Maintenance of muscle myosin levels in adult 
*C. elegans* requires both the double bromodomain protein BET-1 and sumoylation

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Summary
Attenuation of RAS-mediated signalling is a conserved process essential to control cell proliferation, differentiation, and apoptosis. Cooperative interactions between histone modifications such as acetylation, methylation and sumoylation are crucial for proper attenuation in *C. elegans*, implying that the proteins recognising these histone modifications could also play an important role in attenuation of RAS-mediated signalling. We sought to systematically identify these proteins and found BET-1. BET-1 is a conserved double bromodomain protein that recognises acetyl-lysines on histone tails and maintains the stable fate of various lineages. Unexpectedly, adults lacking both BET-1 and SUMO-1 are depleted of muscle myosin, an essential component of myofibrils. We also show that this muscle myosin depletion does not occur in all animals at a specific time, but rather that the penetrance of the phenotype increases with age. To gain mechanistic insights into this process, we sought to delay the occurrence of the muscle myosin depletion phenotype and found that it requires caspase activity and MEK-dependent signalling. We also performed transcription profiling on these mutants and found an up-regulation of the FGF receptor, *egl-15*, a tyrosine kinase receptor acting upstream of MEK. Consistent with a MEK requirement, we could delay the muscle phenotype by systemic or hypodermal knock down of *egl-15*. Thus, this work uncovered a caspase- and MEK-dependent mechanism that acts specifically on ageing adults to maintain the appropriate net level of muscle myosin.

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Key words: Bromodomain, Sumo, Body wall muscle, *C. elegans*

Introduction
Controlling RAS-mediated signalling is crucial to promote or inhibit cell growth, differentiation, and apoptosis in vertebrates. Loss of control at the level of attenuation can lead to hyperactivation of the pathway and in the worst cases tumourigenesis. Over two decades of studies on RAS-mediated signalling in *C. elegans* have shown that the epigenetic landscape can impact on attenuation of the LET-60 (RAS) signalling pathway and cell fate (Andersen and Horvitz, 2007; Ceol and Horvitz, 2001; Fay and Yochem, 2007; Fisher et al., 2010; Lipstick, 2004; Lu and Horvitz, 1998; Poulin et al., 2005; Solari and Ahringer, 2000). Many of the chromatin complexes depositing or removing histone modifications have since been shown to act redundantly to prevent ectopic expression of LIN-3 (EGF) (Andersen and Horvitz, 2007; Andersen et al., 2008; Cui et al., 2006). Ectopic expression of LIN-3 can lead to over-activation of the receptor tyrosine kinase, LET-23 (EGFR), and its conserved downstream cascade: LET-60/LIN-45/MEK-2/MPK-1, RAF/MEK/MAPK in mammals (Sundaram, 2006). It has also been shown that the sumoylation pathway genetically interacts with many of these chromatin complexes to attenuate LET-60 (RAS)-mediated signalling (Leight et al., 2005; Poulin et al., 2005). SUMO is a conserved short polypeptide transferred onto specific substrates (Gareau and Lima, 2010; Johnson, 2004), which can be recognised by effector proteins through SUMO interacting motifs (SIMs) (Geiss-Friedlander and Melchior, 2007; Kerscher, 2007). These effector proteins can in turn regulate specific functions such as transcription, chromatin structure, genome integrity, and DNA repair (Cuberes-Potts and Matunis, 2013; Geiss-Friedlander and Melchior, 2007). Collectively, these studies raised the possibility that post-translational modifications of histones, such as sumoylation, methylation, and acetylation, could form a combinatorial code recognised by specialised proteins referred to as readers of the epigenetic code, which in turn would regulate transcription of genes that prevent hyperactivation of the LET-60 signalling pathway.
We set out to identify readers that recognise chromatin modifications and genetically interact with the sumoylation pathway to prevent hyperactivation of the LET-60 signalling cascade. We used RNAi to deplete all predicted readers and identified CHD-3, HPL-2, and BET-1. CHD-3 and HPL-2 are chromodomains proteins recognising methylated histone tails and were previously shown to play a role in LET-60 attenuation (Coustham et al., 2006; Solari and Ahringer, 2000). BET-1 is a conserved double bromodomian protein of the BET family required for establishment and maintenance of stable fate in various lineages (Shibata et al., 2010). BET-1 shares homology with both human BRD2 and BRD4, and is a likely homolog of BRD4 because of a putative P-TEFb interaction motif not present in BRD2 (Bisgrove et al., 2007). BET-1, like other BET proteins, physically associates with acetyl-lysines on histone tails (Shibata et al., 2010).

Low molecular weight inhibitors such as JQ1 and I-BET151 can efficiently target acetyl-lysine binding sites of BET proteins (Dawson et al., 2011; Delmore et al., 2011; Filippakopoulos et al., 2010; Nicodeme et al., 2010; Zuber et al., 2011). In multiple myeloma, the inhibition of BRD4 leads to downregulation of the oncogenes c-MYC and other growth promoting and apoptotic genes (Delmore et al., 2011). This specific transcriptional regulation has recently been attributed to the effect of BRD4 on super-enhancers (Lovén et al., 2013).

Herein we performed a targeted RNAi screen and identified BET-1 as a novel SUMO interactor. Unexpectedly, we found that SMO-1 and BET-1 act together to maintain net muscle myosin levels in ageing adults. We show that muscle myosin depletion requires caspase activities and the FGF receptor/MEK signalling pathway to manifest. Interestingly, human caspases are activated under muscle catabolic conditions induced by insulin resistance (Du et al., 2004).

Identification of putative readers of chromatin marks

To identify genes for use in the targeted RNAi screen, the Pfam accession number for each domain of interest was used to filter the WormBase database (release WS190), using the WormMart data mining tool.

RNAi experiments

RNAi screens of the ~200 gene set were performed similarly to those described previously (Kamath et al., 2003; Poulin et al., 2005). Briefly, individual cultures were used to inoculate three wells on a six-well plate (Poulin et al., 2005), around 10 synchronized smo-1/k22 L3-L4 stage worms were placed in the upper well for each bacterial strain and the plates maintained at 20˚C. After 48 h, 5 worms from the upper well were transferred to the lower well. The F2 progeny were scored for the Mvp phenotype. RNAi clones giving Mvp in one or both strains were selected for bleaching and the eggs put on NGM plates with no food. After 24 hours the synchronized L1 were washed off and placed on plates with food. Once the F2 progeny had reached L4 the worms were harvested, washed once in M9 buffer and frozen at ~80˚C. RNA preparation: Two replicates of bet-1(+), smo-1(+), and bet-1, smo-1 double mutants and the corresponding balancer strain as a control were processed for microarray analysis. Nematode pellets were incubated with 1% beta-mercaptoethanol and 800 μg/ml protease K at 55˚C, 500 rpm shaking during 60 minutes. Total RNA was extracted from these pellets using RNeasy Micro kit (Qiagen) according to manufacturer instructions. Microarray analysis: The extracted RNA was processed for microarray performance: the platform used for that purpose was C elegans (V2) Gene Expression Microarray 4x44K (Agilent technologies), following manufacturer instructions. Raw data (supplementary material Table S3) was extracted from the scanned images by the Agilent feature extract software. Data was normalized in the statistical programming environment “R” using the LIMMA package (Smyth, 2004) or within array normalizations we used the Loess method and for the between array normalization we used the quantile method. No background correction was needed. A linear model was used to determine the differently expressed genes.

Quantitative RT-PCR

Worm pellets were prepared by harvesting synchronized L4 worms, washing the pellet in 1x PBS and freezing at ~80˚C. Total RNA was extracted from these pellets using TRIzol (Invitrogen) and first strand cDNA synthesis was performed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen), according to the manufacturer’s instructions. Quantitative RT-PCR was performed using the FastStart SYBR Green Master (ROX) mix (Roche) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Two biological samples for each strain were prepared, and for every biological replicate, a triplicate of two serial dilutions was analysed. act-1 was used as the internal reference for data normalization. mRNA levels were determined by comparing the unknown samples to a standard curve of known relative amounts. Primers used are listed in supplementary material Table S4.

JQ1 and U0126 treatments of nematodes

Synchronized L1 larvae were transferred onto NGM plates prepared with the indicated concentration of JQ1 (Filippakopoulos et al., 2010) or U0126 (Morgan et al., 2010).

Results

BET-1 genetically interacts with SUMO

Deposition and removal of post-translational modifications (PTMs) on histone tails play an important role in...
transcriptional regulation, which in turn impacts on the process of attenuation of LET-60-mediated signalling in C. elegans (Cui et al., 2006; Lipsick, 2004; Lu and Horvitz, 1998; Poulin et al., 2005). These PTMs can act by either altering the electrostatic interactions between histones and DNA or by creating recognition sites for specialised proteins often referred to as readers of the epigenetic code (Kouzarides, 2007). To test the latter mode of action, we performed an RNAi screen targeting all known predicted readers (~200 genes; supplementary material Table S1). Since the sumoylation pathw ay has been shown to genetically interact with many chromatin complexes involved in attenuation of LET-60 (Leight et al., 2005; Poulin et al., 2005), we performed the screen in a SUMO-compromised strain ( smo-1/y/+). We selected our candidates according to the observation of SUMO-associated phenotypes. The main expected phenotype being the multivulvae (Muv) phenotype (Broday et al., 2004; Leight et al., 2005; Poulin et al., 2005) and its superficial manifestation the multiventral protrusion phenotype (MVP) (Fisher et al., 2010); these phenotypes indicate hyperactivation of the LET-60/LIN-45/MEK-2/MPK-1 signalling cascade. We identified three candidates: two conserved chromodomain proteins: CHD-3 (Solari and Ahringer, 2000) and HPL-2 (Coustham et al., 2006); and the double bromodomain BET-1. We focused this study on BET-1, which was previously shown to recognise acetyl-lysines on histone tails and to maintain cell fate in various lineages (Shibata et al., 2010).

**BET-1 and SUMO prevent muscle myosin depletion in adults**

Following the identification of BET-1 by RNAi screening, we generated the double mutant smo-1/y bet-1/y and assessed whether we could detect a genetic interaction during vulva development. Surprisingly, we could not find an interaction in the vulva. Instead, we found that the single bet-1/y mutant or RNAi against bet-1 can produce the Muv (multiple vulvae) phenotype, but the additional loss of smo-1 does not aggravate the Muv phenotype (data not shown). However, during these investigations, we noticed that an important proportion of these double smo-1/y bet-1/y mutants lost their ability to crawl early in adulthood. We quantified this observation by assessing loss of locomotion. In this established assay (Herndon et al., 2002), locomotion can fall quantified this observation by assessing loss of locomotion. In this established assay (Herndon et al., 2002), locomotion can fall...
treatment ($P=0.001$; Chi square test of association). This experiment using acute RNAi treatments against smo-1 on young bet-1lf adults shows that the depletion of muscle myosin phenotype can occur after the establishment of muscle development and therefore provide further evidence that the depletion of muscle myosin is consistent with a defect during adulthood.

Caspase-dependent depletion of muscle myosin
Muscle myosin levels are regulated by both synthesis and proteolysis. Since the phenotype described herein has an onset in adulthood and that the bulk of muscle myosin is synthesised prior to adulthood, we hypothesised that the depletion of muscle myosin is more consistent with excessive degradation of muscle myosin. There are four proteolysis systems described for mammalian muscles: the proteasome (Mitch and Goldberg, 1996), the lysosome (Sandri, 2013), calpains (Sorimachi and Ono, 2012) and caspases (Du et al., 2004). The first three have been shown to function in C. elegans (Etheridge et al., 2012). We reasoned that the proteasome and lysosome systems are unlikely to be the primary system acting on muscle myosin because of their inefficiency at directly targeting myofibrils components, such as muscle myosin (Du et al., 2004). Calpains are activated by disruption of the integrin attachment complex (Etheridge et al., 2012), which produces a very different muscle phenotype than the muscle myosin depletion phenotype. We therefore investigated whether a caspase-mediated system could be erroneously activated in smo-1lf bet-1lf double mutants. To test this, we blocked the caspase cascade in smo-1lf bet-1lf mutants using ced-3 or ced-4 loss of function mutants. CED-3 (the
downstream caspase) and CED-4 (the apoptotic protease-activating factor 1) are required for most apoptosis events occurring in *C. elegans* (Ellis and Horvitz, 1986; Miura et al., 1993). The triple *smo-1lf bet-1lf; ced-3lf* or *smo-1lf bet-1lf; ced-4lf* mutants were analysed by immunostaining against muscle myosin. We observed that most of these caspase-defective triple mutants maintain muscle myosin levels at day four adult (CED-3: 98% and CED-4: 95%; Fig. 2A; Table 1). We verified this result using Western blots against MYO-3 and confirmed that muscle myosin levels are in average higher in absence of SMO-1, BET-1 and CED-3 than in absence of SMO-1 and BET-1 (127%, SEM 3%, *n* = 4; Fig. 2B), albeit at levels remaining below the wild type levels (Fig. 2B). Similar results were obtained using the anti-paramyosin antibody (131%, SEM 8.6%, *n* = 3; supplementary material Fig. S2D). Furthermore, we performed the locomotion assay on the triple *smo-1lf bet-1lf; ced-3lf* mutants and observe a significant increase in the proportion of animals retaining locomotion for the triple *smo-1lf bet-1lf; ced-3lf* mutants, when compared with the double *smo-1lf bet-1lf* mutants (from 67%, *n* = 45 to 27%, *n* = 45 at *P* = 0.0003; Fisher’s exact Test) (Fig. 2C), indicating that inactivation of the caspase system causes a delay rather than a rescue of the loss of locomotion phenotype. We also present the delay effect as animated box plots for each locomotion category and strain (supplementary material Movie 2). Taken together, these results provide strong evidence that a caspase system is functional to maintain muscle homeostasis in *C. elegans*.

### Table 1. Immunostaining analysis of muscle myosin levels at day four adulthood to assess the muscle myosin depletion phenotype.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>% Demm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
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<td>0</td>
</tr>
<tr>
<td><em>smo-1lf</em></td>
<td>63</td>
<td>0</td>
</tr>
<tr>
<td><em>bet-1lf</em></td>
<td>52</td>
<td>4*</td>
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<tr>
<td><em>smo-1lf bet-1lf</em></td>
<td>104</td>
<td>38</td>
</tr>
<tr>
<td><em>ced-3lf</em></td>
<td>69</td>
<td>3*</td>
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<tr>
<td><em>smo-1lf bet-1lf; ced-3lf</em></td>
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<td>2*</td>
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<tr>
<td><em>ced-4lf</em></td>
<td>61</td>
<td>0</td>
</tr>
<tr>
<td><em>smo-1lf bet-1lf; ced-4lf</em></td>
<td>55</td>
<td>5*</td>
</tr>
<tr>
<td><em>EV (RNAi)</em></td>
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<td>0</td>
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<tr>
<td><em>EV (RNAi); smo-1lf bet-1lf</em></td>
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<td>34</td>
</tr>
<tr>
<td><em>EV (RNAi); smo-1lf bet-1lf; egl-15lf</em></td>
<td>129</td>
<td>22*</td>
</tr>
<tr>
<td><em>sur-7 (RNAi)</em></td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td><em>sur-7 (RNAi); smo-1lf bet-1lf</em></td>
<td>98</td>
<td>10*</td>
</tr>
<tr>
<td><em>sur-7 (RNAi); smo-1lf bet-1lf; egl-15lf</em></td>
<td>188</td>
<td>6*</td>
</tr>
<tr>
<td><em>egl-15 (RNAi)</em></td>
<td>106</td>
<td>15*</td>
</tr>
<tr>
<td><em>egl-15lf</em></td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td><em>clf-1lf</em></td>
<td>64</td>
<td>0</td>
</tr>
</tbody>
</table>

The muscle myosin depletion phenotype is MEK-dependent
Both sumoylation and BET-1 are important regulators of transcription. We therefore postulated that changes in their transcription profiles could provide insights into the muscle myosin depletion phenotype. However, we met a technical problem; we could not extract sufficient materials from single or double *smo-1lf bet-1lf* homozygous escapers. To palliate to this

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**Fig. 2.** Inactivation of CED-3 in double *smo-1lf bet-1lf* mutants prevents depletion of muscle myosin and improves locomotion. (A) Immunostaining against MYO-3 showing that the depletion of muscle myosin phenotype requires the caspase CED-3 to manifest. (B) Western blot analysis showing that muscle myosin levels are increased when compared with the double *smo-1lf bet-1lf* mutants. See Table 1 for immunostaining results. (C) Locomotion assay performed on cloned individual animals (as in Fig. 1A) showing an improvement of locomotion when CED-3 is inactivated. Blue depicts crawling animals, red, animals requiring prodding, green, immobile animals, and purple, dead animals. L1 to L4 stands for larval stages one to four, and D1 to D4 stands for adult day one to four. Scale bar: 100 μm.
issue, we instead used heterozygotes (see materials and methods). As anticipated, transcription is not strikingly affected in these heterozygous animals. However, focusing our analysis on known components of the LET-60 signalling pathway, we found two possibly up-regulated positive regulators of the pathway: egl-15, (the FGF receptor; (DeVore et al., 1995)) and sur-7 (a cation transporter (Yoder et al., 2004)) (Fig. 3A). To verify these results, we performed quantitative RT-PCR on independent biological samples (in the double smo-1\textsuperscript{lf} bet-1\textsuperscript{lf} heterozygous background) and tested the levels of expression of six genes, including the egl-15 and sur-7 candidates (Fig. 3B). We found that both egl-15 and sur-7 are up-regulated significantly by about 2- and 1.5-fold, respectively (Fig. 3B), egl-15 showing a very strong p-value (Fig. 3B). We also detected a slight up-regulation (~1.3-fold) for let-60 (Fig. 3B). In contrast, cdf-1, ptp-2 and egl-17 are not significantly affected (Fig. 3B). The up-regulation of egl-15 is of interest since the FGF receptor can activate the LET-60/MEK signalling pathway and when hyperactivated increase muscle cells proteolysis as measured using a reporter assay (Kokel et al., 1998; Sundaram, 2006; Szewczyk and Jacobson, 2003). On the other hand, SUR-7 has not been linked to proteolysis in muscle cells, yet it acts as a positive regulator of LET-60/MEK signalling by regulating levels of cytoplasmic zinc ions through sequestration in the endoplasmic reticulum (Yoder et al., 2004). It is also known that elevated levels of zinc ions increase phosphorylation of the scaffold protein KSR, preventing its association with RAF and MEK and instead favouring an inhibitory association with 14-3-3 (Müller et al., 2001). Thus, up-regulation of both EGL-15 and SUR-7 are consistent with an increase in LET-60/MEK-mediated signalling.

These results from the expression profile data suggested the possibility that the absence of BET-1 and SMO-1 could lead to...
hyperactivation of the EGL-15/LET-60/LIN-45/MEK-2 signalling pathway that in turn could initiate muscle myosin depletion. We dampened the LET-60 signalling pathway using the MEK inhibitor U0126 (Morgan et al., 2010) and measured the effect on muscle myosin levels at day four adult. Remarkably, we found that U0126-treated smo-1lf bet-1lf double mutants can maintain muscle myosin levels. 32% of DMSO-treated smo-1lf bet-1lf animals displayed depletion of muscle myosin compared with 2% of U0126-treated animals (Fig. 4A). Importantly, this experiment indicates that the conserved FGF receptor/RAS/RAF/MEK signalling pathway is required for the muscle myosin depletion phenotype to manifest. It is also consistent with other studies linking hyperactivation of the LET-60 signalling pathway with protein degradation in muscles (Szewczyk and Jacobson, 2003; Szewczyk et al., 2007; Szewczyk et al., 2002).

Since we found that egl-15 and sur-7 are overexpressed in double mutants (Fig. 3A), we next addressed specifically whether these could play an important role in the muscle myosin depletion phenotype. To this end, we depleted smo-1lf bet-1lf double mutants of egl-15 by either performing RNAi or using a reduced function allele; we found that the penetrance of the muscle myosin depletion phenotype is decreased by 56%, from 34% to 15% (\(P=0.0042\); Table 1), respectively. We also tested sur-7 (RNAi) and observed a decrease in the penetrance by 70%, from 34% to 11% (\(P=0.001\); Table 1). Thus, the overexpression of EGL-15 and SUR-7, the rescue experiments by the MEK inhibitor, and depletion of either EGL-15 or SUR-7 taken together provide strong evidence that the EGL-15/LET-60 signalling pathway is required for the muscle myosin depletion phenotype to fully manifest.

**Hypodermal depletion of egl-15 or sur-7 rescues the muscle myosin phenotype**

It has been previously shown that the EGL-15/LET-60/LIN-45/MEK-2 signalling cascade, in addition to producing the muscle cell proteolysis defect, can also cause a Clr phenotype (Huang and Stern, 2004; Koke et al., 1998). Of note, clr-1 mutants do not display the muscle myosin depletion phenotype (Table 1) and are morphologically different from the double smo-1lf bet-1lf mutants (supplementary material Fig. S3). The exact relationship between the muscle proteolysis defect and the Clr phenotype remains unclear. However, the anatomical locus of activity for the EGL-15 signalling cascade, to produce the Clr phenotype, is the hypoderm (Huang and Stern, 2004) rather than the muscles itself. With this in mind, we sought to identify the tissue in which EGL-15 and SUR-7 are required to produce muscle myosin depletion. To this end, we performed hypodermal and body wall muscle specific RNAi against egl-15 and sur-7. This established tissue specific RNAi system takes advantage of an RNAi insensitive strain lacking RDE-1, in which tissue-specific re-expression of RDE-1 reactivates RNAi sensitivity in the targeted tissue (Qadota et al., 2007). We assessed whether knocking down egl-15 or sur-7 in either the hypoderm or the body wall muscles could rescue the muscle myosin depletion phenotype. To this end, we performed hypodermal and body wall muscle specific RNAi against egl-15 and sur-7. This established tissue specific RNAi system takes advantage of an RNAi insensitive strain lacking RDE-1, in which tissue-specific re-expression of RDE-1 reactivates RNAi sensitivity in the targeted tissue (Qadota et al., 2007). We assessed whether knocking down egl-15 or sur-7 in either the hypoderm or the body wall muscles could rescue the muscle myosin depletion phenotype. We found that only hypodermal RNAi, of either egl-15 or sur-7, can do so. Depleting EGL-15 or SUR-7 reduces the penetrance by 47% and 50%, from 32% down to 17% (\(P=0.0042\)) and 16% (\(P=0.001\)), respectively (Table 2). No rescuing effect could be detected by targeting either egl-15 or sur-7 in body wall muscles. Since we cannot rule out discrepancies in RNAi efficiency, we cannot rule out the possibility of a muscle activity (Table 2). Despite this caveat, these data show that hypodermal EGL-15 (and SUR-7) signalling is implicated in the depletion of muscle myosin phenotype.

**JQ1-treated SUMO mutants display the muscle myosin depletion phenotype**

So far we have shown that BET-1 acts together with the sumoylation pathway to prevent muscle myosin depletion in adults. We next wanted to address whether the recognition of acetyl-lysines is important in the depletion of muscle phenotype. We blocked reading of acetyl-lysines using a small molecule compound inhibitor of BET proteins, JQ1 (Dawson et al., 2011;
Delmore et al., 2011; Filippakopoulos et al., 2010; Nicodeme et al., 2010; Zuber et al., 2011). If recognition of acetyl-lysines is involved, we should detect the muscle myosin depletion phenotype when smo-1Lf animals are treated with increasing amount of JQ1 (2.5, 10 and 25 μM). Using immunostaining, we found that JQ1-treated smo-1Lf animals indeed display the muscle myosin depletion phenotype (Fig. 4B,C). JQ1 treatment of wild type animals did not cause the depletion of muscle myosin phenotype (data not shown). From this, we concluded that recognition of acetyl-lysines is important to prevent depletion of muscle myosin in adults. Since it is likely that most of these recognised acetyl-lysines are on histone tails, the data suggest that the muscle myosin depletion phenotype implicates a defect at the epigenetic level. Of note, we confirmed that JQ1 can block BET-1’s association with acetyl-lysines on histones using FRAP (supplementary material Fig. S4), in accordance with another study showing that BET-1 can associate with acetylated histones (Shibata et al., 2010).

**Discussion**

This study provides novel mechanistic insights into the pathways that ensure maintenance of muscle myosin levels in ageing adults and likely to influence the complex behaviour of locomotion. We present a novel muscle phenotype characterised by the depletion of adult muscle myosin. Our investigation shows a number of specific characteristics associated with this phenotype: it is caspase- and MEK-dependent, it requires hypodermal EGL-15 activity, and the muscle myosin depletion is observed only in adults and getting progressively more severe as the animals are ageing.

**Transcriptional regulation of EGL-15 and SUR-7**

We have found that in absence of BET-1 and SMO-1 the FGF receptor, egl-15, and the cation diffusion facilitator sur-7 are up-regulated. Since BET-1 associates with acetyl-lysines on histone tails (supplementary material Fig. S4) (Shibata et al., 2010), it is a possibility that their expression are regulated by this histone modification and therefore implicating an epigenetic mechanism. This possibility is consistent with our experiments showing that the ability to recognise acetyl-lysines is crucial to prevent muscle myosin depletion in adults (Fig. 4B,C). However, the up-regulation of both sur-7 and egl-15 (Fig. 3) suggests that acetyl-lysines could be interpreted as a signal for repression by BET-1, even though acetyl-lysines on histone tails are generally associated with activation of transcription. An alternative explanation for this repression effect is that BET-1 could be required to maintain the expression of a repressor that in turn acts on sur-7 and egl-15. Further work will be needed to distinguish between these mechanisms.

**Non-cell autonomous EGL-15 activity**

It is intriguing that the depletion of muscle myosin phenotype is apparent only in adults (in non-dividing cells), suggesting an important role for BET-1 and SMO-1 in muscle myosin homeostasis. Furthermore, we show that this phenotype is likely to involve a non-cell-autonomous mechanism. Interestingly, previous mosaic analysis on the C. elegans hypoderm (Huang and Stern, 2004). Similarly, muscle myosin depletion is influenced by hypodermal EGL-15 activity (Table 2). Even though it is not obvious how hyperactivation of the EGL-15 signalling in the hypoderm can lead to muscle myosin depletion, there is a physical association between the muscles and the hypoderm. A recent report has shown that calpains mediate integrin attachment complex maintenance of adult muscles in C. elegans (Etheridge et al., 2012). Integrin attachment complexes fulfils multiple functions in muscles (Moerman and Williams, 2006), one of which is to anchor body wall muscles to the basement membrane. Since hypodermal cells are also linked to the basement membrane (Moerman and Williams, 2006) this physical association could mediate signalling events between muscles and hypodermis. Hence, hyperactivation of the EGL-15/LET-60/MEK signalling pathway in the hypoderm could produce a defect in signalling events between muscle and hypoderm, leading to the muscle myosin depletion phenotype.

**Premature loss of locomotion and depletion of muscle myosin**

We have found that loss of locomotion occurs prematurely in single and especially in double smo-1Lf bet-1Lf mutants. Loss of locomotion can be observed before the depletion of muscle myosin. This sequence of events strongly suggests that another function impacting on locomotion is impaired in these mutants. Locomotion is a complex behaviour involving muscles, neurons and muscle attachments to the cuticle via the hypoderm. We have shown that a defect in signalling occurs in the hypoderm and that muscle cells are depleted in muscle myosin. It remains to be investigated whether neurons are affected, since BET-1 has been shown to act in stabilising neuronal cell fate (Shibata et al., 2010). It is also a possibility that the loss of locomotion is the primary defect, leading to the depletion of muscle myosin. There is however a discrepancy between the percentage of animals losing locomotion and the percentage of animals depleted in muscle myosin; there are at least twice as many animals losing locomotion that there are animals depleted in muscle myosin. Further, inactivation of CED-3 in smo-1Lf bet-1Lf mutants allowed maintenance of muscle myosin levels. However, we observe the same discrepancy aforementioned between loss of locomotion at day four and depletion of muscle myosin. Thus, the depletion of

### Table 2. EGL-15 and SUR-7 are active in the hypoderm.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>% Demm</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV(RNAi); smo-1 bet-1; rde-1; kzis9 (hypoderm)</td>
<td>104</td>
<td>32</td>
</tr>
<tr>
<td>egl-15(RNAi); smo-1 bet-1; rde-1; kzis9 (hypoderm)</td>
<td>51</td>
<td>17</td>
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<tr>
<td>sur-7(RNAi); smo-1 bet-1; rde-1; kzis9 (hypoderm)</td>
<td>85</td>
<td>16</td>
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<tr>
<td>EV(RNAi); smo-1 bet-1; rde-1; kzis20 (muscle)</td>
<td>152</td>
<td>26</td>
</tr>
<tr>
<td>egl-15(RNAi); smo-1 bet-1; rde-1; kzis20 (muscle)</td>
<td>50</td>
<td>24</td>
</tr>
<tr>
<td>sur-7(RNAi); smo-1 bet-1; rde-1; kzis20 (muscle)</td>
<td>82</td>
<td>27</td>
</tr>
</tbody>
</table>
muscle myosin, at day four adult, appears unlikely to be induced by loss of locomotion. Unless, a number of animals have been in an immobilised state longer than others prior to analysis and that those particular animals are depleted in muscle myosin. Finally, the muscle myosin depletion phenotype that we described produces an effect resembling muscle atrophy. However, it is unclear whether this phenotype is actually a premature manifestation of sarcopenia, the loss of muscle mass due to ageing, which occurs naturally in *C. elegans* (Herndon et al., 2002) or whether it is a muscular pathology. Taken together, our study has identified bet-1 and smo-1 as important players in the maintenance of adult muscle myosin levels through a caspase- and MEK-dependent mechanism, which could be relevant to muscle ageing and/or a muscle pathology.

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Competing Interests

The authors have no competing interests to declare.


SUMO and BET-1 maintain muscle myosin levels in adults

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Fig. S1. Locomotion assay as in Fig. 1 but the categories B and C were pooled. The data are displayed by a cumulative plot of locomotory versus locomotory impaired showing that the double smo-1lf bet-1lf mutants are more affected than their respective single mutants. All mutants are affected when compared with the wild type.
Fig. S2. Different antibodies against muscle myosin or paramyosin show results consistent with Fig. 1. (A) Immunostaining using a different antibody against MYO-3 (5–14). (B) Immunostaining using an antibody against paramyosin (5–23). (C,D) Western blots using an antibody against paramyosin (5–23). (C) Western blots using 5–23 (antiparamyosin) were performed in quadruplicates and show that paramyosin is depleted in double smo-1lf bet-1lf mutants as found with muscle myosin. Quantification relative to Wild Type: 66%, SEM +/- 2.8%. (D) Western blots using 5–23 (antiparamyosin) were performed in triplicates and show that paramyosin is depleted in double smo-1lf bet-1lf mutants as found with muscle myosin and expression rescued in the triple smo-1lf bet-1lf; ced-3lf mutant. Quantification of triple relative to double mutant: 131%, SEM +/- 8.6%.

Fig. S3. DIC photographs showing the morphological appearance of the double smo-1lf bet-1lf mutants compared with wild type and the clr-1lf mutant.
Fig. S4. BET-1 can be displaced from chromatin by the bromodomain inhibitor JQ1. (A) Photographs of different time courses of representative cells showing FRAP (Fluorescence Recovery After Photobleaching) for BET-1::GFP. Untreated control human U2OS cells recover rapidly during FRAP, but the recovery rate for SAHA-treated cells is reduced. The HDAC inhibitor SAHA is used to increase the levels of global acetylation (by preventing deacetylation), which stabilises BET-1::GFP association with acetylated chromatin. The principle of the assay is based on the capacity of BET-1::GFP to diffuse freely if not stably associated with histones. When cells are treated with SAHA, BET-1::GFP’s association with acetyl-lysine on histone tails is stabilised and the half time for recovery is increased. When JQ1 is added, we found a faster recovery rate, indicating that JQ1 destabilises BET-1::GFP’s association with acetylated histones. (B) Time course of fluorescence intensity after photobleaching, shown are the mean of at least 10 cells per treatment. (C) Mean time for half-maximal recovery of each treatment group calculated from the time courses of individual cells. These data are consistent with structural studies showing that an acetyl-lysine molecule is recognized by a central hydrophobic cavity and anchored by a hydrogen bond using a conserved asparagine residue present in bromodomains (Owen et al., 2000). This asparagine residue is present in BET-1 and our results are therefore consistent with these structural predictions.
Movie 1. Animated box plots showing the life histories of the wild type and mutant worm populations at each day of the 21-day locomotion assay. Each frame represents one day of 21 days of analysis. The life history is presented in terms of days spent in any of the four categories from the locomotion assay depicted in Fig. 1A. Each box plot describes the distribution of the days spent in that category by the indicated worm populations. The central bar in bold represents the median of days spent in a particular category. The upper and lower limits of the box represent the 3rd quartile and 1st quartile respectively. The ends of the whiskers above and below denote the highest and lowest values in the data, although anything beyond 1.5-fold of inter-quartile range above the third quartile or below the first quartile is defined as an outlier and plotted as circles. For example, by selecting day 10 of the assay, we see that the wild type population of worms has spent all 10 days in category A and none in any of the other categories. As for the double smo-1;bet-1 mutant, we see that the majority of the population has only spent 5–7 days of their lives in category A. Of those that progress to either category B or C, they have spent a median of 3 days in each. An outlier of the population has progressed to category D and spent 1 day like this, although the majority have not progressed so far yet.
Movie 2. Animated box plots (as in supplementary material Movie 1) showing the life histories of the wild type and mutant worm populations at each day of the 21-day locomotion assay. Each frame represents one day of 21 days of analysis. The life history is presented in terms of days spent in any of the four categories from the locomotion assay depicted in Fig. 2C. Each box plot describes the distribution of the days spent in that category by the indicated worm populations. The central bar in bold represents the median of days spent in a particular category. The upper and lower limits of the box represent the 3rd quartile and 1st quartile respectively. The ends of the whiskers above and below denote the highest and lowest values in the data, although anything beyond 1.5-fold of inter-quartile range above the third quartile or below the first quartile is defined as an outlier and plotted as circles.