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The *Mycobacterium tuberculosis* Phagosome

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Summary

Tuberculosis is currently the most devastating human bacterial disease, causing millions of deaths annually and infecting an overwhelming percentage of the global population. Its success as a scourge lies in the ability of *Mycobacterium tuberculosis* to prevent normal phagolysosome biogenesis, essential to the destruction of invading microorganisms, inside macrophages. Recent work has identified host GTPases involved in the block of normal phagolysosome biogenesis during mycobacterial infection and has provided a set of methods, in particular efficient macrophage transfection, which will prove essential in examining the role of host effectors in this process.

Key Words: *M. tuberculosis*; phagosome; Rabs; maturation; Rab conversion.

1. Introduction

Mycobacterium tuberculosis is the world's most lethal bacterium, causing nearly 2 million deaths worldwide on a yearly basis, and it currently infects more than one third of the world's population (http://www.who.int/inf-fs/en/fact104.html). The fundamental capability of this bacterium to cause so much destruction and survive in such a large percentage of the population resides in its ability to modify membrane trafficking and organelle biogenesis inside host macrophages. Essentially, the bacterium is maintained in a phagosome that cannot mature into the acidified microbicidal phagolysosome normally enriched with lysosomal hydrolases, which, in turn, is important for tuberculosis latency, disease activation, and spread ***(1)***. The lack of interaction of *M. tuberculosis* with the host immune system is therefore dominated by the ability of the pathogen to prevent phagosome-lysosome fusion ***(1,2)***.

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Despite this block in normal organellar trafficking, mycobacterial phagosomes are able to access transferrin-bound iron ***(3)*** and even accumulate transferrin receptors ***(4)***, suggesting that the mycobacterial phagosome is not a static organelle. The mechanism(s) by which *M. tuberculosis* is able to finely adjust the intracellular environment to its preference involves both host proteins and mycobacterial effectors. This chapter will focus on the altered maturation of mycobacterial phagosomes and the donation of Rabs to forming this specialized intracellular niche that allows for *M. tuberculosis* survival and host immune evasion.

Rabs are small GTPases, belonging to the Ras superfamily of GTPases, that control the compartmentalization of intracellular organelles and direct membrane trafficking and cargo destination in all eukaryotic cells ***(5,6)***. More detail on the interplay of small GTPases and phagosomes can be found in a separate chapter of this book. Only a select few Rabs have been shown to play an important role in the trafficking program driven by *M. tuberculosis* ***(7,8)***. Early examinations of mycobacterial phagosomal maturation demonstrated that mycobacterial phagolysosomal biogenesis is stalled between the maturation stages controlled by Rab5 (early endocytic) and Rab7 (late endosomal) ***(2)***. Rab5 was detected on mycobacterial phagosomes, while phagosomes were devoid of Rab7 at times anticipated for its acquisition ***(2)***. Several additional studies confirmed the observation of the presence of Rab5 and absence of Rab7 on mycobacterial phagosomes ***(8–10)***. A recent fundamental development in endosomal trafficking and maturation shows that Rab5 and Rab7 virtually "switch" on endocytic organelles as they progress from "early" to "late" stages ***(11)***. This switch is referred to as Rab conversion ***(11)*** and has offered a novel perspective on the original observations of Rab5 and Rab7 presence, or lack thereof, on mycobacterial phagosomes ***(2,12)***. In essence, the inhibition of Rab conversion defines the block in phagolysosome biogenesis generated by mycobacteria.

Recently, Rab22a was identified as a Rab that controls the exchange of Rab5 for Rab7 on mycobacterial phagosomes ***(13)***. Four-dimensional microscopy has shown that phagosomes harboring mycobacteria recruited increasing amounts of Rab22a for extended periods of time as compared to other, similar, Rabs. Immunofluorescence laser scanning confocal microscopy (ILSM) combined with siRNA-mediated Rab knockdown in macrophages demonstrated an enhanced maturation profile of live mycobacterial phagosomes, evidenced by increased colocalization with the late endosomal marker CD63 specifically upon Rab22a knockdown, as well as the early acquisition of Rab7 on mycobacterial phagosomes indicative of positive Rab conversion **(Fig. 1)**. *M. tuberculosis*, therefore, retains Rab22a to inhibit the conversion of Rabs on its phagosome, in turn, preventing phagolysosomal biogenesis.

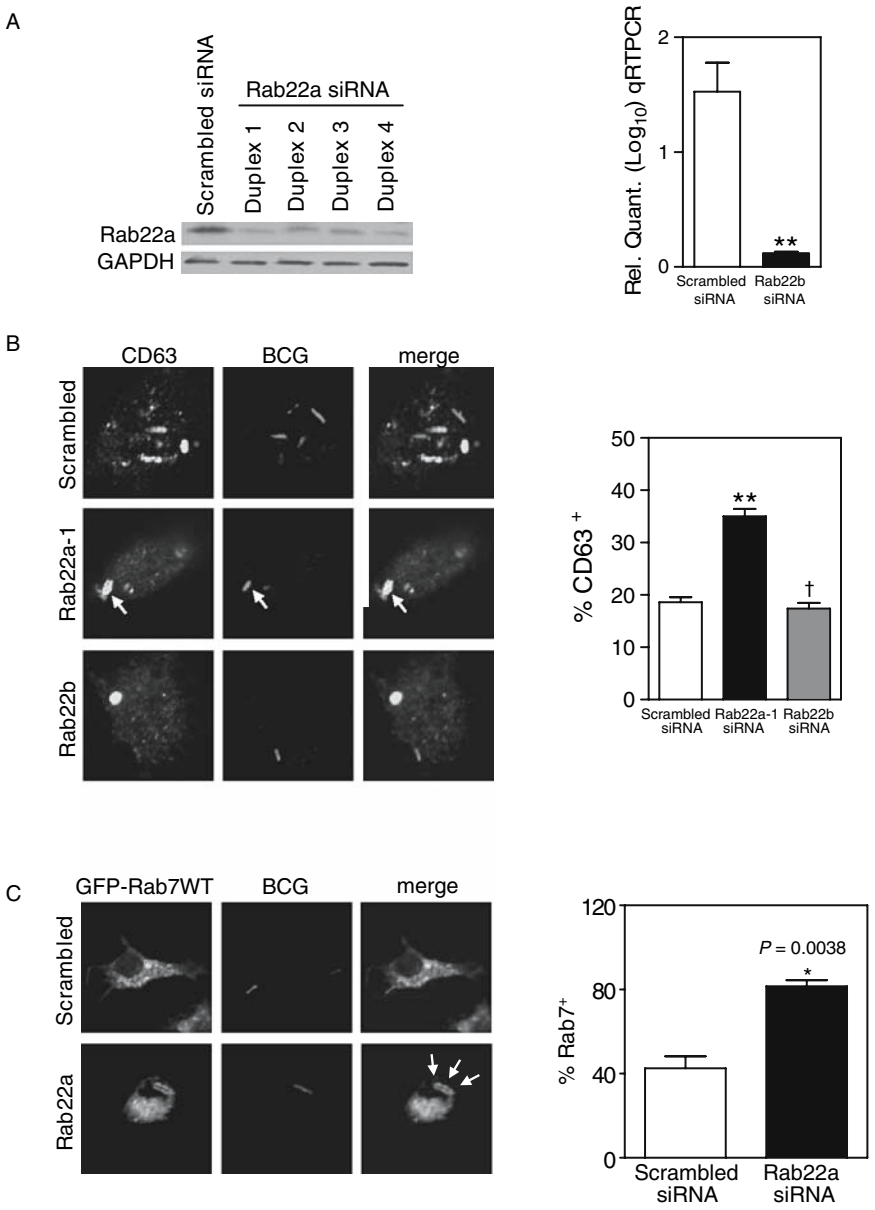

Fig. 1. (A) siRNA-mediated knockdown of Rab22a and Rab22b as shown by immunoblot and qRT-PCR, respectively. **(B)** Rab22a knockdown, specifically, enhances the maturation profile of live mycobacterial phagosomes. This includes the acquisition of the late organellar marker Rab7 **(C)** indicating abnormal Rab conversion on live mycobacterial phagosomes upon Rab22a silencing. ** $p < 0.001$; † $p > 0.05$.

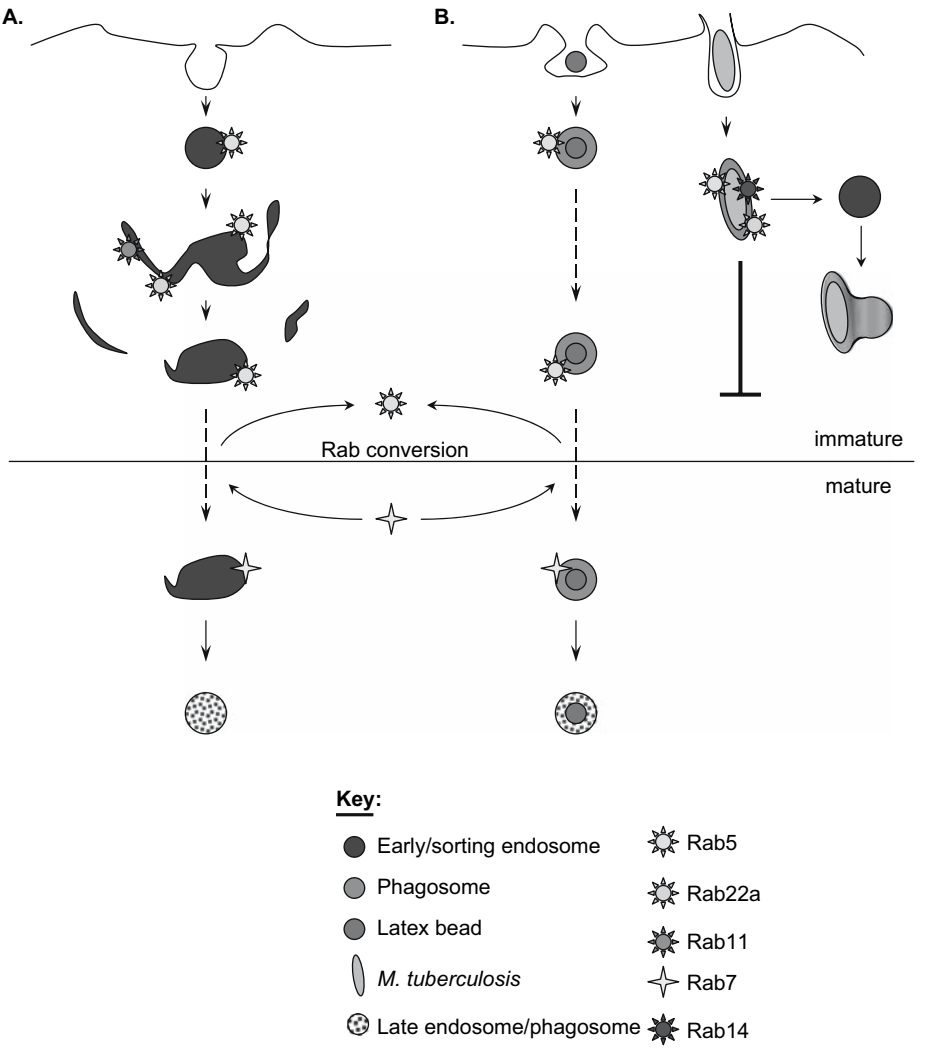

Fig. 2. The endosomal pathway **(A)** begins by the demarcation of early/sorting endosomes with the early endocytic Rabs, Rab5 and Rab22a. It is also a site of Rab11-dependent recycling. Both endosomes and model latex bead phagosomes mature into late organelles upon the synchronous exchange of Rab5 for Rab7 **(A** and **B)**, termed Rab conversion. However, *M. tuberculosis* recruits Rab22a to its phagosome to prevent Rab conversion **(B)**. In addition, Rab14 is recruited to the mycobacterial phagosome to stimulate the fusion of these organelles with early endosomes and also to inhibit phagolysosome biogenesis **(B)**.

A critical role for the small GTPase Rab14 in maintaining mycobacterial phagosome maturation block and in stimulating the interaction of phagosomes with early endosomes was also recently uncovered ***(14)***. As in the case of Rab22a, phagosomes containing live mycobacteria accumulated Rab14 following phagocytosis and siRNA-mediated depletion of Rab14 reversed the normal maturation block, leading to the progression of live mycobacterial phagosomes into phagolysosomes. Importantly, mechanistic studies using in vitro fusion assays (*see* Chapter 17) demonstrated a role for Rab14 in stimulating the fusion of phagosomes with early endosomes but not with late endosomes. This study was able to show that Rab14 enables mycobacterial phagosomes to maintain early endosomal characteristics and avoid the late endosomal/lysosomal degradative pathway. **Figure 2** outlines the general role of Rabs in the endocytic pathway and the differences observed between model phagosomes and those containing *M. tuberculosis*.

The nucleoporation protocol developed by Amaxa currently allows the efficient transfection of macrophages and has been the cornerstone technique in the analysis of Rabs with respect to mycobacterial phagolysosome biogenesis ***(13,14)***. Transient transfection rates of 30–40% for plasmids 10 kb or less, as evidenced by GFP expression, and 90% or greater for siRNAs, as determined by immunoblotting, are commonplace after 24 h, particularly in RAW 264.7 macrophages. Prior to optimized nucleoporation, transient transfection efficiencies of this magnitude in RAW 264.7 macrophages were only achieved after 48 h and with incubation of 1 μmol/L of 5-azacytidine using the DEAE-Dextran transfection method ***(15)***. Future work to define novel Rabs and Rab effectors that mediate the inhibition of mycobacterial phagosomal maturation will, therefore, likely utilize efficient nucleoporation of macrophages in conjunction with the other techniques outlined in this chapter.

2. Materials

1. RAW 264.7 cells (ATCC TIB-71) are grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS, Hyclone, Ogden, UT) and 4 m*M* L-glutamine (BioWhittaker, Walkersville, MD).
2. Amaxa Nucleofector Device and Nucleofector V solution (Amaxa, Cologne Germany).
3. *Mycobacterium tuberculosis* var. *bovis BCG* (BCG) is grown in Middlebrook 7H9 broth supplemented with albumin-dextrose-catalase enrichment (ADC, Gibco/BRL, Grand Island, NY) and 0.05% Tween 80 (Sigma, St. Louis, MO) at 37°C, 5% CO_2 on a rolling apparatus (Stovall, Greensboro, NC).

4. Immunofluorescence blocking solution: 10% skim milk, 6% BSA Fraction V (Sigma), and 2% of appropriate serum matching secondary antibody host mixed in phosphate-buffered saline (PBS).
5. Immunoblotting lysis buffer composition: Nonidet-P40 buffer supplemented with 1 m*M* phenylmethylsulfonylfluoride (PMSF).
6. Bichinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL).
7. RNA extraction: Versagene RNA Cell Kit (Gentra Systems, Minneapolis, MN).
8. cDNA synthesis: Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA).
9. Amplification and detection: qPCR Mastermix Plus for SYBR green I (Eurogentec, San Diego, CA), 96-well plate (Applied Biosystems, Foster City, CA), ABI Prism Optical Adhesive Cover (Applied Biosystems), and ABI 7300 Real Time PCR System (Applied Biosystems).

3. Methods

3.1. Latex Bead Labeling with Texas Red

1. Texas-Red sulforhodamine 101 sulfonyl chloride (Tx-Red, Sigma S3388) is dissolved in enough 100% dimethylsulfoxide (DMSO) to obtain a red color (i.e., a change from black to red). Then this stock is diluted with PBS to a concentration of less than 0.1 mg/mL.
2. Remove 50 µL of Streptavidin 1 µm latex beads (Sigma L7405) to microcentrifuge tube.
3. Pellet beads at 13,000 g/5 min.
4. Wash three times with 1 mL of PBS, removing supernatant carefully with pipetman.
5. Spin down Tx-Red briefly to remove precipitate and add 200 µL to beads, incubate at 4°C overnight (*see* **Note 1**).
6. Next day, spin and wash as in **steps 2** and **3**.
7. Add 200 µL Tx-Red and incubate at least 2 h at room temperature with shaking.
8. Spin and wash as in **step 5**.
9. Resuspend in 50 µL of PBS.
10. If you are performing l-µm Tx-R latex bead infections, add 5 µl/mL of DMEM inoculum.

3.2. Live/Dead M. tuberculosis var. bovis BCG (BCG) Labeling with Tx-Red

1. Remove 10 mL from a rolling BCG culture with an OD_{600} = 0.5–1.0 into a 15-mL conical tube. Pellet mycobacteria at 2500 g/10 min.
2. Remove supernatant and resuspend in 1 mL PBS (for dead mycobacteria) or let stand (live mycobacteria) until finished processing dead mycobacteria.
3. Heat-kill mycobacteria in a 1.5-mL microcentrifuge tube at 85–100°C for 10 min.
4. Spin at 13,000 g for 5 min and remove supernatant.

5. Resuspend live/dead mycobacterial pellet in 200 μL of un-mixed Tx-R (make sure you do not pipet the Tx-R precipitate, you can avoid this by prespinning the Tx-R briefly). Add more Tx-R if the color is too milky. Transfer to a 1.5-mL microcentrifuge tube.
6. Wrap tubes in foil and allow the mycobacteria to bathe in Tx-R by shaking for 30–60 min at room temperature.
7. Pellet mycobacteria at 13,000 g for 5 min, remove the Tx-R, and wash three times with 1 mL PBS with 5-min spins in between washes.
8. Resuspend mycobacterial pellet in 7 mL complete DMEM in a 15-mL conical tube, transfer to a 7-mL Dounce homogenizer, and homogenize using 30 strokes.
9. Pipet homogenized mycobacteria into 15-mL polypropylene conical tube, wrap lid with parafilm, and sonicate 10 min in waterbath sonicator.
10. Spin at 1200 g for 5 min to attain, a monodispersion (clumps will pellet).
11. To prepare the mycobacterial inocula, dilute 1 mL of monodispersion into 5 mL DMEM prewarmed to 37°C in a new 15 mL conical tube. The dilution here is subjective and dependent on the density of the monodispersion. The ideal inocula should look as clear as DMEM.

3.3. Transfection of Macrophages with Plasmids or siRNAs

1. Transfection with either plasmid DNA constructs or siRNA duplexes is performed using an Amaxa Nucleofector Device in conjunction with the protocol outlined by the manufacturer for the specific cell type (http://www.amaxa.com).
2. Split RAW 264.7 macrophages grown to 80% confluency in a T-175 flask 1:4 in DMEM with 10% FBS and grow for 2 d at 37°C.
3. Scrape cells with a cell scraper into 6 mL DMEM and aliquot 1.5 mL per transfection (approximately 4–5 × 10^6 cells) into 15 mL conical tubes.
4. Pellet cells at 1000 g/5 min and completely remove DMEM.
5. Nucleoporate 5–10 μg of plasmid DNA or 1.5 μg of siRNA using program D-032 on the Nucleofector Device with Nucleofector V solution according to the manufacturer's protocol (http://www.amaxa.com).
6. Resuspend transfected cells in 5 mL prewarmed DMEM.
7. Remove 10 μL, mix with 5 μL 0.4% Trypan Blue stain, and count cells in hemocytometer.
8. Plate 2 × 10^5 cells/well in a 12-well plate containing sterile glass cover slips and allow expression or knockdown to proceed for 24 h at 37°C, 5% CO_2.

3.4. Macrophage Infection

1. Remove the media from macrophages and add 1 mL of respective inoculum to each well of RAW 264.7 macrophages in a 12-well plate (Corning, Acton, MA).
2. Spin plate 1 min at 1000 g to settle mycobacteria/latex beads onto macrophages and allow the infection to incubate at 37°C, 5% CO_2 for the specified time (this is considered the pulse period).

3. Remove the inoculum, wash three times using PBS prewarmed to 37°C quickly, and incubate in DMEM at 37°C, 5% CO_2 for the specified period (this is considered the chase period).

3.5. Immunofluorescence Laser Scanning Confocal Microscopy (ILSM)

1. Quickly wash cover slips three times in PBS.
2. Fix 10 min using 2% paraformaldehyde in PBS.
3. Wash three times in PBS.
4. Permeabilize cells using 0.5 mL of either 0.2% saponin for 10 min or 0.5% Triton X-100 for 5 min at room temperature.
5. Block for 30 min using blocking solution.
6. Prepare primary antibody in blocking solution to appropriate dilution for IF according to the manufacturer or begin with a 1:200 dilution if uncertain.
7. Add 300 µL per well or, alternatively, invert cover slips onto 70-µL droplets placed onto parafilm wrapped over a hard, flat surface. Cover samples, and seal with parafilm to prevent evaporation. Incubate at 4°C overnight (*see* **Note 2**).
8. Wash three times in PBS/5 min.
9. Prepare secondary fluorophore-labeled antibody in blocking solution at 1:500–1000.
10. Add 0.5 mL/well and incubate at room temperature for at least 2 h.
11. Wash cover slips three times in PBS/5 min.
12. Mount onto microscope slides using Permafluor (Thermo Shandon, Waltham, MA).
13. Collect 1-µm-thick optical sections using a 63x oil objective on a LSM 5 Pascal, 510, or META system, according to the manufacturer's protocol (Carl Zeiss, Thornwood, NY).
14. Prepare images using Adobe Photoshop V. 7.0 in conjunction with the Zeiss LSM Image Browser version 3.5.0.223.

3.6. Analysis of siRNA-Mediated Knockdown by Immunoblotting

1. After transfection with siRNA, plate 1×10^6 cells per well in a 6-well plate.
2. After 24 h, wash cells 3X with 1X PBS, scrape cells into 12 mL of 1X PBS using a cell scraper, and pellet cells at 1000 g/5 min.
3. Lyse cells using 200–400 µL of immunoblotting lysis buffer.
4. Determine protein concentration using Bicinchoninic acid (BCA) Protein Assay Kit according to the manufacturer's protocol (Pierce).
5. Using standard procedure, prepare protein samples in SDS loading buffer and load 30–50 µg of total protein/well in a 12% pre-cast BioRad minigel (BioRad, Hercules, CA).
6. Run gel and perform Western blot using a MiniProtean 3 system according to the manufacturer's suggestions (BioRad).

7. Develop blot using SuperSignal West Dura Extended Duration Substrate according to the manufacturer's protocol (Pierce) and standard autoradiography using GAPDH as a loading control.

*3.7. Analysis of siRNA-Mediated Knockdown by qRT-PCR (***see** *Note 3)*

3.7.1. RNA Extraction and cDNA Synthesis

1. Perform **steps 1** and **2** described for Western blot after siRNA transfection.
2. Extract total RNA from macrophages using the Versagene RNA Cell Kit (Gentra Systems) according to the manufacturer's protocol.
3. Perform the optional DNAse treatment using the Versagene DNase Kit (Gentra Systems).
4. Measure the total amount of DNA using standard procedures.
5. Synthesize cDNA from 1 μg of total RNA from both scrambled control and target siRNA total RNA using Superscript III First-Strand Synthesis Sysytem for RT-PCR (Invitrogen) in conjunction with random hexamers according to the manufacturer's protocol.

*3.7.2. qRT-PCR Primer Set Validation (***see** *Note 4)*

1. Use 2 μL of cDNA produced from 1 μg of resting RAW264.7 macrophage total RNA as described above and aliquot 2 μL of a twofold serial dilution series into at least four wells of a 96-well plate in triplicate for each primer set to be tested (i.e., you should amplify product from at least 12 wells per primer set).
2. Prepare a sufficient volume of qPCR reaction master mix using qPCR Mastermix Plus for SYBR green I (Eurogentec) according to the manufacturer's protocol, except add primers to 400 n*M* final concentration. Add 25 μl of master mix per well.
3. Spin 96-well plate briefly to collect reaction contents and seal the plate using a ABI Prism Optical Adhesive Cover (Applied Biosystems).
4. Perform qRT-PCR using the default parameters on an ABI 7300 Real Time PCR System (Applied Biosystems) using the Relative Quantification Plate software according to the manufacturer's protocol.
5. Plot the results of the validation experiment and determine the degree of experimental variation between the primer sets according to the guidelines given under the Comparative C_T Method for Relative Quatification section in the Sequence Detection System Chemistry Guide (Applied Biosystems).

3.7.3. qRT-PCR of siRNA Knockdown Samples

1. Aliquot 2 μL per well of cDNA in triplicate for each sample to be amplified.
2. Prepare a sufficient volume of qPCR reaction master mix using qPCR Mastermix Plus for SYBR green I (Eurogentec) according to the manufacturer's protocol, except add primers to 400 n*M* final concentration. Add 25 μL of master mix per well.

3. Spin 96-well plate briefly to collect reaction contents and seal the plate using an ABI Prism Optical Adhesive Cover (Applied Biosystems).
4. Perform qRT-PCR using the default parameters on an ABI 7300 Real Time PCR System (Applied Biosystems) using the Relative Quantification Plate software according to the manufacturer's protocol.
5. Analyze the amplification results using the Relative Quantification Study software according to the manufacturer's protocol, plotting the results in logarithmic fashion.

4. Notes

1. Overnight incubations of Texas Red with latex beads at 4°C are performed for convenience. Alternatively, incubation at room temperature for a minimum of 4 h can be performed.
2. Primary antibody incubations at 4°C overnight are performed for convenience. Alternatively, primary antibody incubations can be performed at room temperature or 37°C for a minimum of 1 h.
3. Many times, antibodies for a specific protein of interest are unavailable. In these instances, it remains imperative to maintain some degree of measurement of siRNA-mediated knockdown of a specific target. While measuring a decrease in the amount of mRNA does not necessarily translate into a knockdown of the protein per se, it becomes the next best measurement of siRNA-targeted inhibition. Therefore, qRT-PCR of siRNA knockdown is a useful tool in determining the net effect of siRNA activity.
4. In order to accurately measure the relative amount of target mRNA in a given sample to an internal control mRNA, the two primer sets to be used for comparison need to be validated. This procedure is necessary to determine the amplification efficiency of the two primer sets, as a large difference in amplification efficiency can yield a "false-positive" effect (i.e., differences in mRNA amounts may actually be due to a gross difference in amplification efficiency between the two primer sets). Use standard primer design software to aid in the development of compatible primers and primer sets, such as ABI Prism Primer Express v. 2.0.

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References

1. Russell, D. G., Mwandumba, H. C., and Rhoades. E. E. (2002) Mycobacterium and the coat of many lipids. *J. Cell Biol.* **158**, 421.

2. Via, L. E., Deretic, D., Ulmer, R. J., Hibler, N. S., Huber, L. A., and Deretic, V. (1997) Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. *J. Biol. Chem*. **272**, 13326.
3. Sturgill-Koszycki, S., Schaible, U. E., and Russell, D. G. (1996) Mycobacterium-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis. *EMBO J*. **15**, 6960.
4. Clemens, D. L., and Horwitz, M. A. (1996) The *Mycobacterium tuberculosis* phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. *J. Exp. Med.* **184**, 1349.
5. Pereira-Leal, J. B., and Seabra, M. C. (2001) Evolution of the Rab family of small GTP-binding proteins. *J. Mol. Biol.* **313**, 889.
6. Pfeffer, S. R. (2005) Structural clues to Rab GTPase functional diversity. *J. Biol. Chem.* **280**, 15485.
7. Fratti, R. A., Backer, J. M., Gruenberg, J., Corvera, S., and Deretic, V. (2001) Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. *J. Cell Biol.* **154**, 631.
8. Kelley, V. A. and Schorey, J. S. (2003) Mycobacterium's arrest of phagosome maturation in macrophages requires Rab5 activity and accessibility to iron. *Mol. Biol. Cell* **14**, 3366.
9. Clemens, D. L., Lee, B. Y., and Horwitz, M. A. (2000) *Mycobacterium tuberculosis* and *Legionella pneumophila* phagosomes exhibit arrested maturation despite acquisition of Rab7. *Infect. Immun.* **68**, 5154.
10. Clemens, D. L., Lee, B. Y., and Horwitz, M. A.. (2000) Deviant expression of Rab5 on phagosomes containing the intracellular pathogens *Mycobacterium tuberculosis* and *Legionella pneumophila* is associated with altered phagosomal fate. *Infect. Immun.* **68**, 2671.
11. Rink, J., Ghigo, E., Kalaidzidis, Y., and Zerial, M. (2005) Rab conversion as a mechanism of progression from early to late endosomes. *Cell* **122**, *735*.
12. Deretic, V. (2005) Ay, there's the Rab: organelle maturation by Rab conversion. *Dev. Cell* **9**, 446.
13. Roberts, E. A., Chua, J., Kyei, G. B., and Deretic, V. (2006) Higher order Rab programming in phagolysosome biogenesis. *J. Cell Biol.* **174**, 923.
14. Kyei, G. B., Vergne, I., Chua, J., et al. (2006) Rab14 is critical for maintenance of *Mycobacterium tuberculosis* phagosome maturation arrest. *EMBO J.* **25**, 5250.
15. Escher, G., Hoang, A., Georges, S., et al. (2005) Demethylation using the epigenetic modifier, 5-azacytidine, increases the efficiency of transient transfection of macrophages. *J. Lipid Res.* **46**, 356.