

Age-related change in γ H2AX of *Drosophila* muscle: its significance as a marker for muscle damage and longevity

Ho-Jun Jeon · Young-Shin Kim · Jung-Sun Park · Jung-Hoon Pyo · Hyun-Jin Na · In-Joo Kim · Cheol-Min Kim · Hae Young Chung · Nam Deuk Kim · Robert Arking · Mi-Ae Yoo

Received: 3 December 2014 / Accepted: 6 April 2015 / Published online: 10 April 2015
© Springer Science+Business Media Dordrecht 2015

Abstract Muscle aging is closely related to unhealthy late-life and organismal aging. Recently, the state of differentiated cells was shown to be critical to tissue homeostasis. Thus, understanding how fully differentiated muscle cells age is required for ensuring healthy aging. Adult *Drosophila* muscle is a useful model for exploring the aging process of fully differentiated cells. In this study, we investigated age-related changes of γ H2AX, an indicator of DNA strand breaks, in adult *Drosophila* muscle to document whether its changes are correlated with muscle

degeneration and lifespan. The results demonstrate that γ H2AX accumulation increases in adult *Drosophila* thoracic and leg muscles with age. Analyses of short-, normal-, and long-lived strains indicate that the age-related increase of γ H2AX is closely associated with the extent of muscle degeneration, cleaved caspase-3 and poly-ubiquitin aggregates, and longevity. Further analysis of muscle-specific knockdown of heterochromatin protein 1a revealed that the excessive γ H2AX accumulation in thoracic and leg muscles induces accelerated degeneration and decreases longevity. These data suggest a strong correlation between age-related muscle damage and lifespan in *Drosophila*. Our findings indicate that γ H2AX may be a reliable biomarker for assessing muscle aging in *Drosophila*.

Electronic supplementary material The online version of this article (doi:10.1007/s10522-015-9573-0) contains supplementary material, which is available to authorized users.

H.-J. Jeon · Y.-S. Kim · J.-S. Park · J.-H. Pyo · H.-J. Na · I.-J. Kim · M.-A. Yoo (✉)
Department of Molecular Biology, Pusan National University, Busan 609-735, Republic of Korea
e-mail: mayoo@pusan.ac.kr

C.-M. Kim
Research Center for Anti-Aging Technology Development, Pusan National University, Busan 609-735, Republic of Korea

H. Y. Chung · N. D. Kim
Molecular Inflammation Research Center for Aging Intervention (MRCA), College of Pharmacy, Pusan National University, Busan 609-735, Republic of Korea

R. Arking
Department of Biological Sciences, Wayne State University, Detroit, MI 48202, USA

Keywords γ H2AX · Muscle · Aging · DNA damage · *Drosophila* · Longevity

Introduction

Tissue aging is linked with longevity. Particularly, muscle aging is closely related to the occurrence of age-related diseases and unhealthy late-life conditions (Nair 2005; Demontis et al. 2013). A cohort study revealed that decline in muscle function can elevate mortality (Newman et al. 2006). Skeletal muscle is a tissue with low cellular turnover and high regenerative potential (Rando 2006). Recently, the state of

differentiated muscle cells was shown to be critical to muscle homeostasis (Gopinath and Rando 2008).

Drosophila muscle is a suitable model for studying aging of fully differentiated muscle cells. Adult *Drosophila* muscle is composed of highly differentiated muscle cells and contains no stem cells (Daczewska et al. 2010). Moreover, adult flies have a short lifespan, and longevity strains with different lifespans have revealed that climbing activity is dependent on lifespan (Piazza et al. 2009). Additionally, muscular degenerative markers such as poly-ubiquitin aggregates are available for studying muscle aging in *Drosophila* (Demontis and Perrimon 2010).

DNA damage is an important contributor to aging (Li et al. 2008; Moskalev et al. 2013). Progressive accumulation of DNA damage induces physiological changes that disrupt various regulatory mechanisms that maintain cellular or tissue integrity (Freitas and de Magalhães 2011). Among the various types of DNA damage, strand breaks are known to trigger apoptotic cell death (Kaina 2003). In fact, compared to other organs, muscle is especially sensitive to DNA damage (Garcia et al. 2010), and progressive loss of muscle mass and function accompanying apoptotic cell death is regarded as a key feature of muscle aging (Du et al. 2004; Zheng et al. 2005; Marzetti and Leeuwenburgh 2006; Augustin and Partridge 2009; Cruz-Jentoft et al. 2010). However, the correlation between muscle degeneration and nuclear DNA damage in differentiated muscle cells remains unclear.

Histone variant H2AX is phosphorylated on Serine 139 by ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3-related (ATR) in response to DNA strand breaks to form γ H2AX (Rogakou et al. 1998; Tanaka et al. 2006). Due to its role in sensing DNA damage, γ H2AX formation is regarded as an early indicator of damage (Mah et al. 2010). Gamma-H2AX foci are reversibly generated a few minutes after application of a genotoxic agent and subsequently eliminated in 24–48 h in typical cells (Redon et al. 2011). Recent studies have reported that γ H2AX is a marker for aging in various tissues and organisms (Wang et al. 2009; Mah et al. 2010; Park et al. 2012; Bopp et al. 2013).

DNA damage accumulation is dependent on chromatin state (Burgess et al. 2012). Loss of heterochromatin components induces genomic instability phenotypes and increases DNA double-strand breaks (DSBs) (Peng and Karpen 2009), and heterochromatin

levels have been reported to decline with age (O'Sullivan and Karlseder 2012). Recent studies using flies to examine the knockdown of heterochromatin protein 1 (HP1) reported that loss of heterochromatin shortens lifespan (Larson et al. 2012). Furthermore, loss of heterochromatin-associated proteins, including HP1, makes DNA susceptible to damage (Falk et al. 2008) and can induce genomic instability due to their roles in chromatin compaction and genome protection (Yan et al. 2011; Papamichos-Chronakis and Peterson 2013). A study in *Drosophila* larval tissues reported that loss of HP1a induces the increase of γ H2AX formation (Yan et al. 2011).

In this study, we investigated age-related changes in the levels of γ H2AX in adult *Drosophila* muscle and their significance with respect to muscle aging and longevity.

Materials and methods

Fly stocks

Fly stocks were maintained at 23–25 °C on standard food [79.2 % water, 1 % agar, 7 % cornmeal, 2 % yeast, 10 % sucrose, 0.3 % bokinin (butylparaben, anti-bacterial and anti-fungal additive), and 0.5 % propionic acid] under a 12/12 h light/dark cycle. Female flies were used in the experiments, and Oregon-R (OR) flies were used as wild-type. w^{1118} , long-lived (La) and normal-lived (Ra) strains were carefully maintained as previously reported (Arking 1987). w^{1118} flies were used as control for La and Ra. *Mef2-Gal4* (#27390), *Mhc-Gal4* (#38464), and *UAS-GFP* (#4775) strains were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN, USA). *UAS-HP1a-RNAi* (#31994), *UAS-ATM-RNAi* (#22502), and *UAS-ATR-RNAi* (#103624) strains were obtained from the Vienna *Drosophila* RNAi Center (VDRC, Vienna, Austria).

Immunocytochemistry

To obtain thorax muscles, thorax tissues were fixed in 4 % paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 2 h at room temperature and infiltrated with 20 % sucrose overnight at 4 °C. After freezing in Tissue-Tec OCT medium (SAKURA, West Chester, PA, USA), 12- μ m

thick sections were obtained by Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany). Sections were blocked in 1 % bovine serum albumin (BSA) for 30 min and incubated overnight with primary antibodies, washed with phosphate-buffered saline (PBS), and then incubated for 1 h with secondary antibodies. After washing with PBS, samples were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and analyzed using a Zeiss AxioSkop 2plus microscope (Carl Zeiss, Göttingen, Germany).

Dissected mid-leg muscles were fixed for 30 min in 4 % paraformaldehyde at room temperature then washed with PBS supplemented with 0.1 % Triton X-100 (PBST). The samples were incubated overnight with primary antibodies at 4 °C. After washing with PBST, the samples were incubated for 1 h with secondary antibodies at room temperature, washed in PBST, mounted with Vectashield, and analyzed using a Zeiss AxioSkop 2plus microscope.

Antisera

Antibodies for immunostaining were diluted as follows: rabbit anti- γ H2AvD (the *Drosophila* ortholog of γ H2AX; Rockland Immunochemicals, Limerick, PA, USA, 1:1000), mouse anti-mono and poly-ubiquitin (Enzo Life Sciences, Farmingdale, NY, USA, 1:1000), rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA, 1:400), mouse anti-HP1 (DSHB, Iowa City, IA, USA, 1:100), rabbit anti-4-hydroxynonenal (Abcam, Cambridge, UK, 1:400), and rhodamine-phalloidin (Invitrogen, Grand Island, NY, USA, 1:400). Secondary antibodies included Cy3-conjugated goat anti-rabbit, FITC-conjugated goat anti-rabbit, and FITC-conjugated goat anti-mouse (Jackson ImmunoResearch, West Grove, PA, USA, 1:400). DAPI (Molecular Probes, Grand Island, NY, USA, 1:400) was used to detect DNA.

Ex vivo paraquat treatment

Dissected leg muscles were treated with 10 mM paraquat (PQ) in Shields and Sang M3 Insect Medium (WelGene, Daegu, Korea) for 1 h. Samples were subsequently fixed and immunostained as described above. Control samples were treated only with M3 media.

γ -Irradiation

Adult flies were irradiated with a γ -irradiation machine (^{137}CS , 21.275tBq [575 Ci], MDS Nordion International, Kanata, Canada) at a rate of 2.55 Gy/min.

Climbing assay

Analysis of climbing activity was performed as previously described (Gargano et al. 2005). Adult flies were anesthetized, and approximately 30 flies were loaded into plastic vials. After 12 h, flies were tapped to the bottoms of the vials, and climbing flies were recorded by Sony NEX-F3 digital camera (Sony, Tokyo, Japan). The heights of flies 4 s after initiation of climbing were measured by analyzing the recordings.

Measurement of lifespan

To measure lifespans, 25–30 adult flies were cultured in a vial and transferred into new vials containing fresh food every 2–3 days. Dead flies were counted every 2–3 days.

Measurement of mean fluorescence

To measure the mean fluorescence of γ H2AvD signals, 15–20 nuclei per sample were selected and analyzed by Adobe Photoshop CS3 extended software (Adobe Systems, San Jose, CA, USA). The value was normalized to randomly selected background fluorescence intensity.

Quantitative analysis of foci number

To quantitatively analyze the number of poly-ubiquitin and cleaved caspase-3 foci, images were analyzed by ImageJ (NIH, Bethesda, MD, USA). The number of foci was counted automatically by the Analyze Particle tool.

Statistical analysis

Quantified data are expressed as the mean \pm SEM values. Significance testing was conducted using Student's *t* test (unpaired, two-sided *t* test). All data

analyses and plotting were conducted by GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

Results

Increased γ H2AX in aged *Drosophila* muscles

To determine whether γ H2AX accumulates in *Drosophila* muscle with age, we stained the thoracic and leg muscles of OR flies with an anti- γ H2AvD antibody. To confirm the γ H2AvD signal in muscle, we treated flies with γ -irradiation, a DSB inducer. After 20 Gy irradiation, we detected increased γ H2AvD foci in leg muscles of OR young (5-days-old) flies (Fig. S1). The level of γ H2AvD also increased after ex vivo treatment with PQ, an oxidative stress inducer (Fig. S2a). Then, we stained the thoracic and leg muscles of OR young and aged flies with an anti- γ H2AvD antibody. The level of γ H2AvD in aged (30-days-old) flies was increased compared to young (5-days-old) flies in thoracic muscle (Fig. 1a, b). Similarly, we observed a gradual increase of γ H2AvD in 30- and 60-days-old leg muscles compared to 5-days-old leg muscles (Fig. 1c–e). To compare γ H2AvD levels accurately, we quantified the mean γ H2AvD fluorescence in each DAPI region (Fig. 1f). The age-related increase of γ H2AvD was only absent in muscle with ATM knockdown (Fig. S3a), indicating an ATM-dependent increase of γ H2AvD in muscle but no dependence on ATR. Leg muscles of aged (40-days-old) flies showed much stronger level of γ H2AvD than young (5-days-old) flies after 20 Gy irradiation (Fig. S1) indicating increased sensitivity to DNA damage in aged muscle. These results indicate that γ H2AX levels increase in adult thoracic and leg muscles during aging.

Age-related degenerative markers in *Drosophila* muscle

To understand the significance of increased γ H2AX in muscle, we set up muscular degenerative markers. We first measured the activity of caspase-3, the key protease in apoptosis (Nicholson and Thornberry 1997), as a muscular degenerative marker. OR thoracic muscles were stained with an anti-cleaved caspase-3 antibody, which detects the active form of caspase-3 (Kumar 2007). As expected, the number of cleaved

caspase-3 foci showed an age-dependent increase. A robust increase in the number of foci was detected in muscle of 50-days-old flies, exceeding that in 5- and 30-days-old flies (Fig. 2a–c, g). This indicates that caspase-3 activity increases in aged thoracic muscle.

We also assessed poly-ubiquitin aggregates for damaged protein accumulation. As previously reported (Demontis and Perrimon 2010), we observed an age-dependent increase of poly-ubiquitin aggregates in OR thoracic muscle (Fig. 2d–f, h). Because caspase-3 affects muscular protein degradation through its protease activity associated with the ubiquitin–proteasome degradation pathway (Du et al. 2004; Wang et al. 2010), we co-immunostained cleaved caspase-3 and poly-ubiquitin in aged thoracic muscle. Interestingly, cleaved caspase-3 foci co-localized with poly-ubiquitin aggregates (Fig. 2i). Four-hydroxynonenal (4-HNE) foci, a marker of oxidative damage (Dalleau et al. 2013), also showed an age-related increase and co-localized with poly-ubiquitin aggregates (Fig. S2b). These results indicate that the levels of cleaved caspase-3 and poly-ubiquitin aggregates are useful as muscular degenerative markers in this study.

Correlation among γ H2AX levels, muscle degenerative markers, and organismal aging in longevity strains

We investigated whether the γ H2AX level is correlated with muscular degenerative markers and organismal aging. For this purpose, we used finely selected longevity strains with short- (w^{1118}), normal- (Ra), or long-lived (La) lifespans (Arking 1987; Soh et al. 2007).

The age-related changes of γ H2AX levels were examined in 5-, 30-, and 50-days-old thoracic and leg muscles of longevity strains by using the anti- γ H2AvD antibody. In immunohistochemical images and quantified data, La shows no increase in γ H2AvD, whereas w^{1118} and Ra show age-related increases of γ H2AvD in thoracic muscle (Fig. 3a–c, g, S4). In leg muscle, all three strains showed age-related increases of γ H2AvD; however, the lowest and highest rates of increase were observed for La and w^{1118} , respectively (Fig. 3d–f, h, S5). These results indicate that the age-related increase of γ H2AvD in muscle is related to organismal aging.

Then, we examined the age-related changes of caspase-3 activity and ubiquitin aggregates in longevity strains. In w^{1118} and Ra, age-related increases of the

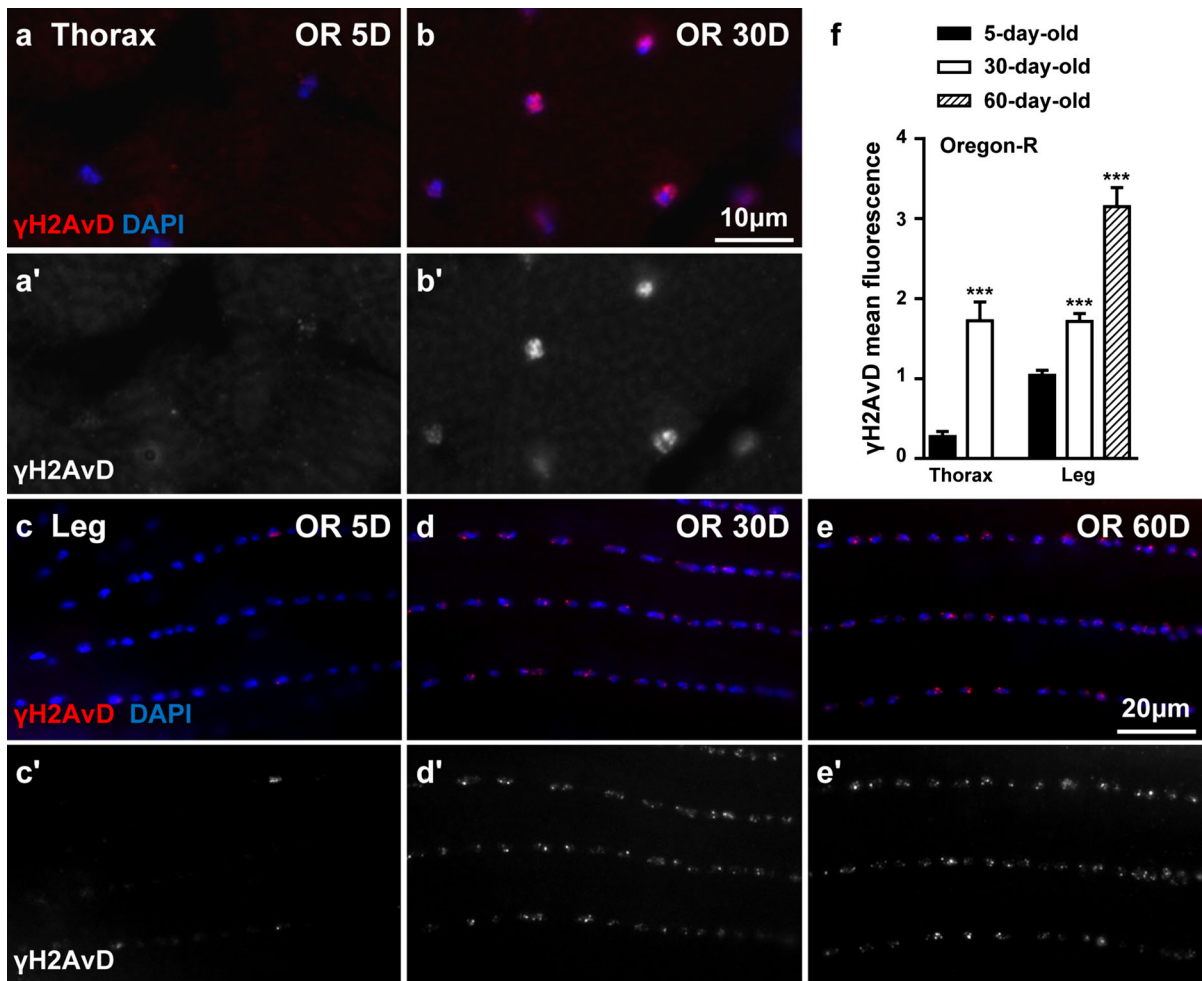


Fig. 1 γ H2AvD accumulation in thoracic and leg muscles with age. **a, b** Immunostaining with anti- γ H2AvD antibody (red) was performed in thoracic muscles of OR adult females. The level of γ H2AvD in thoracic muscle nuclei was increased in 30-days-old compared to 5-days-old adult. **c–e** The age-related increase of γ H2AvD was also detected in leg muscle. The γ H2AvD level increased gradually from 5- to 60-days-old tissue. **f** The

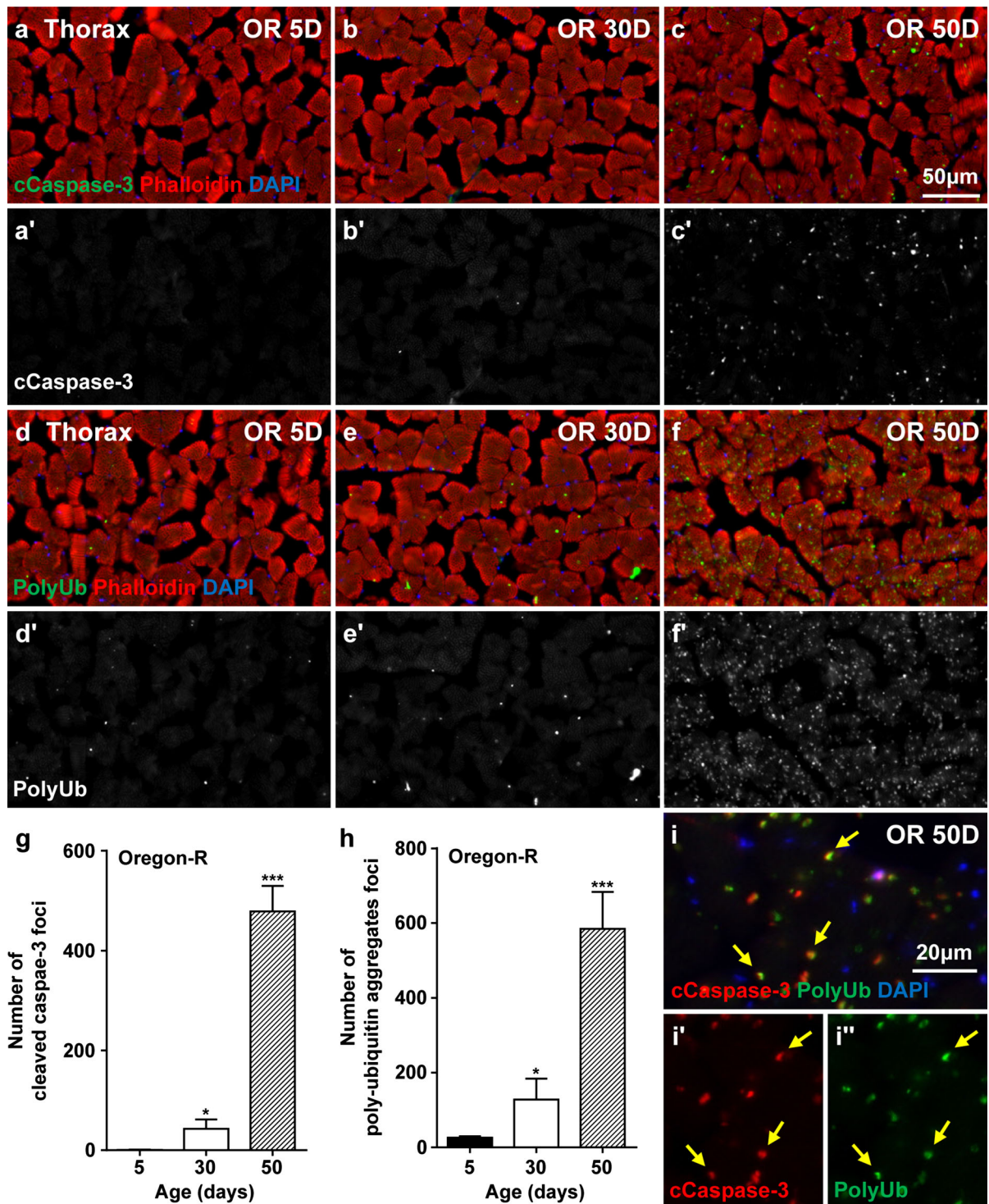
γ H2AvD mean fluorescence was measured in selected nuclei of thoracic and leg muscles (5- and 30-days-old thorax, $n = 64$ and 80, respectively; 5-, 30-, and 60-days-old leg, $n = 150$, 150, and 150, respectively). Data are given as mean \pm SEM, *** $p < 0.001$, two-tailed unpaired t test. Asterisks indicate significance compared to the 5-days-old control. (Color figure online)

number of cleaved caspase-3 foci were observed in thoracic muscle. w^{1118} showed the most rapid increase in the number of foci, but the La strain showed no significant increase in aged muscle (Fig. 4a–c, g, S6). Age-related increases in poly-ubiquitin aggregates were detected in all three longevity strains. Whereas w^{1118} has the most abundant foci in aged muscle, La showed only a slightly increased level of poly-ubiquitin aggregates (Fig. 4d–f, h, S7). These levels of cleaved caspase-3 and poly-ubiquitin aggregates in the three longevity strains indicate that age-related increases of caspase-3 and poly-ubiquitin aggregates

in muscle are related to organismal aging. Altogether, these results indicate that the γ H2AX level is correlated with muscular degenerative markers as well as organismal aging.

Correlation among γ H2AX level, muscle degenerative markers, and organismal aging in muscle-specific HP1a-knockdown flies

To test whether γ H2AX level is correlated with muscular degenerative markers and organismal aging, we also examined flies with muscle-specific



knockdown of HP1a by using muscle-specific Gal4 drivers (Fig. S8). Flies with muscle-specific HP1a knockdown showed significant loss of HP1 in thoracic

and leg muscles (Fig. 5, S9a). Moreover, we observed that muscle-specific knockdown of HP1a shortens lifespan compared to control flies (Fig. S10a). We

◀ **Fig. 2** Age-related increase of degenerative markers in thoracic muscle. Immunostaining was performed on cryosections in 5-, 30-, and 50-days-old OR adult female thoracic muscle. Thoracic muscle was stained for nuclei (DAPI, *blue*), F-actin (rhodamine-phalloidin, *red*), **a–c** cleaved caspase-3 (*green*), and **d–f** poly-ubiquitin (*green*). Abundant foci were observed in aged thoracic muscle. **g, h** The foci were counted in a selected region of thoracic muscle (5-, 30-, and 50-days-old thorax, $n = 8, 6,$ and $8,$ respectively). **i** Co-localization of cleaved caspase-3 (*red*) and poly-ubiquitin (*green*) is shown. *Arrows* indicate co-localization. All images display transverse views. Data are given as the mean \pm SEM, $*p < 0.05,$ $***p < 0.001,$ two-tailed unpaired t test. *Asterisks* indicate significance compared to the 5-days-old control. (Color figure online)

observed that flies with muscle-specific HP1a knockdown have a rapid, age-related increase of the γ H2AvD signal in thoracic and leg muscles compared to control flies (Fig. 5). This indicates that HP1 depletion in muscle induces increased γ H2AvD levels.

We next examined muscular degeneration by observing levels of cleaved caspase-3 and ubiquitin aggregates. The number of cleaved caspase-3 foci in 30-days-old muscle with HP1a knockdown strikingly increased compared to the control, whereas 5-days-old thoracic muscle with HP1a knockdown was similar to control (Fig. 6a–c). Similarly, 30-days-old flies with muscle-specific knockdown of HP1a showed a great increase in the number of ubiquitin aggregate foci in thoracic muscle compared to the control, although there was no significant difference between 5-days-old control and HP1a-knockdown flies (Fig. 6d–f, S9b, S10b). This further supports the correlation between high levels of γ H2AvD and increased degenerative markers in muscle.

In addition, we assessed the effects of HP1a depletion on climbing activity. Control flies showed decreases in climbing activity between 1- and 5-week-old female adults. However, the climbing activity of HP1a-knockdown flies decreased more sharply than that of the controls (Fig. 6g). These results support our previous findings that increased γ H2AX is correlated with muscular aging as well as organismal aging.

Discussion

In the present study, we demonstrated an age-related increase of DNA damage in fully differentiated *Drosophila* muscle cells as well as a robust correlation between muscle damage and longevity.

The age-related increase of γ H2AX in muscle is likely to be proximately due to oxidative stress. Oxidative stress is regarded as a significant cause of DNA damage and physiological decline in function during aging (Finkel and Holbrook 2000), and muscle tissue shows spontaneous production of oxidative stress (Powers et al. 2010). Our finding that 4-HNE shows an age-dependent increase in muscle further supports the role of endogenous oxidative damage in muscle tissue (Fig. S2b). The La strain exhibits a significantly lower level of γ H2AX in aged muscle and has higher expression of genes involved in antioxidant defense than the normal-lived Ra strain, which accounts for the extended longevity phenotype of the La strain (Fig. 3, S4, S5) (Arking 2001). Mitochondria from the La strain produce significantly lower levels of ROS in vitro relative to Ra mitochondria (Ross 2000). Cybrid flies with Ra nuclear genomes and La mitochondrial genomes showed an extended longevity phenotype, suggesting mitochondria as a key determinant of longevity (Soh et al. 2007). Additionally, our data showed that ex vivo treatment with PQ induces a significant increase of γ H2AX levels in muscle (Fig. S2a). Therefore, age-related mitochondrial dysfunction may be a factor underlying the age-related increase of γ H2AX in muscles.

One of our major questions we were asking is whether the γ H2AX levels can be used as a biomarker of muscle function in *Drosophila*. Our data showed an age-related increase of γ H2AX accompanying the increase of degenerative markers. As shown in the La strain, when the γ H2AX level did not increase significantly, the number of foci indicating degenerative markers also did not increase (Fig. 4, S6, S7). Conversely, excessive γ H2AX formation induced by muscle-specific HP1a knockdown leads to increased accumulation of muscle degenerative markers (Fig. 6, S9). These results are similar to previous work showing that loss of heterochromatin with excessive DNA damage accumulation and genomic instability is a crucial cause of neurodegenerative disease in *Drosophila* brain tissues, which is also composed of fully differentiated cells (Frost et al. 2014). In murine study, it was reported that DNA damage in muscle occurs prior to muscle degeneration (Sandri et al. 1995). And decreased DNA damage accumulation in myostatin knockout mice showed decreased muscle degeneration (Sriram et al. 2014). Therefore, our data

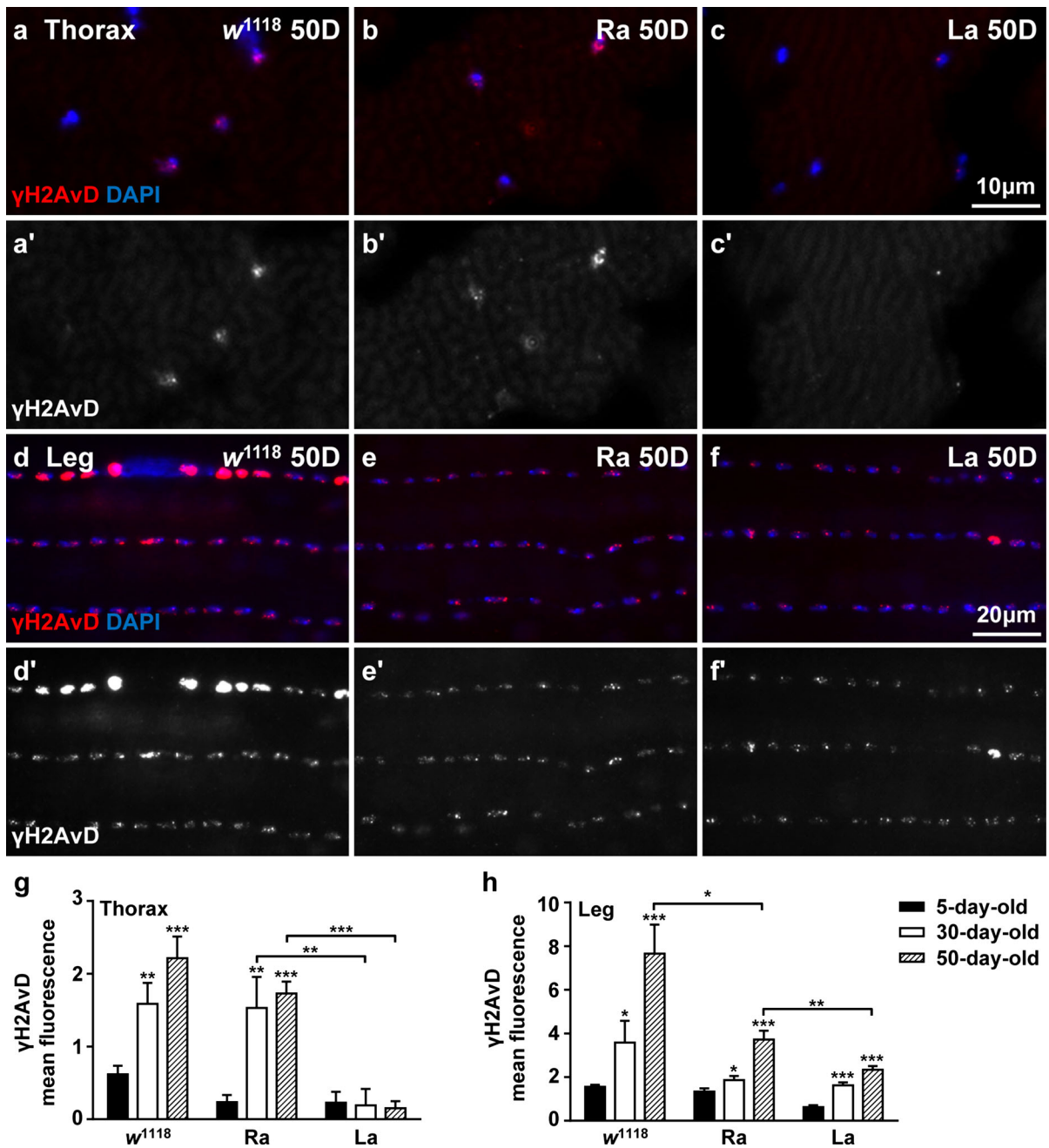


Fig. 3 γ H2AvD accumulation in thoracic and leg muscles of longevity strains. Immunostaining with anti- γ H2AvD antibody (red) was performed in **a–c** thoracic and **d–f** leg muscles of three longevity strains: w^{1118} , Ra, and La. The level of γ H2AvD in 50-days-old leg muscle is lowest in La and highest in w^{1118} . **g**, **h** The exact comparison was achieved by measuring the γ H2AvD fluorescence in thoracic (w^{1118} , Ra, and La: 5-days-

old, $n = 50, 40, \text{ and } 40$; 30-days-old, $n = 50, 40, \text{ and } 40$; 50-days-old, $n = 50, 60, \text{ and } 60$, respectively) and leg muscle (w^{1118} , Ra, and La: 5-days-old, $n = 75, 60, \text{ and } 60$; 30-days-old, $n = 60, 60, \text{ and } 60$; 50-days-old, $n = 60, 45, \text{ and } 45$, respectively). Nuclei (DAPI, blue) were visualized. Data are given as the mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-tailed unpaired t test. (Color figure online)

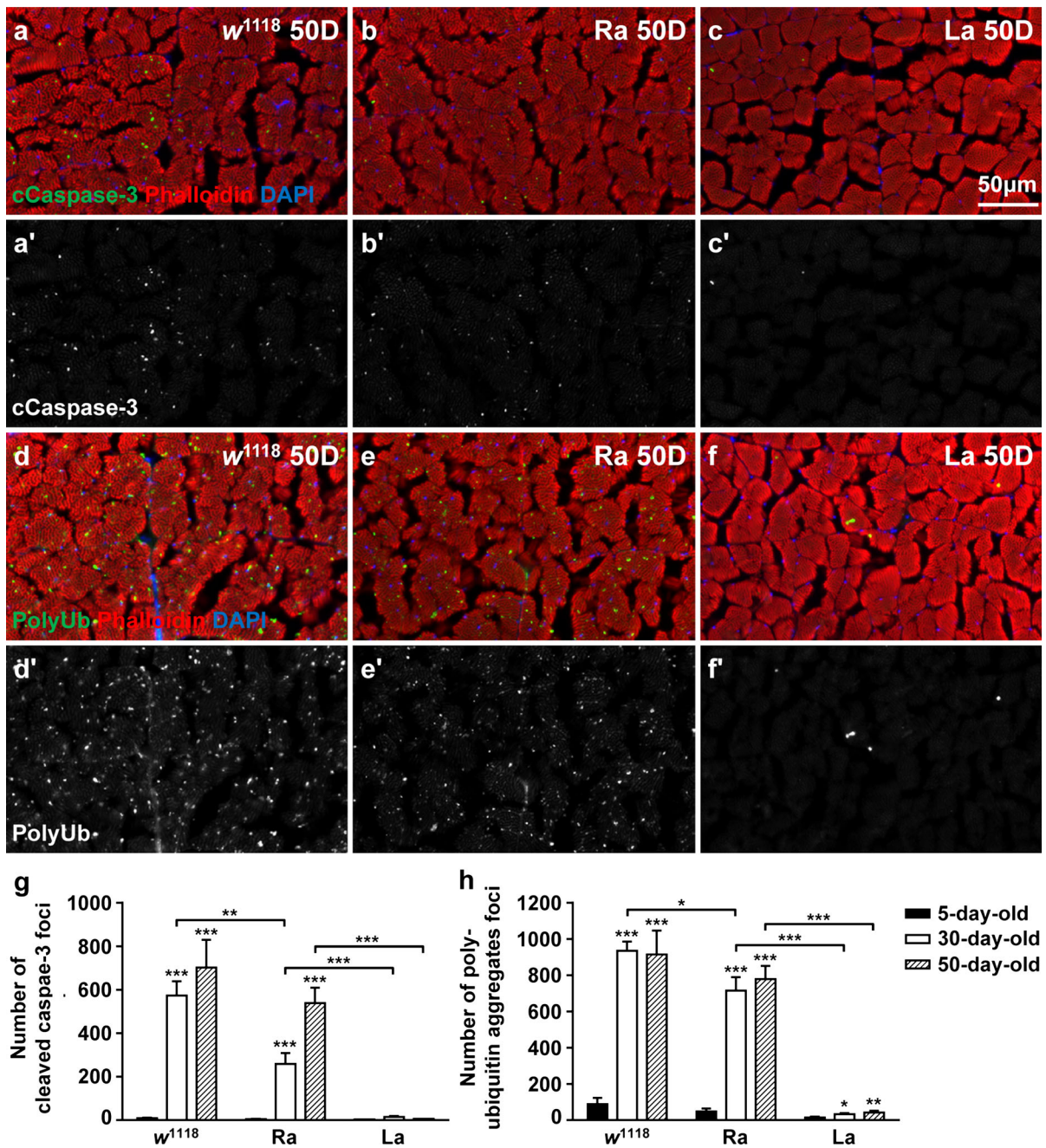


Fig. 4 Increase of degenerative markers in thoracic muscle of longevity strains. Immunostaining was performed on cryosections from *w*¹¹¹⁸, Ra, and La thoracic muscle. Nuclei (DAPI, blue) and F-actin (rhodamine-phalloidin, red) were visualized. **a–c** Cleaved caspase-3 (green) was stained in 50-days-old thoracic muscle. *w*¹¹¹⁸ tissue exhibits the most abundant foci while La tissue has few foci. **d–f** Poly-ubiquitin (green) was stained in 50-days-old thoracic muscle. La tissue has remarkably fewer foci than *w*¹¹¹⁸ and Ra. For quantification, **g** cleaved

caspase-3 (*w*¹¹¹⁸, Ra, and La: 5-days-old, n = 15, 11, and 12; 30-days-old, n = 12, 11, and 12; 50-days-old, n = 7, 6, and 8, respectively) and **h** poly-ubiquitin foci (*w*¹¹¹⁸, Ra, and La: 5-days-old, n = 14, 12, and 12; 30-days-old, n = 13, 12, and 11; 50-days-old, n = 7, 6, and 7, respectively) were counted in selected regions of thoracic muscle. Data are given as the mean ± SEM, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, two-tailed unpaired *t* test. (Color figure online)

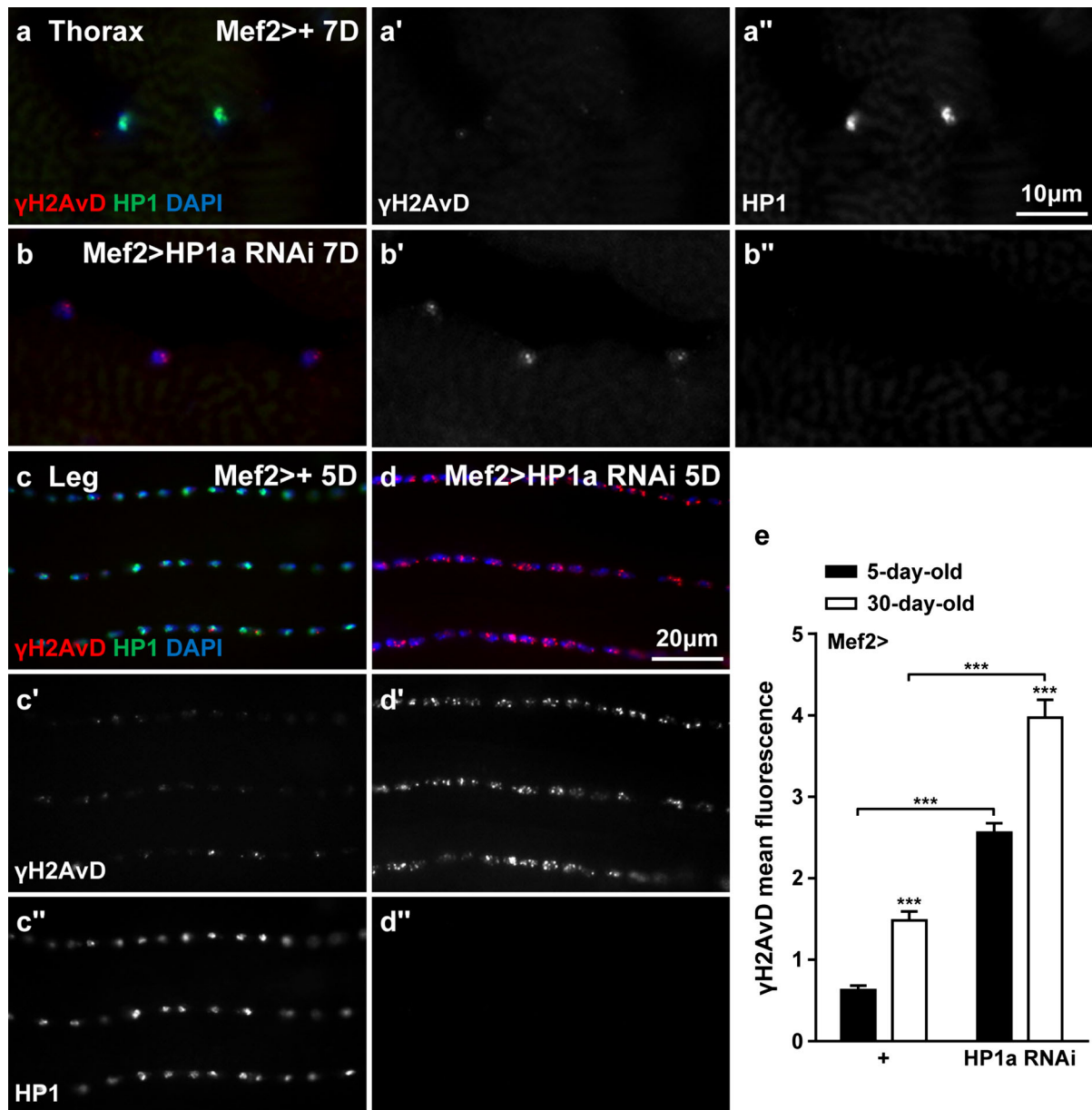


Fig. 5 Increased γ H2AvD in flies with muscle-specific HP1a knockdown. Immunostaining with anti- γ H2AvD (red) and anti-HP1 (green) antibodies was performed for flies with muscle-specific knockdown of HP1a knockdown (+/+; *Mef2-Gal4/UAS-HP1a-RNAi*). **a, b** In 7-days-old thoracic muscle of HP1a-knockdown flies, HP1 was successfully depleted, and the γ H2AvD level was higher than in the control (+/+; *Mef2-*

Gal4+). **c, d** Increased γ H2AvD was also detected in 5-days-old leg muscle of HP1a-knockdown flies with depletion of HP1 in nuclei. **e** The γ H2AvD fluorescence in leg muscle nuclei was measured (5- and 30-days-old: control, n = 90 and 90, respectively; HP1a knockdown, n = 90 and 90, respectively). Data are given as the mean \pm SEM, ****p* < 0.001, two-tailed unpaired *t* test. (Color figure online)

suggest that nuclear DNA damage, specifically DSBs represented by γ H2AX is closely linked to age-related macromolecular changes represented by degenerative markers.

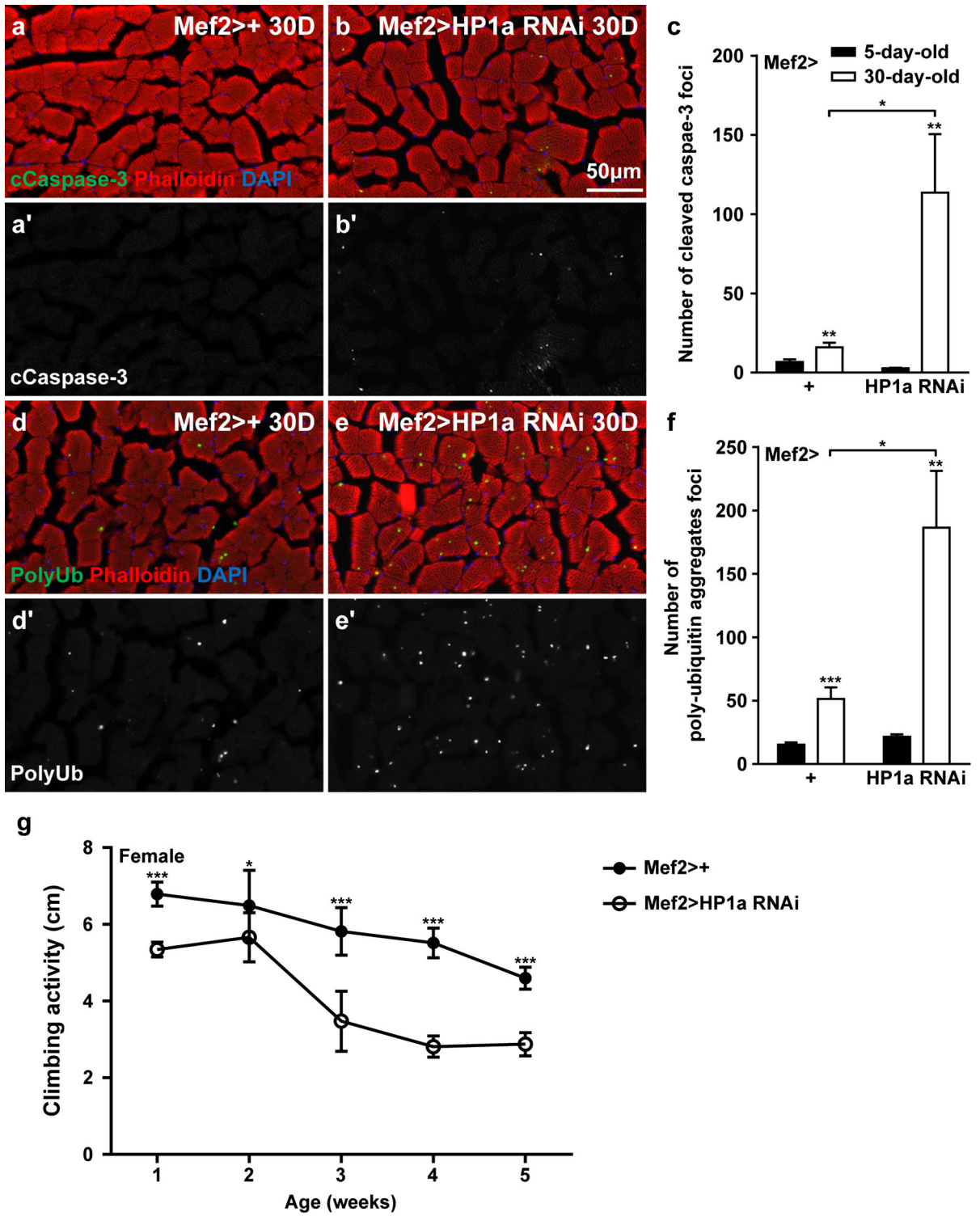
The correlation between γ H2AX and muscle degeneration can be explained in terms of the DNA damage repair mechanism in differentiated cells. Age-related phenotypes differ between proliferative

midgut stem cells and differentiated muscle cells with increased γ H2AX formation, and this difference may be associated with the DNA repair capacity and the outcome of the DNA damage response. Formation of γ H2AX is an early response of DSB repair (Tanaka et al. 2006), after which cells repair damage and then re-enter either the cell cycle or senescence and death (Bartek and Lukas 2007). Pyo et al. (2014) showed that γ H2AX disappeared within 24 h after irradiation of highly proliferative *Drosophila* intestinal stem cells (ISCs), indicating a rapid repair process that is consistent with other observations (Redon et al. 2011). Moreover, aged *Drosophila* ISCs show increased proliferative activity despite increased γ H2AX formation (Park et al. 2012; Na et al. 2013). In differentiated muscle cells, however, factors involved in DNA damage repair are downregulated compared to their counterparts in proliferative cells (Fortini and Dogliotti 2010; Szczesny et al. 2010) and muscle cells have slow kinetics of γ H2AX formation after DNA damage (Fortini et al. 2012). Our finding that irradiation-induced γ H2AX remained present for 168 h in *Drosophila* muscle (Fig. S3) further indicates a slow and inactive repair process. Generally, DNA repair ends 48 h after inducing damage, and persistent damage repair foci can promote senescence-associated gene expression changes (Fortini et al. 2012; Rodier et al. 2011). Despite cellular senescence is generally termed in proliferating cells, it was reported that fully differentiated neuron cell showed senescence-like phenotype with age (Jurk et al. 2012). Therefore, although it needs more precise and diverse approaches for the possibility of senescence phenotypes in differentiated muscle cell, tissue degeneration may result from distinct DNA damage repair in fully differentiated muscle cells.

We observed that flies with muscle specific ATM knockdown are defective in γ H2AX formation in muscle. These flies show more rapid increase of degenerative markers in muscle with age than control or ATR knockdown (Fig. S3b–e). The phenotypes of flies with muscle specific ATM knockdown may indicate that γ H2AX formation is required in muscle during aging. Increased degenerative markers in muscle with ATM knockdown may be also due to γ H2AX independent role of ATM, since it was reported that extranuclear localized ATM negatively regulates cellular oxidative stress level (Reichenbach et al. 2002; Watters et al. 1999).

Fig. 6 Increase of degenerative markers in flies with muscle-specific HP1a knockdown. Degenerative markers, nuclei (DAPI, *blue*), and F-actin (rhodamine-phalloidin, *red*) were stained in 30-days-old thoracic muscles of flies with muscle-specific knockdown of HP1a. **a, b** 30-days-old flies had a higher number of cleaved caspase-3 foci (*green*) in HP1a-knockdown muscle than in control muscle. **c** Cleaved caspase-3 foci were counted in a selected region of the transverse view (5- and 30-days-old: control, n = 15 and 13, respectively; HP1a knockdown, n = 14 and 15, respectively). **d, e** The number of poly-ubiquitin aggregate foci (*green*) was also higher in HP1a knockdown muscle than in control muscle. **f** The poly-ubiquitin foci were counted in a selected region of the transverse view (5- and 30-days-old: control, n = 15 and 13, respectively; HP1a knockdown, n = 14 and 15, respectively). Data are given as the mean \pm SEM, * p < 0.05, ** p < 0.01, *** p < 0.001, two-tailed unpaired t test. **g** Decreased climbing activity was detected in 1- to 5-week-old flies with muscle-specific HP1a knockdown (control and HP1a knockdown: 1-week-old, n = 10 and 5; 2-week-old, n = 14 and 10; 3-week-old, n = 15 and 10; 4-week-old, n = 10 and 6; 5-week-old, n = 5 and 5, respectively). Data are given as the mean \pm SD, * p < 0.05, *** p < 0.001, two-tailed unpaired t test. (Color figure online)

It is well known that tissue interactions seem to coordinate and regulate systemic aging via endocrine signals (Russell and Kahn 2007). Recent work in *Drosophila* has shown that muscle has the capacity to significantly alter organismal longevity by either autonomous or non-autonomous processes involving other types of signals (Demontis et al. 2014; Bai et al. 2013; Owusu-Ansah et al. 2013). It seems as if muscle-autonomous signal can result in preserving muscle functions, but that muscle non-autonomous signals can result in extended longevity of the organism. In the present study, we demonstrated a close association between the γ H2AX level in muscle and longevity: longer-lived strains experience a much slower increase of γ H2AX in muscle tissue (Fig. 3, S4, S5). Furthermore, DNA damage accumulation in muscle by muscle-specific HP1a knockdown supports the association between γ H2AX and longevity, implying accelerated organismal aging (Fig. S10). Our findings are in line with previous evidence that excessive DNA damage accumulation can shorten organismal longevity (reviewed in Moskalev et al. 2013). The γ H2AX level provides a cross-sectional molecular biomarker which can be usefully combined with the longitudinal climbing assay to deliver an integrated view of flight and/or leg muscle function in *Drosophila*. Its use also should allow the specific identification of the signaling pathways involved in



the experimental modulation of the DNA damage response in *Drosophila* muscle and other tissues.

Notably, cleaved caspase-3 was detected in thoracic muscle but not in leg muscle (data not shown). *Drosophila* thoracic and leg muscles are similar to mammalian slow-twitch and fast-twitch muscles, respectively (Taylor 2006). Our results may indicate a difference in the caspase-3 pathways of thoracic and leg muscle, and further investigation is required to determine the source of these differences.

In summary, we showed an age-related increase of γ H2AX in fully differentiated *Drosophila* muscle and its correlation with degenerative markers, cleaved caspase-3 and poly-ubiquitin aggregates, and longevity. We suggest that aging in differentiated muscle cell is a crucial determinant of organismal aging. Our data indicates that γ H2AX can be a biomarker of muscle aging in *Drosophila* differentiated muscle cells in further studies for interventions of muscle aging and healthy aging.

Acknowledgments We thank Prof. Byung P. Yu (University of Texas Health Science Center) for invaluable comments on the manuscript. This work was supported by the R&D program of MOTIE/KEIT (10040391, Development of Functional Food Materials and Device for Prevention of Aging-associated Muscle Function Decrease).

References

- Arking R (1987) Successful selection for increased longevity in *Drosophila*: analysis of the survival data and presentation of a hypothesis on the genetic regulation of longevity. *Exp Gerontol* 22:199–220
- Arking R (2001) Gene expression and regulation in the extended longevity phenotypes of *Drosophila*. *Ann NY Acad Sci* 928:157–167
- Augustin H, Partridge L (2009) Invertebrate models of age-related muscle degeneration. *Biochim Biophys Acta* 1790:1084–1094
- Bai H, Kang P, Hernandez A, Tatar M (2013) Activin signaling targeted by Insulin/dFOXO regulates aging and muscle proteostasis in *Drosophila*. *PLoS Genet* 9:e1003941
- Bartek J, Lukas J (2007) DNA damage checkpoints: from initiation to recovery or adaptation. *Curr Opin Cell Biol* 19:238–245
- Bopp A, Wartlick F, Henninger C, Kaina B, Fritz G (2013) Rac1 modulates acute and subacute genotoxin-induced hepatic stress responses, fibrosis and liver aging. *Cell Death Dis* 4:e558
- Burgess RC, Misteli T, Oberdoerffer P (2012) DNA damage, chromatin, and transcription: the trinity of aging. *Curr Opin Cell Biol* 24:724–730
- Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, Martin FC, Michel JP, Rolland Y, Schneider SM, Topinková E, Vandewoude M, Zamboni M (2010) Sarcopenia: European consensus on definition and diagnosis: report of the European working group on sarcopenia in older people. *Age Ageing* 39:412–423
- Daczewska M, Picchio L, Jagla T, Figeac N, Jagla K (2010) Muscle development and regeneration in normal and pathological conditions: learning from *Drosophila*. *Curr Pharm Des* 16:929–941
- Dalleau S, Baradat M, Gueraud F, Huc L (2013) Cell death and diseases related to oxidative stress: 4-hydroxynonenal (HNE) in the balance. *Cell Death Differ* 20:1615–1630
- Demontis F, Perrimon N (2010) FOXO/4E-BP signaling in *Drosophila* muscles regulates organism-wide proteostasis during aging. *Cell* 143:813–825
- Demontis F, Piccirillo R, Goldberg AL, Perrimon N (2013) The influence of skeletal muscle on systemic aging and lifespan. *Aging Cell* 12:943–949
- Demontis F, Patel V, Swindell W, Perrimon N (2014) Intertissue control of the nucleolus via a myokine-dependent longevity pathway. *Cell Rep* 12:1481–1494
- Du J, Wang X, Miereles C, Bailey JL, Debigare R, Zheng B, Price SR, Mitch WE (2004) Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions. *J Clin Invest* 113:115–123
- Falk M, Lukasova E, Kozubek S (2008) Chromatin structure influences the sensitivity of DNA to gamma-radiation. *Biochim Biophys Acta* 1783:2398–2414
- Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. *Nature* 408:239–247
- Fortini P, Dogliotti E (2010) Mechanisms of dealing with DNA damage in terminally differentiated cells. *Mutat Res* 685:38–44
- Fortini P, Ferretti C, Pascucci B, Narciso L, Pajalunga D, Puggioni E, Castino R, Isidoro C, Crescenzi M, Dogliotti E (2012) DNA damage response by single-strand breaks in terminally differentiated muscle cells and the control of muscle integrity. *Cell Death Differ* 19:1741–1749
- Freitas AA, De Magalhães JP (2011) A review and appraisal of the DNA damage theory of ageing. *Mutat Res* 728:12–22
- Frost B, Hemberg M, Lewis J, Feany M (2014) Tau promotes neurodegeneration through global chromatin relaxation. *Nat Neurosci* 17:357–366
- Garcia A, Calder R, Dollé M, Lundell M, Kapahi P, Vijg J (2010) Age- and temperature-dependent somatic mutation accumulation in *Drosophila melanogaster*. *PLoS Genet* 6:e1000950
- Gargano JW, Martin I, Bhandari P, Grotewiel MS (2005) Rapid iterative negative geotaxis (RING): a new method for assessing age-related locomotor decline in *Drosophila*. *Exp Gerontol* 40:386–395
- Gopinath S, Rando T (2008) Stem cell review series: aging of the skeletal muscle stem cell niche. *Aging Cell* 7:590–598
- Jurk D, Wang C, Miwa S, Maddick M, Korolchuk V, Tsolou A, Gonos ES, Thrasivoulou C, Saffrey MJ, Cameron K, von Zglinicki T (2012) Postmitotic neurons develop a p21-dependent senescence-like phenotype driven by a DNA damage response. *Aging Cell* 11:996–1004
- Kaina B (2003) DNA damage-triggered apoptosis: critical role of DNA repair, double-strand breaks, cell proliferation and signaling. *Biochem Pharmacol* 66:1547–1554

- Kumar S (2007) Caspase function in programmed cell death. *Cell Death Differ* 14:32–43
- Larson K, Yan SJ, Tsurumi A, Liu J, Zhou J, Gaur K, Guo D, Eickbush TH, Li WX (2012) Heterochromatin formation promotes longevity and represses ribosomal RNA synthesis. *PLoS Genet* 8:e1002473
- Li H, Mitchell JR, Hastay P (2008) DNA double-strand breaks: a potential causative factor for mammalian aging? *Mech Ageing Dev* 129:416–424
- Mah LJ, El-Osta A, Karagiannis T (2010) GammaH2AX as a molecular marker of aging and disease. *Epigenetics* 5: 129–136
- Marzetti E, Leeuwenburgh C (2006) Skeletal muscle apoptosis, sarcopenia and frailty at old age. *Exp Gerontol* 41:1234–1238
- Moskalev AA, Shaposhnikov MV, Plyusnina EN, Zhavoronkov A, Budovsky A, Yanai H, Fraifeld VE (2013) The role of DNA damage and repair in aging through the prism of Koch-like criteria. *Ageing Res Rev* 12:661–684
- Na HJ, Park JS, Pyo JH, Lee SH, Jeon HJ, Kim YS, Yoo MA (2013) Mechanism of metformin: inhibition of DNA damage and proliferative activity in *Drosophila* midgut stem cell. *Mech Ageing Dev* 134:381–390
- Nair KS (2005) Aging muscle. *Am J Clin Nutr* 81:953–963
- Newman AB, Kupelian V, Visser M, Simonsick EM, Goodpaster BH, Kritchevsky SB, Tyllavsky FA, Rubin SM, Harris TB (2006) Strength, but not muscle mass, is associated with mortality in the health, aging and body composition study cohort. *J Gerontol A Biol Sci Med Sci* 61: 72–77
- Nicholson DW, Thornberry NA (1997) Caspases: killer proteases. *Trends Biochem Sci* 22:299–306
- O'Sullivan RJ, Karlseder J (2012) The great unravelling: chromatin as a modulator of the aging process. *Trends Biochem Sci* 37:466–476
- Owusu-Ansah E, Song W, Perrimon N (2013) Muscle mitochondrial hormesis promotes longevity via systemic repression of insulin signaling. *Cell* 155:699–712
- Papamichos-Chronakis M, Peterson C (2013) Chromatin and the genome integrity network. *Nat Rev Genet* 14:62–75
- Park JS, Lee SH, Na HJ, Pyo JH, Kim YS, Yoo MA (2012) Age- and oxidative stress-induced DNA damage in *Drosophila* intestinal stem cells as marked by Gamma-H2AX. *Exp Gerontol* 47:401–405
- Peng JC, Karpen GH (2009) Heterochromatic genome stability requires regulators of histone H3 K9 methylation. *PLoS Genet* 5:e1000435
- Piazza N, Gosangi B, Devilla S, Arking R, Wessells R (2009) Exercise-training in young *Drosophila melanogaster* reduces age-related decline in mobility and cardiac performance. *PLoS One* 4:e5886
- Powers SK, Duarte J, Kavazis AN, Talbert EE (2010) Reactive oxygen species are signalling molecules for skeletal muscle adaptation. *Exp Physiol* 95:1–9
- Pyo JH, Park JS, Na HJ, Jeon HJ, Lee SH, Kim JG, Park SY, Jin YW, Kim YS, Yoo MA (2014) Functional modification of *Drosophila* intestinal stem cells by ionizing radiation. *Radiat Res* 181:376–386
- Rando T (2006) Stem cells, ageing and the quest for immortality. *Nature* 441:1080–1086
- Redon CE, Nakamura AJ, Martin OA, Parekh PR, Weyemi US, Bonner WM (2011) Recent developments in the use of gamma-H2AX as a quantitative DNA double-strand break biomarker. *Aging* 3:168–174
- Reichenbach J, Schubert R, Schindler D, Müller K, Böhles H, Zielen S (2002) Elevated oxidative stress in patients with ataxia telangiectasia. *Antioxid Redox Signal* 4:465–469
- Rodier F, Muñoz D, Teachenor R, Chu V, Le O, Bhaumik D, Coppé JP, Campeau E, Beauséjour CM, Kim SH, Davalos AR, Campisi J (2011) DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *J Cell Sci* 124:68–81
- Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM (1998) DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* 273:5858–5868
- Ross RE (2000) Age-specific decrease in aerobic efficiency associated with increase in oxygen free radical production in *Drosophila melanogaster*. *J Insect Physiol* 46:1477–1480
- Russell S, Kahn C (2007) Endocrine regulation of ageing. *Nat Rev Mol Cell Biol* 8:681–691
- Sandri M, Carraro U, Podhorska-Okolov M, Rizzi C, Arslan P, Monti D, Franceschi C (1995) Apoptosis, DNA damage and ubiquitin expression in normal and mdx muscle fibers after exercise. *FEBS Lett* 373:291–295
- Soh JW, Hotic S, Arking R (2007) Dietary restriction in *Drosophila* is dependent on mitochondrial efficiency and constrained by pre-existing extended longevity. *Mech Ageing Dev* 128:581–593
- Sriram S, Subramanian S, Juvvuna P, McFarlane C, Salerno M, Kambadur R, Sharma M (2014) Myostatin induces DNA damage in skeletal muscle of Streptozotocin-induced type 1 diabetic mice. *J Biol Chem* 289:5784–5798
- Szczesny B, Tann A, Mitra S (2010) Age- and tissue-specific changes in mitochondrial and nuclear DNA base excision repair activity in mice: susceptibility of skeletal muscles to oxidative injury. *Mech Ageing Dev* 131:330–337
- Tanaka T, Halicka H, Huang X, Traganos F, Darzynkiewicz Z (2006) Constitutive histone H2AX phosphorylation and ATM activation, the reporters of DNA damage by endogenous oxidants. *Cell Cycle* 5:1940–1945
- Taylor MV (2006) Comparison of muscle development in *Drosophila* and vertebrates. In: Sink H (ed) *Muscle development in Drosophila*. Landes Bioscience, Springer, New York, pp 169–203
- Wang C, Jurk D, Maddick M, Nelson G, Martin-Ruiz C, Von Zglinicki T (2009) DNA damage response and cellular senescence in tissues of aging mice. *Aging Cell* 8:311–323
- Wang XH, Zhang L, Mitch WE, LeDoux JM, Hu J, Du J (2010) Caspase-3 cleaves specific 19 S proteasome subunits in skeletal muscle stimulating proteasome activity. *J Biol Chem* 285:21249–21257
- Watters D, Kedar P, Spring K, Bjorkman J, Chen P, Gatei M, Birrell G, Garrone B, Srinivasa P, Crane D, Lavin M (1999) Localization of a portion of extranuclear ATM to peroxisomes. *J Biol Chem* 274:34277–34282
- Yan SJ, Lim SJ, Shi S, Dutta P, Li WX (2011) Unphosphorylated STAT and heterochromatin protect genome stability. *FASEB J* 25:232–241
- Zheng J, Edelman SW, Tharmarajah G, Walker DW, Pletcher SD, Seroude L (2005) Differential patterns of apoptosis in response to aging in *Drosophila*. *Proc Natl Acad Sci USA* 102:12083–12088