Age-associated de-repression of retrotransposons in the *Drosophila* fat body, its potential cause and consequence

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**Introduction**

Epigenetic modifications of chromatin are critical for many biological functions ranging from organism development to tissue homeostasis. Studies in multiple organisms have shown that maintaining the repressive epigenetic state of heterochromatin is important for lifespan extension. For example, pioneering studies in budding yeast have shown that replicative aging is correlated with heterochromatin loss in telomeric regions, mating type loci, and rDNA repeats (Kim et al., 1996; Smeal et al., 1996; Kennedy et al., 1997). Importantly, overexpression of Sir2, a histone deacetylase required for heterochromatin formation and gene silencing, induces an increased lifespan in yeast (Rogina & Helfand, 1999). The connection between heterochromatin maintenance and lifespan extension is further strengthened by studies in Caenorhabditis elegans and *Drosophila* (Rogina & Helfand, 2004; Hashimoto et al., 2010; Jiang et al., 2013; Whitaker et al., 2013). Disruption of UTX-1, a histone demethylase that reduces the heterochromatin epigenetic modification called histone H3 K27 trimethylation (H3K27me3), in *C. elegans* leads to increased H3K27me3 levels and prolonged lifespan (Jin et al., 2011; Maures et al., 2011). Moreover by modulating the expression of HP1, a protein required for the maintenance of heterochromatin, researchers have shown that increase or decrease HP1 levels leads to lifespan extension or shortening, respectively, in *Drosophila* (Larson et al., 2012). Consistently, old *Drosophila* exhibits both HP1 reduction and the reduction of pericentric heterochromatin as judged by a decrease in H3K9me3 modification (Wood et al., 2010; Larson et al., 2012; Chen et al., 2014).

Despite a clear connection between heterochromatin maintenance and lifespan extension (Feser et al., 2010; Meister et al., 2011; Larson et al., 2012; Jiang et al., 2013), the cause of heterochromatin loss and how it leads to cellular damage and aging is not well understood. Generally speaking, heterochromatin loss would lead to a mis-expression of genes that are normally repressed, which could result in age-associated cellular defects. Heterochromatin is also enriched for transposable elements (TE). As mobile elements, TEs, including both the retrotransposons and DNA transposons, can transpose from one chromatin location to another (Wicker et al., 2007). In doing so, they create DNA breaks that can lead to genome damages. Since accumulation of DNA damage is known to cause cellular senescence and organismal aging (Best, 2009), activation of TEs could contribute to organismal aging. Although TE activation during development has also been implicated as important for proper development of the nervous system (Reilly et al., 2013), in most settings TEs are repressed by the heterochromatin state to prevent uncontrolled transposition (Levin & Moran, 2011; Wood & Helfand, 2013). Therefore, in addition to de-repression of genes, the age-associated heterochromatin loss could also cause an increased expression of TEs, which would in turn contribute to age-associated cell and organ defects.

Consistent with the idea that TE activation could contribute toward aging (De Cecco et al., 2013b; Sedivy et al., 2013), increased expression of *Ty1* retrotransposon is correlated with increased chromosome rearrangements and genome instability in old yeasts (Maxwell et al., 2011; VanHoute & Maxwell, 2014; Patterson et al., 2015).

Additionally, increased expression of *Cer1*, which belongs to the gypsy/Ty3 family of retrotransposons, is observed in old *C. elegans* (Dennis et al., 2012). Studies of the old *Drosophila* brains have also revealed an increased expression of several TEs, including the gypsy retrotransposon (Li et al., 2013). More recent studies have reported increased expression of *Alu* retrotransposons in the retinal pigmented
epithelial cells from old humans, which can contribute to age-associated macular degeneration (Kaneko et al., 2011; Tarallo et al., 2012).

Interestingly, increased expression of Alu retrotransposons also occur when human adult stem cells undergo senescence (Wang et al., 2011; De Cecco et al., 2013a), which correlates with increased formation of DNA damage foci within the TE-enriched chromatin regions.

Considering the connection between TE activation and aging, it is important to understand what cellular changes during aging may contribute to heterochromatin loss and TE activation. Since mutations in some heterochromatin modifying enzymes have been shown to regulate the lifespan of C. elegans and Drosophila (Siebold et al., 2010; Jin et al., 2011; Maures et al., 2011), it is possible that age-associated changes of chromatin regulators could contribute to heterochromatin loss and TE activation. Nuclear lamins, the major structural components of the nuclear lamina that associate with the nuclear peripheral heterochromatin, are believed to contribute to the establishment and maintenance of the heterochromatin (Dechat et al., 2008; Bank & Gruenbaum, 2011; Kim et al., 2011; Meuleman et al., 2013). For example, lamins can help maintain heterochromatin by interacting with other heterochromatin regulators such as HP1 (Dechat et al., 2008; Bank & Gruenbaum, 2011). Interestingly, of the two A- and B-types of lamins, lamin-B1 (B-type) has been shown to undergo age- associated reduction in human skin keratinocytes (Dreesen et al., 2013). A similar reduction of the single Drosophila lamin-B protein, LAM (also called lamin Dm0), is observed in the old Drosophila fat body, a humoral immune organ that is equivalent to the liver and adipose tissue in mammals (Chen et al., 2014). The age-associated LAM reduction in fat body leads to gut hyperplasia in Drosophila. This suggests that LAM loss upon aging could contribute to disease. Although LAM reduction in fat body is shown to lead to the de-repression of immune responsive genes, it remains unclear whether such loss also influences TE repression.

Using genome-wide transcriptome analyses (RNA sequencing, RNA-seq), we have obtained the expression profiles of retrotransposons in young and old Drosophila fat bodies. Our analyses show that the old fat bodies exhibit a significant up-regulation of a large number of retrotransposons. Our further studies suggest that LAM loss upon aging could contribute to increased retrotransposon expression and DNA damage due, in part, to the loss of heterochromatin. We will discuss our finding in the context of age-associated pathologies.

Results

Increased expression of retrotransposons in old Drosophila fat bodies

Retrotransposons represent a major population of TEs in mammals and their de-regulation leads to many of the reported TE-associated human diseases (Hancks & Kazazian, 2012). Since retrotransposon activation is associated with their increased transcription, we performed RNA-seq using dissected Drosophila fat bodies (Fig. 1A) from young (5 days) and old (50 days) animals. By analyzing the differential expression of 111 annotated Drosophila retrotransposons, we found a 2.7-fold higher level of overall expression of total retrotransposons in old fat bodies than that of the young (Fig. 1B). Further analyses showed that 18 retrotransposons were significantly up-regulated in old fat bodies (fold change > 2, FDR < 0.05, Fig. 1C). These include 14 long terminal repeat (LTR) retrotransposons and four non-LTR retrotransposons. We also found 18 down-regulated retrotransposons upon aging, but both their overall expression level and the degree of down-regulation are low (Fig. 1B, Table S1). By contrast, the fold increase is high in the 18 up-regulated retrotransposons (Fig. 1B,C). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses further confirmed the age- associated de-repression of retrotransposons in fat bodies (Fig. 1D). Thus upon aging, ~16% of the annotated retrotransposons exhibit significant up-regulation in the Drosophila fat bodies.

Increased retrotransposon expression correlates with elevated DNA damage signature in old Drosophila fat bodies

Retrotransposon expression can lead to their replication and insertion back into the genome, which would lead to increased DNA breaks. Therefore, we compared DNA damage foci between the old and young fat bodies. Using an antibody to Drosophila γ-H2AvD (homologous to mammal γ-H2AX) known to label double stranded DNA breaks, we visualized and quantified the total intensity of the nuclear DNA damage foci. These analyses revealed a gradual increase of nuclear γ-H2AvD foci in 10, 30, and 50-day old fat bodies (Fig. 2A–C,H). Therefore, the increased expression of retrotransposons upon aging is correlated with an increased accumulation of γ-H2AvD foci in the nuclei of old Drosophila fat body cells.

We then asked whether forced activation of retrotransposons in young fat bodies could also lead to increased DNA damage as marked by γ-H2AvD. The endogenous siRNA pathway mediated by Dicer-2 (Dcr-2) and Argonaute2 (AGO2) have been shown to repress retrotransposon expression in Drosophila somatic cells. Previous studies have shown that AGO2 mutant flies or shRNA-mediated depletion of AGO2 caused increased expression of retrotransposons in Drosophila brain and S2 cells (Czech et al., 2008; Ghildyal et al., 2008; Li et al., 2013). Thus, we depleted AGO2 in the fat body (Cg-gal4>AGO2 shRNA) by shRNA (mediated by Dicer-1- AGO1) using two different fly RNAi lines (line 1: Bloomington Stock Center #34799, and line 2: Bloomington Stock Center #55672) that target different AGO2 sequences. We found that both lines resulted in a similar AGO2 depletion (Fig. 2D). Using qRT-PCR, we found that depleting AGO2 in 10-day-old fat bodies caused a significant increase in the expression of two retrotransposons (Fig. 2E) that we found to undergo age-associated up-regulation (see Fig. 1C,D). This increased expression of retrotransposons was accompanied by increased γ-H2AvD foci in the nuclei of fat bodies depleted of AGO2 (Fig. 2A,F,H). Therefore, retrotransposon up-regulation in fat bodies may lead to increased DNA damage.

Next, we asked whether DNA damage could be reduced in old fat bodies by repressing retrotransposon expression. We utilized an AGO2 EP line (Bellen et al., 2004) that allowed Cg-Gal4-induced overexpression of AGO2 in fat bodies. qRT-PCR confirmed an elevated expression of AGO2 (Fig. S1). AGO2 elevation resulted in a noticeable decrease of the number of nuclear γ-H2AvD foci in the old fat bodies (compare Fig. 2G to 2C). Our quantification using randomly selected nuclei showed a small but significant decrease of the average nuclear γ-H2AvD intensity in these old fat bodies (Fig. 2H). These findings suggest that increased expression of retrotransposons could contribute toward the increased DNA damage in old fat bodies.

LAM may repress the expression of multiple retrotransposons in fat bodies

Our previous studies have shown that Drosophila aging is accompanied by a gradual reduction of lamin-B, LAM (encoded by the gene Lam) (Chen et al., 2014). Since LAM is involved in maintaining heterochromatin, its loss could contribute to the age- associated increase in retrotransposon expression and DNA damage in fat bodies. We
therefore asked whether LAM depletion in young fat bodies could lead to a similar up-regulation of retrotransposons as seen in old fat bodies. The global expression profiles of retrotransposons appeared similar between old fat bodies from wild-type flies and young fat bodies depleted of LAM (Cg-Gal4/+; tub-Gal80ts/Lam RNAi) (Fig. 3A).

By comparing the retrotransposon expression profiles between 5-day old control fat bodies (Cg-Gal4/+; tub-Gal80ts/+) and the 5-day old flies with LAM depleted in fat bodies, we found that 17 retrotransposons were up-regulated at least 2-fold upon LAM depletion (Table S2). Importantly, 11 of these 17 de-repressed retrotransposons were also found to be de-repressed in wild-type fat bodies from old flies (Fig. 3B).

Additionally, most (64.7%) of these de-repressed retrotransposons exhibited similar degrees of up-regulation in both the young fat bodies depleted of LAM and the old fat bodies (Fig. 3C). qRT-PCR analyses further confirmed that LAM depletion in young fat bodies resulted in increased retrotransposon expression, whereas control depletion by LacZ RNAi or GFP RNAi in fat bodies had no effect (Fig. 3D and Fig. S2). This suggests that LAM could play a role in repressing the retrotransposons in the fat bodies.

Age-associated LAM loss may contribute to the formation of DNA damage foci in the nuclei of fat body cells

Since up-regulation of retrotransposons in the fat body resulted in increased DNA damage foci formation in aging flies and since LAM depletion in young fat bodies resulted in retrotransposon de-repression, we asked whether LAM depletion in young fat bodies could lead to increased DNA damage foci formation. Compared to young fat bodies from control flies (Cg-Gal4/+; tub-Gal80ts/+, Fig. 4A,E), we found that LAM-depleted young fat bodies (Cg-Gal4/+; tub-Gal80ts/Lam RNAi) exhibited a significant increase of nuclear γ-H2AvD foci (Fig. 4C,E). The degree of increase by LAM depletion was similar to those observed in the 50-day old control fat bodies (Cg-Gal4/+; tub-Gal80ts/+, Fig. 4A,E), whereas the control RNAi depletion of LacZ or GFP in fat bodies had no effect (Fig. 3E). Next, we asked whether expressing LAM in old fat bodies could reduce the DNA damage foci. LAM was expressed in the old fat body using the Gal4/Gal80ts-UAS system. We found that induced expression of LAM in old fat bodies (Cg-Gal4/+; tub-Gal80ts/UAS-Lam) resulted in noticeable decrease in the number and intensity of the DNA damage foci, suggesting that LAM could play a role in regulating DNA damage foci formation.

Fig. 1 Age-associated up-regulation of retrotransposon expression in Drosophila fat bodies. (A) A cartoon illustration of adult Drosophila dorsal abdomen. Adult fat body cells (green) align the inner cuticle surface of abdominal segments (A1-6). The oenocytes (pink) and cardiac tube (orange) are also shown. All immunofluorescence images of the fat body cells in this manuscript were captured from the A4 region. (B) MA-plot comparing the retrotransposon RNA-seq data between young and old fly fat bodies. Each dot represents one retrotransposon. X-axis shows the mean RNA-seq expression level of retrotransposons in young and old fat bodies, calculated by log2 (reads per million reads). Y-axis shows the mean log2 expression change of retrotransposons in old fat bodies compared to young. The red line is the linear regression, showing that retrotransposons expressed at high levels in young and/or old fat bodies are more likely to be further up-regulated in old fat bodies. (C) A list of retrotransposons that are up-regulated by more than 2-folds in old fat bodies compared to young. The numbers and color codes indicate the fold changes. (D) qRT-PCR analyses of the indicated retrotransposons that was found to be up-regulated by RNA-seq in old fat bodies. The fold expression change of 50-day fat bodies was plotted relative to 5-day fat bodies, which was set to 1. Error bars, standard error of the mean (SEM) based on three independent experiments. Student’s t-tests: *P < 0.05, **P < 0.01.

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Fig. 2 Retrotransposon de-repression leads to DNA damage in old fat bodies. (A–C) Representative immunostaining images of γ-H2AvD (green) and DNA (DAPI, red) labeling in fat bodies (from the A4 region). 10-day-old young control fat body cells (A, Cg-gal4/+; tub-gal80ts/+) had a few faint nuclear γ-H2AvD signals, which was gradually increased by 30 (B) and 50 days (C). Nuclei outlined by white squares are enlarged to the right. White arrows indicate γ-H2AvD staining foci. Scale bars, 20 μm. (D) Two independent fly lines carrying fat body specific RNAi (Bloomington Stock Center, #34799 and #55672) driven by Cg-gal4;tub-gal80ts exhibited significant reduction of AGO2 in fat body. The fold change of AGO2 expression was determined relative to the Gal4 control flies (Cg-gal4/+; tub-gal80ts/+), which was set to 1. Error bars, SEM based on three independent experiments. Student’s t-tests: *P < 0.05, **P < 0.01. (E) Depletion of AGO2 in the fat body by the two different AGO2 RNAi allele (Cg-gal4/AGO2 RNAi#1; tub-gal80ts/+ and Cg-gal4/AGO2 RNAi#2; tub-gal80ts/) significantly increased the expression of two retrotransposons (BLASTOPIA and DM1731) that were found to undergo age-associated up-regulation in fat body. The fold expression change was determined relative to the Gal4 control flies (Cg-gal4/+; tub-gal80ts/+), which was set to 1. Error bars, SEM based on three independent experiments. Student’s t-tests: *P < 0.05, **P < 0.01. (F–G) Depletion of AGO2 (Cg-gal4/AGO2 RNAi#1; tub-gal80ts/) by RNAi in fat bodies increased γ-H2AvD signal in 10-day-old young fat bodies (F), whereas the expression of AGO2 (Cg-gal4/+; tub-gal80ts/UAS-AGO2EP) in fat bodies reduced γ-H2AvD signal in 50-day old young fat bodies (G). Nuclei outlined by white squares are enlarged to the right. White arrows indicate γ-H2AvD staining foci. Scale bars, 20 μm. (H) Quantification for γ-H2AvD intensities in fat body cells from 10-, 30-, or 50-day old Gal4 control flies (Cg-gal4/+; tub-gal80ts/+), UAS control flies (for AGO2 RNAi#1, AGO2 RNAi#2, and AGO2 EP line), 10-day-old flies with AGO2 depleted in fat body (Cg-gal4/AGO2 RNAi#1; tub-gal80ts/+ and Cg-gal4/AGO2 RNAi#2; tub-gal80ts/+), and 50-day old flies with extra AGO2 expression in fat body (Cg-gal4/+; tub-gal80ts/UAS-AGO2EP). The γ-H2AvD signal was determined by measuring the total nuclear immunofluorescence signal from the images captured using the same microscopy settings (see the detailed description in the experimental procedures). Nuclei from the A4 region (see Fig. 1A) were measured to allow the comparison among different fat bodies. Error bars, SEM. Student’s t-test: *P < 0.05, **P < 0.01. The numbers (n) of fat body cells analyzed shown were from three independent experiments.

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Lamin-B may repress retrotransposons in vivo, H. Chen et al.

Previous studies show that retrotransposons are regulated by both chromatin structure and RNAi machinery. Since Dicer-2-AGO2-mediated endogenous small interfering RNA (esiRNAs) pathway regulates the expression of TEs in Drosophila somatic cells, we examined whether Dicer-2 and AGO2 undergo age-associated expression change. Both RNA-seq and RT-qPCR revealed similar expression of Dicer-2 and AGO2 in young and old fat bodies (Fig. S3A, B). Therefore, retrotransposon derepression upon natural aging or upon LAM depletion in fat body is not due to decreased expression of the two proteins required for the esiRNA pathway.

Since LAM could contribute toward heterochromatin maintenance by binding to chromatin regulators such as HP1, we wish to determine whether LAM reduction in old fat bodies might contribute to retrotransposon de-repression by reducing the histone H3 lysine 9 trimethylation (H3K9me3) modification. Unfortunately, it is very difficult to obtain enough pure fat body cells from adult flies for genome-wide analyses based on chromatin immunoprecipitation sequencing (ChIP-seq). On the other hand, one can obtain relatively large quantities of pure third instar larva (L3) fat bodies (Fig. S5A) for ChIP-seq. Since Lam<sup>−/−</sup> (Lam<sup>Dros</sup>/Lam<sup>2</sup>) flies could survive to third instar larvae stage, we first analyzed whether the Lam<sup>−/−</sup> fat bodies exhibited retrotransposon derepression by RNA-seq. We identified 51 up-regulated retrotransposons (fold change >2, FDR < 0.05) in Lam<sup>−/−</sup> larvae fat bodies compared to those of the wild-type (Table 1). Among these up-regulated retrotransposons, 14 were also up-regulated either in the old fat bodies or in the LAM-depleted young fat bodies. Next, we dissected fat bodies from wild-type and Lam<sup>−/−</sup> third instar larvae and performed H3K4me3 or H3K9me3 ChIP-seq. We found a significant decrease of H3K9me3 levels in the chromatin regions containing the de-repressed retrotransposons, whereas the H3K4me3 was increased in these regions for most retrotransposons. This finding can be demonstrated by plotting the log2 fold change of H3K4me3 or H3K9me3 for each retrotransposon on the y-axis using the box plot (Fig. S3C), which shows that upon LAM depletion, most retrotransposons exhibit H3K4me3 increase (>0 on y-axis) and H3K9me3 decrease (<0 on y-axis). By analyzing H3K9me3 and H3K4me3 levels on the de-repressed retrotransposons, we found that the de-repression is accompanied by a significant increase and decrease of H3K4me3 and H3K9me3, respectively (Table 1). We further confirmed these findings by ChIP-qPCR analyses of four retrotransposons selected from Table 1 using dissected fat bodies from wild-type and Lam<sup>−/−</sup> third instar larvae (Fig. S4A). Moreover, we present the genome browser views for retrotransposon RNA, H3K4me3, and H3K9me3 reads for two de-repressed retrotransposons, BLASTOPIA and Invader4, obtained from the wild-type and Lam<sup>−/−</sup> L3 larvae (Fig. S8B–E).

Although it is difficult to obtain enough pure fat bodies from old flies for ChIP-seq, we succeeded to use ChIP-qPCR to study H3K9me3 and H3K4me3 modifications of four selected de-repressed retrotransposons (BLASTOPIA, Gypsy6, Invader4 and QUASIMODO) due to aging or LAM deletion in the dissected control young or old wild-type fat bodies and the LAM-depleted young fat bodies. We found that these retrotransposons indeed exhibited decreased H3K9me3 and increased H3K4me3 upon aging or upon LAM depletion (Fig. S5F and Fig. S4B). Taken together, our findings suggest that LAM reduction upon aging could contribute to the elevated DNA damage foci formation in the nuclei of this tissue.
contribute to the loss of heterochromatin and retrotransposon activation in the fat bodies.

Discussion

By analyzing a few selected retrotransposons, previous studies have shown that organismal aging can lead to the activation of retrotransposons in several tissues. There has been, however, no report on genome-wide analyses of retrotransposon de-repression in dissected old tissues thus far. Here we report that ~16% of retrotransposons undergo over 2-fold increase in expression in the old Drosophila fat bodies. Our analyses suggest the possibility that retrotransposon up-regulation could contribute to the increased formation of DNA damage foci in this Drosophila tissue. By analyzing the Lam/C0 fat bodies from the third instar larvae, the LAM-depleted young fat bodies, and the old fat bodies known to exhibit LAM reduction, our findings suggest that LAM loss could contribute to the loss of heterochromatin-based repression of retrotransposons. Based on our findings, we propose a speculative model that increased formation of DNA damage foci in the old fat body, caused in part by the increased retrotransposon expression as a result of LAM reduction, contribute to fly aging phenotypes (Fig. 6). It will be important to further establish whether and how LAM directly contributes to the establishment and maintenance of the heterochromatin in the fat body. Further studies will be required to determine whether the increased retrotransposon expression is accompanied by increased retrotransposition in the fat body.

Previously, we have shown that age-associated LAM reduction in fat bodies leads to increased systemic inflammation, which contributes to gut hyperplasia in old flies (Chen et al., 2014). Repression of activities of the immune deficiency (IMD) pathway in old fat bodies can only partially reverse the hyperplastic phenotype in the gut. While the partial rescue could be due to insufficient RNAi-mediated silencing, it is also possible that the age-associated LAM reduction in the fat body could contribute to gut hyperplasia due to increased DNA damage in fat body cells. Interestingly, DNA damage is known to trigger alterations of metabolism in cells (Shimizu et al., 2014). As the major organ that controls sugar and lipid metabolism, increased DNA damage in fat bodies may contribute to gut hyperplasia independent of the inflammatory pathways. Despite the potential connection between DNA damage and disease, we stress that the increased DNA damage foci formation as revealed by the γ-H2AvD...
staining may not reflect an actual increase in double strand DNA breaks. Therefore, it will be important to further explore whether the age-associated increase in retrotransposon expression in fat bodies indeed leads to increased DNA damage, how the DNA damage may contribute to the metabolic dysfunction of this organ, and how this may in turn influence the gut and other organs.

The declined ability to maintain heterochromatin has been established as one of the major culprit for aging phenotypes. A number of studies have shown that Lamin-B may repress retrotransposons in vivo, H. Chen et al. 2016.

Fig. 5 LAM inhibits retrotransposon expression by maintaining heterochromatin. (A) A cartoon illustration of the third instar larvae. Fat body (orange) aligns the inner cuticle surface of most of the larval body. The mouth hook (black) and salivary gland (blue) are also shown. (B–E) RPKM (Reads Per Kilobase per Million mapped reads) values of RNA expression, H3K4me3, and H3K9me3 modification for the indicated two retrotransposons (BLASTOPIA and Invader4) in the wild-type and Lam advertise (Lam2395/Lam152) third instar fat bodies. Increased retrotransposon expression was accompanied by decreased H3K9me3 and increased H3K4me3 modifications. (F) Chromatin immunoprecipitation (ChIP)-qPCR analysis of selected retrotransposons (BLASTOPIA and Invader4) of fat body dissected from the 5-day or 50-day wild-type (w1118), 5-day Lam advertise (Lam2395/Lam152) flies. Chromatin was immunoprecipitated with antibodies to H3K4me3, H3K9me3, or control IgG. Primers corresponding to BLASTOPIA, Invader4, and rp49 (control) were used to amplify precipitated DNA. ChIP samples were normalized to the input DNA. Error bars, SEM, based on three independent experiments. Student’s t-tests: ^P > 0.05, *P < 0.05, **P < 0.01.
have shown that heterochromatin loss can lead to the activation of TEs. Age-associated decline of histones or chromatin regulators known to control the heterochromatin state have been shown to contribute to the loss of heterochromatin and TE activation. More recently, DICER1 decline in aging retina has been linked to Alu retrotransposon activation and macular degeneration (Tarallo et al., 2012). Our studies add another protein, lamin-B, into the list of proteins whose age-associated decline may contribute to retrotransposon activation. It will be important to further dissect how these proteins work together to maintain the heterochromatin-mediated silencing of TE.

Additionally, it is not clear why aging is associated with the decline of some proteins but not the others. It is possible that the decline of one of these nuclear proteins leads to the reduction of other nuclear and cytoplasmic proteins through transcriptional regulation. Alternatively, different pathways may regulate the decline of different nuclear proteins. Finally, it is unclear whether the age-associated decline in protein levels as detected by Western blotting or immunostaining is due to protein reduction or protein modification change, as altered modifications may not only block antibody recognition but could also result in a dysfunctional protein. Research toward addressing how aging leads to the reduction of proteins or protein functions required for maintaining heterochromatin will help to answer these questions.

### Experimental procedures

**Fly stocks, genetic manipulations, and culture condition**

We used w^{1118} (Bloomington Stock Center #3605) as the wild-type fly. All other alleles were backcrossed into w^{1118} flies for at least five generations. LAM lines: LamD395 (null) (Munoz-Alarcon et al., 2007), Lam^{2} (LAM truncation, Bloomington Stock Center #25093) (Patterson et al., 2004), Lam^{395}/Lam^{2} transheterozygous (as Lam^{−/−}), and Lam cDNA transgenic line (UAS-Lam354) (Munoz-Alarcon et al., 2007). RNAi alleles: Lam RNAi (Vienna Drosophila Resource Center, #v45635), AGO2 RNAi (Bloomington Stock Center, #34799 and #55672), LacZ RNAi (from Allan Spradling), and GFP RNAi (Bloomington Stock Center, #9330). UAS alleles: AGO2EY04479 (Bloomington Stock Center, #16608). Gal4 alleles used to drive UAS lines: Cg-Gal4 (Bloomington stock center # 7011) for fat body. The crosses were maintained at 19°C to repress the UAS-GAL4 system. One day or five days after eclosion, the adult were shifted to 29°C to turn on the UAS-Gal4 system, which induces RNAi. These flies were then incubated at 29°C for 5 days followed by immunostaining, qRT-PCR, RNA-seq, or Western blotting analyses. The Gal4 lines and UAS lines, which were treated in the same way in parallel, were used as controls.

To inhibit AGO2 expression in fat body of 5-day old flies, Cg-Gal4; tub-Gal80^{D18} was used to drive AGO2 shRNA alleles. The crosses were maintained at 19°C to repress the UAS-GAL4 system. One day before eclosion, the pharate adult were shifted to 29°C and the eclosed flies were maintained at 29°C for 5 days followed by dissection of fat bodies for immunostaining or qRT-PCR. The Gal4 lines (Gal4s with tub-Gal80^{D18}) and

| Retroposon name | RNA expression | H3K4me3 | H3K9me3 |
|-----------------|----------------|---------|---------|
| ACCORD*         | 8.80           | 1.33    | −1.60   |
| ACCORD2^†       | 3.90           | 7.67    | −2.32   |
| Baggins1        | 3.32           | 2.53    | −2.04   |
| BATUMI          | 3.51           | 3.20    | −2.25   |
| BEL             | 2.33           | 3.11    | −1.66   |
| BLASTOPIA^*     | 4.83           | 1.43    | −1.40   |
| BLOOD           | 3.90           | 2.14    | 0.92    |
| BS2             | 2.30           | 2.08    | −1.97   |
| Copia2          | 11.09          | 2.69    | −1.83   |
| DIVER           | 5.45           | 1.88    | −1.34   |
| DM176           | 8.16           | 1.46    | −1.51   |
| DM297^*         | 11.11          | 2.71    | −0.88   |
| DMCR1^A         | 4.55           | 1.85    | −2.02   |
| DMLR^S          | 2.31           | 1.40    | −2.12   |
| DMTO1^M         | 3.58           | 2.03    | −1.51   |
| DOC             | 2.27           | 1.72    | −2.18   |
| DOCS            | 2.79           | 3.06    | −1.37   |
| GSA             | 10.05          | 1.80    | −2.02   |
| Gypsy10         | 2.72           | 1.44    | −1.69   |
| Gypsy11         | 2.24           | 3.46    | −1.84   |
| Gypsy12^*       | 3.06           | 10.71   | −1.93   |
| Gypsy4          | 4.53           | 2.61    | −2.52   |
| Gypsy5          | 2.49           | 1.63    | −2.82   |
| Gypsy6^†        | 4.81           | 1.88    | −2.12   |
| HMRB1AGE-I       | 3.27           | 1.38    | −1.47   |
| I-Element        | 4.20           | 4.32    | −1.21   |
| Invader1        | 3.62           | 2.25    | −1.73   |
| Invader2        | 2.81           | 2.10    | −1.94   |
| Invader3        | 2.32           | 2.10    | −1.99   |
| Invader4^†       | 9.54           | 1.90    | −1.55   |
| Invader5        | 6.30           | 4.40    | −1.55   |
| LINE1           | 4.19           | 0.79    | −1.78   |
| MDG1            | 3.31           | 1.41    | −2.41   |
| MICROPIA^†       | 19.44          | 2.05    | −9.00   |
| NOMAD           | 4.80           | 1.31    | −3.60   |
| QUASIMODOS2     | 2.22           | 3.19    | −1.22   |
| QUASIMODOS2^†   | 2.75           | 3.08    | −1.49   |
| R1-2            | 12.99          | 2.70    | −2.24   |
| R2              | 12.05          | 6.87    | −1.74   |
| ROO             | 2.88           | 1.23    | −1.83   |
| ROOA            | 4.42           | 2.15    | −1.63   |
| ROVER^*         | 11.91          | 2.41    | −1.16   |
| Stalker2        | 12.53          | 1.18    | −1.17   |
| STALKER4^†      | 6.07           | 1.94    | −1.69   |
| TAHRE^†         | 21.65          | 1.38    | 0.87    |
| TAR             | 2.19           | 0.80    | 0.70    |
| TRAN^†          | 5.65           | 3.55    | −1.02   |
| TOM             | 14.55          | 2.99    | −1.23   |
| TTV1            | 6.55           | 3.93    | −0.88   |
| ZAM^*           | 2.46           | 3.04    | −1.34   |

* Red and blue color codes indicate the fold changes. ** or † mark the retrotransposons that were also up-regulated in old or LAM-depleted young adult fat bodies, respectively.

Table 1 A list of retrotransposons whose expressions are increased in the Lam^{−/−} (Lam^{395}/Lam^{D18}) L3 larvae along with the changes of H3K4me3 and H3K9me3 type.
UAS lines, which were treated in the same way in parallel, were used as controls.

To rescue aging phenotype by ectopic LAM or AGO2 expression in fat body, we used Cg-Gal4; tub-Gal80 to drive UAS-Lam cDNA or AGO2 EP line, respectively. The crosses were performed at 19 °C and shifted to 25 °C one day before eclosion. To induce LAM or AGO2 expression in adult fat body, we shifted the flies from 25 °C to 29 °C for 24 h on days 15, 25, 35, and 45. The fat bodies were then dissected on day 50 and processed for immunostaining. The Gal4 lines (Gal4s with tub-Gal80ts) and UAS lines, which were treated in the same way in parallel, were used as controls.

Adult fat body dissection, qRT-PCR, RNA-seq and analyses

Fat body dissection, RNA isolation, qRT-PCR, RNA-seq and analyses were described previously (Chen et al., 2014).

qRT-PCR primers

Blastopia:
Forward primer (F): GAGCAGTCAATCGTCCGTAA
Reverse primer (R): TCTATAGTCCACGCAAACGC

Burdock:
F: GGACGCGTTACGTGTATTTG
R: ATAAGGGCGAATTGGTAACG

ADO2:
F: ATTACCATTACCGCCTCAGC
R: GGACAGGTCAAGGTCCAGAT

Dicer-2:
F: ACTATGTGCGCATTCGCTC
R: TGGGACTCCTTTCTCTGCTT

DM1731:
F: GAGAAATCACTTTGGGCCAT
R: TCGTCGCTGGTCTACAGTTC

G6:
F: AAAGGACTCCACCACTCCAC
R: GTTCTTCTGCCAATGGGATT

Gypsy12:
F: TGACTCGGCTGATGTTTCTC
R: GAAACACAGGTGGAATCGTG

IDES:
F: CGCTCTAGTGACAGAACCA
R: TGAATGAGGCCATTGTGGGTA

Invader4:
F: TCCGATCGAGTGGTACATAAA
R: CGACGTCAGCAGTCAAACCT

IVK:
F: ACTCTGAGGTCCAGATCATC
R: GGTCCTTGAGTAAAAACGGA

QUASIMODO:
F: TCTCAGTGCCATCGAGAGG
R: TAGTCAGCAGCAGTCAAACGGA

ROVER:
F: CAGACCGCAACTCAAATCCCT
R: CGTTGTCGCTGCTCGTCT

TAHRE:
F: ATCCAGCCCAACTCAATCCT
R: CGTTGTCGCTGCTCGTCT

ZAM:
F: GATGGTACTCCAGGCCAAGCT
R: AGAGCTTCGTCTGCTGTA

rp49:
F: TACAGGGCCAAAGTCGTGA
R: TCTCCTTGGCCTTCTTGGA

Immunofluorescence microscopy and quantifications

Adult fat bodies with attached cuticles were dissected in PBs. The details of the staining procedure have been described previously (Chen et al., 2014). All primary antibodies were preabsorbed using embryos fixed by 4% paraformaldehyde. The following primary antibodies were used: rabbit anti-Histone H2AvD pS137 (anti-γ-H2AvD), Rockland #600-401-914, 1:5000), mouse anti-LAM (DSHB #ADL67, 1:100). The secondary antibodies used, Alexa 488 and Alexa 568 from Molecular Probes/Invitrogen (1:1000). DNA was stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma) at 1 μg/mL.

A Leica TCS-SP5 confocal microscope was used to acquire all immunofluorescence images. For each set of experiments, images were acquired as confocal stacks using the same setting. Adobe Photoshop and Adobe Illustrator CS3 were used to assemble the images.
To quantify the nuclear immunofluorescence intensity of γ-H2AvD, the fat body nuclei were imaged using an Epitome microscope (Zeiss ApoTome.2). We used the same method as described in our previous paper (Wan et al., 2015) to determine the nuclear fluorescence signal from the epifluorescence images. Briefly, we defined the nuclear regions by drawing a circle X based on the DAPI staining. Then, we draw a larger circle around the nucleus as Y. The areas of X and Y were determined as Xa and Ya. The mean fluorescence intensity for X and Y were measured as Xf and Yf.

The signal intensity for each nucleus was defined ad Xi and was determined as Xi = Xf – (Yf × Ya – Xf × Xa)/(Ya – Xa). All experiments were performed three times. About 50 nuclei were quantified from these experiments for each genotype and plotted.

ChiP-seq analyses and additional detailed descriptions of all methods can be found in the Supporting information.

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Conflict of interest
None declared.

Author contributions
Haiyang Chen designed and performed all experiments, Xiaobin Zheng analyzed all the RNA-seq and ChiP-seq data, Haiyang Chen and Yixin Zheng conceived the project, interpreted the data, and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1 (related to Figure 2). The expression of AGO2 significantly increased in fat bodies in the Cg-gal4-driven AGO2 EP line compared to controls.

Fig. S2 (related to Figure 3). The expression of retrotransposons does not significantly change in young fat bodies in the control flies with Cg-gal4-driven GFP RNAi as compared to flies carrying no GFP RNAi.

Fig. S3 (related to Figure 5). The expression of Dicer-2 or AGO2 did not significantly change either in aged fat bodies or lamin-B depleted young fat bodies.

Fig. S4 (related to Figure 5). ChIP-qPCR analyses of H3K4me3 and H3K9me3 on selected retrotransposons in fat bodies.

Table S1 (related to Figure 1). A list of retrotransposons that were down regulated in wild type 50-day-old fat bodies as compared to that of the young (5-day).

Table S2 (related to Figure 1). A list of retrotransposons that were up regulated in 5-day old fat bodies upon LAM depletion.

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