The fruit fly acetyltransferase chameau promotes starvation resilience at the expense of longevity

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Abstract

Proteins involved in cellular metabolism and molecular regulation can extend lifespan of various organisms in the laboratory. However, any improvement in aging would only provide an evolutionary benefit if the organisms were able to survive under non-ideal conditions. We have previously shown that Drosophila melanogaster carrying a loss-of-function allele of the acetyltransferase chameau (chm) has an increased healthy lifespan when fed ad libitum. Here, we show that loss of chm and reduction in its activity results in a substantial reduction in weight and a decrease in starvation resistance. This phenotype is caused by failure to properly regulate the genes and proteins required for energy storage and expenditure. The previously observed increase in survival time thus comes with the inability to prepare for and cope with nutrient stress. As the ability to survive in environments with restricted food availability is likely a stronger evolutionary driver than the ability to live a long life, chm is still present in the organism's genome despite its apparent negative effect on lifespan.

Keywords acetylation; chameau; Drosophila; HBO1; starvation

Subject Categories Chromatin, Transcription, & Genomics; Metabolism; Post-translational Modifications & Proteolysis

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Introduction

Organisms respond to a changing environment by complex physiological and behavioral adaptations (Flatt, 2020). These are triggered by changes in gene expression or metabolism. The availability of nutrients is one of the most dynamically changing extrinsic parameters. Therefore, a tight regulation of nutrient storage and energy expenditure is essential for survival (Zinke et al., 2002; Hsieh et al., 2022). While hormonal regulation of metabolism at an organismic level is well established, the existence of intracellular feedback loops that regulate storage and usage of metabolites is largely underexplored. Metabolic pathways are often regulated by positive- or negative-feedback loops mediated by key metabolites. While these loops result in fast changes in metabolic fluxes, organisms can also adapt to long-term changes in nutrient availability by transcriptional and posttranscriptional regulation of key enzymes (Carthew, 2021). The coupling of metabolite concentrations to transcriptional regulators has been suggested to constitute an important and yet understudied principle of regulation (Katada et al., 2012). Several transcription factors have been shown to respond to nutrients via the mTOR pathway, CAMP signaling, or even by direct binding of metabolites to transcription factors. However, nutritional changes can also result in an altered activity of enzymes or transcription factors by changing their posttranslational modification status. Such modifications can result not only in transient changes in the metabolic flux in case of enzymes (Shi & Tu, 2015; Ye & Tu, 2018; Hsieh et al., 2022) but also in alterations of gene expression programs that result in more sustainable adaptation (Rodrigues et al., 2021). The latter is often affected by modifications of histones or sequence-specific transcription factors. In fact, multiple transcription factors, such as C/EBPα (Zaini et al., 2018), PGC1 (Jeninga et al., 2010), (spargel), or dFoxo (Molaei et al., 2019), have been shown to be dynamically acetylated upon metabolic variation.

Chameau (chm) is one of four MYST domain-containing acetyltransferases in Drosophila and is involved in the specification of replication origins and in JNK/AP-1-dependent transcription during fly development (Aggarwal & Calvi, 2004; Miotto et al., 2006; McConnell et al., 2012). Its human ortholog HBO1 (HAT bound to ORC1; Grienenberger et al., 2002) also plays a role in DNA replication (Iizuka & Stillman, 1999) and JNK-mediated transcriptional activation (Miotto et al., 2006). Moreover, HBO1 and chm are also involved in metabolic reprogramming metastasizing colorectal cancer cells (Wu et al., 2015) and during the differentiation of hematopoietic progenitors (Tiwari et al., 2020), respectively. Consistent
Figure 1. Loss of chm and reduction in its activity results in physiological changes in weight/size.

A Image of 8- to 9-day-old male control (w¹¹¹⁸, left) and chmRNAi (right) flies upon ubiquitous chm knockdown (left) and the corresponding quantification (right; N = 6, paired t-test was performed for statistical significance).

B Image of 8- to 9-day-old male control (w¹¹¹⁸, left) and chmRNAi (right) flies upon fat body–specific chm knockdown (left) and the corresponding quantification (right; N = 4, paired t-test was performed for statistical significance).

C Image of 8- to 9-day-old male control (w¹¹¹⁸, left) and chmRNAi (right) flies upon neuron-specific chm knockdown (left) and the corresponding quantification (right; N = 3, paired t-test was performed for statistical significance).

D Image of 8- to 9-day-old male control (w¹¹¹⁸, left) and chmRNAi (right) flies upon muscle-specific chm knockdown (left) and the corresponding quantification (right; N = 3, paired t-test was performed for statistical significance).

E Image of 8- to 9-day-old male control (w¹¹¹⁸, left) and chmMYST/+ (right) flies (left) and the corresponding quantification (right; N = 5, paired t-test was performed for statistical significance).

F Comparison of weight difference between enzymatic mutant and RNAi flies with varying specificity of chm knockdown (N = 3 (neuron- and muscle-specific RNAi), 4 (fat body-specific RNAi), 5 (MYST/+ mutants), and 6 (ubiquitous RNAi); Tukey post-hoc test was performed for statistical significance, ANOVA P-value = 0.012).

Data information: All replicates are independent biological replicates and error bars indicate standard error of the mean (SEM). Paired t-test or Tukey test was performed as indicated and non-significant values are not shown (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

Source data are available online for this figure.
with the hypothesis that HBO1/chm evolved to sense environmental changes, chm exhibits a strong sequence variation between tropical and temperate populations of Drosophila melanogaster (Levine & Begun, 2008). A homozygous deletion of chm in Drosophila results in pupal lethality of the organism (Grienenberger et al., 2002) but a partial reduction in chm in Drosophila or HBO1 in mice extends lifespan (Peleg et al., 2016a; Wang et al., 2021) when animals are fed ad libitum. In Drosophila, this lifespan extension is caused by a lower responsiveness of chm mutant flies to potentially dangerously increased levels of acetyl-CoA in aging animals (Peleg et al., 2016a). Therefore, we wondered whether the ability of flies to cope with longer lifespan of chm mutant flies is accompanied by an impaired response during conditions of limiting nutrients.

**Results**

**Loss of chameau (chm) results in a substantial weight loss in Drosophila melanogaster**

To investigate chm’s physiological function in adult flies, we reduced chm levels by crossing flies carrying an UAS-driven chm RNAi transgene with a ubiquitous da-Gal4 driver line (Fig EV1A). In addition to a fewer number of eclosed flies, loss of chm resulted in a reduction in body weight and size of both male and female flies as compared to the isogenic control flies of the same age or a control RNAi line (Figs 1A and EV1B). As chm is highly expressed in neuronal tissue and in fat body (Jenkins et al., 2022), we selectively removed chm from these tissues by crossing the RNAi flies with the corresponding driver lines (elav-Gal4 for neurons and r4-Gal4 for fat body). While we did not observe an effect on fly development, a reduction in chm expression in neurons as well as in fat body showed a substantial loss in size and weight (Fig 1B and C). However, when crossed with mel-Gal4 (muscle-specific driver), no difference in size or weight was observed between control and RNAi flies (Fig 1D).

**Enzymatic activity of chm is important for the organisms’ normal physiology**

Since chm is an acetyltransferase, we wanted to assess if its enzymatic activity is important for this phenotype. To do this, we used a fly line heterozygous for a mutant control allele with partial deletion of the MYST domain (Grienenberger et al., 2002), referred to as chmMYST+/−. While flies homozygous for this allele do not survive, heterozygous chmMYST+/− flies are viable and even show an extended lifespan independent of the ambient temperature (Peleg et al., 2016a; Fig EV1C). The mutant allele did not affect the protein levels of chm (Fig EV1D), but a qPCR assay using primers hybridizing with the sequence of the mutated MYST domain showed a 50% reduction (Fig EV1E). Consistent with the reduced chm activity, a reduced bulk level of H4K12ac was measured by mass spectrometry (Feller et al., 2015; Peleg et al., 2016a; Fig EV1F). The heterozygous deletion mutant did not show any noticeable difference in development but showed a significant weight reduction compared to the controls of same age (Figs 1E and EV1G), indicating that the acetyltransferase activity of chm is important for the organism’s physiology. Interestingly, this phenotype is only observed at temperatures of 23°C and not higher, which is why we performed all following experiments at 23°C.

In summary, lack of chm and its activity affects the physiology of flies as indicated by the thin phenotype. This effect is mainly caused by the expression of active chm in the fat body or neurons (Fig 1F).

**Transcriptomic analysis predicts the role of chm in regulation of starvation**

As chmMYST+/− flies have reduced histone acetylation levels, we decided to assess the transcriptome of these flies. Principle component analysis (PCA) and a Spearman correlation clustering of the transcriptome separate into two clear clusters indicating the two genotypes but (Fig 2A and B) only few individual genes showed significantly different expression levels (Fig 2C; Dataset EV1). To check whether the moderate effect of chm on individual genes may nevertheless result in cumulative effect on entire pathways, we performed a gene set enrichment analysis (GSEA) on the data set. This analysis revealed a significant enrichment and downregulation of most biological processes related to metabolism (Fig 2D; Dataset EV2). Interestingly, and consistent with the observed weight reduction, genes involved in processes such as response to starvation and maintenance of nutrient levels were also downregulated in chmMYST−/− flies (Fig 2E). These observations suggest a modulatory role of chm in response to nutrient-limiting conditions by affecting the expression of genes involved in metabolic pathways.

**Loss of chm reduces proteome and acetylome of metabolic proteins**

As transcriptional effects were rather moderate compared to the strong phenotype, we wondered whether chm might also have a transcription-independent effect on the proteome. We therefore measured proteomic changes upon chm knockdown. We identified approximately 4,000 proteins, of which 158 proteins showed...
significant differences (Figs 3A and EV2A; Dataset EV3). Similar to the transcriptional changes, GESA revealed a reduction in proteins involved in metabolic processes such as amino acid, small molecule, lipid, and carbohydrate metabolism upon loss of chm. Moreover, chm knockdown flies had higher levels of several immune response proteins (Fig 3B; Dataset EV4).
Figure 3.
Subsequently, we wondered how chm affects the cellular acetylome being an acetyltransferase. Analysis of acetylome upon chm knockdown identified a total of 182 acetylation sites (of approx. 2,400) that were significantly altered (Figs 3C and EV2B; Dataset EV5). Similar to the effects observed for the transcriptome and the proteome, the differential acetylated proteins were frequently involved in metabolic processes of carbohydrate, lipid, and amino acids (Fig 3D; Dataset EV6), suggesting that loss of chm leads to misregulated acetylation of proteins involved in metabolism. Overall, these results indicate a role of chm in regulating metabolism. As changes in metabolism may affect the organism’s ability to respond to nutritional challenges, we wondered whether chm changes the flies’ ability to cope with metabolic stress.

Chameau (chm) and its enzymatic activity are required for regulating nutritional stress response independent of its role in development

To test if chm is required to cope with starvation, we subjected flies compromised for chm expression to wet starvation. Consistent with the prediction from changes in transcriptome, proteome, and acetylome, we observed a strong decrease in survival during starvation in both male and female flies of chmRNAi compared to their corresponding controls (Figs 4A and EV3A). This effect is independent of the RNAi line used (Fig EV3B, C and H). Furthermore, selective loss of chm in neurons and fat body also increased the flies’ sensitivity to starvation (Fig 4B and C). Muscle-specific knockdown of chm in flies showed no differences compared to its control flies (Fig 4D) further supporting the hypothesis that chm exerts its function in both neurons and fat body (Fig EV3D).

Earlier studies have shown that chm is required for proper development (Aggarwal & Calvi, 2004; Hainaut et al., 2012; McConnell et al., 2012). We therefore wanted to assess if the observed sensitivity toward starvation is dependent on its role in developmental regulation. We therefore employed the GeneSwitch (Osterwalder et al., 2001) system to induce an adult-specific knockdown of chm. Flies that were not fed with RU486 (or ethanol fed; Fig 4E) and flies that were fed with RU486 for 2 days before starvation (Fig EV3E) showed no differences in starvation response. However, when fed with RU486 for 4 days before the start of starvation, chmRNAi showed increased susceptibility to starvation as compared to the controls (Fig 4F). These data indicate that regulation of nutrient stress by chm is not coupled to its role in development. Finally, we also observed sensitivity to starvation in chmMYST+ male and female flies (Figs 4G and EV3F), suggesting that the resilience towards metabolic stress requires at least the partial role of chm’s enzymatic activity (Fig EV3G). In order to be sure, we also tested the starvation response using a GFP-RNAi line. In contrast to the chm RNAi line, the GFP-RNAi showed an even higher survival than the w1118 male flies (Fig EV3H). As the GFP-RNAi was not backcrossed with w1118, while da-gal4, the other RNAi lines and the MYST+/ mutant flies were, we used w1118 as control in all our experiments.

Chm mutant flies show a dampened transcriptomic response upon starvation

To test whether chm modulates the expression of genes upon starvation, we analyzed the transcriptional response of control and chmMYST mutant flies to wet starvation (Fig 5A). Consistent with a modulatory role of chm activity in response to starvation, PCA of the
transcriptome of fed and starved flies separated primarily based on its nutrient status (PC1) and only secondarily on their corresponding genotypes (PC2; Fig 5B). Furthermore, we noticed that chm mRNA levels were unaffected by starvation (Fig EV4A).

In accordance with this finding, we did not observe many differences in gene expression when comparing the transcriptional response to starvation between chmMYST+/+ and control flies (Figs 5B and EV5C; Dataset EV7 and EV8), such that a large overlap of 745 significant genes were shared between the genotypes upon starvation (Fig 5C). To investigate whether chm modulates the amplitude of transcriptional change, we performed GSEA for change in transcript levels between control and chmMYST+/+ flies in response to starvation. Although most GO terms were shared (Fig EV4C; Dataset EV9 and EV10), lack of chm activity clearly dampened the transcriptional response of genes involved in stress and starvation response and carbohydrate and lipid metabolism, upon starvation (Fig 5D–G).

Interestingly, genes in autophagy were upregulated upon starvation as expected but they did not show genotype-specific differences (Fig EV4D). We, therefore, conclude that chmMYST+/+ flies respond to starvation but much less efficiently than wild-type flies. Overall,
these data indicate that enzymatic activity of chm is required to mount a full transcriptional response when the organism is exposed to novel and stressful conditions, such as starvation.

**Overexpression of chm improves weight and starvation susceptibility in Drosophila**

We then wanted to address whether the metabolic phenotypes can be rescued by restoring the full activity of chm. For this, we used chm\(^{MYS^{+}}\) flies carrying a UAS-chm.myc transgene. We used da-Gal4 for strong and arm-Gal4 for weak expression and measured the weight of flies expressing different levels of wild-type chm. Consistent with chm playing a role in storing metabolic energy, we noticed a gain of weight that was dependent on the overall expression levels (Figs 6A and EV5A). Both drivers also improved starvation resistance in a chm\(^{MYS^{+}}\) background (Fig 6B and C) supporting the hypothesis that both phenotypes are caused by similar mechanisms. Furthermore, da-Gal4-driven expression resulted in a stronger increase in survival upon starvation as compared to arm-Gal4, further indicating significance of active chm in regulating

**Figure 5.** Mutant chm flies show a dampened transcriptomic profile upon starvation.

A Experimental design for transcriptomic analysis of fed and starved control (\(w^{1118}\)) and chm\(^{MYS^{+}}\) flies.
B PCA of the transcriptome between fed and starved control (\(w^{1118}\)) and chm\(^{MYS^{+}}\) flies (\(N = 5\), unpaired). Colors indicate the genotype and shapes indicate the nutrient status.
C Venn diagram of significant genes (FDR < 0.05 and II < 0.05) between control (\(w^{1118}\)) and chm\(^{MYS^{+}}\) in response to starvation.
D–G Box plot (left) and heatmap (right) of annotated genes from GO terms of D) cellular response to stress (ANOVA P-value: \(6.49 \times 10^{-6}\)); E) Response to starvation (ANOVA P-value: \(3.09 \times 10^{-6}\)); F) Carbohydrate metabolic process (ANOVA P-value: \(5.46 \times 10^{-6}\)); G) Lipid metabolic process (ANOVA P-value: \(7.38 \times 10^{-9}\)). Box plot shows the average vst normalized read counts of all annotated genes within the GO term averaged over the replicates. Boxes indicate interquartile range with central band as the median and central filled dot as the average, and the whiskers indicate the maximum and minimum values across five biological replicates. Heatmap shows the scaled values of vst normalized read counts in each replicate for all conditions.

Data information: All replicates are independent biological replicates and error bars indicate standard error of the mean (SEM). Tukey test was performed and non-significant values are not shown (*\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\), ****\(P < 0.0001\)). Source data are available online for this figure.
stress response (Fig EV5B). In addition, we also performed rescue experiments using tissue-specific driver lines (r4-Gal4 for fat body, elav-Gal4 for neurons, and mef-Gal4 for muscles). As expected, neuronal and fat body expression but not muscle-specific expression increased the survival consistently, validating the role of chm in neuronal and fat body regulation of starvation (Fig 6D–F). Furthermore, chm expression in fat body showed a stronger increase in survival as compared to neuronal tissue-specific expression, therefore bolstering the role of chm in fat body and metabolism (Fig EV5C).

In summary, these results validate chm as an important modulator of metabolism that could influence the organisms’ phenotype under non-ideal conditions at multiple levels.

Discussion

Our current data show that the acetyltransferase chm is important for maintenance of physiology and starvation resilience in Drosophila melanogaster. We show that a knockdown of chm perturbs the physiology of the fly as they are thinner than control flies. This phenotype is also observed in flies that have a reduced chm catalytic activity, suggesting a key role of chm’s acetyltransferase activity in this process. This is not only due to chm’s ability to acetylate H4K12ac but also due to its function in regulating protein levels and its ability to acetylate other non-histone substrates. In fact, the latter might be more relevant for chm’s role in modulating the response to starvation as we did not observe a strong effect of starvation on H4 acetylation (Appendix Fig S1A and B).

The fact that selective loss of chm in neurons and fat body is sufficient for the phenotype suggests that behavioral as well as metabolic changes in these flies could contribute to their thin body and response to starvation. To investigate whether the weight loss is primarily due to a behavioral change, we tested the feeding behavior of the flies ubiquitously expressing a chm RNAi and in a chmMYST/+ background. While we observed reduced feeding in chmRNAi flies upon ubiquitous knockdown, MYST/+ mutants showed no such

![Figure 6](https://www.embopress.org)

**Figure 6. Overexpression of chm improves weight and survival.**

A  Weight of 8- to 9-day-old male chmMYST/+ and chmMYST+;UAS-chmmyc flies upon weak (left, N = 5, paired) and strong (right, N = 4, paired, log-rank test was performed for each biological replicate. All replicates had P-values < 0.0001) chm overexpression with arm-Gal4 and da-Gal4, respectively (paired t-test was performed). B  Average survival curve of chmMYST/+ and chmMYST+;UAS-chmmyc male flies upon ubiquitous weak expression of chm with arm-Gal4 (N = 4, paired, log-rank test was performed for each biological replicate. All replicates had P-values < 0.0001). C  Average survival curve of chmMYST/+ and chmMYST+;UAS-chmmyc male flies upon ubiquitous strong expression of chm with da-Gal4 (N = 3, paired, log-rank test was performed for each biological replicate. All replicates had P-values < 0.0001). D  Average survival curve of chmMYST/+ and chmMYST+;UAS-chmmyc male flies upon fat body expression of chm with r4-Gal4 (N = 4, paired, log-rank test was performed for each biological replicate. All replicates had P-values < 0.0001). E  Average survival curve of control chmMYST/+ and chmMYST+;UAS-chmmyc male flies upon neuronal expression of chm with elav-Gal4 (N = 5, paired, Log-rank test was performed for each biological replicate. All replicates had P-values < 0.0001). F  Average survival curve of chmMYST/+ and chmMYST+;UAS-chmmyc male flies upon muscle expression of chm with mef-Gal4 (N = 5, paired, log-rank test was performed for each biological replicate. All replicates had P-values ranged between 0.6 and 0.0001 across replicates).

Data information: All are independent biological replicates and error bars indicate standard error of the mean (SEM). Paired t-test was performed for the fly weight data and non-significant values are not shown (***P < 0.001, ****P < 0.0001). For survival curves, log-rank test was performed for each biological replicate. The displayed P-value is based on all biological replicates. Source data are available online for this figure.
differences. However, both cases showed a strong starvation susceptibility suggesting that feeding behavior does not explain the reduced starvation resilience. Moreover, previous studies from our lab have shown that chm-MYST+ mutants are more active than its corresponding control of same age (Peleg et al., 2016a). In addition, the fact that effect of a chm loss on the response to starvation can also be observed when chm is removed in adults using the GeneSwitch system. The latter experiments also show that the effect is not due to chm’s role in fly development (Aggarwal & Calvi, 2004; Hainaut et al., 2012; McConnell et al., 2012).

The increased survival under normal conditions and the higher sensitivity toward starvation seem contradictory at first glance. However, they are very likely caused by the same regulatory mechanism. The leaner body mass of flies lacking chm is similar to what is seen during caloric restriction. While this state can result in lifespan extension (Peleg et al., 2016a,b) as long as sufficient nutrients are available, the system does not respond appropriately when exposed to less favorable conditions (Appendix Fig S2). A similar inability to mount a proper response to environmental challenges has recently been shown to lead to a selective growth advantage of epigenetically disrupted cancer cells (Loukas et al., 2023).

The phenotypic inertia is a consequence of the dampened response in flies lacking chm activity. This resilience to changing nutrient supplies is likely mediated on several levels as we observe an effect of a reduced chm activity on the transcriptome, the proteome, and the acetylome. While we are unable to identify a single pathway that is responsible for this effect, the affected transcripts and proteins show a clear enrichment for GO terms associated with many metabolic processes. Such interplay between an epigenetic enzyme and metabolism, especially upon starvation, is not uncommon and has been shown previously in Drosophila (Nakajima et al., 2022; Wang et al., 2023).

Finally, to our knowledge, this is probably the first evidence of an enzyme having opposing effects on longevity and nutrient stress. We speculate that while having less chm increases longevity, the requirement to survive under stress in a novel environment outweighs the benefit of a long and healthy lifespan. Our study therefore provides an explanation for the evolutionary conservation of chm in Drosophila melanogaster.

Materials and Methods

Fly maintenance

Fly lines were maintained in an incubator (Panasonic, MLR-235H-PE) at 25°C with a 12 h/12 h light–dark cycle at 60% relative humidity. The composition of the fly food is given in Table 1. Details of fly lines used in the study are given in Table 2.

Starvation assay

As age, sex, temperature, light–dark cycle, and humidity could affect starvation response and resistance (Jang & Lee, 2015, 2018; Chauhan et al., 2021), we kept all these constant by using flies of age 8–9 days old developed and starved at 23°C with 60% relative humidity and 12 h light/12 h dark cycle. Approx. 50–60 flies were transferred to an empty fly bottle containing a tissue with 4 ml of water. Total number of flies for each experiment ranged from 100 to 250 for each genotype/condition tested. Readings were taken until flies were dead in all genotypes with dead flies counted every 10–12 h.

For GeneSwitch, 7- to 10-day-old males from the cross of ActSC-GeneSwitch (males) and w1118;UAS-chmRNAi (females) were used. RU486 (mifepristone; Sigma-Aldrich) was administrated with food for 4–5 days prior to the food deprivation, and then with water during the deprivation. RU486 was dissolved in ethanol (10 mg/ml) and mixed with melted food or water in a final concentration of 0.2 mM. The same amount of ethanol was added to the food or water for the control groups. Males and females were separated 3 days prior to the food deprivation.

Longevity assay

Male flies of age 8–9 days old developed and aged at 23°C with 60% relative humidity and 12 h light/12 h dark cycle. Approx. 30 male

<p>| Table 1. Composition of fly food. |</p>
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<th>Components</th>
<th>Amount</th>
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<tr>
<td>Agarose</td>
<td>130 g</td>
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<tr>
<td>Corn meal</td>
<td>1,300 g</td>
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<tr>
<td>Soy flour</td>
<td>150 g</td>
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<tr>
<td>Yeast</td>
<td>300 g</td>
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<tr>
<td>Maltase</td>
<td>650 g</td>
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<tr>
<td>Molasses</td>
<td>1,300 g</td>
</tr>
<tr>
<td>Methyl 4-Hydroxybenzoate (Nipagin)</td>
<td>415 ml Ethanol (10%)</td>
</tr>
<tr>
<td>Acid mix</td>
<td>295 ml</td>
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<tr>
<td>Water</td>
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<table>
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<th>Table 2. Details of fly lines used in this experiment.</th>
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<td>Fly lines</td>
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</tr>
<tr>
<td>w1118</td>
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<tr>
<td>+;cyoG</td>
</tr>
<tr>
<td>chm1024Aa</td>
</tr>
<tr>
<td>chm1024Aa*</td>
</tr>
<tr>
<td>chm144</td>
</tr>
<tr>
<td>chm144;cyoGFP</td>
</tr>
<tr>
<td>chm144;cyoGFP;UAS-chm144</td>
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<td>da-Gal4</td>
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<tr>
<td>mef-Gal4</td>
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<tr>
<td>ActSC-GAL4Switch</td>
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<td>GFPMos</td>
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*Flies have been backcrossed to w1118 for at least seven generations.
flies were used per bottle and the total number of flies ranged from 100 to 150 for each genotype tested. The food was changed and readings were taken once a week initially and once in 2–3 days when flies started dying. Readings were taken until all flies were dead in both genotypes.

Weight measurement

Flies of the fed conditions were transferred to a 2.0 ml tube and snap frozen in liquid nitrogen. Following this, weight of each empty tube was measured. Heads and bodies were obtained by passing through sieves. First sieve (width: 710 μm) separates the bodies from remaining and the second (width: 355 μm) separates heads from wings/legs (Analysensieb). A total of 20–30 heads and bodies were transferred to the corresponding weight of 20–30 flies from which weight per fly was calculated. Weights were measured using KERN ABJ 120-4NM weighing machine. Samples and all the components were kept in dry ice for the entire duration of the experiment.

Post-translational modification of histones

Sample preparation

Fly heads were obtained following the same procedure given in the Body and head weight measurement section. Approx. 300 μl of homogenization buffer (60 mM KCl, 15 mM NaCl, 4 mM MgCl₂, 15 mM HEPES [pH 7.5], 0.5% Triton-X-100, 0.5 mM DTT, 20 mM sodium butyrate, and 1 tablet protease inhibitor) was added to 30–50 fly heads and were homogenized extensively with an electrical stirrer (5 × 10 s ON and 15 s OFF). Following this, sonication was performed with Bioruptor® Pico for 3 × 10 s ON and 45 s OFF at 4°C. The obtained lysate was centrifuged at 20,817 g for 30 min. Obtained pellet was resuspended in 200 μl of 0.2 M H₂SO₄, vortexed heavily, and rotated overnight at 15 and 4°C. Subsequently, overnight incubated lysate was centrifuged at 20,817 g (max. Speed) for 10 min at 4°C. Histone was precipitated by adding trichloroacetic acid (TCA; ThermoScientific, Cat. No 85183) to reach 26% final concentration. Tubes were mixed and incubated at 4°C for 2 h and spun at 20,817 g for 15 min. Pellets were washed thrice with ice-cold 100% acetone (VWR, Cat. No AA22928-K2; 5 min rotation at 4°C and 15 min of 20,817 g spin at 4°C between washes), dried for 15 min at room temperature, and resuspended in 20 μl of 1× Laemmli sample buffer for million cells and boiled at 95°C for 5 min. Samples were stored at –20°C until further use. The histones corresponding to 0.5 million cells were separated into 4–20% precast polyacrylamide gels (SERVA, Cat. No 43277.01). Gels were briefly stained with InstantBlue Coomassie Protein Stain (Abcam, Cat. No ab119211). For targeted mass-spectrometry analysis, histones bands were excised, washed once with MS-grade water (Sigma-Aldrich, Cat. No 1153331000) and de-stained twice (or until transparent) by incubating 30 min at 37°C with 200 μl of 50% acetonitrile (ACN; Carl Roth, Cat. No 8825.2) in 50 mM ammonium bicarbonate (NH₄HCO₃; Carl Roth, Cat. No T871.1). Gel pieces were then washed twice with 200 μl MS grade and twice with 200 μl of 100% ACN to dehydrate them. Histones were in-gel acylated by first adding 20 μl of d6 acetic anhydride (Sigma-Aldrich, 175641-5G), followed by 40 μl of 100 mM NH₄HCO₃. After 5 min, 140 μl of 1 M NH₄HCO₃ was slowly added to the reaction. pH of the final solution should be around 7 (in cases where pH was acidic, few microliters of 1 M NH₄HCO₃ was added). Samples were incubated at 37°C for 45 min at 550 rpm. Following this, samples were washed five times with 200 μl of 100 mM NH₄HCO₃, four times with 200 μl of MS-grade water, and four times with 200 μl of 100% ACN. They were spun down briefly and all remaining ACN was removed. Gel pieces were rehydrated in 50 μl of trypsin solution (25 ng/ml trypsin in 100 mM NH₄HCO₃; Promega, Cat. No V5111) and incubated at 4°C for 30 min. After the addition of 150 μl of 50 mM NH₄HCO₃, histones were in-gel digested overnight at 37°C at 550 rpm. Peptides were sequentially extracted by incubating 10 min at room temperature with 150 μl of 50 mM NH₄HCO₃, twice with 150 μl of 50% ACN (in MS-grade water) 0.1% trifluoroacetic acid (TFA) and twice with 100 μl of 100% ACN. During each of the above washing steps, samples were sonicated for 3 min in a water bath followed by a brief spin down. Obtained peptides were dried using a centrifugal evaporator and stored at –20°C until resuspension in 30 μl of 0.1% TFA. For desalting, peptides were loaded in a C18 Stagetect (prewashed with 20 μl of methanol followed by 20 μl 80% ACN 0.1% TFA and equilibrated with 20 μl of 0.1% TFA), washed twice with 20 μl of 0.1% TFA, and peptides were eluted three times with 10 μl of 80% ACN 0.25% TFA. Flow through obtained from loading of peptides in C18 was further desalted with TopTip Carbon (glygen, Cat. No TT1CAR.96) by loading the flow through thrice (prewashed thrice with 30 μl of 100% ACN followed by equilibration thrice with 30 μl of 0.1% TFA), washed five times with 30 μl of 0.1% TFA, and eluted thrice with 15 μl of 70% ACN and 0.1% TFA. Eluted peptides from both desalting steps were combined and evaporated in a centrifugal evaporator, resuspended in 15–17 μl of 0.1% TFA, and stored at –20°C until mass spectrometry analysis.

Targeted mass spectrometry

Desalted histone peptides in 0.1% TFA were injected in an RSLNano System (Thermo Fisher Scientific) and separated in a 15 cm analytical column (75 μm ID home packed with ReproSil-Pur C18-AQ 2.4 μm from Dr. Maisch) with a 50 min gradient from 4 to 40% ACN in 0.1% formic acid at 300 nl/min flowrate. The effluent from the HPLC was electrospreyed into Q Exactive HF mass spectrometer (Thermo Fisher Scientific). The MS instrument was programmed to target several ions except for the MS3 fragmentation (22). Survey full-scan MS spectra (from m/z 270 to 730) were acquired with resolution R = 60,000 at m/z 400 (AGC target of 3 × 106). Targeted ions were isolated with an isolation window of 0.7 m/z to a target value of 2 × 105 and fragmented at 27% normalized collision energy. Typical mass spectrometric conditions were as follows: spray voltage, 1.5 kV; no sheath and auxiliary gas flow; and heated capillary temperature, 250°C.

Data analysis

Raw data from mass spectrometry were analyzed using Skyline (Pino et al., 2020) v21.1. Peak integration was performed for H3 and H4 peptides for each of its corresponding modifications. Relative levels of each PTM were calculated from the obtained intensities using R environment based on the formula given in Tiwari et al., 2020.
Western blot

For protein extraction, 30–50 fly heads were homogenized in RIPA buffer using a mechanical homogenizer, followed by sonication with Bioruptor® Pico (Diagenode, B01060010) for 4× 10 s ON, 30 s OFF. This was followed by centrifugation at maximum speed for 30 min and at 4°C. The obtained supernatant was transferred to a new tube and Laemelli sample buffer was added. Samples were then boiled for 5 min at 95°C.

For Western blot, samples were then loaded onto a 4–20% pre-cast gel (SERVA, 43277.01) and run at 90 V for 30 min followed by 160 V for 2 h (SDS running buffer (10×): Tris 30.2 g, glycine 142 g, and SDS 10 g, make it to 1 l with double-distilled water). Wet transfer was performed for 2 h and 30 min at 175 V and 4°C (transfer buffer [10×]: Tris 30.3 g and glycine 144 g, make it to 1 l with double-distilled water; for 1×:10× transfer buffer 100 ml, methanol 150 ml, and 20% SDS 1 ml, make it to 1 l with double-distilled water). Following this, blocking was performed by incubating the membrane with 5% milk in PBS for 60–90 min at room temperature. Primary antibody incubation was performed at 4°C overnight. PBS-T (0.1%) washes were then carried out for 3 min at room temperature and secondary antibody incubation was performed at room temperature for 60 min. Following this, PBS-T (0.1%) washes were carried out for 3 min at room temperature and membrane was developed by chemiluminescence method using ECL detection kit (BioRad, Clarity™ Western ECL Substrate, 170-5061), and ImageLab™ (v6.0) was used for the analysis. Information about the antibodies used is given in Table 3.

Quantitative reverse transcriptase PCR

RNA extraction

Thirty heads were homogenized with an electrical stirrer with 500 µl of Trizol (Thermo Fisher; cat. no. 15596026). Chloroform was added at the ratio of 1:5 with Trizol and the solutions were mixed for 15 s by inverting the tubes. After 5 min incubation at room temperature, samples were centrifuged at 12,000 g for 15 min. Aqueous phase was transferred to a new tube from the centrifuged sample to which isopropanol was added at 1:1 ratio of the obtained aqueous phase, vortexed briefly, and incubated for 10 min at room temperature. They were then centrifuged for 10 min at 12,000 g. Supernatant was discarded and obtained pellet was washed with 750 µl of 80% ethanol. After brief vortexing, samples were centrifuged at 8,000 g for 5 min. Obtained supernatant was discarded and pellet was air-dried for 5 min inside the hood and resuspended in RNase-free water. RNA concentration and A260/280 ratio were measured with NanoDrop.

DNase treatment, cDNA synthesis, and qRT–PCR

DNase treatment (Roche, DNase I recombinant, RNase-free from bovine pancreas, Cat. No. 04716728001) was performed with 1 µg of RNA as starting material following manufacturer’s instructions. cDNA synthesis was performed using SuperScript III First Strand Synthesis System (Invitrogen, cat. No: 18080051, Random hexamer priming) using DNase-treated RNA. Each reaction was set up with/without Superscript III reverse transcriptase. Obtained cDNA was treated with RNaseH to remove the RNA–DNA duplex and diluted 1:5 with RNase-free water. Diluted cDNA was used for qPCR reaction with Fast SYBR™ Green Master Mix (Thermo Fisher Scientific, cat no: 4385612) following manufacturer’s instructions and ran on a Lightcycler 480 II (Roche) instrument. Primer efficiency was calculated using serial dilutions and the corresponding melt curves were also assessed. Sequences of qPCR primers used are given in Table 4.

RNA sequencing

Library preparation

One microgram of RNA, obtained from fly heads, was used for library preparation. Both total and mRNA quality was assessed on a 2100 Bioanalyzer (Agilent Technologies, Cat. No G2939BA) using RNA pico assay kit (Agilent RNA 6000 Pico Kit, cat. No: 5067-1513) using manufacturer’s protocol. RNA depletion was performed using NEBNext rRNA Depletion Kit (Human/Mouse/Rat; NEB #E6310), and library preparation for RNA sequencing was performed using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760) following manufacturer’s protocol. Libraries were sequenced on an Illumina HiSeq 1500 instrument at the Laboratory of Functional Genomic Analysis (LAFUGA, Gene Center Munich, LMU).

Data analysis

A total of 50 bp paired-end reads were aligned to the D. melanogaster reference genome (release 6) using STAR aligner (version 2.5.3a) with providing GTF annotation (dmel-all-r6.17.gtf). Reads with multiple alignments were filtered by setting outFilterMultimapNmax parameter to 1. Reads were counted per gene with parameter –quantMode GeneCounts. BAM files were converted to normalized bedgraph coverages using genomeCoverageBed command (bedtools version 2.27.1) with scale parameter set to divide by the total number of reads and multiplied by a million. Bedgraph files were converted to tdf files (igvtools version 2.3.98) to visualize in the IGV browser. Count tables (read counts per gene) were read into R environment and low-count genes were filtered out (at least three read per gene in 10% of the samples analyzed together). Differential expression analysis was performed by DESeq2 (Love et al, 2014) package (version 1.24) by adding replicate information as batch variable. Samples that were directly compared to each other were fitted in the same DESeq2 (Love et al, 2014) model. Log2FoldChange estimates and adjusted P-values were obtained by the results function (DESeq2) and an FDR cutoff < 0.05 was applied. In addition, the

Table 3. List of antibodies used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Raised</th>
<th>Dilution</th>
<th>Company/Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td>chm 3G3</td>
<td>Mouse</td>
<td>1:1</td>
<td>Homemade</td>
</tr>
<tr>
<td>Lamin</td>
<td>Mouse</td>
<td>1:1,000</td>
<td>Homemade</td>
</tr>
<tr>
<td>H3</td>
<td>Rabbit</td>
<td>1:5,000</td>
<td>Abcam (ab18521)</td>
</tr>
<tr>
<td>Anti-mouse</td>
<td>Sheep</td>
<td>1:5,000</td>
<td>RRID: AB772210</td>
</tr>
<tr>
<td>Anti-rabbit</td>
<td>Donkey</td>
<td>1:5,000</td>
<td>RRID: AB772206</td>
</tr>
</tbody>
</table>

Table 4. List of primer pairs used for qPCR.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin-5c</td>
<td>CAGACCACCGGTGTTACCTC</td>
<td>GTGTGCTGCGATCTTCCT</td>
</tr>
<tr>
<td>chm-Exon8</td>
<td>CAATATCCACCGACGCATCAT</td>
<td>AGCCAAGATCTCGATCTCGT</td>
</tr>
</tbody>
</table>

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less stringent \( p \)-value that includes both statistical and biological information was used. \( p \)-value takes into consideration log2FoldChange and \( p \)-value to obtain values between 0 and 1 (Xiao et al., 2014; Hostrup et al., 2022). For principal component analysis (PCA), batch effect was corrected by the remove batch effects function from limma (Ritchie et al., 2014; package version 3.52.0) on the normalized read counts.

Gene set enrichment analysis was performed on the obtained results from different conditions/comparisons using the gseGO function from clusterProfiler (Yu et al., 2012; package version 3.12.0) by ranking the genes based on \( t \)-statistic value without any log2FoldChange or \( P \)-adjusted cut-off. GSEA plots with selected GO terms were also generated with R environment with these selected GO terms having FDR < 0.05 cut-off.

**Proteome and acetylome**

**Protein extraction**

Approximately 1,000 male fly heads were collected and homogenized in an electrical homogenizer with 250 \( \mu \)l of lysis buffer (20 mM HEPES pH 8.0, 9 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM \( \beta \)-glycerophosphate, 1 mM Na-butyrate, and 60 \( \mu \)M sirtinol). After homogenization, sample volume was made up to 700 \( \mu \)l with lysis buffer. The obtained homogenate was rotated for 30 min (8 revolutions per min) at 4°C. Samples were then sonicated in a Bioruptor for six cycles (10 s ON, 45 s OFF) and again rotated for 10 min (8 revolutions per min) at 4°C. Centrifugation was performed at 14,000 g and 4°C for 20 min and the supernatant was transferred to another Eppendorf. This was followed by another centrifugation to remove the debris completely. The final supernatant was then transferred to a 15 ml falcon and the volume was made up to 2 ml with lysis buffer.

**Acetylome sample preparation**

Sample preparation for acetylome was performed with PTMScan® Acetyl-Lysine Motif [Ac-K] kit (Cell Signaling, 13416), and manufacture’s protocol was followed with minor modifications. To the sample, 0.1% TFA was added and vortexed for 30 s, and supernatant was transferred to a new vial for further use. The sample was then vortexed for 1 min and centrifuged at 2,000 g for 5 min at room temperature, resuspended with 1.4 ml of the IAP buffer (provided by the manufacturer), and centrifuged again at 10,000 g at 4°C for 5 min. Resuspended samples were then quantified by BCA to make sure equal amount of peptides was used as input for proteome and acetylome. Ten percent of the cleared solution was taken as input for proteome. Lysine antibody beads (provided by the manufacturer) were washed with 1 ml of 1× PBS, centrifuged at 2,000 g, and resuspended with 40 \( \mu \)l of 1× PBS. To the cleared solution, half the volume of antibody bead slurry was used and incubated in a rotator for 2 h at 4°C. Incubated samples were then centrifuged at 2,000 g for 30 s, and supernatant was transferred to a new vial for further use. Beads were then washed twice with 1 ml of IAP buffer, mixed by inverting, centrifuged at 2,000 g for 30 s, and followed by similar washes, thrice with 1 ml chilled HPLC water. Peptides bound to the beads were eluted twice by adding 55 \( \mu \)l of 0.15% TFA, vortexing, incubating for 10 min at room temperature, and centrifuging for 30 s at 2,000 g.

Desalting of the eluted peptide was performed with AttractSPE® Disks Tips C18 column (affinisep, Tips-C18.T2.200.96). The column was first equilibrated with 50 \( \mu \)l 0.1% TFA twice. Input proteome and IP sample was then added to the C18, followed by washes with 0.1% TFA twice. Peptides were eluted with 10 \( \mu \)l of 0.1% TFA and 40% ACN, dried with vacuum concentrator, and resuspended with 15 \( \mu \)l of 0.1% TFA for mass spectrometry analysis.

**Mass spectrometry**

Samples were evaporated to dryness, resuspended in 15 \( \mu \)l of 0.1% formic acid solution, and injected in an Ultimate 3000 RSLCnano system (Thermo) separated in a 25 cm Aurora column (lIonopticks) with 150 min gradient from 0 to 43% of 80% acetonitrile in 0.1% formic acid. The effluent from the HPLC was directly electrosprayed into an Orbitrap Exploris 480 (Thermo) operated in data-dependent mode to automatically switch between full-scan MS and MS/MS acquisition. Survey full-scan MS spectra (from m/z 350–1,200) were acquired with resolution \( R = 60,000 \) at m/z 400 (AGC target of 3 × 106). The 20 most intense peptide ions with charge states between 2 and 6 were sequentially isolated to a target value of \( 1 \times 10^{5} \), and fragmented at 30% normalized collision energy. Typical mass spectrometric conditions were as follows: spray voltage, 1.5 kV; no sheath and auxiliary gas flow; heated capillary temperature, 275°C; and intensity selection threshold, 3 × 105.

**Data analysis**

Raw flies from mass spectrometry were aligned using MaxQuant (Cox & Mann, 2008; v2.1.3.0) with Drosophila fasta from UniProt. Output files of proteome (proteinGroups.txt) and acetylome (Acetyl (K) sites.txt) from MaxQuant were analyzed in R environment. Proteins and acetylation sites were only used for further analysis when they were detected and quantified in at least three out of four replicates of at least one condition. Following filtering, MinProb imputation algorithm with \( q = 0.01 \) was performed to impute the missing values, and limma (Ritchie et al., 2014)-based differential expression analysis was performed. Intensities of acetylome were not normalized to the proteome LFQ intensities and were used directly for
differential expression analysis. As with transcriptome, both q-value and t-value (Xiao et al., 2014; Hostrup et al., 2022) were considered for significance with a cut-off of 0.05 in both cases. Gene set enrichment analysis was performed for the proteome from using the gseGO function clusterProfiler (Yu et al., 2012; package version 3.12.0) by ranking the genes based on t-statistic value without any log2FoldChange or P-adjusted cut-off (FDR < 0.05 cut-off for predicted GO terms). Overrepresentation analysis was performed without a background set for significant proteins in the acetylome by separating them based on log-fold change to obtain the up- and downregulated sites and their corresponding proteins (FDR < 0.05 cut-off for predicted GO terms). Corresponding GO plots were also generated with R environment. The script for data analysis was written based on DEP package (Zhang et al., 2018; v1.18.0) but was modified to be used for protein modification analysis. This will be provided on request as there is a possibility of publishing it as an R package in the future.

Plots and statistical analysis

All statistical analyses were performed in R (R Core Team, 2022; R: A language and environment for statistical computing and R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/) environment unless otherwise mentioned. Graphics in all figures were created using Biorender.com. Statistical tests were decided based on the experimental design and measurements and have been mentioned in each of the figure legends. Plots and graphs generated for all experiments were also generated in R environment unless otherwise mentioned.

Data availability

The datasets produced in this study are available in the following databases:
- Histone PTM mass spectrometry data: ProteomeXchange Consortium via the PRIDE20 partner repository PXD035947 (http://www.ebi.ac.uk/pride/archive/projects/PXD035947).
- Acetylation and proteome mass spectrometry data: ProteomeXchange Consortium via the PRIDE20 partner repository PXD042471 (http://www.ebi.ac.uk/pride/archive/projects/PXD042471).

Expanded View for this article is available online.

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Author contributions

Anuroop Venkateswaran Venkatasubramani: Conceptualization; data curation; investigation; visualization; methodology; writing – original draft; writing – review and editing. Toshiharu Ichinoise: Investigation; visualization; methodology. Mai Kanno: Methodology; writing – review and editing. Ignasi Forne: Formal analysis; visualization; methodology; writing – review and editing. Hiromu Tanimoto: Supervision; funding acquisition; methodology. Shahaf Peleg: Conceptualization; supervision; writing – review and editing. Axel Imhof: Conceptualization; resources; supervision; funding acquisition; writing – original draft; project administration; writing – review and editing.

Disclosure and competing interests statement

AI is a co-founder and shareholder of EpiQMAx. The other authors declare no competing interests.

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