of the following genotypes: y^2wct^6; *Ser*::Gal4/+; T7-*Cp190*, $mod(mdg4)^{u1}$/+, $mod(mdg4)^{u1}$ (0), y^2wct^6; pUAST7 25A/*Ser*::Gal4; T7-*Cp190*, $mod(mdg4)^{u1}$/+, $mod(mdg4)^{u1}$ (1 and 2), y^2wct^6; pUAST7 25A/*Ser*::Gal4; T7-*su(Hw)*, $mod(mdg4)^{u1}$/+, $mod(mdg4)^{u1}$ (3 and 4). Note that all T7 transcript transgenes are paternally transmitted. See also supplementary Fig S5 online. mRNA, messenger RNA; RT, reverse transcription; RT-PCR, reverse transcription polymerase chain reaction; Su(Hw), Suppressor of Hairy wing.

level similar to *su(Hw)* and *Cp190* and shares sequence similarity to *Cp190* but is not enriched in Su(Hw)/CP190 RIP purifications (supplementary Tables S1,S2 online). When T7-*su(Hw)*, T7-*Cp190* and T7-*mod(mdg4)2.2* are expressed by inducing T7 polymerase with *Ser*::Gal4, the *myc*-tagged transcripts are readily detected by RT–PCR from total RNA at similar levels for each transgene (Fig 4B). We also verified that T7 transcripts are visible in the nucleus but not cytoplasm by *in situ* hybridization to *myc* in whole mount tissue in the expression patterns of *Ser*::Gal4, *GMR*::Gal4, or *nrv2*::Gal4, indicating transcripts are highly expressed in a Gal4 and T7 polymerase-dependent manner (unpublished data). No change in endogenous CP190, Su(Hw) or Mod(mdg4)2.2 protein levels is observed by western blotting on induction of T7 transcripts with *Ser*::Gal4 (Fig 4C; supplementary Fig S3 online). Furthermore, immunofluorescence of whole mount tissues and western blotting failed to detect Myc antigen from T7 transcript expressing larvae. These results indicate that T7 transcripts remain untranslated within the nucleus.

Functional significance of *gypsy*-associated transcripts

We next tested whether T7-*su(Hw)* and T7-*Cp190* transcripts affect insulator complex localization. We examined the distribution of insulator bodies by indirect immunofluorescence of endogenous CP190 protein in whole mount larval imaginal discs and brains. Compared with no T7 transcript or T7-*mod(mdg4)2.2* expression using *Ser*::Gal4, T7-*su(Hw)* causes formation of ectopic and more pronounced insulator bodies relative to overall nucleoplasmic signal, particularly in peripheral cells of the leg discs (Fig 4D,E, top panel; observed in 10 of 12 experiments) as well as wing discs. Expression of T7-*Cp190* causes a less penetrant and less pronounced but similar phenotype to T7-*su(Hw)* (observed in four of seven experiments). Importantly, no effect on overall development of disc or brain tissue is observed as a result of T7 polymerase expression with or without T7 transcript (supplementary Fig S4 online). No change in insulator bodies is observed in the eye disc, which does not express *Ser*::Gal4 (Fig 4D,E, bottom panel). Moreover, no change is detected in the brain, in which expression is much lower than the leg and wing discs (supplementary Fig S4 online). Similarly, no effects on insulator body localization in the eye or brain are observed when T7 transcripts are expressed by *GMR*::Gal4 or *nrv2*::Gal4, respectively (supplementary Fig S4 online). Resultant changes in insulator body morphology using *Ser*::Gal4 indicate that expression of untranslatable *su(Hw)* or *Cp190* transcripts are sufficient to affect the overall nuclear distribution of insulator complexes in certain tissues. These RNAs might alter higher-order insulator interactions or those between insulators and factors that regulate their activities. An alternative explanation is that T7-*su(Hw)* or T7-*Cp190* transcripts change the relative distribution of insulator proteins on chromatin versus storage sites either by competing with endogenous transcripts or nucleating ectopic insulator bodies.

Finally, we examined whether T7 transcripts also alter enhancer-blocking activity using *gypsy*-dependent phenotypic reporters. We scored enhancer-blocking activity of the well-characterized *yellow*^{2} (y^2) and *cut*^{6} (ct^6) alleles, which both result from a *gypsy* retrotransposon insertion between an enhancer and the promoter. Insertion results in loss of *y* or *ct* expression and corresponding loss of abdominal pigmentation or proper wing margin development, respectively [19]. In wild-type flies, ubiquitous expression of T7 polymerase using *Act5C*::Gal4 results in lethality, and expression in the wing using *Ser*::Gal4 causes blistering and subsequent necrosis of adult wings, preventing analysis of the y^2 and ct^6 phenotypes (supplementary Fig S4 online). However, in the sensitized *mod(mdg4)*^{u1} background, expression of T7-*su(Hw)* with *Ser*::Gal4 reduces wing blistering (supplementary Fig S4 online), allowing assessment of *gypsy* insulator activity at ct^6. Compared with matched controls without T7 polymerase, expression of T7-*su(Hw)* results in dramatically increased wing notching whereas expression of T7-*Cp190* produces a milder but similar phenotype, signifying improvement in insulator enhancer-blocking activity (Fig 4F). No change of y^2 expression in the wing was observed using *Ser*::Gal4, and no effects on y^2 expression in the wing or body were observed using a *y*::Gal4 driver that includes wing and body enhancers ([20]; supplementary Fig S5 online). Changes in both insulator activity and insulator body localization due to ectopic expression of these transcripts strongly suggest the functional importance of insulator-associated mRNA.

Core insulator proteins are not known to bind RNA directly, predicting the necessity for protein adapters to recruit RNAs to insulator complexes. On the basis of its antagonistic effect on insulator activity, it is unlikely that Shep mediates interaction between *su(Hw)* or *Cp190* transcripts and insulator complexes. Future work to identify this putative adapter as well as the sequence specificity of its associated transcripts will provide important insights into this novel mechanism.

Insulator-associated mRNAs might be reminiscent of long noncoding RNA (lncRNA) that have been proposed to act as stabilizers of chromatin conformation or scaffolds for recruitment of chromatin-modifying factors to specific loci. Binding of a particular long noncoding RNA can provide a signal or induce a conformational change to alter the interaction potential of its associated protein [21]. Likewise, mRNA associated with *gypsy* insulator complexes could alter the likelihood of assembly at particular sites or affect insulator-dependent interactions that modulate their activities. Our results not only add to the growing list of dual coding/noncoding transcripts [22] but further suggest the potential for coevolution of bifunctionality.

METHODS

Quantitative PCR. Transcripts from Su(Hw) RIP and preimmune RIP were reverse transcribed using gene-specific primers (supplementary Table S4 online) and quantified on an Applied Biosystems Real-Time PCR System using SYBR-green incorporation (Affymetrix/USB). PCR primers were tested for specificity and efficiency by electrophoresis and real-time PCR of serially diluted templates. Error is represented as the standard deviation of four technical replicates of each PCR reaction, removing statistical outliers. Transcript quantities were normalized to a 5-point genomic DNA serial dilution standard verified to be amplified in the linear range and expressed as percent of total nuclear RNA. Two independent biological replicates showed similar results.

Immunofluorescence. Brains and imaginal discs were dissected from at least five larvae of each genotype and stained as described previously [11] and were imaged using a Leica DM5000B epifluorescent microscope and captured using OpenLab

software. See supplementary Methods online for antibody descriptions.

RIP-seq and computational analysis. See supplementary Methods online.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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Author contributions: L.H.M. and E.P.L. designed and performed the experiments, R.K.D. performed the computational analyses and all authors analysed the data and generated figures. L.H.M. and E.P.L. wrote the paper with input from R.K.D.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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