**SUPPLEMENTARY INFORMATION**

# Non-coding transcripts in the *H19* imprinting control region mediate gene silencing in transgenic *Drosophila*

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## LIST OF SUPPLEMENTARY INFORMATION



## SUPPLEMENTARY RESULTS AND DISCUSSION

### The mouse *H19* ICR is bi-directionally transcribed from both parental chromosomes

We performed additional double label RNA-FISH experiments on mouse fetal liver cells to confirm our results. Here, we used strand-specific probes against the non-coding ICR transcripts in combination with probes against the maternal *H19* gene transcripts. Cells with monoallelic *H19* expression and ICR transcripts were grouped into three categories: expression from the maternal chromosome (supplementary Fig 1A,D: one ICR signal overlapping with the *H19* signal), expression from the paternal chromosome (supplementary Fig 1B,E: one ICR signal non-overlapping with the *H19* signal), and biallelic expression

(supplementary Fig 1C,F: two ICR signals, one of which overlaps with the *H19* signal). Sense and antisense ICR transcripts were detected at comparable frequencies originating from both parental chromosomes, possibly indicating that they might be co-regulated, as has been demonstrated for other sense-antisense transcription pairs (Cawley *et al*, 2004).

**Sense and antisense transcription in a *Drosophila H19* ICR transgene**

We performed reverse transcription reactions on RNA derived from *H19* ICR transgenic flies and wild-type control flies. We detected transcripts from the *H19* locus in fly lines carrying the *H19* ICR transgene, but not in wild-type control flies (supplementary Fig 2A). This result was reproduced in three independently derived *Drosophila H19* ICR transgenic lines (out of three lines analyzed, data not shown), excluding the possibility that regulatory elements (for example enhancers) located in the vicinity of the transgene's genomic integration site could mediate transcription in the transgenic *H19* ICR locus. We next performed strand specific reverse transcription reactions. We detected sense and antisense transcripts in *H19* ICR transgenic *Drosophila*, which were absent in flies not carrying the *H19* ICR transgene (supplementary Fig 2B). Our results show that the transcriptional activity at the *H19* ICR is conserved between mouse and transgenic flies, and they strongly suggest that the *H19* ICR harbours regulatory elements that are targeted by the transcriptional machinery in both species.

**Gene silencing conferred by the *H19* ICR in transgenic flies is independent of the RNAi pathway**

We detected sense and antisense RNAs with the potential to form double-stranded RNA at the *H19* ICR in both the endogenous mouse locus and at a transgenic *Drosophila* locus (Fig 2, supplementary Fig 2). In the process of RNA interference (RNAi), double stranded RNAs are cleaved to small interfering RNAs (siRNAs), which in turn catalyze cleavage of RNAs with the equivalent sequence. Studies in *S. pombe* and *Drosophila* have shown that the RNAi machinery is involved in the establishment of heterochromatin and transcriptional gene

silencing (Volpe *et al*, 2002; Pal-Bhadra *et al*, 2004). Notably, some mutations in *Su(var)* genes, whose gene products are components of heterochromatin, partially relieve *H19* ICR mediated gene silencing at the transgenic *Drosophila* locus, consistent with the idea of a heterochromatin-like structure involved in gene silencing at the transgenic *Drosophila H19* locus (Schoenfelder & Paro, 2004). In light of these observations, we explored the possibility that an RNAi mechanism might be involved in reporter gene silencing at the *Drosophila H19* ICR transgene. To test this hypothesis, we first genetically combined flies carrying the ICR transgene with mutations in genes of the RNAi pathway. These mutations have been demonstrated to interfere with RNAi gene silencing processes (Kennerdell *et al*, 2002; Liu *et al*, 2003; Lee *et al*, 2004; Pal-Bhadra *et al*, 2004). However, when combined with the *H19* ICR transgene, they failed to result in a significant relief of *mini-white* silencing (supplementary Fig 3A-F). We next attempted to detect small interfering RNAs originating from the *H19* ICR. We obtained RNA samples enriched for low molecular weight RNAs from wild-type and *H19* ICR transgenic lines at different stages of *Drosophila* development (adults: supplementary Fig 3G, lanes 3, 4; embryos: supplementary Fig 3G, lanes 5, 6; adult heads: supplementary Fig 3G, lanes 8, 9), as well as from mouse embryos at day 13.5 (supplementary Fig 3G, lane 7). We were unable to detect any small RNAs resulting from the *H19* ICR by Northern blot (supplementary Fig 3G, lanes 3 to 9). We cannot rule out the possibility that siRNAs are expressed from the *H19* locus at a level that is below the detection limit of the method we have employed. However, as a positive control for the Northern blot experiment, we expressed a 430 base pair fragment from the *H19* ICR as an inverted repeat using the UAS GAL4 system in transgenic flies (see schematic representation in supplementary Fig 3H). In RNA samples obtained from heads of transgenic flies expressing this hairpin RNAi vector, we were able to detect 21-23 nucleotide siRNAs originating from the *H19* ICR (supplementary Fig 3G, arrow lane 10), proving that the RNA isolation and detection protocols we have employed are suitable to detect small RNA species.

In conclusion, three lines of evidence presented here argue against an RNAi mechanism mediating transcriptional repression at the *Drosophila H19* ICR locus. First, we were unable

to detect small interfering RNAs from the *H19* ICR. Second, mutations in genes of the RNAi pathway do not relieve *H19* ICR conferred reporter gene repression in *Drosophila*. Finally, rather than enhancing *mini-white* silencing as would be predicted if an RNAi like mechanism was repressing reporter gene transcription at the *Drosophila* transgenic *H19* locus, the transgenic expression of siRNAs directed against the *H19* ICR has the opposite effect, resulting in the loss of reporter gene silencing (Fig 3). Collectively, these results point to an RNAi independent mechanism regulating gene expression at the *H19* locus in transgenic *Drosophila*.

**Expression of the *H19* ICR RNAi transgene leads to specific loss of silencing of an *H19* ICR controlled reporter gene**

To control for the specificity of the effect on *mini-white* gene function we observed upon *H19* ICR RNAi vector expression, we carried out genetic crosses. Combining the GAL4 driver with the *H19* ICR transgene in the absence of the ICR RNAi vector did not change *mini-white* expression (supplementary Fig 4B, C). As an additional control, we expressed the ICR RNAi transgene in a fly line carrying the *H19* ICR transgene in which the silencer element has been deleted (ICR transgene Δ silencer, supplementary Fig 4D). We did not detect an increase in *mini-white* transcription upon expression of the RNAi vector in this line compared to the *Drosophila* line carrying the ICR transgene Δ silencer only (supplementary Fig 4E, F). This result demonstrates that, in the absence of their target sequence, siRNAs originating from the RNAi vector transgene have no effect on the expression status of *mini-white*.

**Transcription at the *H19* ICR transgene locus in a heterochromatin mutant that relieves *H19* ICR mediated gene silencing**

We have previously used the *Drosophila H19* ICR transgene to screen for genetic suppressors of ICR mediated gene silencing. We found that some mutations in *Su(var)* genes, which encode heterochromatin components, partially relieve *H19* ICR mediated gene silencing at

the transgenic *Drosophila* locus (Schoenfelder & Paro, 2004). One of these genes is *Su(var)3-9* (supplementary Fig 5A), a histone methyltransferase responsible for setting up repressive histone marks in heterochromatic regions (Schotta *et al*, 2002). Remarkably, the strongest genetic suppressor identified in our study, *su(Hw)* (supplementary Fig 5A, Schoenfelder & Paro, 2004), has also been implicated in the establishment of a heterochromatin-like structure (Gerasimova et al., 1995). Collectively, our findings suggest that an interplay between noncoding RNAs and heterochromatin proteins operates at the transgenic *H19* locus to confer gene silencing to reporter genes. However, it is not clear whether the heterochromatin components are targeted by ncRNAs, or conversely, whether they might be required for the establishment or maintenance of transcription in the *H19* ICR. To address this question, we decided to analyze the transcriptional status of the *H19* ICR in the absence of Su(Hw). The ICR transcripts could be detected in a genetic background deficient for *su(Hw)*, ruling out the possibility that Su(Hw) is required for the generation of the nc ICR transcripts (supplementary Fig 5B). However, this result is consistent with the idea of targeting heterochromatin components to the transgenic *Drosophila H19* locus by ncRNAs.

## SUPPLEMENTARY MATERIALS AND METHODS

### RNA isolation, reverse transcription, PCR

Three embryonic day 15.5 livers were collected from F1 progeny of female C57BL6 and male SD7 (SD7 is a C57BL6 congenic line which bears a spretus allele at the distal part of chromosome 7) and directly snap frozen. RNA was obtained by purification with Qiagen RNeasy mini columns with Qiagen DNaseI treatment on column, followed by a second DNase digest (Ambion Turbo DNase) and re-purification by Qiagen MinElute RNA cleanup columns. cDNA was synthesised using Invitrogen Superscript III. Total random primed cDNA was obtained in a 90 minute reaction at 50°C, starting with 1.5 ug ultrapure RNA. PCRs were performed with Invitrogen native Taq DNA polymerase and Invitrogen PCR Optimizer buffers. RT-PCR amplifications were usually done with $1/80^{th}$ of the total random cDNA produced, with an amplification of 36 cycles (1 cycle 94 °C 2 min 30 sec., 36 cycles of 94°C 30 sec., 60°C 30 sec., 72°C 35 sec., and 1 cycle 72°C 8 min). For cDNA reactions with specific primers, between 125 and 500 ng of ultrapure RNA were used per synthesis. Three reactions were set up per cDNA synthesis with specific primers: one with reverse transcriptase and with specific primer, one with reverse transcriptase but without primer (to control for potential endogenous priming), and one without reverse transcriptase and without primer (to control for potential DNA contamination). After the RNA denaturation step at 65°C, tubes were kept at 55°C throughout the procedure to prevent endogenous random priming. For cDNA synthesis, reactions were incubated at 55°C for 60 minutes with 2 ul of Superscript III (Invitrogen).

Nuclear and cytoplasmic RNA fractions were obtained from mouse embryonic fibroblasts collected from 14.5 gestational day embryonic bodies. After tissue trypsinisation (mean of 4-5 bodies per flask), cells were dispersed on standard TC 175 culture flasks in DMEM and 10% FBS, expanded and collected before passage 6. To purify the MEF nuclear and cytoplasmic fractions, we used the Ambion Paris columns. Two nuclear and cytoplasmic fractions of MEF

obtained from two different litters were collected. The primers used for the PCR, single stranded cDNA and sequencing are as follows (PCR fragment as depicted in Figure 1A): PCR 1: 5'-CCAACTGAGAGGGCCATAGT-3' and 5'- GGCTCTTGGGGTCAAGTCTA-3'; PCR 2: 5'-GCTCTATCCCATCGAAATGC-3' and 5'-CTGATTCAGCAGACGTCCAA-3'; PCR 3: 5'-GGACACATGCATTTTCTAGGC-3' and 5'-ACCATGCTTAGTGGGGTCTG-3'; PCR 4: 5'- GGGGTTCATGCTAGTCCTTG -3' and 5'-GAACCCCAACTTTGCCATAA-3'; PCR 5: 5'- ATGCCAGAAAGCACAAAAGC-3' and 5'-CTCGGCAACTTCGGTCTTAC-3'; PCR 6: 5'-AGGGGTGGTAAGATGTGTGC-3' and 5'-AATGCCTGATCCCTTTGTTG-3'; PCR 7: 5'-TACATATTGCTCGGCAGACG-3' and 5'-ACCCTCCTGCTTCACTTCAA-3'; PCR 8: 5'-GGGAGACAGAGACCAACTGC-3' and 5'-ACAAATCAGGGCACCAGAGA-3'; PCR 9: 5'-CCACTAGGCTGAGGATCTGC-3' and 5'-CCCCAGCCTTTGTCCTAGTC-3'; PCR 10: 5'-GGTGAGGAGTGCCCAAATTA-3' and 5'-GCCCCTACTCTGTCAACCAA-3'; PCR 11: 5'- GGAAAACATCGGAGTGAAGC-3' and 5'-CAACCCTGCACCTCTTCTTT-3'

RNA was isolated from *Drosophila* tissues by adding 1 ml of Trizol reagent (Invitrogen) per 100 mg of tissue. After homogenizing (Polytron Ultra-Turrax), centrifugation (12000xg, 10 min) and chloroform extraction, the RNA was precipitated with isopropanol. RNA was subsequently treated with DNase I (Roche) for 30 minutes at 37°C. Reverse transcription reactions were performed using M-MLV Reverse Transcriptase (Promega, M3681) following the manufacturer's instructions. Briefly, 1 ug of RNA was incubated with either 250 ng of random hexamer primers (Roche) or 2 pmol of a gene-specific primer at 50°C for 60 min, in the presence of dNTPs and RNasin (Promega, N2511). Primers sequences used for reverse transcription reactions and subsequent PCRs with *Drosophila* tissue are available upon request.

**RNA FISH**

RNA FISH was performed as described in Osborne *et al*, 2004. E14.5 mouse fetal liver cells were fixed in formaldehyde/acetic acid. Probes were generated by first cloning the respective

sequences into pGEMTeasy (Promega). After *in vitro* transcription, reverse transcription was performed in the presence of DIG-dUTP (Roche) or DNP-dUTP (Perkin-Elmer) using Superscript II (Invitrogen) to generate labelled single-stranded DNA probes. After hybridization, transcripts were detected using anti-DIG (Roche, 1333089) and anti-DNP (Serotec, MCA 1212) antibodies, followed by TR- and FITC-conjugated secondary and tertiary antibodies. In the case of *H19*, a probe against the entire gene was used. *Igf2* probes were described in Osborne *et al*, 2004. To detect *H19* ICR transcripts, a probe covering the ICR and the silencer region (base pairs -3979 to -1789 in relation to the *H19* transcriptional start site) was generated. We examined RNA FISH signals on an Olympus BX41 epifluorescence microscope. Pictures of representative nuclei were captured with a CCD camera. Primers sequences used for cloning are available upon request.

***Drosophila* stocks and genetic crosses**

The *H19* ICR RNAi transgenic line was generated by standard techniques, using the *yellow* gene as transformation marker in flies with a *yw* genetic background. In order to express the ICR RNAi transgene, transgenic flies were crossed to flies carrying the P(GMR-GAL4w-) transgene (a kind gift from Steven Henikoff). This strain lacks a functional *white* gene and expresses GAL4 in the developing and adult *Drosophila* eye (Ahmad & Henikoff, 2001). As a consequence, when this line is genetically combined with the *H19* ICR RNAi transgenic line, GAL4 drives the expression of the RNAi transgene in the same tissues. RNAi mutants used in this study have all been demonstrated to interfere with RNAi gene silencing. More specifically, mutations in *aubergine*, *dicer-2*, *homeless* and *piwi* lead to a derepression of heterochromatin-associated reporter genes (Lee *et al*, 2004; Pal-Bhadra *et al*, 2004). Furthermore, upon injection of double-stranded RNA, embryos with mutations in *aubergine*, *homeless*, or *r2d2* fail to display a normal RNAi response (Kennerdell *et al*, 2002; Liu *et al*, 2003). More information on RNAi mutants is available on Flybase.

**Cloning, Plasmids**

P(UASTyellowH19IR), the plasmid used to generate the *H19* ICR RNAi transgene, was generated by first cloning a 430 bp fragment from the *H19* imprinting control region (bp -2915 to -2486 in relation to the *H19* transcriptional start site) in inverted orientation upstream and downstream of an intronic sequence into the P(WIZ), which allows the expression of a hairpin RNA (Lee & Carthew, 2003). Cloning was essentially performed as described in Lee and Carthew, 2003. Briefly, a NotI-XbaI fragment, composed of the 430 bp *H19* ICR fragment in sense orientation, the intron of P(WIZ), and the 430 bp *H19* ICR fragment in antisense orientation, was subcloned into P(UASTyellow), a gift from Laurent Perrin.

pGEMTeasy-H19 ICR and pGEMTeasy-H19 were used to generate probes for RNA FISH detection of ICR and *H19* transcripts, respectively. Both constructs were generated by amplifying the respective PCR fragments from genomic DNA and cloning them into pGEMTeasy (Promega). Primer sequences used for cloning are available upon request.

**Northern Blot analysis**

We used the Decade marker system (Ambion) as RNA size marker according to the manufacturer's instructions. As a control for the hybridization conditions, we used a 21 nt RNA oligo with complementary sequence to the probe. Low molecular weight enriched RNA was isolated using the mirVana miRNA isolation kit (Ambion). 3 ug of RNA were loaded per lane and separated on a TBE/urea/acrylamide gel (Protean II xi Cell system, Biorad) in 0.5xTBE buffer. Transfer was done overnight in a Trans Blot Cell (Biorad) onto a Hybond N+ membrane (Amersham). After the transfer, the membrane was baked for 5 minutes at 80°C and UV crosslinked (UV Stratalinker 2400, Stratagene). At this stage, the RNA size marker lane was separated from the rest of the membrane for subsequent exposure to control the efficiency of the transfer. The membrane was then prehybridised for two hours at 37°C in LMW buffer (45% formamide, 7% SDS, 50 mM sodium phosphate buffer, 300 mM NaCl, 1x Denhardt's solution, 100 ug/ml salmon sperm DNA). The probe was generated by ligating a

T7 promoter fragment to a PCR fragment covering the entire *H19* silencer region using the Lign' Scribe Kit (Ambion). The probe was radioactively labelled in an *in vitro* transcription reaction following the manufacturer's instructions (MAXIscript-T7-Kit, Ambion). Before addition to the membrane in LMW buffer, the probe was hydrolysed in sodium carbonate buffer at 60°C to an average length of 50 nucleotides. For the hybridization, the probe was incubated with the membrane in 15 ml LMW buffer at 37°C overnight. The membrane was washed twice at 50°C in wash buffer (2xSSPE/0.5% SDS) before exposure.

**Chromatin Immunoprecipitation**

ChIP was performed essentially as described in Umlauf *et al*, 2004, with minor modifications: Mouse fetal liver cells were fixed with 2 % formaldehyde in DMEM/FBS (Gibco), and Dynabeads Protein A (Invitrogen) were used to pull down cross-linked fragments. Real-time PCR was performed with an ABI PRISM 7000 Sequence Detection System using SYBR green PCR Master Mix (Applied Biosystems). An average of three assay replicates is presented, error bars depict SD. The real time PCR value for α-globin is set to 100. One representative ChIP profile is shown, a second independent experiment gave highly similar results (data not shown). Primer sequences are available upon request.

**Eye pigment measurements**

For each line, ten male and ten female flies were collected 24 hours after hatching. Quantitative pigment measurements were performed as described in Schmitt *et al*, 2005. Mean values for three independent experiments are shown.

**SUPPLEMENTARY REFERENCES**


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**SUPPLEMENTARY FIGURE LEGENDS**

Supplementary Fig 1. Biallelic sense and antisense transcription in the *H19* ICR

Detection of *H19* transcripts in combination with ICR sense (A)-(C) and antisense (D)-(F) transcripts in mouse E14.5 fetal liver cells by double label RNA–FISH. Green signals are ICR transcripts, red signals are *H19* gene transcripts. DAPI staining is blue. Bars below the pictures show frequencies of the respective signals.

Supplementary Fig 2. *H19* ICR non-coding transcripts in *Drosophila* lines carrying a transgene containing the *H19* ICR

(A) Reverse transcription reaction primed with random primers. Primers for the subsequent PCR amplify sequences from the *H19* imprinting control region (lanes 1, 3, 5, and 6) or the *Tubulin* gene (lanes 2, 4, and 7). Flies not carrying the ICR transgene (-) are wild-type control flies. RT: Reverse transcriptase; RNase: Ribonuclease. (B) Reverse transcription reaction primed with strand specific primers against the antisense (lanes 1, 2, and 3) or the sense (lanes 4, 5, and 6) strand of the ICR transcripts. Subsequent PCR reactions were performed with primers to amplify a sequence from the *H19* imprinting control region. Flies not carrying the ICR transgene (-) are wild-type control flies. PCR products correspond to amplicons 5 and 6 in Fig 1, respectively.

Supplementary Fig 3. The *H19* ICR confers reporter gene silencing through an RNAi independent mechanism in *Drosophila*

(A)-(F) Eyes of flies carrying the *H19* ICR transgene combined with mutations in RNAi pathway genes: (A) wild-type genetic background, (B) *piwi* mutation $piwi^{1}/piwi^{2}$, (C) *dicer 2* mutation $dcr\text{-}2^{L811fsX}$, (D) *aubergine* mutation $aub^{\Delta P\text{-}3a/Qc42}$, (E) *r2d2* mutation $r2d2^{1}$, (F) *homeless* mutation $hls^{E1/E616}$.

(G) Detection of small RNAs in the *H19* ICR by Northern Blot

The source of the RNA loaded in each lane is indicated above the gel. The position of a band with the size of 21 to 23 nucleotides is indicated by the arrowhead. A signal present in all *Drosophila* RNA preparations resulting from non-specific hybridization to the *H19* probe is marked by the asterisk.

(H) Schematic representation of the GAL4 driven RNAi vector used in this study to express siRNAs directed against the *H19* ICR.

Supplementary Fig 4. Genetic crosses demonstrate the specificity of the GAL4 driver and ICR RNAi vector transgenes

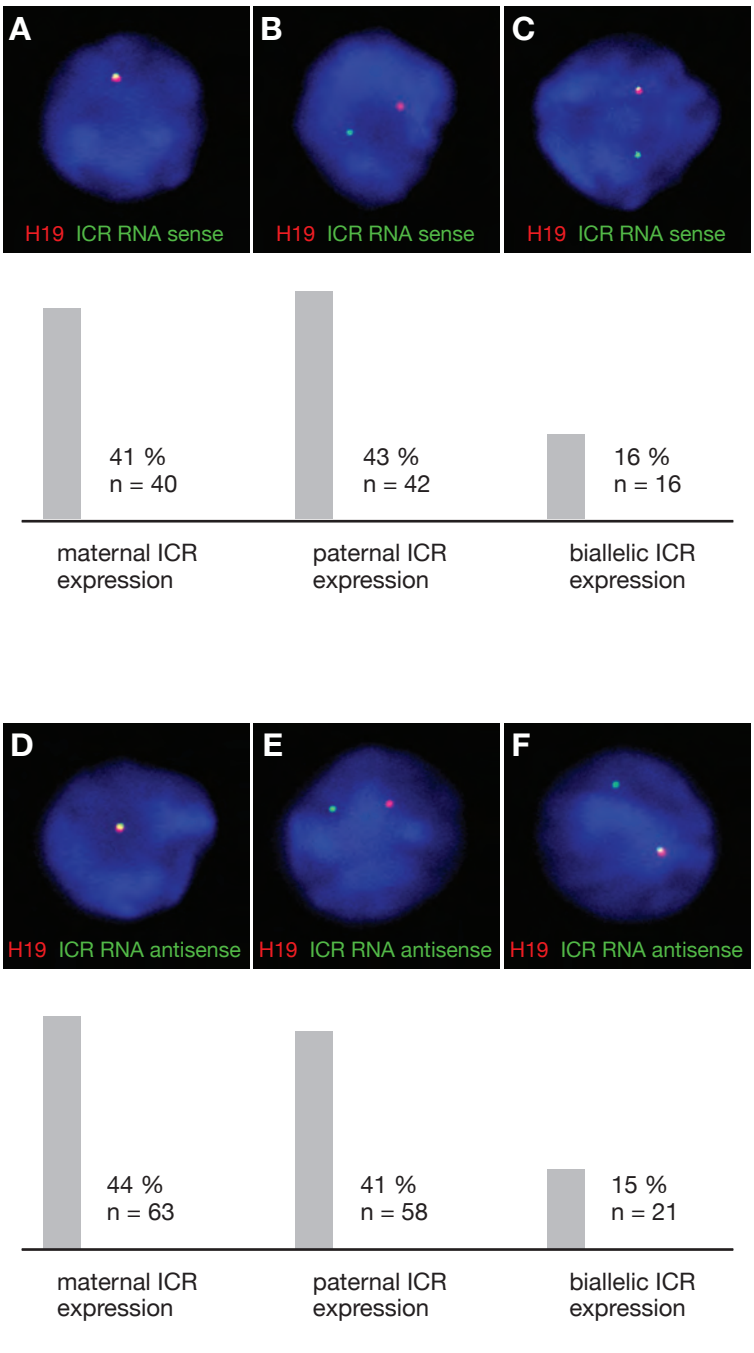
(A) Schematic representation of the *H19* upstream sequence controlling *mini-white* expression in transgenic *Drosophila* carrying the ICR transgene. The red bar symbolises the target sequence of the RNAi vector, the ICR and the silencer are represented by a yellow and a red box, respectively. (B) *H19* ICR transgene. (C) *H19* ICR transgene combined with GAL4 driver. (D) Schematic representation of the *H19* upstream sequence controlling *mini-white* expression in transgenic *Drosophila* carrying the ICR transgene Δ silencer. (E) *H19* ICR transgene Δ silencer. (F) *H19* ICR transgene Δ silencer combined with the RNAi vector transgene and the GAL4 driver transgene.

Supplementary Fig 5. Transcription in the transgenic *H19* ICR locus is independent of Su(Hw)

(A) Eyes of flies carrying the *H19* ICR transgene combined with mutations in genes encoding heterochromatin components: wild-type genetic background (left picture), *Su(var)3-9* mutation $Su(var)3\text{-}9^{19}$ (middle picture), *su(Hw)* mutation $su(Hw)^{v/f}$ (right picture).

(B) Strand specific reverse transcription reaction with primers in the *H19* ICR with (+RT) or without (-RT) reverse transcriptase. RNA samples are obtained from embryos of the respective *Drosophila* lines shown in (A): wild-type *H19* ICR transgenes (upper panel), and

*H19* ICR transgenes carrying a mutation in the *su(Hw)* gene (lower panel). PCR products correspond to amplicon 6 in Fig 1.

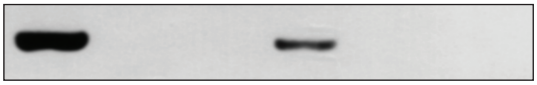



Supplementary Figure 1, Schoenfelder et al.

**A**

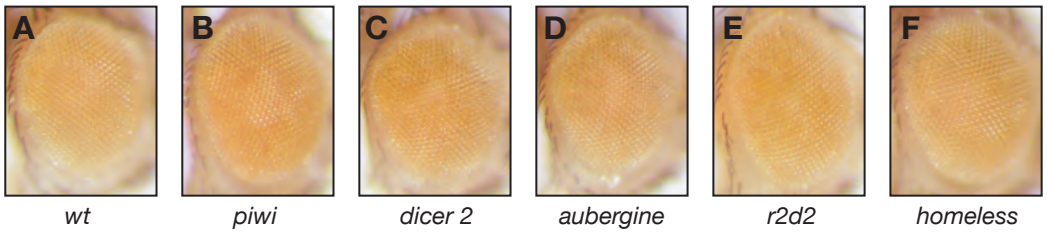
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| ICR transgene | + | + | + | + | + | - | - |
| RT | + | + | - | - | + | + | + |
| RNase | - | - | - | - | + | - | - |
| PCR primer | ICR | Tub | ICR | Tub | ICR | ICR | Tub |

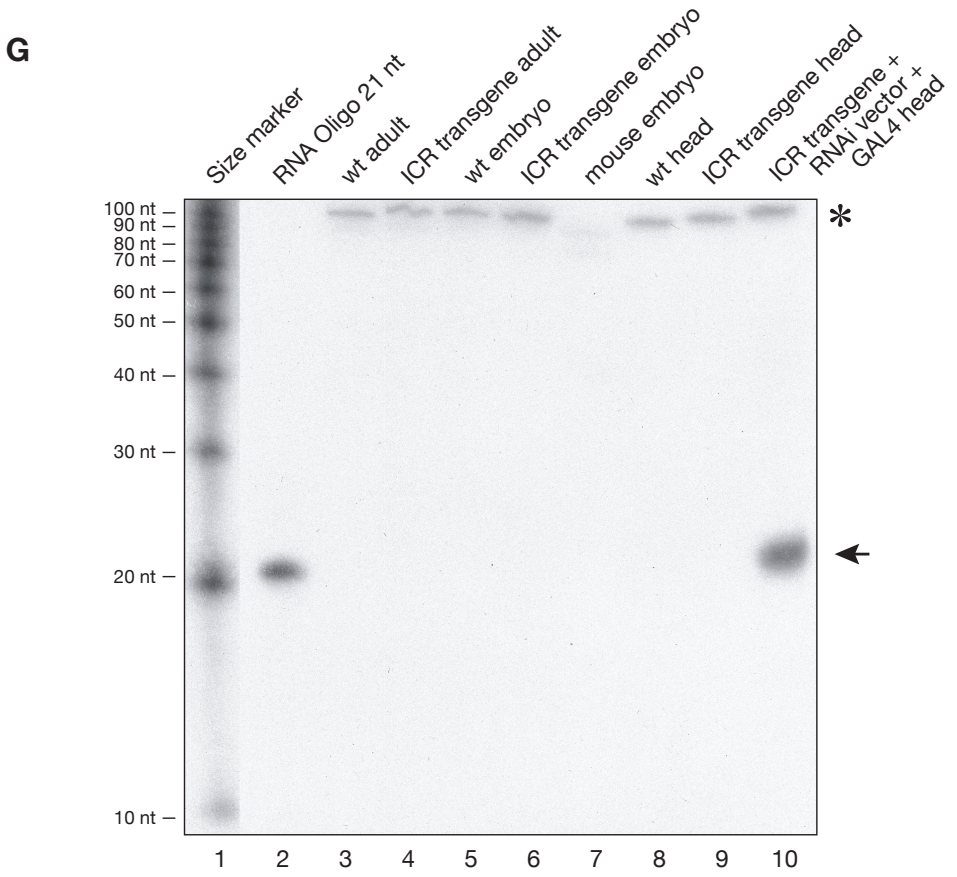
**B**

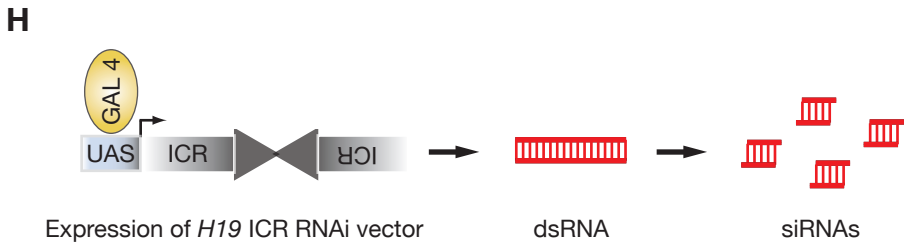


| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| ICR transgene | + | + | - | + | + | - |
| RT | + | - | + | + | - | + |
| | ICR RNA antisense | | | ICR RNA sense | | |

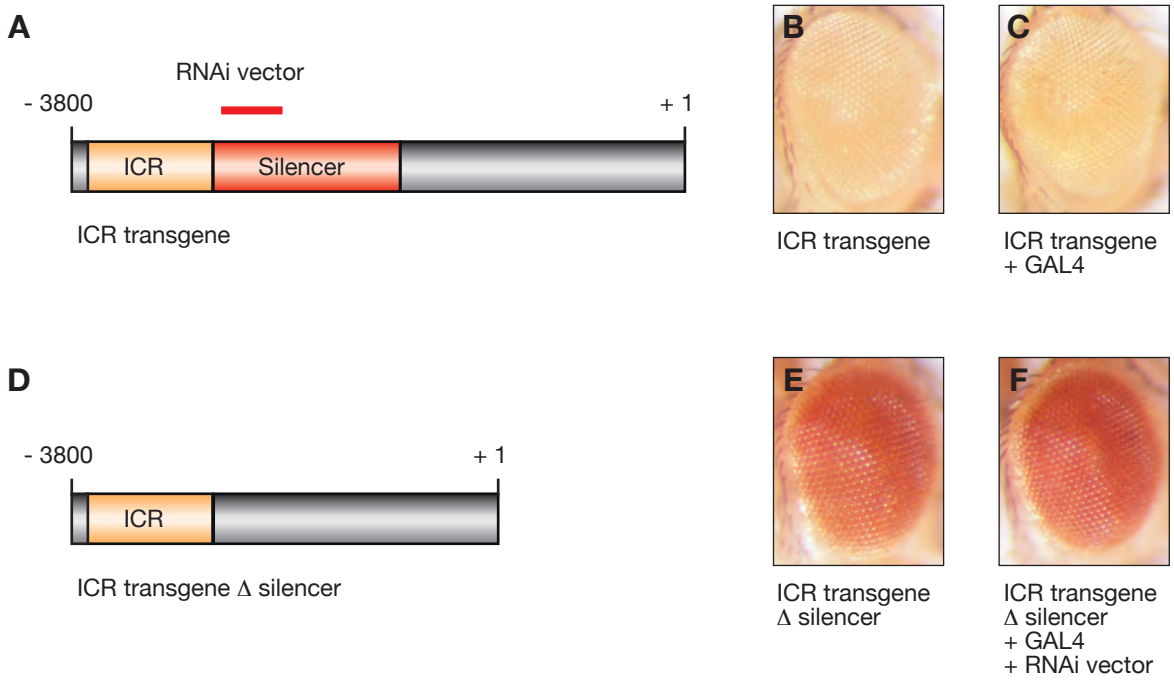
Supplementary Figure 2, Schoenfelder et al.



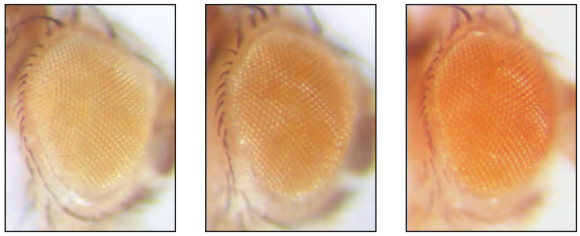




Supplementary Figure 3, Schoenfelder et al.

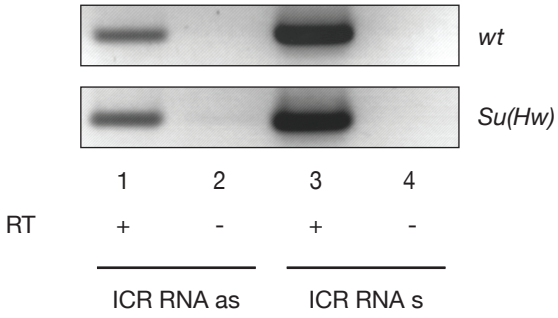



Supplementary Figure 4, Schoenfelder et al.

**A**




**B**




Supplementary Figure 5, Schoenfelder et al.