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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**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; Lipsick, 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, RAS/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 (Cubañas-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 bromodomain 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 oncopgenes 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 wells on a six-well plate (Poulin et al., 2005), around 10 synchronized were used to inoculate three wells on a six-well plate (Poulin et al., 2005), around 10 synchronized were used to inoculate three wells on a six-well plate (Poulin et al., 2005), around 10 synchronized were used to inoculate three wells on a six-well plate (Poulin et al., 2005). Briefly, RNAi was performed using JQ1 and U0126 treatments of nematodes (Dawson et al., 2011; Delmore et al., 2011; Filippakopoulos et al., 2010). Worm pellets were prepared by harvesting synchronized L4 worms, washing the pellet in 1x PBS and boiling in 1x sample buffer containing 100 mM DTT, anion muting. Quantification performed using Imaged software.

Microarrays
Sample preparation: Whole worm extract for microarray analysis was prepared by placing ~50 mothers on 10 cm NGM plates for each strain. Gravid progeny were bleached and the eggs put on NGM plates with no food. After 24 hours the synchronised 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/+ mutants, and smo-1, bet-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 proteinase 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). For within array normalization we used the Limma 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 replicate of two serial dilutions was analysed. act-1 was used as the internal reference for data normalization. mRN 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

Fluorescence Recovery After Photobleaching (FRAP)
For FRAP studies, full length bet-1 ORF was cloned into the pcDNA3.2-N-EmGF-DEST vector and verified by sequencing. Full length GFP-tagged bet-1 was reverse-transfected into U2OS cells using Fugene HD (Roche) and plated into glass bottom dishes (World Precision Instruments, USA). Bromodomain FRAP assays have been previously described (Filippakopoulos et al., 2010). Briefly, FRAP was performed 24 hours after transfection using a Zeiss LSM 710 scanhead coupled to an inverted Zeiss Axio Observer.Z1 microscope (40x oil immersion objective) and an argon-ion laser (488 nm) with PMT detection set to 500–550 nm. The HDAC inhibitor SAHA was added (5 µM, 4 hours post transfection) to increase the assay window (BET binding increased) and JQ1 (2.5 µM) was added 1 hour prior to FRAP. Approximately half of the GFP positive nucleus was selected for bleaching and a time lapse series was taken to record GFP recovery using 1% of the power used for bleaching. The image datasets and fluorescence recovery data were exported from the microscope control software (ZEN 2009) into GraphPad Prism to determine the half-time for full recovery for individual cells and averages were calculated from 10–20 cells per treatment point.

Western blot analysis
Whole worm protein extracts at the indicated stages were prepared by harvesting synchronized worms, washing the pellet in 1x PBS and boiling in 1x sample buffer containing 100 mM DTT, anion muting. Quantification performed using Imaged software.

Expression Microarray 4
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 pathway 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-1y/+). We selected our candidates according to the observation of SUMO-associated phenotypes. The main expected phenotype being the multivulva (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 chromodomains 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-1y bet-1y 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-1y 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-1y bet-1y 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 into three exclusive categories: fully mobile (A), mobile following prodding (B), immobile (C). This latter category is defined by animals incapable of producing a full body movement following prodding whilst remaining alive, which is determined by head movements. We measured loss of locomotion from larval stage 1 (L1) for each single mutant, the double mutant, and wild type. We found that a proportion of the single bet-1y or smo-1y mutant lost locomotion earlier than wild type (4.5%, n=44; and 23%, n=48, respectively). However, the decline in locomotion is accelerated and more penetrant in the double smo-1y bet-1y mutants (73%, n=45). The double smo-1y bet-1y mutants are statistically different (Fisher’s exact test) from N2 and the singles (P=4x10^-9 (compared with N2), 0.1x10^-9 (compared with bet-1y), 3x10^-6 (compared with smo-1y)), Fig. 1A; supplementary material Fig. S1). Further, to provide an overview of the life history of each population, we present the entire dataset as box plots from day 1 till day 21. The number of days spent in each category for each strain at a specific time point can be selected (supplementary material Movie 1) or alternatively the entire 21-day assay can be viewed as a movie (supplementary material Movie 1) showing how the population for each category changes according to time. In conclusion, the locomotion assay shows that BET-1 and SMO-1 can act individually and in cooperation to prevent loss of locomotion in adults.

Time-associated loss of locomotion is a natural phenomenon attributed to a failure of maintaining a functional ageing muscle mass (Herndon et al., 2002). We therefore explored the possibility that the bet-1y and smo-1y mutants are experiencing a decline in muscle functionality. To test this, we directly measured muscle myosin (MYO-3), a major component of myofibrils, using immunofluorescence stainings and Western blot analysis. We first surveyed the levels of adult muscle myosin by immunostainings on wild type animals versus singles and double mutants. We used a modified freeze-crack method, which involves cutting open the mothers roughly in the middle, allowing the antibody to penetrate thoroughly the muscles. All wild type animals are successfully stained using this adaptation. At day one of adulthood, we found that the MYO-3 signal was depleted in 4.7% of double smo-1y bet-1y mutants while all wild type animals and the respective single mutants all remained positive for MYO-3 (Fig. 1B). Interestingly, at day four of adulthood, the phenotype reached a penetrance of 37.5% for smo-1y bet-1y animals. A slight increase was observed with single mutants (bet-1y mutants: 2.7%; smo-1y mutants: 4.8%) but no effect was found on wild type (Fig. 1B; Table 1). One day later at day five, the double smo-1y bet-1y reached 42.5% and the respective single mutants about 10% (Fig. 1B). Depletion was never observed in late embryos (Fig. 1C). Taken together, these experiments indicate a time-associated defect in net levels of muscle myosin for all mutants.

We next verified whether the observations obtained by immunostainings could also be detected by Western blot analysis, hence eliminating possible artefacts due to the staining procedure. We prepared whole worm extracts from double smo-1y bet-1y mutants and compared with extracts from wild type animals at three time points: L4, day-one adults and day-four adults. We found that muscle myosin levels are similar at L4 and day-one adults, but depleted at four-day adults (Fig. 1D). We quantified this effect relative to wild type sample and found that levels of myosin are down to 68.8% in average for smo-1y bet-1y mutants (SEM 6.5%; n=4). This is consistent with our immunostaining experiments (Fig. 1B) and thus validates the occurrence of the muscle myosin depletion phenotype. We also observed degradation products from day-one adults, but only in wild type animals, we do not know the significance of this reproducible effect. Of note, we also confirmed the muscle myosin depletion phenotype using a different antibody against MYO-3 (supplementary material Fig. S2A). Moreover, we used an antibody against paramyosin (supplementary material Fig. S2B,C) and observed the same effect, i.e. immunofluorescence shows that ~30% of double smo-1y bet-1y mutants are depleted in paramyosin and Western blot analysis shows that paramyosin is down to 66% in average (SEM 2.8%; n=4).

Finally, our data from immunostaining and Western blot analysis show that the muscle myosin depletion phenotype has an onset in adults, but it does not rule out that the casual defect occurs prior to adulthood, since the genetic deletions are always present. To circumvent this problem, we performed acute smo-1(RNAi) treatments on young bet-1y adults followed by immunostainings against MYO-3 on day four adults. We observed that 28.1% (n=34) of bet-1y, smo-1(RNAi) are MYO-3 depleted compared with 0% (n=32) for the control RNAi
treatment \((P=0.001;\) Chi square test of association). This experiment using acute RNAi treatments against smo-1 on young bet-1\(lf\) 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-1\(lf\) bet-1\(lf\) double mutants. To test this, we blocked the caspase cascade in smo-1\(lf\) bet-1\(lf\) 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*.

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

| Genotype             | N  | % Demm |
|----------------------|----|--------|
| Wild type            | 43 | 0      |
| *smo-1lf*            | 63 | 0      |
| *bet-1lf*            | 52 | 4*     |
| *smo-1lf* + *bet-1lf*| 104| 38     |
| *ced-3lf*            | 69 | 3*     |
| *smo-1lf* + *ced-3lf*| 58 | 2*     |
| *ced-4lf*            | 61 | 0      |
| *smo-1lf* + *ced-4lf*| 55 | 5*     |
| *EV (RNAi)*          | 32 | 0      |
| *EV (RNAi) + *smo-1lf*| 162| 34     |
| *EV (RNAi) + *ced-3lf*| 129| 22*    |
| *sur-7 (RNAi)*       | 42 | 0      |
| *sur-7 (RNAi) + *smo-1lf*| 98 | 10*    |
| *sur-7 (RNAi) + *ced-3lf*| 188| 6*     |
| *egl-15 (RNAi)*      | 106| 15*    |
| *egl-15 (RNAi) + *ced-3lf*| n/a| n/a   |
| *sur-7 (RNAi) + *sur-7 (RNAi) + *egl-15 (RNAi) + *egl-15 (RNAi) + *ced-3lf*| n/a| n/a |

**Table 1. Immunostaining analysis of muscle myosin levels at day four adulthood to assess the muscle myosin depletion phenotype.** Not applicable (n/a) for *egl-15* mutant or RNAi, since the worms are egg laying defective and eventually burst before any analysis can be conducted. Sterility avoids this problem. The * indicates a *P* value < 0.01 by Chi square test of association. Demm: Depletion of muscle myosin.

**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-1lf bet-1lf 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-1<sup>lf</sup> bet-1<sup>lf</sup> double mutants can maintain muscle myosin levels. 32% of DMSO-treated smo-1<sup>lf</sup> bet-1<sup>lf</sup> 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-1<sup>lf</sup> bet-1<sup>lf</sup> double mutants of egl-15 by either performing RNAi or using a reduced function allele; we found that the penetrance of the myosin depletion phenotype is decreased by 56%, from 34% to 15%, (P<0.001; Table 1) and by 35%, from 34% to 22%, (P=0.042; 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/MEK 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; Kokel et al., 1998). Of note, cls-1 mutants do not display the muscle myosin depletion phenotype (Table 1) and are morphologically different from the double smo-1<sup>lf</sup> bet-1<sup>lf</sup> 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=0.016) 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;
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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 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).

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 Clear phenotype caused by hyperactivation of LET-60 signalling revealed that the anatomical locus of activity for the EGL-15 signalling cascade is hypodermal (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 calpain complexes mediate integrin attachment complex maintenance of adult muscles in C. elegans (Etheridge et al., 2012). Integrin attachment complexes fulfil 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 membranes (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. The depletion of muscle myosin (Demm) phenotype was assessed using immunostaining against muscle myosin. Significant p values of 0.0111 and 0.0162 by chi square test of association.

| Genotype                        | N | % Demm |
|--------------------------------|---|--------|
| EV(RNAi); smo-1 bet-1; rde-1; kizs9 (hypoderm) | 104 | 32 |
| egl-15(RNAi); smo-1 bet-1; rde-1; kizs9 (hypoderm) | 51 | 17 |
| sur-7(RNAi); smo-1 bet-1; rde-1; kizs9 (hypoderm) | 85 | 16 |
| EV(RNAi); smo-1 bet-1; rde-1; kizs20 (muscle)   | 152 | 26 |
| egl-15(RNAi); smo-1 bet-1; rde-1; kizs20 (muscle) | 50 | 24 |
| sur-7(RNAi); smo-1 bet-1; rde-1; kizs20 (muscle) | 82 | 27 |

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).

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 acteyl-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.

Further work will be needed to distinguish between these mechanisms.
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.

References

Andersen, E. C. and Horvitz, H. R. (2007). Two C. elegans histone methyltransferases repress lin-3 EGF transcription to inhibit vulval development. Development 134, 2991-2999.

Andersen, E. C., Saffer, A. M. and Horvitz, H. R. (2008). Multiple levels of redundant processes inhibit Caenorhabditis elegans vulval cell fates. Genetics 179, 2001-2012.

Bisgrove, D. A., Mahmoudi, T., Henklein, P. and Verdin, E. (2007). Conserved P-TEFb-interacting domain of BRD4 inhibits HIV transcription. Proc. Natl. Acad. Sci. USA 104, 13690-13695.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics 77, 71-94.

Broday, L., Kolotuev, I., Didier, C., Bhoumik, A., Gupta, B. P., Sternberg, P. W., Ellis, H. M. and Horvitz, H. R. (2006). synMuv genes redundantly inhibit lin-3 EGF expression to prevent inappropriate vulval induction in C. elegans. Dev. Biol. 291, 102-104.

Cuben˜as-Potts, C. and Matunis, M. J. (2006). SUMO junction-what’s your function? New insights through analysis of the Caenorhabditis elegans genome using RNAi. Nat. Rev. Mol. Cell Biol. 7, 550-555.

Kokel, M., Borland, C. Z., DeLong, L., Horvitz, H. R. and Stern, M. J. (1998). clr-1 encodes a receptor tyrosine phosphatase that negatively regulates an FGF receptor signaling pathway in Caenorhabditis elegans. Genes Dev. 12, 1425-1437.

Morgan, C. T., Lee, M. H. and Kimble, J. (1990). The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase gcn5p. EMBO J. 9, 1411-1419.

Muller, J. O., Deas, A., Copeland, T., Piwnica-Worms, H. and Morrison, D. K. (2000). The SUMO-dependent protein ligase Ubc9 interacts with a large complex implicated in vulval development. Cell 102, 329-339.

Qadota, H., Inoue, M., Hikita, T., Koppen, M., Hardin, J. D., Amano, M., Liu, X. and Horvitz, H. R. (2007). Conserved P-TEFb-interacting domain of BRD4 inhibits HIV transcription. Dev. Biol. 314, 166-173.

Rabouille, C-TAK1 regulates Ras signaling by phosphorylating the MAPK scaffold, KSR1. Annu. Rev. Biochem. 73, 355-382.

Rabouille, C-TAK1 regulates Ras signaling by phosphorylating the MAPK scaffold, KSR1. Annu. Rev. Biochem. 73, 355-382.
SUMO and BET-1 maintain muscle myosin levels in adults

Sibata, Y., Takeshita, H., Sasakawa, N. and Sawa, H. (2010). Double bromodomain protein BET-1 and MYST HATs establish and maintain stable cell fates in C. elegans. Development 137, 1045-1053.

Smyth, G. K. (2004). Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3, Article3.

Solari, F. and Ahringer, J. (2000). NURD-complex genes antagonise Ras-induced vulval development in Caenorhabditis elegans. Curr. Biol. 10, 223-226.

Sorimachi, H. and Ono, Y. (2012). Regulation and physiological roles of the calpain system in muscular disorders. Cardiovasc. Res. 96, 1-22.

Sundaram, M. V. (2006). RTK/Ras/MAPK signaling. WormBook 2006, 1-19.

Szewczyk, N. J. and Jacobson, L. A. (2003). Activated EGL-15 FGF receptor promotes protein degradation in muscles of Caenorhabditis elegans. EMBO J. 22, 5058-5067.

Szewczyk, N. J., Peterson, B. K. and Jacobson, L. A. (2002). Activation of Ras and the mitogen-activated protein kinase pathway promotes protein degradation in muscle cells of Caenorhabditis elegans. Mol. Cell. Biol. 22, 4181-4188.

Szewczyk, N. J., Peterson, B. K., Barma, A. J., Parkinson, L. P. and Jacobson, L. A. (2007). Opposed growth factor signals control protein degradation in muscles of Caenorhabditis elegans. EMBO J. 26, 935-943.

Wang, S., Fisher, K. and Poulin, G. B. (2011). Lineage specific trimethylation of H3 on lysine 4 during C. elegans early embryogenesis. Dev. Biol. 355, 227-238.

Yoder, J. H., Chong, H., Guan, K. L. and Han, M. (2004). Modulation of KSR activity in Caenorhabditis elegans by Zn ions, PAR-1 kinase and PP2A phosphatase. EMBO J. 23, 111-119.

Zuber, J., Shi, J., Wang, E., Rappaport, A. R., Herrmann, H., Sison, E. A., Magoun, D., Qi, J., Blatt, K., Wunderlich, M. et al. (2011). RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. Nature 478, 524-528.