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C. elegans Rab GTPase activating protein TBC-2 promotes cell corpse degradation by regulating the small GTPase RAB-5

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During apoptosis, dying cells are quickly internalized by neighboring cells or phagocytes, and are enclosed in phagosomes that undergo a maturation process to generate the phagoslysosome, in which cell corpses are eventually degraded. It is not well understood how apoptotic cell degradation is regulated. Here we report the identification and characterization of the *C. elegans tbc-2* gene, which is required for the efficient degradation of cell corpses. *tbc-2* encodes a Rab GTPase activating protein (GAP) and its loss of function affects several events of phagosome maturation, including RAB-5 release, phosphatidylinositol 3-phosphate dynamics, phagosomal acidification, RAB-7 recruitment and lysosome incorporation, which leads to many persistent cell corpses at various developmental stages. Intriguingly, the persistent cell corpse phenotype of *tbc-2* mutants can be suppressed by reducing gene expression of *rab-5*, and overexpression of a GTP-locked RAB-5 caused similar defects in phagosome maturation and cell corpse degradation. We propose that TBC-2 functions as a GAP to cycle RAB-5 from an active GTP-bound to an inactive GDP-bound state, which is required for maintaining RAB-5 dynamics on phagosomes and serves as a switch for the progression of phagosome maturation.

KEY WORDS: *C. elegans*, TBC-2, RAB-5, GAP, Phagosome maturation, Cell corpse degradation

INTRODUCTION

The phagocytosis of apoptotic cells involves the specific recognition, followed by internalization, of cell corpses. The resulting intracellular vesicles containing ingested apoptotic cells, termed phagosomes, undergo a series of maturation steps leading to the destruction of cell corpses. Although the specific factors and mechanisms controlling maturation of apoptotic-cell-containing phagosomes are not well understood, recent studies in *C. elegans* indicate that cell corpse clearance through phagosome maturation follows a general path that requires similar components and cellular events to that of phagosomes carrying opsonized particles or microorganisms (Kinchen and Ravichandran, 2008; Vieira et al., 2002; Zhou and Yu, 2008). For example, Rab GTPases, the key regulators of endocytic transport and maturation of phagosomes with ingested opsonized cells or microbes, are also found to play important roles in the maturation of phagosomes containing apoptotic cells in both mammals and *C. elegans* (Kinchen et al., 2008; Kitano et al., 2008; Lu et al., 2008; Mangahas et al., 2008; Yu et al., 2008). Moreover, cellular events that recapitulate the progression from endosome to lysosome are similarly involved in the maturation of apoptotic-cell-containing phagosomes (Kinchen and Ravichandran, 2008; Vieira et al., 2002).

RAB5, an early endosome marker and key regulator of early endosome traffic, is found to control the early steps of phagosome maturation. RAB5 is recruited transiently to the newly formed phagosomes in intact macrophages, and it is essential for the fusion between isolated sorting endosomes and purified nascent phagosomes in a cell-free system (Desjardins et al., 1994; Desjardins et al., 1997; Henry et al., 2004; Jahraus et al., 1998; Roberts et al., 2000; Vieira et al., 2002). Moreover, RAB5/RAB-5 is found to be transiently associated with apoptotic-cell-containing phagosomes at an early stage and is required for phagosome maturation and apoptotic cell degradation in mammals and *C. elegans* (Kinchen et al., 2008; Kitano et al., 2008; Zhou and Yu, 2008).

Phosphatidylinositol 3-phosphate [PtdIns(3)*P*], which is characteristic of early endosomes and is an essential player in endosome trafficking, is also present on nascent phagosomes to regulate phagosome maturation (Simonsen et al., 2001; Vieira et al., 2002). PtdIns(3)*P* is generated by the RAB5 effector VPS34, a class III phosphatidylinositol 3-kinase required for the maturation of phagosomes carrying various cargoes, including apoptotic cells (Kinchen et al., 2008; Vieira et al., 2001). The effects of PtdIns(3)*P* on phagosome maturation are likely to be mediated by recruiting FYVE or PX domain-containing proteins such as EEA1 and HRS, both of which are implicated in phagosome maturation and are targeted by mycobacteria (Fratti et al., 2001; Vieira et al., 2001; Vieira et al., 2004). However, neither of these two proteins is essential for cell corpse removal in *C. elegans*, suggesting that different factors are used to mediate the functions of RAB-5 or PtdIns(3)*P* in cell corpse degradation (Kinchen and Ravichandran, 2008). The appearance of PtdIns(3)*P* on phagosomes seems to be transient (Ellson et al., 2001; Vieira et al., 2002). However, it is not yet known how the dynamic accumulation of PtdIns(3)*P* is regulated and how it contributes to phagosome maturation.

RAB7 localizes to late endosomes and controls the late steps of endosome traffic. In accordance with its role in endocytosis, RAB7 is found to regulate phagolysosome formation through its effector protein RILP (RAB7-interacting lysosomal protein) in macrophages with ingested opsonized particles (Harrison et al., 2003; Vieira et al., 2002). In *C. elegans*, RAB-7 is essential for cell corpse degradation. It acts downstream of RAB-5 and is required for phagosome fusion with lysosomes (Kinchen et al., 2008; Yu et al., 2008). Interestingly, components of the HOPs complex seem to function downstream of RAB-7 to mediate phagolysosome formation, although no data is currently available on whether any of them could act directly as RAB-7 effectors (Kinchen et al., 2008; Xiao et al., 2009). In addition to RAB-5 and RAB-7, UNC-108/RAB2 also plays a role in cell

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corpse degradation (Lu et al., 2008; Mangahas et al., 2008). Nevertheless, it is still not clear at which specific step(s) UNC-108 acts during phagosome maturation.

Although increasing evidence points to an important role for Rab GTPases in the maturation of phagosomes containing distinct cargoes, it is not clear how they are regulated and coordinated during this process. RAB5 is by far the best characterized Rab in both endosomal traffic and phagosome maturation. However, how RAB5 is regulated and how its function in phagosome maturation is mediated remains elusive. For example, it is not well understood how RAB5 is recruited and activated on nascent phagosomes, and even less is known about its inactivation and release from phagosomes. Moreover, how phagosomes mature through a RAB5-positive to a RAB7-positive stage is not clear. As a small GTPase, the role of RAB5 as a molecular switch relies on it oscillating between inactive GDP-bound and active GTP-bound states, which is coordinated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs activate RAB5 by promoting the exchange of GDP for GTP, whereas GAPs inactivate it by accelerating GTP hydrolysis. In mammals, several VPS9 domain-containing proteins have been found to show GEF activity on RAB5; these proteins either have a general role in endosomal traffic or regulate the trafficking of specific proteins to endosomes (Carney et al., 2006). Among them, however, only gapex 5 seems to be required for RAB5 activation and the maturation of apoptotic cell-containing phagosomes (Kitano et al., 2008). In *C. elegans*, none of the three VPS9 domain-containing RAB-5 GEFs (RME-6, RABX-5 and TAG-333) was found to be essential for apoptotic-cell removal, suggesting that a novel GEF is likely to be involved (Kinchen et al., 2008). On the other hand, more than 50 putative Rab GAPs exist in mammals, but only very few of them have been matched to a specific Rab protein. In the case of RAB5, six proteins, RabGAP5 (SGSM3), RN-TRE, p120-RASGAP (RASA1), p85α (PIK3R1), PRC17 and TSC2, were found to possess GAP activity towards RAB5 in vitro, among which only RN-TRE and RabGAP5 have been reported to regulate RAB5 function in the endocytic pathway (Chamberlain et al., 2004; Haas et al., 2005; Lanzetti et al., 2000; Liu and Li, 1998; Pei et al., 2002; Xiao et al., 1997). Unfortunately, the functional requirement for these RAB5 GAPs in phagosome maturation has not been addressed. It is therefore not known whether one or more of them is required for regulating RAB5 function on phagosomes, or whether a novel GAP is involved. In addition, the GAP of RAB-5 for the regulation of either endocytosis or phagosome maturation in *C. elegans* has not yet been identified.

In the present study, we have identified a TBC domain-containing Rab GTPase activating protein, TBC-2, as an essential regulator for the removal of cell corpses in *C. elegans*. We show that *tbc-2* mutation affects several aspects of phagosome maturation, including RAB-5 release, PtdIns(3)*P* dynamics, phagosomal acidification, RAB-7 recruitment and lysosome incorporation. Moreover, partial inactivation of *rab-5* by RNAi suppresses the persistent cell corpse phenotype of *tbc-2* mutants, whereas overexpression of a GTP-locked RAB-5 mimics its phagosome maturation defects. Our data indicate that TBC-2 is likely to act as a GAP of RAB-5 to cycle it from an active GTP-bound to an inactive GDP-bound state, which serves as a switch for phagosomes to mature through the RAB-5-positive stage.

MATERIALS AND METHODS

C. elegans strains

Strains of *C. elegans* were cultured at 20°C using standard procedures (Brenner, 1974). *qx20* and *tm2241* mutants were kept at 20°C and moved to 25°C 1 day before the phenotype was examined. The N2 Bristol strain was used as the wild-type strain. Mutations used are described in *C. elegans II* (Riddle et al., 1997), unless otherwise indicated. Linkage group I (LG I): *ced-1(e1735)*, *vps-34(h797)*, *dpy-5(e61)*, *unc-13(e450am)*, *unc-108(n3263)* (Mangahas et al., 2008), *unc-108(sm237)* (Lu et al., 2008). LG II: *unc-4(e120)*, *rol-1(e91)*, *lin-31(n301)*, *sqt-2(sc3)*, *bli-2(e768)*, *clr-1(e1745ts)*, *dpy-10(e128)*, *mIn1[dpy-10(e128)mIs14]/bli-2(e768)unc-4(e120)*, *rrf-3(pk1426)* (Simmer et al., 2002), *qx20* and *tm2241* (this study). LG III: *ced-4(n1162)*. LG IV: *ced-3(n717)*. LG V: *unc-76(e911)*. *opIs334* [P_{ced-1}YFP::2×FYVE, a gift of Dr K. S. Ravichandran, University of Virginia, Charlottesville, VA, USA; and Dr M. O. Hengartner, University of Zurich, Zurich, Switzerland (Kinchen et al., 2008)].

Other strains carrying integrated arrays used in this study are as follows: *qxIs65* (P_{ced-1}*gfp::rab-5*), *qxIs66* (P_{ced-1}*gfp::rab-7*), *qxIs68* (P_{ced-1}*mcherry::rab-7*), *qxIs60* (P_{ced-1}*lmp-1::gfp*), *qxIs157* [P_{hsp}RAB-5(Q78L)], *qxIs161* (P_{ced-1}*gfp::tbc-2*) and *qxIs109* (P_{tbc-2}*tbc-2::gfp*).

Molecular cloning of *tbc-2*

qx20 was mapped to linkage group II between *lin-31* (–6.0) and *unc-4* (+1.76). Two more rounds of three-point mapping were then performed by using *bli-2* (–0.99) *unc-4* (+1.76) and *lin-31* (–6.0) *clr-1* (–1.29) *dpy-10* (0), which mapped *qx20* to a small genetic interval (–1.28-0) and near –0.74. Transformation rescue experiments were then performed and one fosmid clone in this region, WRM0621aG07, rescued the persistent cell corpse phenotype of the *qx20* mutant. Long PCR fragments covering different open reading frames within this fosmid were tested and only one fragment that covers ZK1248.10 possessed rescue activity, which corresponds to *tbc-2* gene. The molecular lesion in the *qx20* mutant was determined by sequencing the *tbc-2* locus.

Plasmid construction

The 3.3 kb genomic sequence of the *tbc-2* gene was PCR-amplified from fosmid WRM0621aG07 (Geneservice, UK) and cloned into the *Kpn*I site of the P_{ced-1} vector to get P_{ced-1}*tbc-2*, or into the P_{ced-1}*gfp* and P_{ced-1}*mcherry* vectors to obtain the constructs P_{ced-1}*gfp::tbc-2* and P_{ced-1}*mcherry::tbc-2*, respectively. The R689A mutation was introduced into the P_{ced-1}*tbc-2* construct by site-directed mutagenesis (QuickChange; Stratagene, USA). To generate P_{tbc-2}*tbc-2::gfp*, a 5.4 kb genomic fragment containing the genomic sequence of the *tbc-2* gene, including the 2 kb promoter region, was PCR-amplified from WRM0621aG07 and cloned into the pPD49.26-gfp vector through the *Kpn*I site. The full-length cDNA of *tbc-2* was amplified from a *C. elegans* cDNA library (Invitrogen, USA) and cloned into the pET41b vector through the *Spe*I-*Kpn*I sites. To construct P_{ced-1}GFP::PH (or ::SMC or ::TBC), the genomic fragment covering the PH domain or the cDNA fragment covering the SMC or the TBC domain of TBC-2 was amplified and cloned into P_{ced-1}*gfp* through its *Kpn*I site. To generate P_{ced-1}GFP::ΔPH or P_{ced-1}GFP:: ΔTBC , a 2.7 kb (ΔPH) or a 2.3 kb (ΔTBC) genomic fragment of the *tbc-2* gene was amplified and cloned into P_{ced-1}*gfp* via its *Kpn*I site. To construct P_{ced-1}GFP:: ΔSMC, the cDNA fragment of *tbc-2* covering only the PH domain was first amplified and cloned into the pPD49.83 through the *Kpn*I-*Eco*RV sites. The resulting pPD49.83-PH was then ligated with the cDNA fragment covering only the TBC domain through the *Eco*RV site. Finally, P_{ced-1}GFP:: ΔSMC was generated by releasing the 2 kb *Kpn*I fragment containing both PH and TBC domain from pPD49.83-PH-TBC and inserting this into P_{ced-1}*gfp* through the *Kpn*I site. The full-length cDNA of *rab-5*, *rab-7* or *unc-108* was amplified from a *C. elegans* cDNA library (Invitrogen, USA) and the point mutations that affect the nucleotide binding of Rab GTPases were introduced by site-directed mutagenesis (QuickChange; Stratagene, USA), which were further cloned into the P_{hsp} vectors through the *Nhe*I-*Kpn*I sites, or inserted into P_{ced-1}*gfp* through the *Kpn*I site. To construct pPD129.36-*rab-5*, the full-length cDNA of *rab-5* was cloned into the pPD129.36 vector through the *Xba*I-*Hin*dIII sites.

Quantification of cell corpses

The number of somatic cell corpses in the head region of living embryos and the number of germ cell corpses in one gonad arm at various adult ages were scored as described (Gumienny et al., 1999; Wang et al., 2002). Four-dimensional (4D) microscopy analysis of cell corpse duration was performed at 20-22°C as described (Wang et al., 2003). Acridine Orange (AO) staining was performed as described (Lu et al., 2008).

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Fluorescence microscopy

For fluorescent imaging, the AxioImager M1 equipped with epifluorescence and an AxioCam monochrome digital camera was used, and images were processed and viewed using AxioVision release 4.5 software (Carl Zeiss). Time-lapse imaging of GFP, YFP and mCHERRY was performed at 20-22°C as described (Lu et al., 2008). *C. elegans* embryos around the 400-cell stage were mounted on an agar pad and images in a 20 μm *z*-series (1.0 μm/section) were captured every 3 minutes for 120 minutes using a Zeiss LSM 510 Pascal inverted confocal microscope with 488, 514 and 633 nm lasers (Carl Zeiss). Images were processed and viewed using LSM Image Browser software.

RNAi

The bacterial feeding protocol was used in RNAi experiments as described before (Kamath and Ahringer, 2003). Briefly, L4 larvae of wild type or *tbc-2* mutants were treated with either control (pPD129.36-*gfp* or pPD129.36) or *rab-5* RNAi (pPD129.36-*rab-5*) vectors. The embryonic cell corpses were scored 18 to 20 hours after transferring to RNAi plates and the germ cell corpses were scored either 48 hours post L4/adult molt in control animals or with F1 escapers when treated with *rab-5* RNAi. To perform *rab-5* RNAi in an RNAi-sensitive strain, L4 larvae of *rrf-3(pk1426)* were moved to control or *rab-5* RNAi plates and cell corpses were scored 20-25 hours after treatment. The reduction of *rab-5* expression after RNAi treatment was examined by using the *qxIs65* strain, which contains an integrated *gfp::rab-5* array under the control of the *ced-1* promoter ($P_{ced\text{-}1}$*gfp::rab-5*). Consistent with previous findings (Simmer et al., 2002), loss of *rrf-3* activity enhanced the RNAi efficiency, as *rab-5* RNAi caused greater reduction of *gfp::rab-5* expression in *rrf-3(pk1426);qxIs65* animals, in which all of the F1 embryos (*n*=83) showed significant reduction of GFP fluorescence. However, in *qxIs65* (*n*=72) or *tbc-2(qx20);qxIs65* (*n*=77) worms treated with *rab-5* RNAi, only 74% and 77% of embryos, respectively, displayed decreased *gfp* expression, indicating that *rab-5* expression is only partially blocked in these animals.

For *rab-7* and *unc-108* RNAi, L2 or L3 larvae were treated with either control vectors or *rab-7* (II-8G13) or *unc-108* RNAi (I-1J21) (Kamath and Ahringer, 2003) vectors, and cell corpses were scored in the F1 generation.

Heat-shock experiments

Young adults were moved to fresh NGM plates and cultured at 20°C for 12 hours before they were incubated at 33°C for 1 hour (+HS) or left at 20°C without heat-shock treatment (–HS), followed by recovery at 20°C for 1.5 hours. Adult worms were removed and embryos were incubated at 20°C and scored for the number of cell corpses or the persistence of cell corpses 5 to 10 hours after treatment.

RESULTS

tbc-2 is required for cell corpse clearance

In a forward genetic screen for additional genes involved in *C. elegans* cell corpse clearance, we identified a recessive mutation, *qx20*, which causes an increased number of cell corpses at all developmental stages in both somatic and germ cells (Fig. 1A,B). The appearance of cell corpses in the *qx20* mutant was totally blocked by strong loss-of-function mutations in the *ced-3* and *ced-4* genes that are required for all apoptosis in *C. elegans*, indicating that they are indeed apoptotic cell corpses (data not shown). Moreover, the cell corpse phenotype in *qx20* mutants appeared to be both temperature-sensitive and maternally rescued (see Tables S1 and S2 in the supplementary material). For example, the cell corpse phenotype of *qx20* can be observed at 20°C but not at 16 °C, and is more potent at 25°C (see Table S1 in the supplementary material). This increase in cell corpses did not appear to be a result of ectopic cell death because no extra 'undead' or missing cells were observed in the anterior pharynx or in the ventral cord of *qx20* animals, indicating that the occurrence of cell death is not affected (data not shown). To find out whether the increased number of cell corpses is due to a defect in cell corpse removal, we performed a time-lapse analysis to measure the persistence of cell corpses in both wild-type and *qx20* mutant embryos (Wang et al., 2003). In wild-type animals, the majority of the cell corpses persist between 10 to 40 minutes, with an average duration of 28 minutes. Conversely, most of the cell corpses last between 40 to 130 minutes in the *qx20* mutant, with an average duration of 58 minutes, which is 107% longer than that in wild-type embryos. These results indicate that cell corpse clearance is compromised in the *qx20* mutant (Fig. 1C).

We cloned the gene affected in the *qx20* mutant, which corresponds to *tbc-2* gene encoding a TBC domain-containing Rab GTPase activating protein (GAP) (Neuwald, 1997) (see Fig. S1A and Fig. S2 in the supplementary material). TBC-2 is most similar to the Rab GTPase activating protein TBC1D2B in mouse and *Xenopus*, with 27% and 30% sequence identity, and 47% and 48% sequence similarity, respectively (see Fig. S2 in the supplementary material). These three proteins all contain a conserved C-terminal TBC domain that is required for GAP activity, an N-terminal PH domain and an SMC or a coiled-coil domain in the middle, which might mediate protein-lipid or protein-protein interactions (see Fig. S2 in the supplementary material). The sequence of *tbc-2* in the *qx20* mutant was determined and a C to T transition was identified that resulted in an early stop codon and a truncated TBC-2 protein containing only the PH domain (see Fig. S1A and Fig. S2 in the supplementary material). In addition, *tm2241*, a *tbc-2* deletion mutant that contains a 230 bp deletion plus a 8 bp insertion that results in a truncated TBC-2 protein of 177 amino acids, showed a similar cell corpse phenotype in both somatic and germ cells (Fig. 1A, B; see Fig. S1A and Fig. S2 in the supplementary material). Intriguingly, the cell corpse phenotype of the *tm2241* mutant is also temperature sensitive and exhibits a maternal-rescue effect (see Tables S1 and S2 in the supplementary material).

TBC-2 associates transiently with phagosomes

To understand how TBC-2 regulates cell corpse clearance, we first examined the cellular localization of TBC-2 by expressing a TBC-2 green fluorescent protein (GFP) fusion under the control of the *tbc-2* promoter ($P_{tbc\text{-}2}$TBC-2::GFP), which fully rescued the cell corpse phenotype of *tbc-2* mutants (see Fig. S1A in the supplementary material). TBC-2::GFP was found to be ubiquitously expressed in the embryo, starting from the ~200-cell stage, and throughout the larval and adult stages. The strong TBC-2::GFP expression was observed in several known engulfing cell types, such as pharyngeal muscle cells, hypodermal cells and intestinal cells (see Fig. S1B in the supplementary material). In addition, a weak GFP signal was also seen in the gonadal sheath cells that engulf germ cell corpses (data not shown). Importantly, the expression of TBC-2 driven by the *ced-1* promoter ($P_{ced\text{-}1}$*tbc-2*) but not the *egl-1* promoter ($P_{egl\text{-}1}$*tbc-2*), which drive gene expression specifically in engulfing cells and dying cells, respectively, completely rescued the persistent cell corpse phenotype of the *tbc-2* mutants, indicating that *tbc-2* needs to function in the engulfing cells to regulate cell corpse clearance (see Fig. S1A in the supplementary material) (Conradt and Horvitz, 1998; Zhou et al., 2001). Interestingly, in wild-type embryos carrying either $P_{tbc\text{-}2}$TBC-2::GFP or $P_{ced\text{-}1}$GFP::TBC-2, a strong GFP signal was observed around cell corpses, indicating that TBC-2 might associate with phagosomes in engulfing cells (see Fig. S1B in the supplementary material).

To further dissect which domain is important for TBC-2 to associate with phagosomes, we generated GFP fusions of TBC-2 truncations that contain only one or two domains in different combinations and examined their cellular localizations. We found that GFP::SMC existed on phagosomes, as did the full-length

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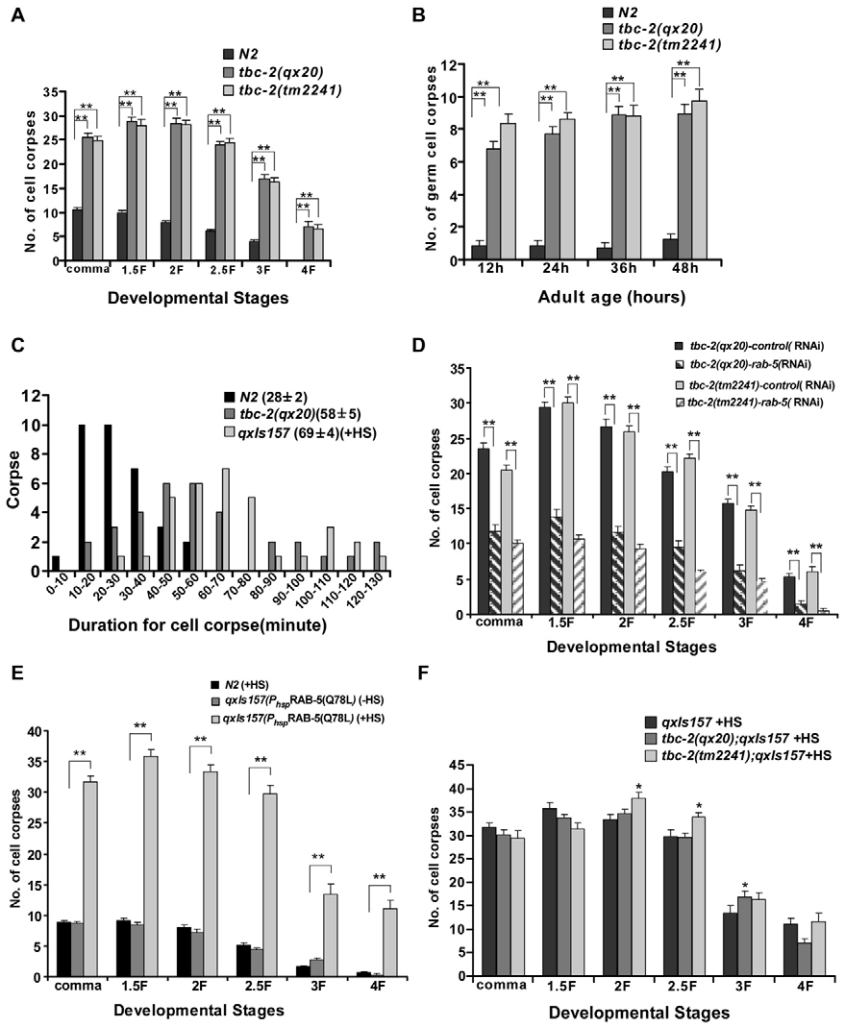

Fig. 1. *tbc-2* promotes cell corpse clearance through *rab-5*. (**A**,**B**) Time-course analysis was performed in somatic cells (A) or germ cells (B) in wild type (N2), *tbc-2(qx20)* and *tbc-2(tm2241)* mutants. (**C**) Four-dimensional microscopy analysis of cell corpse duration was performed in wild type (N2), *tbc-2(qx20)* mutants and *qxIs157* [P_{hsp}RAB-5(Q78L)] worms. The persistence of 33 cell corpses from three embryos of each strain was monitored. The numbers in parentheses indicate the average duration of cell corpses (±s.e.m.). The *y*-axis represents the number of cell corpses and the specific duration range is shown on the *x*-axis. The duration of four cell divisions in the MS cell lineage from MS cell to MS.aaaa cell was followed to ensure that the embryos scored had similar developmental rates. The average duration of four cell divisions is 93±6 minutes in wild type and 103±1.1 minutes in *tbc-2(qx20)* embryos. Pre-comma-stage *qxIs157* embryos were followed after heat-shock treatment as described in the Materials and methods. (**D**) The persistent cell corpse phenotype in *tbc-2* mutants can be suppressed by partial inactivation of *rab-5*. RNAi experiments were performed as described in the Materials and methods, genotypes and treatments are as indicated. Bacteria expressing dsRNA corresponding to *gfp* were used as a control. (**E**,**F**) Overexpression of GTP-locked RAB-5 results in the accumulation of cell corpses and is not enhanced by *tbc-2* mutations. Heat-shock experiments were performed as described in the Materials and methods. In A and D-F, cell corpses from the indicated animals were scored at the following embryonic stages: bean/comma (comma), 1.5-fold (1.5F), 2-fold (2F), 2.5-fold (2.5F), 3-fold (3F) and 4-fold (4F). In panel B, cell corpses were scored every 12 hours after the L4/adult molt from one gonad arm. In A,B and D-F, at least 15 animals were scored at each stage and error bars indicate s.e.m. Data derived from different genetic backgrounds at multiple developmental stages were compared by two-way analysis of variance. Post-hoc comparisons were carried out using Fisher's PLSD (protected least squares difference) test. $^*P<0.05$, $^{**}P<0.0001$, all other points are $P>0.05$.

TBC-2 protein, whereas the phagosome localization pattern was totally abolished when the SMC domain was absent (GFP::ΔSMC), indicating that the SMC domain is both sufficient and necessary for TBC-2 to localize to phagosomes (Fig. 2A-D). By contrast, neither the PH nor the TBC domain alone was associated with phagosomes, and TBC-2 truncations lacking either of these domains still appeared on phagosomes (Fig. 2E-L). However, both of these domains are essential for rescuing the cell corpse phenotype in the *tbc-2* mutant (see Fig. S1A in the supplementary material). Importantly, we found that a point mutation located in the TBC domain, R689A, in which the Arginine residue crucial for GAP activity was replaced by Alanine, completely abolished the rescuing activity of TBC-2, indicating that GAP activity is required for TBC-2 to remove apoptotic cells (see Fig. S1A and Fig. S2 in the supplementary material) (Albert et al., 1999; Pan et al., 2006; Rak et al., 2000).

To better understand the dynamic association of TBC-2 with phagosomes, we performed a time-lapse analysis to monitor the kinetics of phagosomal association of TBC-2 in embryos coexpressing TBC-2 with either RAB-5 or RAB-7, two key factors

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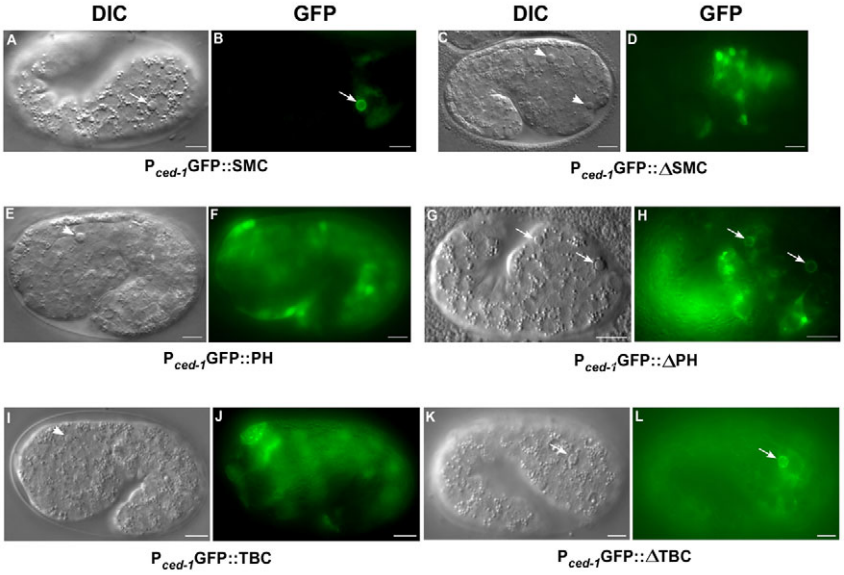

Fig. 2. The SMC domain is required for the phagosomal association of TBC-2. DIC (**A**,**C**,**E**,**G**,**I**,**K**) and fluorescence (**B**,**D**,**F**,**H**,**J**,**L**) images of wild-type embryos transgenic for P_{ced-1}GFP::SMC (A,B), P_{ced-1}GFP::ΔSMC (C,D), P_{ced-1}GFP::PH (E,F), P_{ced-1}GFP::ΔPH (G,H), P_{ced-1}GFP::TBC (I,J) and P_{ced-1}GFP::ΔTBC (K,L) are shown. Cell corpses were clustered by GFP::TBC-2 truncations that contain the SMC domain (indicated by arrows), but not by those that lack the SMC domain (indicated by arrowheads). Scale bars: 5 μm.

that are recruited to phagosomes at different stages during their maturation (Kinchen et al., 2008; Zhou and Yu, 2008). We found that TBC-2 was transiently recruited to phagosomes at a similar stage to RAB-5, which was followed by the recruitment of RAB-7 (Fig. 3). Importantly, TBC-2 was released from phagosomes simultaneously with RAB-5, which preceded the release of RAB-7 (Fig. 3). These results indicate that TBC-2 might act at the same step as RAB-5 to regulate phagosome maturation.

The accumulation of cell corpses in *tbc-2* mutants is likely to be caused by the persistent activation of RAB-5

TBC domain-containing proteins such as TBC-2 are conserved in eukaryotic organisms, and function as GAPs that inactivate Rab GTPases by accelerating their low intrinsic rate of GTP hydrolysis (Bernards, 2003). Therefore, loss of *tbc-2* function might lock a Rab GTPase into an active GTP-bound conformation, which might result in the accumulation of cell corpses. Since RAB-5 is required for cell corpse degradation during phagosome maturation and TBC-2 shows similar phagosomal recruitment kinetics as RAB-5, we investigated whether TBC-2 regulates cell corpse clearance by inactivating RAB-5. In agreement with previous findings, we found that reducing gene expression of *rab-5* by RNAi in wild-type animals resulted in a high percentage of embryonic lethality and an accumulation of germ cell corpses in F1 escapers (see Table S3 in the supplementary material) (Kinchen et al., 2008). However, no abnormal accumulation of cell corpses in wild-type embryos was observed with this treatment (see Table S3 in the supplementary material). Nevertheless, when the RNAi experiment was similarly performed in an RNAi-sensitive mutant, *rrf-3(pk1426)* (Simmer et al., 2002), which caused a greater reduction of *rab-5* expression (see Materials and methods), significantly more cell corpses were found in the *rab-5* RNAi-treated embryos than in control animals, indicating that the loss of *rab-5* function affects cell corpse clearance in both somatic and germ cells (see Table S3 in the supplementary material). Intriguingly, partial inactivation of *rab-5* by RNAi significantly suppressed the persistent cell corpse phenotype in *qx20* and *tm2241* mutants (Fig. 1D; see Table S3 in the supplementary material). For example, *tbc-2 (qx20)* animals treated with control RNAi contained an average of 26.6 cell corpses at the 2-fold embryonic stage, which was reduced to 13.5 when treated with *rab-5* RNAi, displaying a reduction by 49%. In fact, the numbers of cell corpses in *tbc-2* mutants were significantly reduced at all embryonic stages when treated with *rab-5* RNAi (Fig. 1D), suggesting that RAB-5 is required for the accumulation of cell corpses in these mutants. By contrast, reducing or eliminating gene expression of *rab-7* or *unc-108*, the two other Rabs that have been implicated in cell corpse clearance, failed to suppress the cell corpse phenotype of *tbc-2* mutants (see Table S3 in the supplementary material). Given that *tbc-2* encodes a Rab GAP that acts to cycle Rab GTPase from an active to inactive conformation, we therefore speculated that loss of *tbc-2* function arrests RAB-5 at an active GTP-bound state, which affects cell corpse removal.

In order to further test this hypothesis, we examined whether persistent activation of RAB-5 would affect cell corpse removal using a mutant form of RAB-5, RAB-5(Q78L), which is defective in GTP hydrolysis and thus stays in a GTP-locked conformation (Stenmark et al., 1994). Indeed, we found that overexpression of RAB-5(Q78L) driven by *C. elegans* heat-shock promoters [*qxIs157*: P_{hsp}RAB-5(Q78L)] resulted in an increased number of cell corpses at all examined developmental stages, which is not caused by extra cell deaths because no extra or missing cells were observed in either the anterior pharynx region or the ventral cord of *qxIs157* animals after heat-shock treatment (Fig. 1E; data not shown). Moreover, similar to *tbc-2* mutants, the embryonic cell corpses persisted much longer in *qxIs157* animals, with an average duration of 69 minutes, which is 1.5 times longer than that in wild-type embryos, indicating that sustained activation of RAB-5 affected cell corpse removal (Fig. 1C). In addition, the cell corpse phenotype observed in *qxIs157* animals was not significantly enhanced by the loss-of-function mutations of *tbc-2* (Fig. 1F). By contrast, no obvious defect in cell corpse removal was observed when GTP-locked RAB-7 [RAB-7(Q68L)] or UNC-108 [UNC-108(Q65L)] was similarly overexpressed (data not shown). Taken together, our data strongly argue that loss of *tbc-2* function affects the GTP hydrolysis of RAB-5 and thus keeps it in an active conformation, which in turn causes defects in cell corpse clearance.

tbc-2 mutants affect the release of RAB-5 from phagosomes

Our genetic data indicate that TBC-2 targets RAB-5, which controls early steps of phagosome maturation in different systems (Kinchen et al., 2008; Kitano et al., 2008; Nakaya et al., 2006; Vieira et al.,

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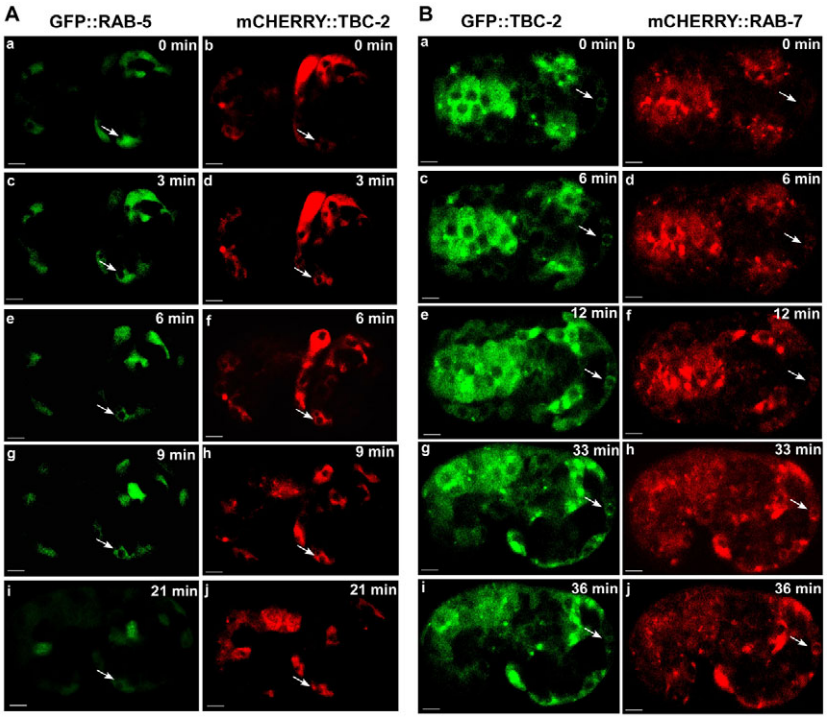

Fig. 3. TBC-2 is recruited to and released from phagosomes with similar kinetics to RAB-5. Confocal time-lapse images of wild-type embryos coexpressing (**A**) $P_{ced\text{-}1}$*gfp::rab-5* (a,c,e,g,i) and $P_{ced\text{-}1}$*mcherry::tbc-2* (b,d,f,h,j) or (**B**) $P_{ced\text{-}1}$*gfp::tbc-2* (a,c,e,g,i) and $P_{ced\text{-}1}$*mcherry::rab-7* (b,d,f,h,j). Arrows indicate the phagosomes followed. Scale bars: 5 μm.

2002; Vieira et al., 2003). Therefore, we investigated whether the phagosome maturation process is affected in *tbc-2* mutants, in which RAB-5 is probably arrested in the active state. We first examined the phagosomal association of RAB-5 in *tbc-2* mutants and found that more cell corpses were labeled by GFP::RAB-5 in *tbc-2* mutants than in wild-type embryos, suggesting that the release of RAB-5 from phagosomes is probably affected in these mutants (Fig. 4). To examine this process more carefully, we performed a time-lapse analysis to monitor the phagosomal release of RAB-5 in both wild type and *tbc-2* mutants. In wild-type embryos, RAB-5 disappeared from 70% of phagosomes within 20 minutes and the average duration time was 18 minutes (Fig. 5A,B; see Movie 1 in the supplementary material). By contrast, the majority of cell corpses were labeled by GFP::RAB-5 for more than 30 minutes in *tbc-2(qx20)* mutants, and some phagosomes failed to release RAB-5 even after 60 minutes (Fig. 5A,B; see Movie 2 in the supplementary material). On average, phagosome-associated RAB-5 was retained for 39 minutes in *tbc-2(qx20)* mutants, which is 116% longer than that in wild-type embryos, indicating that the phagosomal release of RAB-5 is indeed disturbed (Fig. 5B).

The dynamic accumulation of PtdIns(3)*P* on phagosomes is affected in *tbc-2* mutants

During phagosome maturation, RAB-5 regulates the phagosomal accumulation of phosphatidylinositol 3-phosphate [PtdIns(3)*P*] through VPS-34, a class III phosphatidylinositol 3-kinase that is responsible for generating PtdIns(3)*P* and that is essential for cell corpse clearance (Kinchen et al., 2008). Because our genetic analyses indicate that TBC-2 probably acts together with RAB-5, we further examined whether the phagosomal accumulation of PtdIns(3)*P* was also affected in the *tbc-2* mutants using a YFP::2×FYVE marker (*opIs334*: $P_{ced\text{-}1}$YFP::2×FYVE), which specifically binds PtdIns(3)*P* on endosomes as well as on phagosomes containing apoptotic cells (Kinchen et al., 2008). In wild-type embryos, 35% of cell corpses were labeled by YFP::2×FYVE, which persisted on phagosomes only for a short period of time, with an average duration of 11 minutes (Fig. 4; Fig. 5C,D; see Movie 3 in the supplementary material), indicating that the phagosomal accumulation of PtdIns(3)*P* is also transient in *C. elegans*, as it is in macrophages (Ellson et al., 2001; Henry et al., 2004; Vieira et al., 2002). By contrast, about 70% of cell corpses were labeled by YFP::2×FYVE in *tbc-2* mutants and the YFP signals on the phagosomes were brighter than those in wild-type animals (Fig. 4). Moreover, a significantly longer duration of YFP::2×FYVE on phagosomal membrane was observed in *tbc-2(qx20)* mutants, with an average duration of 55 minutes, which is four times longer than that in wild-type embryos, suggesting that either more PtdIns(3)*P* was produced, its turnover was disrupted, or both (Fig. 5C,D; see Movie 4 in the supplementary material). In agreement with previous findings, we found that the phagosomal accumulation of PtdIns(3)*P* was significantly reduced in animals treated with *rab-5* RNAi or when *vps-34* activity was lost, suggesting that RAB-5 modulates PtdIns(3)*P* production through the phosphatidylinositol 3-kinase VPS-34 (Fig. 6A) (Kinchen et al., 2008). Importantly, reducing *rab-5* gene expression by RNAi or loss of *vps-34* function not only reduced the phagosomal accumulation of PtdIns(3)*P* in *tbc-2* mutants, but also suppressed the persistent cell corpse phenotype, indicating that excessive accumulation of PtdIns(3)*P* prevented cell corpse clearance (Fig. 1D; Fig. 6). Collectively, our data suggest that the cycling of RAB-5 from the active GTP-bound to the inactive GDP-bound conformation by TBC-2 is crucial for both releasing RAB-5 from phagosomes and maintaining a proper level of PtdIns(3)*P* on phagosomes, which is required for the efficient removal of cell corpses.

tbc-2 mutants affect phagosome acidification

The acidification of phagosomes occurs gradually during the maturation process and is required for the proper activity of acid hydrolases. However, the stage at which acidification is initiated and whether it requires RAB-5 activation and/or inactivation are not known. We determined whether *tbc-2* mutation also affects phagosome acidification using Acridine Orange staining (AO) that

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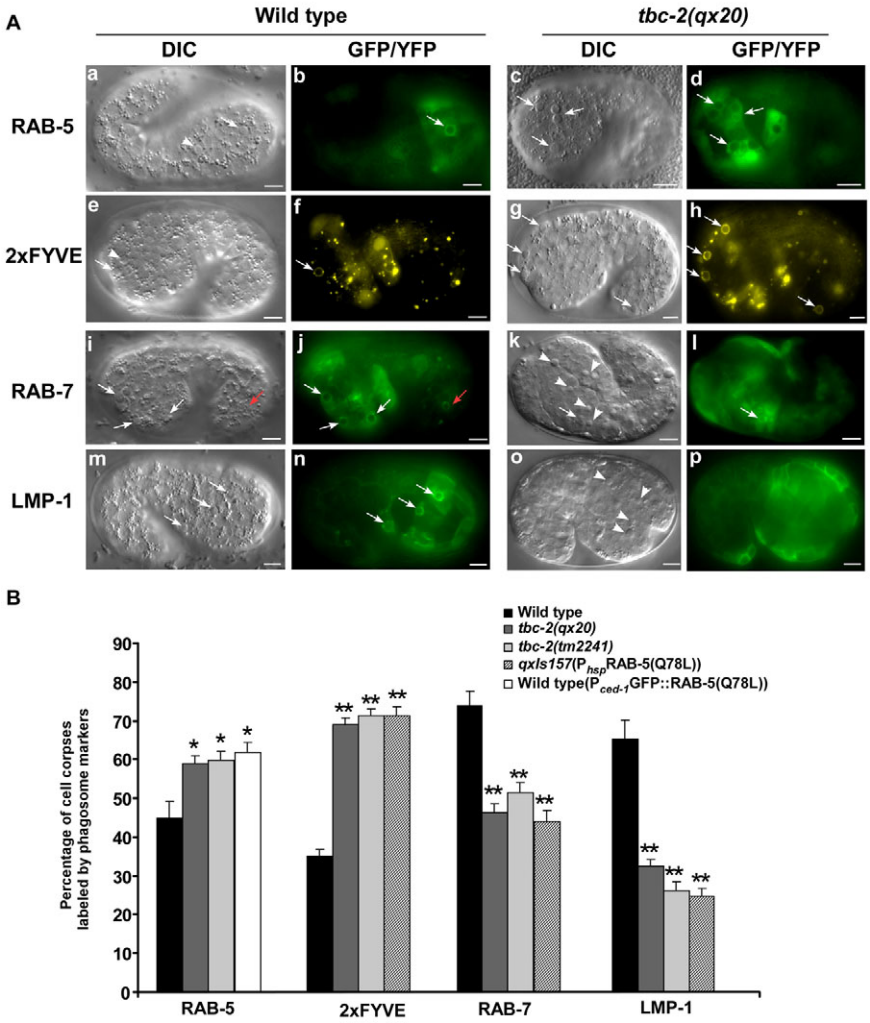

Fig. 4. *tbc-2* mutants exhibit defects in phagosome maturation. (**A**) DIC and fluorescence images of wild-type (a,b,e,f,i,j,m,n) or *tbc-2(qx20)* embryos (c,d,g,h,k,l,o,p) expressing GFP::RAB-5 (a-d), YFP::2×FYVE (e-h), GFP::RAB-7 (i-l) or LMP-1::GFP (m-p). Cell corpses clustered by GFP or YFP are indicated by arrows and those that failed to be labeled are indicated by arrowheads. Red arrow indicates a late stage cell corpse that is being degraded and clustered by GFP::RAB-7. Scale bars: 5 μm. (**B**) Quantification of data shown in A. Data are shown as mean±s.e.m. At least 15 animals were scored for each condition. An unpaired *t*-test was carried out by comparing all other data sets with that of wild type. **P*<0.05, ***P*<0.0001.

selectively stains internalized cell corpses in acidic organelles (Gumienny et al., 1999; Lettre et al., 2004). In wild-type embryos or *unc-108(sm237)* mutant embryos defective in cell corpse degradation, almost all of the cell corpses were stained by AO, indicating that the internalized apoptotic cells were within the acidified phagosomes (Fig. 7; data not shown). Conversely, only 33% and 22% of apoptotic cells were labeled by AO in *tbc-2(qx20)* and *tbc-2(tm2241)* mutants, respectively, suggesting that either the apoptotic cells were not internalized or the acidification of phagosome was disrupted (Fig. 7). Given that more cell corpses were clustered by GFP::RAB-5 and YFP::2×FYVE from engulfing cells in *tbc-2* mutants, the low percentage of AO staining is likely to be due to a defect in phagosome acidification rather than a failure of cell corpse internalization. In fact, in *ced-1(e1735)*; *tbc-2(qx20)* double mutants, in which the internalization of cell corpses is abrogated, the phagosomal recruitment of RAB-5, accumulation of PtdIns(3)*P* and AO staining of cell corpses were totally blocked (Fig. 7B; data not shown).

The phagosomal recruitment of RAB-7 and lysosome incorporation is defective in *tbc-2* mutants

RAB-7 is recruited to phagosomes at a later stage than that of RAB-5 or TBC-2, and is required for lysosome fusion (Fig. 3B) (Henry et al., 2004; Kinchen et al., 2008; Vieira et al., 2003). Since several early events of phagosome maturation were impaired in *tbc-2* mutants, including RAB-5 release, PtdIns(3)*P* dynamics and phagosome acidification, we examined whether *tbc-2* mutation also affected RAB-7 recruitment and lysosome incorporation. In wild-type embryos expressing P_{ced-1}GFP::RAB-7, 74% of cell corpses were surrounded by GFP::RAB-7, indicating that RAB-7 is recruited to the majority of phagosomes (Fig. 4). In *tbc-2(qx20)* embryos, however, the percentage of cell corpses labeled by GFP::RAB-7 was reduced to 46%, indicating that the phagosomal recruitment of RAB-7 is affected (Fig. 4). Consistently, LMP-1, a lysosome-associated membrane protein that is recruited to phagosomes when they fuse with lysosomes, also failed to be efficiently delivered to phagosomes in *tbc-2* mutants, indicating that lysosome incorporation is defective in these animals (Fig. 4). Altogether, our data indicate that *tbc-2* is required for cell corpse degradation through phagosome maturation.

Overexpression of GTP-locked RAB-5 causes defects in phagosome maturation, as seen in *tbc-2* mutants

To further investigate whether the phagosome maturation defects observed in *tbc-2* mutants are caused by the persistent activation of RAB-5, we examined the phagosome maturation process in *qxIs157* animals [P_{hsp}RAB-5(Q78L)], in which a GTP-locked RAB-5 is overexpressed and many persistent cell corpses accumulate (Fig. 1E).

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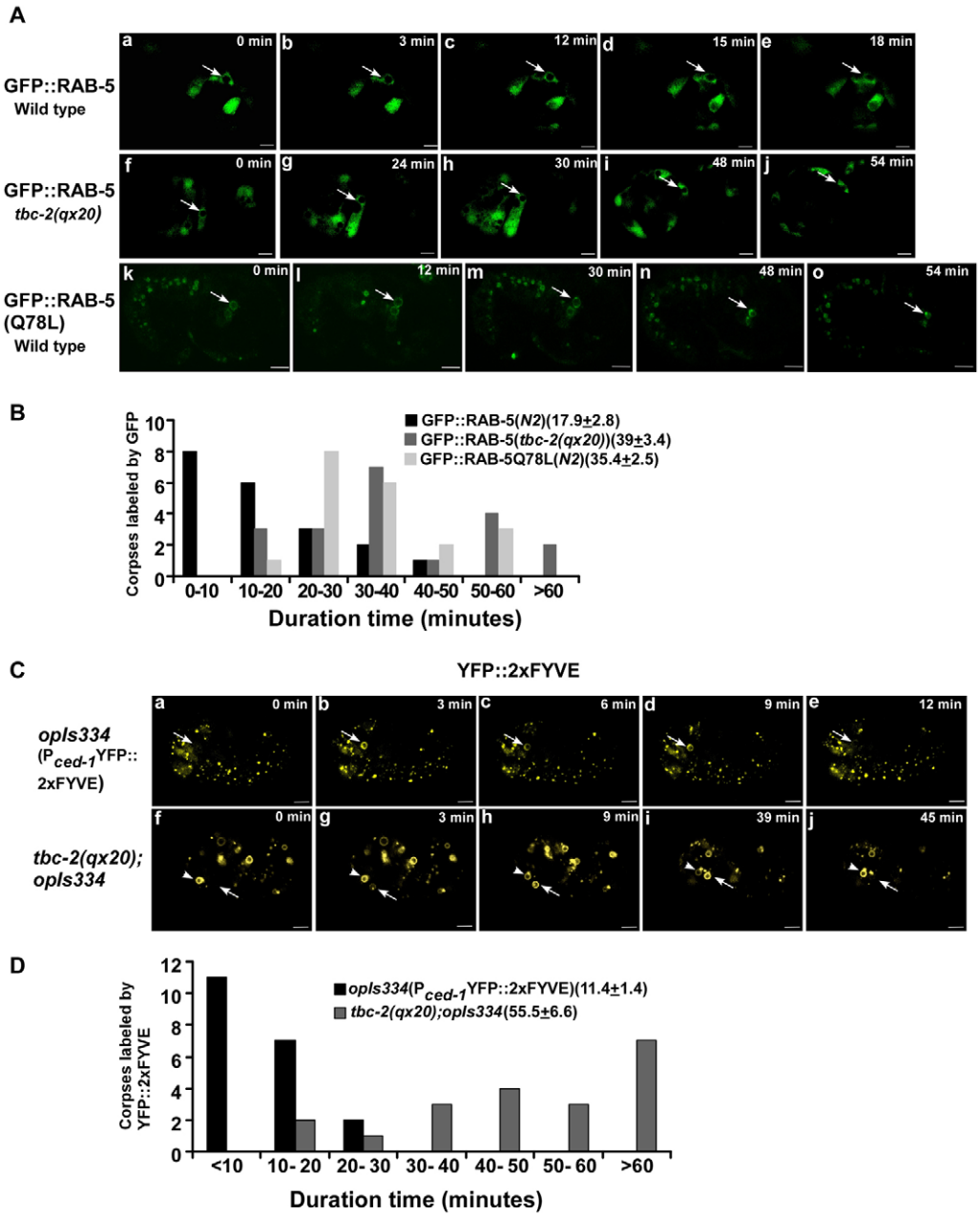

Fig. 5. *tbc-2 (qx20)* affects RAB-5 release and PtdIns(3)*P* dynamics on phagosomes. (**A**) Time-lapse images of wild-type embryos (N2, a-e and k-o) and a *tbc-2(qx20)* mutant embryo (f-j) expressing GFP::RAB-5 (a-j) or transgenic for P_{ced-1}GFP::RAB-5(Q78L) (k-o). Phagosomes followed are indicated by arrows. (**B**) Quantification of data shown in A. Twenty phagosomes in each strain were monitored and the numbers in parentheses indicate the average duration time (±s.e.m). (**C**) Time-lapse images of a wild-type embryo carrying P_{ced-1}YFP::2×FYVE (*opls334*, a-e) and a *tbc-2(qx20)* embryo expressing YFP::2×FYVE (*tbc-2(qx20); opls334*, f-j) are shown. Phagosomes followed are indicated by arrows and a YFP::2×FYVE-positive phagosome that underwent a bright-dark-bright cycle in the *tbc-2(qx20)* mutant is indicated by an arrowhead. (**D**) Quantification of data shown in C. Twenty phagosomes in each strain were monitored and the numbers shown in parentheses indicate the average duration time (±s.e.m.). Scale bars: 5 μm.

Firstly, we examined the phagosomal release of the GTP-locked RAB-5 using GFP::RAB-5(Q78L) driven by the *ced-1* promoter. In agreement with the impaired phagosomal release of RAB-5 in *tbc-2* mutants, we found that more cell corpses in wild-type embryos were labeled by this GTP-locked RAB-5 (Fig. 4B). A time-lapse analysis further revealed that GFP::RAB-5(Q78L) existed on phagosomes with an average duration of 35 minutes, which is 94% longer than that of wild-type RAB-5, indicating that GTP-locked RAB-5 was not properly released from phagosomes (Fig. 5A,B; see Movie 5 in the supplementary material). Consistently, GFP::RAB-5(S33N), a GDP-locked RAB-5, failed to associate with phagosomes (see Fig. S1C in the supplementary material). Secondly, we examined the phagosomal accumulation of PtdIns(3)*P* and found that significantly more cell corpses were labeled by YFP::2×FYVE in *qxIs157* animals, as in *tbc-2* mutants (Fig. 4B). Moreover, the acidification of phagosomes was also disrupted in *qxIs157* animals, in which only 16% of cell corpses were stained by AO (Fig. 7B). Finally, we found that the phagosomal recruitment of RAB-7 and LMP-1 was similarly reduced in *qxIs157* animals, as only 44% and 25% of cell corpses were positive for GFP::RAB-7 and LMP-1::GFP, respectively, compared with 74% and 65% in wild-type animals, indicating that lysosomes are not properly incorporated into phagosomes (Fig. 4B). Taken together, very similar phagosome maturation defects were observed in *qxIs157* animals to those in *tbc-2* mutants, indicating that persistent activation of RAB-5 disrupts phagosome maturation. Therefore, inactivation of RAB-5 by TBC-2 maintains RAB-5 dynamics, which are required for the progression of phagosome maturation.

DISCUSSION

TBC-2 promotes cell corpse degradation by inactivating RAB-5

In this study, we identified *tbc-2*, which encodes a TBC domain-containing Rab GTPase activating protein, as an essential player for the removal of apoptotic cells in *C. elegans*. Several lines of

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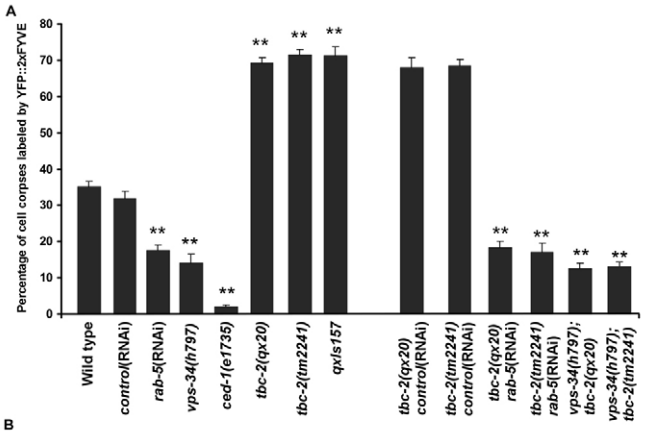

B

Maternal genotype	Zygotic genotype	No. of cell corpses
dpy-5(e61) vps-34(h797); qxEx[vps-34(+)]	*dpy-5(e61) vps-34(h797); qxEx[vps-34(+)]*	11.9 ± 0.5
	dpy-5(e61) vps-34(h797)	19.8 ± 0.9
dpy-5(e61) vps-34(h797); tbc-2(qx20); qxEx[vps-34(+)]	*dpy-5(e61) vps-34(h797); tbc-2(qx20); qxEx[vps-34(+)]*	28.1 ± 1.1
	dpy-5(e61) vps-34(h797); tbc-2(qx20)	15.8 ± 1.2
dpy-5(e61) vps-34(h797); tbc-2(tm2241); qxEx[vps-34(+)]	*dpy-5(e61) vps-34(h797); tbc-2(tm2241); qxEx[vps-34(+)]*	28.5± 0.9
	dpy-5(e61) vps-34(h797); tbc-2(tm2241)	18.1 ± 1.1

Fig. 6. Persistent phagosomal accumulation of PtdIns(3)*P* affects cell corpse clearance. (**A**) *rab-5* RNAi and *vps-34(lf)* significantly reduce the phagosomal accumulation of PtdIns(3)*P* in wild type and *tbc-2* mutants. The percentage of cell corpses labeled by YFP::2×FYVE was quantified in the indicated strains. At least 15 animals were scored for each strain and data are shown as mean±s.e.m. An unpaired *t*-test was carried out for the two sets of experiments by comparing all other data sets with either wild type or *tbc-2(qx20)* treated with control RNAi. $*P<0.05$, $**P<0.0001$, all other points are $P>0.05$. (**B**) *vps-34(lf)* suppresses the persistent cell corpse phenotype in *tbc-2* mutants. At least 15 2-fold stage embryos were scored for cell corpses in each genotype, data are shown as mean±s.e.m. The *vps-34*-deficient strain, *vps-34(h797)*, is maintained as *dpy-5(e61) vps-34(h797)*; *qxEx* [*vps-34*(+); P_{sur-5}*sur-5::gfp*]. Non-fluorescent embryos [*dpy-5(e61) vps-34(h797)*] were scored as *vps-34(lf)* and flurorescent embryos that carry the transgene were scored as wild type for *vps-34*.

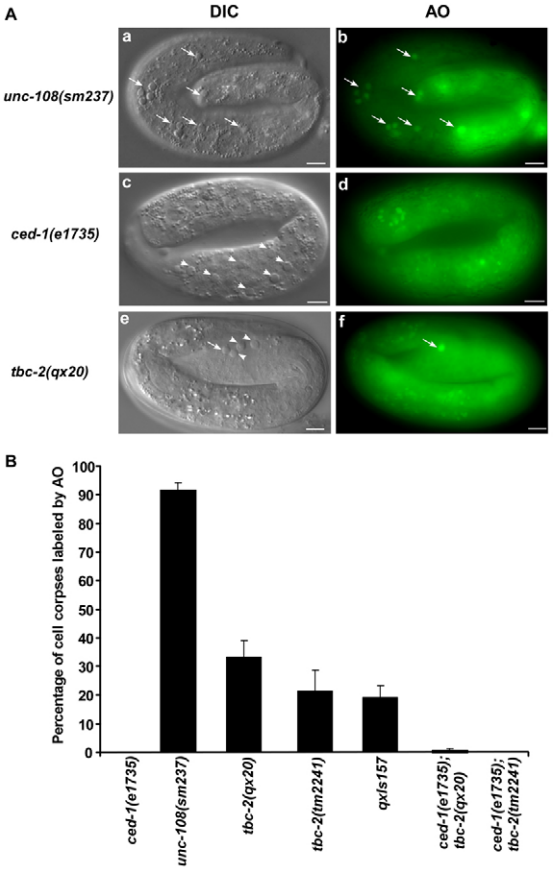

Fig. 7. *tbc-2* mutation affects phagosomal acidification. (**A**) Acridine orange (AO) staining of 4-fold stage *unc-108 (sm237)* (a,b), *ced-1(e1735)* (c,d) and *tbc-2(qx20)* (e,f) embryos. Apoptotic cells labeled by AO are indicated by arrows and those that failed to be stained are indicated by arrowheads. Scale bars: 5 μm. (**B**) Quantification of data shown in A. At least 15 animals were scored for each strain and data are shown as mean±s.e.m.

evidence indicate that TBC-2 functions to regulate phagosome maturation by targeting the small GTPase RAB-5. Firstly, we found that partial inactivation of *rab-5* but not *rab-7* or *unc-108* by RNAi suppressed the persistent cell corpse phenotype of *tbc-2* mutants, which is consistent with the notion that TBC-2 promotes cell corpse clearance by inactivating RAB-5. Secondly, overexpression of a GTP-locked RAB-5, RAB-5(Q78L), resulted in a defect in cell corpse removal, which was not further enhanced by strong loss-of-function mutations of *tbc-2*. By contrast, no defect in cell corpse clearance was observed when GTP-locked RAB-7 or UNC-108 was similarly overexpressed. Thirdly, very similar phagosome maturation defects were observed in *tbc-2* mutants and in animals expressing GTP-locked RAB-5. Moreover, TBC-2 shows very similar kinetics of phagosomal recruitment and release to those of RAB-5. Finally, TBC-2(R689A), in which the Arginine residue crucial for GAP activity was replaced by Alanine, failed to rescue the cell corpse phenotype in *tbc-2* mutants. Therefore, TBC-2 is likely to act as a GAP of RAB-5 to cycle the latter from an active GTP-bound state to an inactive GDP-bound conformation, which is required for the proper progression of phagosome maturation.

TBC-2 is required for maintaining RAB-5 dynamics during phagosome maturation

Although RAB-5 has been shown to be dynamically associated with apoptotic cell-containing phagosomes, such as those in endocytosis or phagocytosis with internalized latex-beads or opsonized cells (Henry et al., 2004; Kinchen et al., 2008; Kitano et al., 2008; Zhou and Yu, 2008), it is not understood how these dynamics are regulated. In our study, we found that GDP-bound RAB-5 [RAB-5(S33N)] failed to localize to phagosomes, whereas GTP-locked RAB-5 [RAB-5(Q78L)] was not properly released from phagosomes, suggesting that the dynamic phagosomal association of RAB-5 is probably regulated through its oscillation between inactive GDP-bound and active GTP-bound states. In agreement with this notion, a recent study in mouse Swiss 3T3 cells suggested that gapex 5, a Rab5 GEF, mediates the recruitment and transient activation of RAB5 on phagosomes through a microtubule-tip-associating protein, EB1 (MAPRE1 – Mouse Genome Informatics) (Kitano et al., 2008). On the other hand, we identified TBC-2 as a Rab GAP required for

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releasing RAB-5 from phagosomes, thereby maintaining its dynamics during phagosome maturation. *C. elegans* TBC-2 is most homologous to TBC1D2B in mammals, the substrate of which has not been defined. Notably, we found that TBC1D2B interacts directly with both worm and mouse RAB-5 in vitro (data not shown). Therefore, it will be interesting to test whether a similar mechanism is used in mammals to regulate RAB5 dynamics and phagosome maturation.

The maintenance of RAB-5 dynamics is crucial for the progression of phagosome maturation

Several events in phagosome maturation are affected when the GTP-to-GDP switch of RAB-5 is blocked, which indicates that inactivation of RAB-5 is required for the progression of phagosome maturation. First, inactivation of RAB-5 is required for maintaining PtdIns(3)*P* dynamics on phagosomes. Although the transient appearance of PtdIns(3)*P* on phagosomes has been previously observed (Ellson et al., 2001; Henry et al., 2004; Vieira et al., 2001), it is not understood how it is regulated and whether it affects phagosome maturation. On the basis of our results and data from other laboratories, it is relatively clear that RAB-5 modulates PtdIns(3)*P* dynamics on apoptotic-cell-containing phagosomes through its oscillation between inactive and active states. On the one hand, activation of RAB-5 promotes the generation of PtdIns(3)*P* on phagosomes through the phosphatidylinositol 3-kinase VPS-34 (Kinchen et al., 2008). On the other hand, inactivation of RAB-5 by TBC-2 downregulates the production of PtdIns(3)*P* and might also promote its turnover. Remarkably, persistent accumulation of PtdIns(3)*P* on phagosomes caused a defect in cell corpse clearance, which can be suppressed by reducing its production, indicating that maintaining PtdIns(3)*P* dynamics is required for the proper removal of apoptotic cells. The effects of PtdIns(3)*P* accumulation and turnover in cell corpse clearance might be achieved by recruiting and releasing certain RAB-5 effectors or effector complexes, thereby allowing continued phagosome maturation. In support of this idea, EEA-1 was found to be transiently recruited to latex bead-containing phagosomes (Fratti et al., 2001).

Second, we found that persistent activation of RAB-5 arrested maturation of apoptotic-cell-containing phagosomes and affected RAB-7 recruitment, as well as lysosome incorporation. These results are distinct from studies of endocytosis or phagocytosis with the internalized *Leishemania* parasite, in which uncontrolled fusion leads to the appearance of giant endosomes or phagosomes (Duclos et al., 2000; Stenmark et al., 1994). Thus, distinct regulatory mechanisms are used during the maturation of phagosomes with different ingested cargoes. However, we currently do not know how RAB-5 release affects RAB-7 recruitment. Further identification of RAB-5 effectors and RAB-7 GEFs involved in apoptotic cell degradation will help to establish the molecular link between these two events. In addition, we found that phagosome acidification was also affected when RAB-5 is locked in a GTP-bound active state. This finding is consistent with the observation in Swiss 3T3 cells that RAB5 inactivation precedes the decrease in pH and breakdown of apoptotic cells (Kitano et al., 2008).

TBC-2 is likely to act as a GAP of RAB-5 to regulate cell corpse clearance

TBC-2 belongs to a large protein family that contains more than 50 members in humans. These TBC domain-containing proteins serve as GAPs for Rab GTPase to determine the lifetime of its GTP-bound state (Bernards, 2003). Twenty TBC proteins are annotated in the *C. elegans* genome, including TBC-17 and TBC-18, which share sequence homology with mammalian RabGAP5 and RN-TRE, the two Rab5 GAPs that regulate RAB5 activity during endocytic traffic (Haas et al., 2005; Lanzetti et al., 2000) (www.wormbase.org). However, no obvious cell corpse phenotype was observed when *tbc-17* or *tbc-18* was inhibited by RNAi (W.Z. and X.W., unpublished). Conversely, we identified TBC-2 as an essential player of cell corpse clearance, and show that it promotes phagosome maturation and cell corpse degradation by inactivating RAB-5. Although interaction between TBC-2 and RAB-5 was observed both in vitro and in 293T cells (data not shown), we did not detect obvious TBC-2 GAP activity towards RAB-5 in vitro by using bacterially expressed recombinant proteins or affinity-purified TBC-2 proteins from 293T cells. However, our in vivo results indicate that TBC-2 promotes phagosome maturation by inactivating RAB-5 and that the GAP activity is required for its function in cell corpse degradation, as mutation of the Arginine residue (R689A) crucial for GAP activity totally abolished its rescue of the cell corpse phenotype in *tbc-2* mutants. The failure in detecting GAP activity in vitro suggests that the catalytic function of TBC-2 might be under tight regulation in vivo. In fact, recent studies indicate that GAPs are regulated by numerous mechanisms, including protein-protein interactions, phospholipid interactions, phosphorylation and proteolytic degradation (Bernards and Settleman, 2004). For example, Bub2p, a TBC domain-containing protein, has an absolute requirement for Bfa1p as a coactivator of GAP activity in budding yeast, which together form a two-component GAP complex to stimulate the GTP hydrolysis of Tem1p (Geymonat et al., 2002). Moreover, GAP phosphorylation can either influence GAP activity or substrate specificity directly, by affecting the structure of the catalytic site, or indirectly, by regulating the subcellular localization or protein degradation (Bernards and Settleman, 2004). Furthermore, phospholipids may stimulate or inhibit GAP activity through binding to the C2 or the PH domain of a GAP to determine when and where it may function (Bernards and Settleman, 2004). Interestingly, TBC-2 contains an N-terminal PH domain, which is not required for locating TBC-2 to phagosomes but is essential for its function in cell corpse degradation. Therefore, it is possible that the PH domain of TBC-2 regulates the GAP activity through its interaction with phospholipids. However, whether any of the proposed regulatory mechanisms are used to control the catalytic activity of TBC-2 in vivo remains to be characterized, and is certainly a challenging task for future investigations.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/14/2445/DC1

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