

TBC-2, a Rab GTPase activating protein, regulates the localization of the HLH-30/TFEB and PQM-1 transcription factors in the *C. elegans* intestine

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Abstract

Insulin/IGF signaling (IIS) inhibits the nuclear localization of the [DAF-16](#)/FOXO transcription factor to regulate longevity and stress resistance in *C. elegans*. In the intestine, IIS promotes [DAF-16](#) localization to endosomes and loss of [TBC-2](#), a [RAB-5](#) GAP, results in increased endomembrane localization of [DAF-16](#) at the expense of nuclear localization, decreased [DAF-16](#) target gene expression, longevity and stress resistance. Here we found that [TBC-2](#) differentially regulates the localization of the IIS-regulated transcription factors [PQM-1](#) and [HLH-30](#)/TFEB. Our results suggest a broader role for [TBC-2](#) in regulating IIS and that [TBC-2](#) likely functions at an upstream point in the IIS pathway.

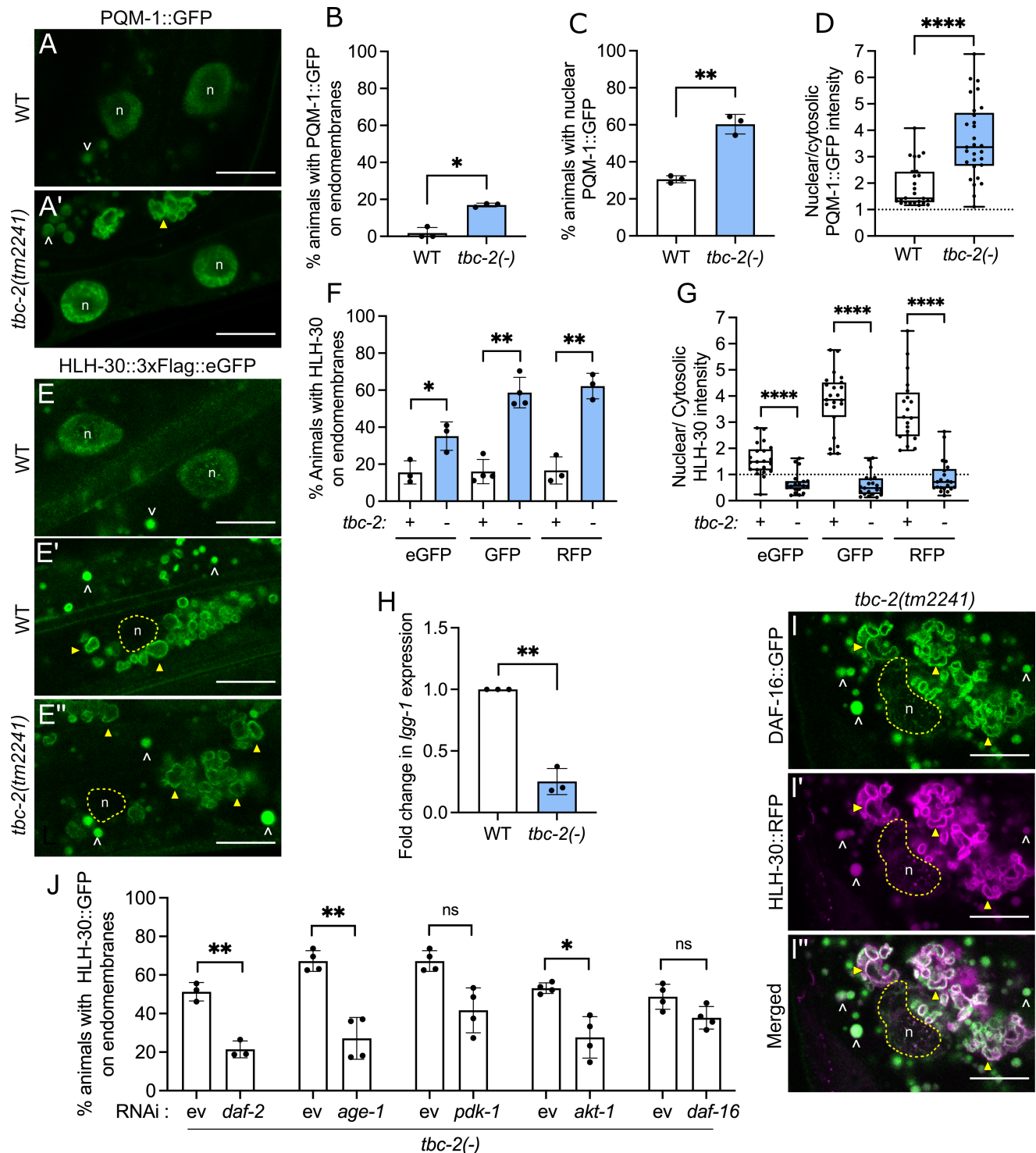


Figure 1. TBC-2 regulates the localization of PQM-1 and HLH-30:

(A-A') Representative confocal images of PQM-1::GFP in the intestine of wild type and *tbc-2(tm2241)* mutants showing nuclear (n) and endomembrane localization (yellow arrow). Δ denotes autofluorescent gut granules. (B, C) Bar graphs showing the percent animals with PQM-1::GFP localization to endomembranes (B) and the nucleus (C). Experimental triplicates with 30-50 animals per replicate. (D) Box and scatter graph representing the ratio of nuclear to cytosolic fluorescence intensity of PQM-1::GFP in wild type ($n=26$) and *tbc-2(tm2241)* mutants ($n=29$), each data point is an individual intestinal cell. (E-E'') Confocal images of endogenously tagged HLH-30::3xFlag::eGFP in the intestine of wild type with and without endomembrane localization (E-E') and in *tbc-2(tm2241)* (E''). Nuclear (n) and endomembrane localization (yellow arrows). (F) Bar graph showing the percent animals with endomembrane localization of HLH-30::3xFlag::eGFP, HLH-30::GFP, and HLH-30::RFP in wild type and *tbc-2(tm2241)* mutants. Experimental replicates with 19-36 HLH-30::3xFlag::eGFP animals per replicate and 30-60 animals per replicate for HLH-30::GFP and HLH-

30::RFP. (G) Box and scatter graphs representing the ratio of nuclear to cytosolic fluorescence intensity of HLH-30::3xFlag::eGFP, HLH-30::GFP, and HLH-30::RFP in wild type and *tbc-2(tm2241)* mutants, $n > 18$ for each condition. (H) Bar graph showing the fold change in *lgg-1* mRNA expression levels between wild type and *tbc-2(tm2241)* mutant, in 3 biological replicates. (I-I'') Representative confocal images DAF-16::GFP and HLH-30::RFP colocalization in *tbc-2(tm2241)* as shown by the yellow arrow. ^ marks the auto fluorescent gut granules and n denotes nuclei. (J) Bar graph showing the percent animals with endosomal HLH-30::GFP in the *tbc-2(tm2241)* background, fed control (ev, empty vector) RNAi or RNAi targeting components of the IIS pathway. Each replicate represents >25 worms per condition. Paired Student T-test was used to analyse data in (B, C, F, H and J) and unpaired Student T-test was used to analyse data in (D and G). Scale bar, 10 μ m. ns, not significant, * $P < 0.05$, ** $P < 0.01$ and **** $P < 0.0001$.

Description

Insulin/Insulin-like Growth Factor (IGF) signaling (IIS) is an evolutionarily conserved regulator of metabolism (Barbieri et al., 2003; Murphy & Hu, 2013). In *C. elegans*, the IIS pathway regulates lifespan, fat storage and resistance to various stresses (Friedman & Johnson, 1988; Kenyon et al., 1993; Kimura et al., 1997; Lithgow et al., 1994; Murphy & Hu, 2013; Ogg et al., 1997). The core pathway is defined by the [DAF-2](#) insulin/IGF receptor, the [AGE-1](#) class I PI3 kinase which generates PI(3,4,5)P which in turn recruits [PDK-1](#) and [AKT-1/2](#) kinases to the plasma membrane where [PDK-1](#) phosphorylates and activates [AKT-1/2](#). A major target of *C. elegans* IIS is the inhibition of the [DAF-16](#)/FOXO transcription (Kimura et al., 1997; Lin et al., 1997; Morris et al., 1996; Ogg et al., 1997; Paradis et al., 1999; Paradis & Ruvkun, 1998). Phosphorylation of FOXO transcription factors by AKT kinases results in cytoplasmic sequestration (Brunet et al., 1999).

The *C. elegans* intestine, while only 20 cells, is a major metabolic organ and site of many IIS-mediated functions (McGhee, 2007; Murphy & Hu, 2013; Zhang et al., 2022). We previously demonstrated that [DAF-16](#) localizes to endosomes in the intestinal cells (Meras et al., 2022). Interestingly, [DAF-16](#) positive endosomes are not present in every cell or every animal and can vary from just a few to hundreds. We found that [TBC-2](#) regulates [DAF-16](#) localization. [TBC-2](#) is a [RAB-5](#) GTPase Activating Protein (GAP) that regulates early to late endosome maturation (Chotard et al., 2010). In *tbc-2(tm2241)* deletion mutants, [DAF-16](#) localizes to irregularly shaped endomembranes to a greater extent than wild type and at the expense of nuclear localization (Meras et al., 2022). As such, [TBC-2](#) antagonizes IIS-mediated gene expression, longevity, fat storage and resistance to several stresses (Meras et al., 2022; Traa et al., 2023). To determine if this localization was specific to [DAF-16](#) we looked at two additional transcription factors that are differentially regulated by IIS, [PQM-1](#) and [HLH-30](#).

[PQM-1](#) is a zinc finger transcription factor required for the long lifespan of *daf-2(-)* mutants (Tepper et al., 2013). Localization of [PQM-1](#) is opposite that of [DAF-16](#) whereby IIS promotes nuclear localization of [PQM-1](#) while it antagonizes [DAF-16](#) (Tepper et al., 2013). In *tbc-2(-)* animals, [PQM-1::GFP](#) showed some localization to endomembranes (Figure 1A-A', B). Despite the endomembrane localization of [PQM-1](#), there was a significant increase in the number of animals with nuclear localized [PQM-1](#) and an increase in the nuclear to cytosolic fluorescence intensity ratio of [PQM-1](#) in *tbc-2(-)* mutants as compared to wild type (Figure 1C, D). This is consistent with the antagonistic genetic relation between [PQM-1](#) with [DAF-16](#) localization (Tepper et al., 2013).

[HLH-30](#) or TFEB (Transcription factor EB) is a transcription factor involved in lysosomal biogenesis and acts in a combinatorial manner with [DAF-16](#) in response to the starvation stress (Lapierre et al., 2013; Lin et al., 2018; O'Rourke & Ruvkun, 2013). In wild type we observed that endogenously tagged [HLH-30::3xFlag::eGFP](#) was mostly nuclear with some cytoplasmic and endomembrane localization (Figure 1E-E', F). In *tbc-2(-)* mutants, we observed an increase in the number of animals with [HLH-30::3xFlag::eGFP](#) localized to endomembranes (Figure 1E'', F). Similar results were seen with [HLH-30::GFP](#) and [HLH-30::RFP](#) transgenic animals (Figure 1F). Like [DAF-16](#), [HLH-30](#) showed reduced nuclear to cytosolic fluorescence intensity in *tbc-2(-)* mutant animals (Figure 1G). To test if the reduced nuclear localization of [HLH-30](#) affected downstream [HLH-30](#) dependent gene expression, we analysed the mRNA expression of *lgg-1*. *LGG-1* is ATG8 in yeast and LC3 in mammals and is responsible for autophagosome formation (Melendez et al., 2003). *LGG-1* expression is dependent on [HLH-30](#), such that *lgg-1* mRNA levels are suppressed in *hlh-30(RNAi)* (Lapierre et al., 2013). There was a significant decrease in the *lgg-1* mRNA levels in *tbc-2(-)* mutants consistent with the reduced nuclear localization of [HLH-30](#) (Figure 1H).

We observed that [DAF-16](#) and [HLH-30](#) co-localized on endomembranes in *tbc-2(-)* mutants indicating that [TBC-2](#) might regulate these transcription factors by a common underlying mechanism (Figure 1 I-I''). To test if IIS regulates [HLH-30](#) localization on endosomes as seen with [DAF-16](#) (Meras et al., 2022), we use RNAi to systematically knock down components of the IIS pathway- [DAF-2](#), [AGE-1](#), [PDK-1](#), [AKT-1](#) and [DAF-16](#) in *tbc-2(-)*; [HLH-30::GFP](#) animals. Compared to empty vector control, RNAi mediated knock-down of *daf-2*, *age-1* and *akt-1* significantly decreased the number of *tbc-2(-)* mutant animals with [HLH-30](#) on endomembranes (Figure 1J). *pdk-1(RNAi)* significantly suppressed [HLH-30](#) endomembrane localization in three of four experiments (Figure 1J). *daf-16* RNAi did not decrease the endomembrane localization of [HLH-30](#) (Figure 1J). Thus, IIS signaling promotes the localization of [HLH-30](#) to endomembrane compartments as it does with [DAF-16](#).

In conclusion, this study builds upon our previous findings that [TBC-2](#) regulates [DAF-16](#) localization to endomembranes (Meras et al., 2022). We demonstrated that [TBC-2](#) regulates the localization of two additional IIS regulated transcription factors, [HLH-30](#) and [PQM-1](#), further supporting a role for [TBC-2](#) in antagonizing IIS in the intestine. Similar to [DAF-16](#), we found that [HLH-30](#) localizes to uncharacterized endomembrane compartments in [tbc-2\(-\)](#) mutants at the expense of its nuclear localization. The reduced expression of the [HLH-30](#) target, [lgg-1](#), in [tbc-2\(-\)](#) mutants suggests that this impacts [HLH-30](#) transcriptional activity.

We found that [HLH-30](#) colocalizes with [DAF-16](#) on the same endomembranes in [tbc-2\(-\)](#) mutants suggesting a similar mode of regulation. [HLH-30](#)/TFEB and [DAF-16](#)/FOXO are inhibited by phosphorylation and binding with 14-3-3 proteins that sequester them in the cytoplasm (Brunet et al., 1999; Colino-Lage et al., 2024; Li et al., 2007; Lin et al., 2018; Martina et al., 2012; Roczniak-Ferguson et al., 2012). We previously demonstrated that [FTT-2](#)/14-3-3 and [DAF-16](#) phosphorylation regulate [DAF-16](#) localization to endomembranes (Meras et al., 2022). We anticipate that [HLH-30](#) is also sequestered in a similar manner. However, we don't know whether 14-3-3 mediates [HLH-30](#) and [DAF-16](#) binding to these endomembrane structures or if there are additional factors involved.

[DAF-16](#) and [HLH-30](#) forms a complex and regulates overlapping stress response genes and lysosome morphology (Lin et al., 2018; Ricaurte-Perez et al., 2025). Whether they form a complex on endomembranes remains unclear. RNAi of [daf-16](#) did not affect the localization of [HLH-30](#) to endomembranes suggesting that there may not be a codependence for endomembrane localization. However, this should be further tested with null alleles.

Like [DAF-16](#), we also found that IIS partially regulates the localization of [HLH-30](#) to endomembranes. Thus, it is possible that other metabolic or stress response pathways such as AMPK, Tor, or JNK could be involved (El-Houjeiri et al., 2019; Greer et al., 2007; Lapierre et al., 2013; Martina et al., 2012; Oh et al., 2005; Roczniak-Ferguson et al., 2012; Settembre et al., 2012). Unlike [DAF-16](#) and [HLH-30](#), IIS promotes nuclear localization of [PQM-1](#) (Tepper et al., 2013). Although we detected some endomembrane localization of [PQM-1](#) in [tbc-2\(-\)](#) mutants, we found that the nuclear versus cytoplasmic localization of [PQM-1](#) was increased suggesting increased nuclear import or possibly an increase in [PQM-1](#) levels. This is consistent with our previous finding that [tbc-2\(-\)](#) mutants had increased expression of [vha-6](#), a top [PQM-1](#) target (Meras et al., 2022; Tepper et al., 2013). Taken together these results suggest that [TBC-2](#) antagonizes IIS at a common point upstream of the [DAF-16](#), [HLH-30](#) and [PQM-1](#) transcription factors.

Methods

All strains were derived from the wild type [N2](#) strain. Worms were maintained at 20°C on NGM plates as described in WormBook (www.wormbook.org). The strains were fed [HB101](#) *E. coli* as a food source.

For RNAi feeding experiments fourth larval stage (L4) worms were placed on RNAi plates and transferred to fresh plates each day for 3 days. L4 progeny from the day 3 plates were scored for the experiment. RNAi feeding clones, except [pdk-1](#), were obtained from the Ahringer RNAi library (Fraser et al., 2000; Kamath et al., 2000; Kamath et al. 2003). [pdk-1](#) RNAi clone (pQcSS01) was generated by PCR of worm lysates using primers SSA38 5' - CAACTAAGATCTATTTCGTG ATCGGACTTGTTG - 3' and SSA39 5' - GTCATACTCGAGGCTAGCATCATTTCCCAAATTCATC -3' and ligated to the L4440 vector from Addgene (<https://www.addgene.org/1654/>).

Live imaging of the L4 hermaphrodite worms was done at room temperature. The worms were transferred to a 2% agarose pad and immobilized with 10 mM levamisole. They were scored for nuclear or endosome localization of [PQM-1](#), [DAF-16](#) and [HLH-30](#) under the fluorescent ZeissA1 axio Imager. To measure the fluorescence intensity of nuclear [PQM-1](#) and [HLH-30](#) L4 worms were imaged using a Zeiss Axio Observer Z1 LSM780 laser scanning confocal microscope in the Molecular Imaging Platform of the RI-MUHC. Image analysis was performed using Fiji (ImageJ).

For quantitative RT-PCR, RNA was extracted from young adult *C. elegans* using TRIZOL as explained in (Meras et al., 2022). [LGG-1](#) was amplified using forward primer 5' - ACCCAGACCGTATTCCAGTG -3' and reverse primer 5' - ACGAAGTTGGATGCGTTTTTC -3'.

Reagents

Strain	Genotype	Source
OP201	unc-119(tm4063) III; wgIs201[pqm-1::TY1::EGFP::3xFLAG(92C12) + unc-119(+)]	CGC; (Sarov et al., 2006; Zhong et al., 2010)
QR1112	tbc-2(tm2241) II; wgIs201[pqm-1::TY1::EGFP::3xFLAG(92C12) + unc-119(+)]	This study
VZ892	hlh-30(syb1452[hlh-30::3xFlag::eGFP]) IV	CGC; (Martina et al., 2021)

QR1138	tbc-2(tm2241) II; hlh-30(syb1452[hlh-30::3xFlag::eGFP]) IV	This study
JIN1679	jinEx10 [hlh-30p::hlh-30::GFP + rol-6(su1006)]	CGC; (Lapierre et al., 2013)
QR1068	tbc-2(tm2241) II; jinEx10 [hlh-30p::hlh-30::GFP + rol-6(su1006)]	This study
WU1598	hlh-30(tm1978) IV; hlh-30p::HLH-30::RFP + rol-6(su1006)	(Murphy et al., 2019)
QR1084	tbc-2(tm2241) II; hlh-30p::HLH-30::RFP + rol-6(su1006)	This study
QR1101	daf-16(hq23[DAF-16::GFP]) I; tbc-2(tm2241) II;; hlh-30p::HLH-30::RFP + rol-6(su1006)	This study

RNAi clone	Gene	Source
L4440	Empty vector control	(Timmons & Fire, 1998)
III-7G12	daf-2	(Kamath et al., 2003)
II-7J02	age-1	(Kamath et al., 2003)
V-7I17	akt-1	(Kamath et al., 2003)
I-5M24	daf-16	(Fraser et al., 2000)
pQcSS01	pdk-1	This study

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