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The Rab GTPase activating protein TBC-2 extends longevity by facilitating release of FOXO/DAF-16 from endosomes

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ABSTRACT

FOXO transcription factors have been shown to regulate longevity in model organisms and are associated with longevity in humans. To gain insight into how FOXO functions to increase lifespan, we examined the subcellular localization of DAF-16 in *C. elegans*. We show that DAF-16 is localized to endosomes and that this endosomal localization is increased by the insulin-IGF signaling (IIS) pathway. Endosomal localization of DAF-16 is also increased by disrupting the Rab GTPase activating protein TBC-2, or decreased by inhibiting the RAB-5 or RAB-7 GTPases, key regulators of early to late endosome maturation. Importantly, the amount of DAF-16 that is localized to endosomes has functional consequences as increasing endosomal localization through mutations in *tbc-2* decreased the lifespan of long-lived *daf-2 IGFR* mutants, depleted their fat stores, and DAF-16 target gene expression. Finally, we show that the ability of TBC-2 proteins, TBC1D2 and TBC1D2B, to regulate FOXO protein localization is conserved in human cells. Overall, this work identifies endosomal localization as a mechanism regulating FOXO/DAF-16, which is important for its functions in metabolism and aging.

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INTRODUCTION

Insulin/insulin-like growth factor signaling (IIS) is an evolutionarily conserved pathway that plays an important role in lifespan, development, metabolism, immunity and stress responses from *Caenorhabditis elegans* to humans (Accili and Arden, 2004; Barthel *et al.*, 2005; Greer and Brunet, 2005; Murphy and Hu, 2013). IIS-mediated regulation of *C. elegans* FOXO, DAF-16, were first identified via genetic characterization of mutants affecting dauer (an alternative stress resistant larval stage) and life span (Riddle *et al.*, 1981; Kenyon *et al.*, 1993; Lin *et al.*, 1997; Ogg *et al.*, 1997). For example, animals that have a mutation in their insulin-like growth factor receptor (IGFR), DAF-2, live almost two times longer than wild type animals in a DAF-16-dependent manner (Kenyon *et al.*, 1993). Under favorable conditions, the *C. elegans* DAF-2 IGFR signals through a conserved PI3K/Akt pathway to phosphorylate and inhibit nuclear accumulation of DAF-16 FOXO (Murphy and Hu, 2013). In short, DAF-2 IGFR activation leads to the activation of AGE-1 PI3K which phosphorylates PI(4,5)P₂ to generate PI(3,4,5)P₃ which can bind and recruit PDK-1 and AKT-1/2 kinases. DAF-18 PTEN, a lipid and protein phosphatase, countacts AGE-1 PI3K and thus negatively regulates signaling. PDK-1 activates AKT-1/2 which in turn phosphorylates DAF-16 creating binding sites for PAR-5 and FTT-2 14-3-3 scaffold proteins that sequester DAF-16 FOXO in the cytoplasm. During adverse conditions such as starvation, IIS is suppressed and DAF-16 FOXO enters the nucleus to induce the expression of stress response genes. As such, DAF-16 FOXO mediates most *daf-2 IGFR* mutant phenotypes.

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Upon activation, the Insulin/IGF receptor is internalized into endosomes, where it can disassociate from its ligand and recycle back to the plasma membrane, or it can be targeted for lysosomal degradation (Bergeron *et al.*, 2016). The identification of activated IGFR on endosomes suggested that endosomes can serve as a platform for signaling (Baass *et al.*, 1995). Subsequently, several components of the IIS pathway have been shown to localize on endosomes. PTEN localizes on PI3P positive endosomes through its C2 domain and has been demonstrated to regulate endosome trafficking via dephosphorylation of Rab7 (Naguib *et al.*, 2015; Shinde and Maddika, 2016). Akt2 localizes to Appl-1 and WDFY-2 positive endosomes to get fully activated and regulate AKT2 specific downstream substrates (Schenck *et al.*, 2008; Walz *et al.*, 2010). 14-3-3 proteins can interact with several AKT phosphorylation targets to regulate their subcellular localization and have been found on endosomes (Jungmichel *et al.*, 2014; Marat *et al.*, 2017). However, a role for endosome trafficking in regulation of FOXO transcription factors has not been demonstrated to the best of our knowledge.

Rab5 and Rab7 GTPases localize to early and late endosomes respectively and are critical regulators of trafficking to the lysosome, an organelle important for cargo degradation and metabolic signaling (Huotari and Helenius, 2011; Settembre *et al.*, 2013). Like many small GTPases, Rabs cycle between a GTP-bound active state and GDP-bound inactive state. This cycling requires guanine nucleotide exchange factors for activation and GTPase Activating proteins (GAPs) to catalyze GTP hydrolysis and hence Rab inactivation. We previously characterized *C. elegans* TBC-2 as having *in vitro* GAP activity towards RAB-5, and some activity towards RAB-7 (Chotard *et al.*, 2010a). Mutations in *tbc-2* result in enlarged late endosomes in several tissues including the intestine, an important site of IIS and metabolic

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regulation (Libina *et al.*, 2003). In addition to early to late endosome maturation, TBC-2 regulates phagosome maturation (Li *et al.*, 2009), dense core vesicle maturation (Sasidharan *et al.*, 2012) endosome recycling as an effector of RAB-10 and CED-10/Rac (Sun *et al.*, 2012; Liu and Grant, 2015) and yolk protein trafficking in oocytes and embryos (Chotard *et al.*, 2010b). Yolk protein is prematurely degraded in *tbc-2* mutants. As such, *tbc-2* mutant larvae hatched in the absence of food had reduced survival during L1 (first larval stage) diapause (Chotard *et al.*, 2010b). TBC-2 has two human homologs TBC1D2/Arms and TBC1D2B (Chotard *et al.*, 2010a). TBC1D2 has *in vitro* GAP activity toward Rab7 and regulates endosome trafficking and autophagy (Frasa *et al.*, 2010; Serva *et al.*, 2012; Carroll *et al.*, 2013; Toyofuku *et al.*, 2015; Jaber *et al.*, 2016). TBC1D2B interacts with Rab22 and Rab31, Rab5 related GTPases, and colocalizes with Rab5 and Rab7, but a specific Rab GTPase target of GAP activity has not been demonstrated (Kanno *et al.*, 2010; Manshouri *et al.*, 2019; Harms *et al.*, 2020; Wei *et al.*, 2021).

Here we explore the subcellular localization of DAF-16 and show that DAF-16 localizes to endosomes in *C. elegans* intestinal cells. We found that endosome localization is regulated by nutrient availability and IIS. We show that endosome localization is increased through mutations in *tbc-2*, at the expense of nuclear localization. The increased endosomal localization of DAF-16 decreases lifespan, fat storage and DAF-16 target gene expression in *daf-2 IGFR* mutant animals. In human cell lines we demonstrated that phosphorylated FOXO1/3a localizes to endosomes and that this localization is regulated by TBC1D2/Arms and TBC1D2B. Combined these results demonstrate a role of endosomal localization in the regulation and function of FOXO, which is conserved between *C. elegans* and humans.

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RESULTS

TBC-2 regulates DAF-16 localization to endosomes in the intestine

We previously reported that *tbc-2* is required for survival during L1 diapause (Chotard *et al.*, 2010b). Since *daf-16* is also required for survival during L1 diapause (Munoz and Riddle, 2003; Baugh and Sternberg, 2006) we sought to test whether TBC-2 might regulate the nuclear versus cytoplasmic localization of DAF-16. Although we determined that loss of *tbc-2* did not result in precocious development during L1 diapause as seen *daf-16* mutants (Baugh and Sternberg, 2006; Chotard *et al.*, 2010b), we found that TBC-2 does in fact regulate DAF-16 localization. Unexpectedly, we found that DAF-16a::GFP (*zIs356*) localized to numerous amorphous vesicles in the intestinal cells of *tbc-2(tm2241)* deletion mutant animals (Figure 1B). Furthermore, we found that DAF-16a::GFP localized to vesicles in the intestine of wild-type animals (Figure 1A). The percentage of hermaphrodites with DAF-16 vesicles increased during larval development, peaking at L4 and young adults (Figure 1I; Figure S1A). *tbc-2(tm2241)* animals were about twice as likely to have DAF-16 vesicles than wild-type (Figure 1J; Figure S1A). We quantified the number of vesicles per animal. Wild-type animals can range from zero vesicles to having hundreds of vesicles (Figure 1I). Vesicles can be distributed throughout the 20 cells of the intestine, or can be present in high numbers in just one or a few intestinal cells. In contrast, *tbc-2(tm2241)* animals typically have many more vesicles than wild-type (Figure 1J; Figure S1A). Although DAF-16a::GFP nuclear localization is low in normal growth conditions, we find that *tbc-2(tm2241)* intestinal nuclei appear to have less nuclear DAF-16a::GFP than wild-type (Figure 1A and B). We quantified the fluorescence intensity of DAF-16a::GFP in nuclei of *tbc-2(tm2241)* intestinal cells comparing nuclei of cells with vesicles versus nuclei of

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cells without vesicles (Figure 1K). We found that nuclei of cells with vesicles have significantly less nuclear DAF-16a::GFP than cells without vesicles. Thus, DAF-16 localizes to vesicles in wild-type and *tbc-2(tm2241)* animals, and *tbc-2(tm2241)* animals have less nuclear DAF-16a::GFP, likely due to sequestration to cytoplasmic vesicles.

To determine if endogenous DAF-16 localizes to vesicles we analyzed *daf-16(hq23)*, a DAF-16::GFP line generated by CRISPR/Cas9 genome editing and generously provided by M.-Q. Dong, and found that endogeneously tagged DAF-16 localized to vesicles in both wild-type and *tbc-2(tm2241)* mutants (Figure S2A-F). We also analyzed three other transgenic strains with lower expression levels than DAF-16a::GFP (*zIs356*): GFP::DAF-16a (*muIs71*), DAF-16a::RFP (*lpIs12*) and DAF-16f::GFP (*lpIs14*, with an alternate N-terminus)(Henderson and Johnson, 2001; Lin *et al.*, 2001; Kwon *et al.*, 2010). While we were unable to detect DAF-16 positive vesicles in wild-type backgrounds, in *tbc-2(tm2241)* mutant animals there were DAF-16 vesicles in all three transgenic animals (Figure S2G-X). To determine if vesicular localization is specific to DAF-16::GFP, we overexpressed GFP by itself in the intestine. Overexpression of GFP from *vha-6* intestinal specific promoter, *vhEx1[Pvha-6::GFP]*, did not show significant vesicular localization in wild-type animals (Figure S1B). In *tbc-2(tm2241)* animals GFP showed some vesicular localization and potential aggregates, but much less than seen with DAF-16a::GFP, indicating that the vesicular localization is due to DAF-16 and not the fluorescent tag. Of note, we found that GFP expression in the *tbc-2* background was visibly stronger than in wild-type (Figure S1C-F), consistent with *vha-6* ranking amongst the top downregulated DAF-16-responsive genes (Tepper *et al.*, 2013) and consistent with TBC-2 facilitating DAF-16 nuclear localization.

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The finding that DAF-16 localizes to enlarged vesicles in *tbc-2* mutants and that DAF-16 vesicles contain autofluorescent lipofuscin (Figure 1E-H), a hallmark of intestinal endolysosomes (Clokey and Jacobson, 1986), suggested that DAF-16 vesicles are endosomal. To determine if the DAF-16 vesicles are endosomes, we co-expressed DAF-16a::GFP with RFP::RAB-5, an early endosomal marker, or with mCherry::RAB-7, a late endosomal marker (Chavrier *et al.*, 1990; Sato *et al.*, 2005; Chen *et al.*, 2006). We found that in wild-type animals, DAF-16a::GFP localizes to a subset of RAB-5 and RAB-7-positive endosomes (Figure 2A-F). Furthermore, we tested the genetic requirements for *rab-5* and *rab-7* for DAF-16 localization by RNAi. We found that *rab-5(RNAi)* and *rab-7(RNAi)* knockdown significantly decreased the number of wild-type and *tbc-2(tm2241)* animals with DAF-16a::GFP vesicles (Figure 2G and H). Together, these data are consistent with DAF-16 localizing to a sub-population of endosomes or endosome-like vesicles in the intestinal cells.

Acute starvation suppresses DAF-16 localization to endosomes

Nuclear localization of DAF-16 is modulated by nutrient availability. As such, starvation promotes DAF-16 cytoplasmic-to-nuclear shuttling (Henderson and Johnson, 2001). Therefore, we tested the effect of acute starvation on DAF-16a::GFP localization to endosomes. We found that starvation strongly suppressed the localization of DAF-16 to endosomes in both wild-type and *tbc-2(tm2241)* mutants (Figure 3A; Figure S3). Upon re-feeding, DAF-16 relocated to endosomes after 1-2 hours, in both wild-type and *tbc-2(tm2241)* mutants. Thus, DAF-16 localization on endosomal membranes is regulated by nutrient availability.

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Insulin/IGF signaling regulates DAF-16 localization to endosomes

To determine if IIS regulates DAF-16 localization to endosomes we analyzed the effect of disrupting IIS on DAF-16a::GFP localization (see Figure 3B for reference). Firstly, we analyzed a hypomorphic mutant of the insulin/IGF receptor, *daf-2(e1370)*, in which IIS is reduced, particularly at higher temperatures (Swanson and Riddle, 1981). We compared DAF-16a::GFP localization in three independent *daf-2(e1370)* strains at 15°C and shifted overnight to 25°C. All three showed a significant reduction in the number of DAF-16 positive vesicles at 25°C, while the wild-type strain did not (Figure 3C), indicating that DAF-2 promotes DAF-16 localization to endosomes.

DAF-18, the homolog of human tumor suppressor protein PTEN, acts as a negative regulator of the IIS pathway counteracting AGE-1 PI3K signaling by dephosphorylating 3'phosphate on the PI(3,4,5)P₃ converting it to PI(4,5)P₂ (Ogg and Ruvkun, 1998). We analyzed DAF-16a::GFP localization in the *daf-18* reference allele, *e1375*. We generated three independent *daf-18(e1375); zIs356* strains, but only two strains had increased endosome localization of DAF-16, but cumulatively the three strains had a statistically significant increase in the number of animals with DAF-16 endosomes (Figure 3D). Since *daf-18(e1375)* is possibly a non-null allele, we analyzed a deletion allele, *daf-18(ok480)*. We found that five independent *daf-18(ok480); zIs356* strains had more DAF-16 endosomes than wild-type that cumulatively was statistically significant (Figure 3D). Since DAF-16 endosome localization is increased in the background of two distinct *daf-18* alleles, it is consistent with increased IIS causing an increase in DAF-16 localization to endosomes.

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C. elegans AKT-1 and AKT-2 function upstream of, and can phosphorylate, DAF-16 (Paradis and Ruvkun, 1998; Hertweck *et al.*, 2004). We found that animals with an *akt-2(ok393)* deletion mutation, there was an insignificant decrease in DAF-16 endosomal localization in two independent strains (Figure 3E). However, in *akt-1(RNAi)* animals, we observed a significant decrease in DAF-16 endosomal localization where DAF-16 localized mainly to the nucleus, compared to the control animals in three independent experiments (Figure 3F). Therefore, AKT-1 is the main kinase regulating DAF-16 localization to endosomes.

Phosphorylation of FOXO proteins by Akt creates binding sites for 14-3-3 proteins, which promote cytoplasmic retention of FOXO (Brunet *et al.*, 1999). *C. elegans* has two genes that encode for 14-3-3 proteins that interact with DAF-16, *par-5* and *ftt-2* (Wang and Shakes, 1997; Morton *et al.*, 2002; Wang *et al.*, 2006). We found that *ftt-2(RNAi)*, but not *par-5(RNAi)*, suppressed the localization of DAF-16 to endosomes (Figure 3G), suggesting that FTT-2 regulates DAF-16 endosomal localization.

To determine if AKT phosphorylation regulates DAF-16 localization to endosomes we tested if DAF-16a^{AM}::GFP *mulS113*, in which the four consensus AKT phosphorylation sites (T54A, S240A, T242 and S314A) are mutated to alanine (Lin *et al.*, 2001), could still localize to vesicles in *tbc-2(tm2241)* mutants. Since the expression levels are lower than DAF-16a::GFP *zIs356*, we used GFP::DAF-16a *mulS71* with comparable expression levels as control. We found that the number of *tbc-2(tm2241)* animals with DAF-16a^{AM}::GFP positive vesicles was significantly less than the GFP::DAF-16a control (Figure 3H). Together, these data demonstrate that IIS regulates endosomal DAF-16 through AKT-1 specific phosphorylation and FTT-2 14-3-3.

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TBC-2 is required for lifespan extension and increased fat storage of *daf-2(e1370)* mutants

To determine if the increased endosomal localization of DAF-16 seen in *tbc-2* mutants affects DAF-16 activity, we tested if TBC-2 regulates adult lifespan. We found that the *tbc-2(sv41)* and *tbc-2(tm2241)* deletion alleles had similar lifespans as compared to wild-type (Figure 4A and B). As expected, *daf-2(e1370)* animals lived significantly longer than wild-type. We found that both *tbc-2* alleles significantly shortened the lifespan of *daf-2(e1370)* animals (Figure 4A and B). Thus, TBC-2 is not required for normal lifespan, but is partly required for the extended lifespan of *daf-2(e1370)* mutants.

To assess TBC-2's contribution to other *daf-2* mutant phenotypes, we tested whether TBC-2 is required for the increased fat storage of *daf-2(e1370)* mutants. Consistent with previous findings we found that *daf-2(e1370)* had significantly more fat than wild-type animals as determined by Nile Red and Oil Red O staining of fixed L4 larvae (Kimura *et al.*, 1997; O'Rourke *et al.*, 2009) (Figure 4C-F). We found that *tbc-2(tm2241)* larvae had less fat than wild-type with Nile Red staining, but not with Oil Red O staining (Figure 4D and F). This difference might reflect the lower sensitivity of Oil Red O for quantifying lipid abundance as compared to Nile Red (Escorcia *et al.*, 2018). Interestingly, we found that *tbc-2(tm2241)* significantly suppressed the *daf-2(e1370)* increased lipid staining by both Nile Red and Oil Red O (Figure 4C-F). Thus, TBC-2 is required for the increased fat storage of *daf-2(e1370)* mutants.

The fact that TBC-2 is required for the increased longevity and increased fat storage of *daf-2(e1370)* mutants suggests that TBC-2 could be a negative regulator of IIS. Therefore, we tested if *tbc-2* mutants regulate DAF-16 target gene expression in *daf-2(e1370)* animals. We

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used qRT-PCR to measure the expression of DAF-16 target genes in wild-type, *daf-2(e1370)*, *tbc-2* mutants and *tbc-2; daf-2* double mutants. Consistent with previous reports, the expression of six of the DAF-16 target genes were upregulated in *daf-2(e1370)* animals (Figure 5) (Honda and Honda, 1999; Murphy *et al.*, 2003; Ackerman and Gems, 2012; Tepper *et al.*, 2013). While *tbc-2(tm2241)* and *tbc-2(sv41)* did not appreciably alter DAF-16 target gene expression relative to wild-type, both reduced the expression of *sod-3*, *dod-3*, *gpd-2* and *icl-1* in *daf-2(e1370)* mutants, while *mtl-1* and *ftn-1* were not significantly decreased (Figure 5). The fact that TBC-2 was required for the increased expression of four of six DAF-16 target genes with elevated expression in *daf-2(e1370)* mutants suggests a more specific role for TBC-2 in regulating DAF-2 to DAF-16 signaling.

TBC1D2 and TBC1D2B regulate phospho-FOXO1/3a localization to endosomes

Towards understanding if endosomal regulation of FOXO proteins occurs in human cells, we tested if FOXO proteins localize to endosomes and if this localization is regulated by the human homologs of TBC-2, TBC1D2 and TBC1D2B. We confirmed that TBC1D2 and TBC1D2B were expressed in Henrietta Lacks (HeLa) and Human Embryonic Kidney 293 (HEK293) cells. TBC1D2 mRNA and protein were more highly expressed in HeLa cells while TBC1D2B mRNA and protein are more highly expressed in HEK293 cells (Figure S4). siRNAs targeting TBC1D2 and TBC1D2B specifically and strongly reduced the expression in both cell lines (Figure S5). We assessed the levels of total FOXO1 and FOXO3a as well as the relative levels of phosphorylated FOXO1 (pFOXO1) and pFOXO3a in HeLa and HEK293 cells treated with siRNA targeting TBC1D2, TBC1D2B or both (Figure S6). We did not detect a significant

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difference in the levels of total FOXO1 or FOXO3a relative to Actin, indicating that TBC1D2 and TBC1D2B do not regulate FOXO1/3a protein levels. Furthermore, the ratio of pFOXO1 and pFOXO3a relative to total FOXO1 and FOXO3a respectively is not significantly different suggesting that the phosphorylation status of FOXO1 and FOXO3a is not regulated by TBC1D2 and TBC1D2B. We assayed the colocalization of FOXO3a, the predominant FOXO in HEK293 and HeLa cells and closest DAF-16 homolog, with endogenous Rab5 and Rab7 early and late endosomal GTPases. FOXO3a is primarily found in the nucleus of HeLa cells and is both cytoplasmic and nuclear in HEK293 cells, as such there is a higher correlation coefficient of FOXO3a with Rab5 and Rab7 in untreated HEK293 cells than in HeLa cells (Figure S7). Thus, FOXO3a localizes to endosomes in human cells. Knockdown of TBC1D2 and TBC1D2B by siRNA had no effect on FOXO3a colocalization with Rab5 and Rab7 in HEK293 cells (Figure S7 A-D). In HeLa cells there was a modest, but significant increase in colocalization of FOXO3a with Rab5 and Rab7, but with only one set of siRNAs targeting both TBC1D2 and TBC1D2B (Figure S7 E-H).

Since our data with DAF-16 suggests that endosome localization correlates with phosphorylated DAF-16, we analyzed the localization of pFOXO1/3a using an antibody that recognizes both phosphorylated FOXO1 and FOXO3a. In HEK293 cells there was a clear increase in colocalization of phosphorylated FOXO1/3a with Rab5 and a moderate increase with Rab7 when both TBC1D2 and TBC1D2B were knocked down with two distinct siRNAs (Figure 6 A-D). This was even more evident in HeLa cells where individual knockdown of TBC1D2 or TBC1D2B was sufficient to significantly increase colocalization of pFOXO1/3a with Rab5 and knockdown of both TBC1D2 and TBC1D2B strongly increased colocalization with both Rab5

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and Rab7 (Figure 6 E-H). These results show that FOXO proteins localize to endosomes in human cells and that similar to the *C. elegans* intestinal cells, this localization is regulated by the TBC1D2 family of RabGAPs.

The modest colocalization seen in HeLa cells appeared to coincide with increased cytoplasmic FOXO3a in TBC1D2 and TBC1D2B knockdown cells (Figure S7). Therefore, we measured the nuclear fluorescence intensity of pFOXO1/3a and total FOXO3a relative to DAPI and found that siRNA knockdown of TBC1D2 and TBC1D2B together or individually both reduced the nuclear localization of FOXO3a in HeLa cells (Figure 7F, H). As expected the correlation of coefficient of pFOXO1/3a with DAPI was low in comparison to FOXO3a and not altered by knockdown of TBC1D2 and TBC1D2B (Figure 7E, G). In HEK293 cells, knockdown of TBC1D2B but not TBC1D2 was sufficient to reduce nuclear localization of both total FOXO3a and pFOXO1/3a (Figure 7A-D), consistent with TBC1D2B being more highly expressed in HEK293 cells than TBC1D2. These data indicate that the increased endosomal localization of FOXO protein correlates with decreased nuclear localization mirroring our data with DAF-16 in *C. elegans* intestinal cells and that there is a conserved role for TBC-2/TBC1D2 family of RabGAPs in regulation of FOXO proteins.

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DISCUSSION

Endosome trafficking and signal transduction are intimately linked processes regulating signal propagation, specificity and attenuation (Miaczynska, 2013; Bergeron *et al.*, 2016). However, there remains a large knowledge gap in the spatial regulation of cell signaling and where downstream transcription factors are regulated. We identified a previously unknown localization for FOXO transcription factors on endosomes in *C. elegans* and in human cell lines. Endosome localization is limited by the TBC1D2 family of Rab GAPs. Loss of *C. elegans* TBC-2 or knockdown of human TBC1D2 and TBC1D2B results in increased endosomal localization of FOXO proteins at the expense of nuclear localization. As such, *C. elegans* TBC-2 is partly required for several *daf-2 IGFR* mutant phenotypes including lifespan extension, increased fat storage, and increased DAF-16 target gene expression that result from DAF-16 nuclear translocation. DAF-16 endosome localization is largely dependent on IIS consistent with this being a phosphorylated, inactive pool of DAF-16. Together our data show that the TBC1D2 family of Rab GAPs have an evolutionarily conserved role in regulating the balance of nuclear versus endosomal localization of FOXO transcription factors.

DAF-16 localizes to endosomes

We were surprised to find that DAF-16 FOXO localizes to a subset of RAB-5 and RAB-7 endosomes in wild-type animals. Many studies have used DAF-16 cytoplasmic versus nuclear localization to assess IIS activity under various conditions. We assume that DAF-16 positive endosomes were not discovered earlier as nuclear translocation can be assessed at low magnification, and DAF-16 positive endosomes are not present in every cell or every animal.

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Furthermore, we first took notice of DAF-16 positive endosomes in the *tbc-2* mutant background where they are more prominent. These are likely not an artefactual consequence of overexpression, as we see these vesicles in the endogenously tagged *daf-16::GFP* and we do not see similar vesicles in a GFP overexpression strain. Additionally, DAF-16-vesicles are regulated by IIS and we detect endogenous FOXO on endosomes in human cells (discussed further below).

Many components of the IIS pathway localize to endosomes in mammalian cells including active insulin receptor and downstream signaling components such as PI3K, Akt, PTEN and 14-3-3 proteins (Khan *et al.*, 1986; Christoforidis *et al.*, 1999; Balbis *et al.*, 2000; Braccini *et al.*, 2015; Naguib *et al.*, 2015; Bergeron *et al.*, 2016; Marat *et al.*, 2017; Kim *et al.*, 2018). In the case of Akt and PTEN, both have demonstrated roles in regulation of endosome trafficking independent of IIS (Shinde and Maddika, 2016, 2017; Mondin *et al.*, 2019; Hsu *et al.*, 2020). On the other hand, the PI3Ks are Rab5 effectors and Rab5 has been shown to promote Akt activity on endosomes (Christoforidis *et al.*, 1999; Shin *et al.*, 2005; Su *et al.*, 2006; Cheng *et al.*, 2009; Braccini *et al.*, 2015). While endosomal localization of FOXO proteins has not been reported to the best of our knowledge, knockdown of Rab5 in mouse liver results in a strong increase in phosphorylated FOXO1 (Zeigerer *et al.*, 2015). This is contrary to the finding that Rab5 promotes Akt phosphorylation (Su *et al.*, 2006; Braccini *et al.*, 2015), which could be a consequence of indirect regulation or suggest tissue-specific regulation. The fact that TBC-2 is a RAB-5 GAP is consistent with increased RAB-5 activity promoting DAF-16 localization on endosomes. This is further supported by the fact that *rab-5* and *rab-7* RNAi knockdown reduces the number of animals with DAF-16 vesicles in both wild-type and *tbc-2* mutants. Given the

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importance of RAB-5 for endosome trafficking, it is difficult to parse whether RAB-5 is promoting a platform for DAF-16 localization or if it also has a role in IIS.

We demonstrated that DAF-16 localized to a subset of RAB-5 and RAB-7 positive endosomes. Since RAB-5 and RAB-7 promote trafficking to the lysosome and promote receptor tyrosine kinase degradation, it is possible that DAF-16-positive endosomes are signaling endosomes, in which case we would expect other upstream signaling components might be present. Consistent with that hypothesis, knockdown of IIS reduces the number of animals with DAF-16 vesicles. However, when analyzing DAF-16 localization in *daf-2(e1370)* mutants at 15°C vs. 25°C, we find that while there is a reduction in the number of DAF-16 endosomes, these endosomes are noticeably fainter at 25°C. This suggests that there are not necessarily less endosomes being generated, but rather less DAF-16 on the vesicles which may be inconsistent with these being signaling endosomes derived from DAF-2 internalization at the plasma membrane. Future studies should test for additional IIS components on DAF-16 positive endosomes.

If these are not signaling endosomes, then what are the DAF-16 endosomes? Since RAB-5 and RAB-7 are also regulators of autophagy, so it is possible that these endosomes contribute to degradation of inactive excess DAF-16. There is precedent for selective autophagy in the degradation of the GATA4 transcription factor (Kang *et al.*, 2015). Alternatively, these endosomes could serve as a reservoir of inactive DAF-16 that can be quickly mobilized if environmental stress is encountered. For example, we found that acute starvation is a potent regulator of DAF-16 endosome localization, even in the *tbc-2* mutant background.

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We find it interesting that there is such variability within the population, and amongst the intestinal cells in a given animal, as to whether there will be DAF-16 positive endosomes or not. It suggests that each intestinal cell autonomously senses changes in IIS, or possibly other nutrient and stress sensing pathways, to regulate DAF-16 localization. Then, why is endosomal DAF-16 more prominent in *tbc-2* mutants? One explanation would be that the expansion of endosomal membranes in a *tbc-2* mutant create more storage space for inactive DAF-16. Another would be that IIS or other pathways are more active in *tbc-2* mutants, or some combination of the two.

A direct role for TBC-2 in regulating *daf-2(e1370)* *IGFR* mutant phenotypes

Our finding that *tbc-2* was required for the extended lifespan and increased fat storage of *daf-2(e1370)* mutants suggests that TBC-2 might have a more specific role related to IIS. However, TBC-2 is not the first endosomal regulator required for lifespan extension of *daf-2(e1370)* mutants. *C. elegans* BEC-1, a homolog of human Beclin1, is a regulator of autophagy and endosome trafficking, and *bec-1* mutants accumulate large late endosomes in the intestinal cells (Melendez *et al.*, 2003; Ruck *et al.*, 2011; Law *et al.*, 2017). Mutations in *bec-1* suppress the increased lifespan of *daf-2(e1370)*, and were reported to be required for the increased fat storage (Melendez *et al.*, 2003). Similarly, other autophagy regulators, *atg-7* and *atg-12*, have been shown to be required for *daf-2* longevity (Hars *et al.*, 2007). An RNAi screen identified regulators of endosome to lysosome trafficking, including RAB-7, and components of the ESCRT and HOPS complexes as being required for the lifespan extension phenotypes of *daf-2 IGFR* mutants (Samuelson *et al.*, 2007). However, the mechanisms by which they regulate lifespan are not known. In *daf-2(e1370)* mutants, there is an increase in autophagy and lysosome

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function, both of which are required for the extended lifespan (Melendez *et al.*, 2003; Lapierre *et al.*, 2013; Guo *et al.*, 2014; Sun *et al.*, 2020), consistent with increased stress resistance contributing to increased longevity. Since the human homologs of TBC-2 are implicated in autophagy (Behrends *et al.*, 2010; Popovic *et al.*, 2012; Carroll *et al.*, 2013; Toyofuku *et al.*, 2015), and *tbc-2* mutants accumulate autophagy protein LGG-1 (LC3/Atg8) in enlarged endosomes (Chotard *et al.*, 2010a), it is possible that TBC-2 also regulates autophagy and thus *daf-2(-)* longevity. However, our findings that TBC-2 regulates DAF-16 nuclear vs. endosome localization and that TBC-2 is required for the DAF-16 target gene expression in *daf-2* mutants demonstrate that TBC-2 has a more direct role in IIS, as opposed to being required for downstream cellular responses. It will be interesting to test if TBC-2 regulates DAF-16-independent mechanisms of longevity as well as to determine if other endosome and autophagy-regulating genes can regulate DAF-16 localization to endosomes.

FOXO localization to endosomes in human cells

We demonstrated that FOXO proteins can localize to Rab5 and Rab7 endosomes in two commonly used human cell lines, HeLa and HEK293. This localization was most prominent with pFOXO1/3a, the inactive pool that is mostly excluded from the nucleus. Consistent with our finding in *C. elegans* we found that siRNA knockdown of TBC1D2 and TBC1D2B increased the endosomal localization of pFOXO1/3a and this effect was strongest with double knockdown, indicating that TBC1D2 and TBC1D2B have overlapping functions. The increased endosomal localization was accompanied by a decrease in nuclear FOXO3a and pFOXO1/3a. These data suggest that the mechanisms in *C. elegans* are conserved in human cells. We did not detect

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significant changes in protein expression levels or of the phosphorylation status of FOXO1 or FOXO3a in TBC1D2 and TBC1D2B knockdown. This suggests that the TBC1D2 proteins do not regulate FOXO expression or phosphorylation state. Given that Rab5 regulates IIS on endosomes, further studies will be needed to determine if TBC1D2 and TBC1D2B functionally impact IIS signaling outcomes in mammalian cells. Interestingly, TBC1D2 and TBC1D2B were shown to interact with PI3,4,5P and that TBC1D2 was recruited to the plasma membrane upon PDGFR/PI3K signal activation (Jungmichel *et al.*, 2014), suggesting the possibility of TBC1D2 and TBC1D2B regulation by IIS.

Endosomal localization of FOXO proteins is reminiscent of lysosomal regulation of the TFEB family of transcription factors. Similar to FOXO, nuclear translocation of TFEB is inhibited by phosphorylation and interactions with 14-3-3 proteins (Settembre *et al.*, 2013). In this case, TFEB is phosphorylated by the nutrient sensing mTOR kinase on lysosomes. Interestingly, the nuclear localization of *C. elegans* TFEB homolog, HLH-30, is inhibited by DAF-2. IGFR and HLH-30 is required for the full lifespan extension of *daf-2(e1370)* mutants, as well as lifespan extension by other perturbations and regulates fat storage (Lapierre *et al.*, 2013; O'Rourke and Ruvkun, 2013). In fact HLH-30 and DAF-16 can interact in the nucleus to combinatorially regulate expression of genes involved in longevity and oxidative stress resistance (Lin *et al.*, 2018). We predict that regulators of endolysosomal trafficking, such as TBC1D2 family of Rab GAPs, would perturb TFEB/HLH-30 regulation on lysosomes.

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In conclusion, we demonstrate that DAF-16/FOXO transcription factors localize to endosomes in *C. elegans* and in human cell lines. This endosomal localization of FOXO is regulated by both IIS and the TBC-2 family of Rab5 and Rab7 GAPs. TBC-2/TBC1D2 promotes the nuclear localization of DAF-16/FOXO, and in *C. elegans* this localization has functional effects on both longevity and metabolism through modulation of DAF-16 target gene expression. Our data suggest that endosomes serve as an important location for FOXO transcription factor regulation, and suggest that endomembranes may function as a site of transcription factor regulation.

METHODS

C. elegans genetics and strain construction

C. elegans strains were cultured as described in Wormbook (www.wormbook.org). The *C. elegans* N2 Bristol strain was the wild-type parent strain and HB101 *E. coli* strain was used as a food source. Both were obtained from the Caenorhabditis Genetic Center (CGC) as were many of the strains used in this study (Table S1). New strains were constructed using standard methods and the presence of mutations were confirmed by PCR and DNA sequencing.

Cell culture and transfection

HEK293 (CRL-1573) or HeLa (CCL-2) cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (growth medium) at 37°C with 5% CO₂. Typically, cells were plated at a density (~150,000 cells/well in

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a six-well plate) that would result in 70-75% confluence the following day, unless otherwise noted.

RNAi experiments

C. elegans RNAi feeding experiments were conducted essentially as described in (Kamath *et al.*, 2001). The following RNAi feeding clones were obtained from the Ahringer RNAi library: *rab-5* (I-4J01), *rab-7* (II-8G13), *akt-1* (V-7I17), *ftt-2* (X-5F07) and *par-5* (IV-6E06) and confirmed by sequencing. The L4440 empty vector was used as a negative control (AddGene).

For siRNA transfection, HEK293 or HeLa cells at ~70% confluence were transfected with 25 nM oligonucleotides (IDT; Table S2) using JetPrime Reagent (Polyplus Transfection) and following the manufacturer's protocol. The transfection mixture was replaced with fresh growth medium the day following transfection and the cells were incubated for an additional 18-24 hours before being harvested.

Microscopy

DAF-16 vesicular localization was analyzed in the intestinal cells of hermaphrodites. Hermaphrodite worms at the L4 stage were imaged alive at room temperature unless it is stated otherwise. Animals were picked and mounted onto 4% agarose pads, animals were anesthetized with levamisole.

Differential interference contrast (DIC) and fluorescent imaging were performed with an Axio Imager A1 compound microscope with a 100×1.3 NA Plan-Neofluar oil-immersion objective lens (Zeiss) and images were captured by using an Axio Cam MRm camera and

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AxioVision software (Zeiss). Confocal microscopy was performed on an Axio Observer Z1 LSM780 and LSM510 laser scanning confocal microscopes with a 63×1.4 NA Plan-Apochromat oil-immersion objective lens (Zeiss) in a multi-track mode using an argon multiline laser (405 nm excitation for autofluorescence, 488 nm excitation for GFP and a 561/ 594 nm excitation for mCherry/RFP). Images were captured with a 32 channel GaAsP detector and ZEN2010 image software. Raw data was analyzed using Fiji (ImageJ) or Zen 2010 Lit programs, and images were modified by using Fiji (ImageJ).

To compare DAF-16 (*zIs356*) nuclear intensity in cells with or without DAF-16 vesicles, animals at L4 stage were imaged using an LSM780 scanning laser microscope. To ensure consistency, only the anterior most intestine cells were imaged. First, the nucleus of intestinal cells were focused under bright field and without changing the position, GFP, autofluorescence and bright field signals were imaged. Each animal was imaged using the same confocal settings. After data collection, each nucleus was categorized as a nucleus with adjacent DAF-16-positive vesicles or a nucleus without any DAF-16 positive vesicles. Total GFP intensity inside the nucleus was measured using Fiji (Image J) software. The nucleus is circled using DIC/Bright Field and autofluorescence channels as reference. Since intestinal cells have two nuclei per cells, if there are two nuclei within the focus, their GFP intensity is averaged for statistical analysis. Prism 8 (GraphPad) were used to graph the data and determine statistical analysis using an unpaired t-test.

HEK293 or HeLa cells (~60,000) were seeded on glass coverslips in 24-well plates and incubated overnight in growth medium. The next day, cells were transfected with siRNA as described under Cell culture and transfection. For immunofluorescence analysis, cells were

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rinsed once in DMEM containing 10 mM HEPES and then fixed in 4% paraformaldehyde for 20 min. The cells were then rinsed twice in DMEM containing 10 mM HEPES, followed by a 1-h incubation in DMEM containing 10 mM HEPES, 2% FBS, 0.1% Triton X-100, and primary antibody (Table S3). Following three washes in DMEM containing 10 mM HEPES, the cells were incubated for 1 h in DMEM containing 10 mM HEPES, 2% FBS, and fluorescently labeled secondary antibody. The cells were washed three times, incubated for 10 min with 4',6-diamidino-2-phenylindole (DAPI) in PBS, rinsed two times with distilled water, and mounted on glass slides using ProLong Gold anti-fade reagent (Life Technologies, Carlsbad, CA). Slides were visualized as described using an Axio fluorescence microscope (40X or 63X magnification; Zeiss, Oberkochen, Germany) and Zen imaging software.

Starvation-Refeeding and Temperature Shift Experiments

For starvation and refeeding experiments animals were synchronized at the L1 stage and grown on NGM plates with HB101 *E. coli* till the L4 stage. Then animals were collected washed 3 times for 5 mins with M9 buffer to remove bacteria in their gut. After the third wash animals were plated to regular NGM plates with or without HB101 *E. coli*, and incubated for 4-5 hours before scoring. Animals are scored for the presence of DAF-16::GFP-positive vesicles the intestine using an A1 Zeiss microscope. After 4h-5h of starvation, animals were harvested with M9 buffer from the starved plates and washed once with M9 buffer and plated to NGM plates with HB101 *E. coli* and incubated for 1-2 hours at 20°C before scoring.

Life span analysis

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Replicate strains were maintained for several generations prior to beginning the lifespan assays which were conducted at 20°C. For each strain 25 young adult hermaphrodites were picked to two NGM plates without FUDR seeded with HB101 *E. coli* for three independent replicates totaling 150 animals. Strains were coded and scored blindly to reduce bias. Animals were transferred to fresh NGM plates to avoid contamination and getting crowded out by their progeny. Animals were scored every 2-3 days and were considered dead when they stop exhibiting spontaneous movement and fail to move in response to 1) a gentle touch of the tail, 2) a gentle touch of the head, and 3) gently lifting the head. Animals that die of unnatural causes (internal hatching of embryos, bursting, or crawling off the plate) are omitted. Graphs and statistics were done using Graphpad Prism. None of the strains used in the lifespan assays carry the *fln-2(ot611)* mutation found in a N2 male stock strain and found to extend median lifespan (Gems and Riddle, 2000; Zhao *et al.*, 2019).

Fat staining

L4 animals were fixed for staining with Nile Red (Invitrogen) or Oil Red O (Sigma-Aldrich) as previously described (Escorcia *et al.*, 2018). Imaging and analysis was done as previously described (Ratemi *et al.*, 2019). Graphs and statistics were done using Graphpad Prism.

Quantitative real-time RT-PCR

C. elegans RNA was isolated from young adults maintained at 15°C using TRIZOL reagent (Invitrogen). RNA from HEK293 and HeLa cells was isolated by solubilizing cells in 4 M guanidinium isothiocyanate and phenol-chloroform extraction. RNA concentration was

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determined using a NanoDrop LITE spectrophotometer (Thermo Fisher Scientific). 1 ug of RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). Quantitative real-time PCR was performed using 1 µl of the cDNA preparation with SYBR-Green Reagents and a Vii7 qPCR analyzer (Applied Biosystems). Each DAF-16 target gene was amplified using PCR primers as described in (Senchuk *et al.*, 2018) and compared to *act-3* (Table S4). TBC1D2 and TBC1D2B were compared to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (Table S4).

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism software. For the analysis of two groups, a student t test was performed using two-tailed distribution for analysis involving two groups of samples. Fishers' exact test was used for comparing groups of four. For each analysis, $P < 0.05$ was considered as significant.

For mammalian work: Student's t-test was used to compare the difference between two groups, one-way analysis of variance (ANOVA) was used to compare the difference between three or more groups, and two-way ANOVA was used to compare the difference between multiple groups under different treatment conditions. A probability $P < 0.05$ was considered significant, with specific values of significance provided the figure legends.

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Figure 1. DAF-16 FOXO localizes to vesicles in intestinal cells

(A-H) Representative confocal and differential interference contrast (DIC) images of an intestinal cell of wild-type (A, C, E, G) and *tbc-2(tm2241)* (B, D, F, H) animals expressing DAF-16a::GFP (*zIs356*). DAF-16a::GFP (green) is present on vesicles in both wild-type and *tbc-2(tm2241)* intestinal cells (A and B) that are positive for autofluorescence (blue) present in the the endolysosomal system (E and F). Corresponding DIC (C and D) and merged (G and F) images are shown. A representative vesicle is shown (arrow head) and the two nuclei of the binucleate cell are marked (n). Note that DAF-16a::GFP is excluded from the large nucleoli (A).

(I-J) Grouped bar graphs quantifying the percentage of wild-type (I) and *tbc-2(tm2241)* (J) animals with 0, 1-10, 11-50 or >50 DAF-16a::GFP (*zIs356*) positive vesicles at the different larval stages (L1-4) or 1 and 3 day old adults (A1 and A3). (K) A Tukey boxplot of the nuclear intensity in artificial units (a.u.) of DAF-16a::GFP in *tbc-2(tm2241)* intestinal cells with and without DAF-16a::GFP positive vesicles. *n*, total number of animals *** $P < 0.001$ in an unpaired t test (Prism 8). Scale bar (A), 10 μ m.

Figure 2. RAB-5 and RAB-7 GTPases promote DAF-16 FOXO localization to endosomes

(A-F) Representative confocal images of the intestinal cells of animals expressing DAF-16a::GFP (*zIs356*) (green) and either RFP::RAB-5 (*pwIs480*) (A-C) or mCherry::RAB-7 (*pwIs429*) (magenta) (D- F). Arrowheads mark examples of vesicles positive for both DAF-16a::GFP and either RFP::RAB-5 or mCherry::RAB-7. Arrows mark examples of DAF-

824 16a::GFP vesicles that are not positive for either RFP::RAB-5 or mCherry::RAB-7. (G and H)

825 Bar graphs displaying the mean (and SEM) of the percent wild-type and *tbc-2(tm2241)* animals

826 with DAF-16a::GFP (*zIs356*) vesicles fed bacteria expressing control empty vector (ev) RNAi

827 (black bars) compared to animals fed *rab-5(RNAi)* or *rab-7(RNAi)* (white bars) from three

828 independent experiments. Fisher's exact test (graphpad.com) was used to determine the

829 statistical difference between conditions. *n*, total number of animals. * $P < 0.05$, *** $P < 0.001$,

830 **** $P < 0.0001$.

Figure 3. DAF-16 FOXO endosome localization is regulated by IIS and nutrient availability

(A) Bar graphs of the percent wild-type (black) and *tbc-2(tm2241)* (red) animals with DAF-16a::GFP (*zIs356*) vesicles in fed animals, animals starved between 4 and 5 hours and starved animals that have been refed for 1 to 2 hours. (B) Diagram of IIS-mediate regulation of DAF-16/FOXO. (C) Grouped bar graphs quantifying the percentage of wild-type and *daf-2(e1370)* L4 larvae (cumulative data from 3 independent strains) with 0, 1-10, 11-50 or >50 DAF-16a::GFP

(*zIs356*) positive vesicles at 15°C or shifted overnight to 25°C. (D) Bar graphs of percent wild-type (black) and *daf-18(e1375)* (gray, 3 independent strains) and *daf-18(ok480)* (gray, 5 independent strains) with DAF-16a::GFP (*zIs356*) vesicles. (E) Bar graphs of percent wild-type (black) and *akt-2(ok393)* (gray, 2 independent strains). (F) Bar graphs of percent wild-type animals treated with control empty vector RNAi (black) and *akt-1(RNAi)* (gray). (G) Bar graphs of the percent wild-type (black) and *tbc-2(tm2241)* (red) animals fed control empty vector RNAi, *par-5(RNAi)* and *fit-2(RNAi)* with DAF-16a::GFP (*zIs356*) vesicles. (H) Bar graph of the percent *tbc-2(tm2241)* animals with GFP::DAF-16a (*muIs71*) or DAF-16a^{AM}::GFP (*muIs113*)-positive vesicles. Representative images of GFP::DAF-16a (*muIs71*) (top) and DAF-16a^{AM}::GFP (*muIs113*) (bottom) are shown. GFP::DAF-16a vesicles (arrows) and the nuclei (n). Fisher's exact test (graphpad.com) was used to determine the statistical difference between conditions. *n*, total number of animals. ns, not significant, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

Figure 4. TBC-2 is required for full lifespan extension and increased fat storage resulting from decreased insulin/IGF signaling

(A,B) Percent survival curve of adult animals of wild-type and *daf-2(e1370)* with either *tbc-2(tm2241)* and *tbc-2(tm2241); daf-2(e1370)* (A) or *tbc-2(sv41)* and *tbc-2(sv41); daf-2(e1370)* (B) genotypes. The survival of all strains are statistically different from *daf-2(e1370)* animals ($P < 0.001$) with the exception of *tbc-2(sv41); daf-2(e1370)* ($P = 0.0314$) as determined by a Mantel-Cox log-rank test. Cumulative data from three independent replicates of 50 young adults

for a total of 150. (C,D) Log2 fold change in Nile Red (NR) fluorescence intensity (C) or Oil Red O staining intensity (D) comparing wild-type, *daf-2(e1370)*, *tbc-2(tm2241)* and *tbc-2(tm2241); daf-2(e1370)* fixed L4 hermaphrodites. Statistical analysis was done using a student's t-test with a one way analysis of variance (ANOVA). ns, not significant. ** $P < 0.01$, **** $P < 0.0001$.

Figure 5. TBC-2 is required for the increased DAF-16 target gene expression resulting from decreased insulin/IGF signaling

Quantitative RT-PCR analysis of DAF-16 target gene expression in wild-type, *daf-2(e1370)*, *tbc-2(tm2241)*, *tbc-2(tm2241); daf-2(e1370)*, *tbc-2(sv41)*, and *tbc-2(sv41); daf-2(e1370)*. Statistical analysis was performed using an Ordinary one-way ANOVA – Tukey's multiple comparisons

873 test. Shown are comparisons of *tbc-2(tm2241); daf-2(e1370)* and *tbc-2(sv41); daf-2(e1370)*
874 versus *daf-2(e1370)* single mutants. ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

875

876 **Figure 6. Depletion of the human TBC-2 homologs enhances localization of phosphorylated**

877 **FOXO/DAF-16 to endosomes.** HEK293 (A-D) and HeLa (E-H) cells were transfected with
878 nonspecific control (CTL) siRNA oligonucleotides or independent oligonucleotides towards
879 TBC1D2, TBC1D2B, or both. Cells were fixed and probed for endogenous phospho-FOXO1/3a
880 (recognizes both FOXO1 and FOXO3a), Rab5 or Rab7 endosomal markers using indirect
881 immunofluorescence and visualized using a confocal microscope (63X-oil magnification with
882 4X digital zoom) 48 hours following transfection. (A,B,E,F): Representative images of indirect
883 immunofluorescence for visualization of phospho-FOXO1/3a (green), and Rab5 (A,E) or Rab7
884 (B,F) (magenta). (C,D,G,H): Coefficient of correlation between phospho-FOXO1/3a and Rab5
885 or Rab7 fluorescence intensity; a minimum of 6 images per group (n=3) with mean $\pm$ SE. * $P <$
886 0.05, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (one-way ANOVA).

887

888 **Figure 7. Depletion of TBC1D2 and TBC1D2B modulates nuclear-cytosolic shuttling of**

889 **FOXO.** HEK293 (A-D) and HeLa (E-H) cells were transfected with nonspecific control (CTL)
 890 siRNA oligonucleotides or independent oligonucleotides towards TBC1D2A, TBC1D2B, or
 891 both. Cells were fixed and probed for pFOXO1/3a or FOXO3a using indirect
 892 immunofluorescence and visualized using a confocal microscope (63X-oil magnification) 48
 893 hours following transfection. (A,B,E,F): Representative images of indirect immunofluorescence
 894 for visualization of endogenous pFOXO1/3a (A,E) or FOXO3a (B,F) (green) or DAPI
 895 (magenta). (C,D,G,H): Coefficient of correlation between pFOXO1/3a (C,F) or FOXO3a (D,H)
 896 and DAPI fluorescence intensity; a minimum of 6 images per group ($n \geq 3$) with mean $\pm$ SE. * $P <$
 897 0.05, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA).