Detection of Autophagy in *Caenorhabditis elegans* by Western Blotting Analysis of LGG-1

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A common way to measure the induction of autophagy in yeast and mammalian cells is to compare the amount of Atg8/LC3-I with that of Atg8-PE/LC3-II by using western blot analysis. This is because changes in the amount of LC3-II correlate closely with changes in the number of autophagosomes present in cells. Atg8/LC3 is initially synthesized as an unprocessed form, which is proteolytically processed to form Atg8/LC3-I, and then this is modified into the phosphatidylethanolamine (PE)-conjugated Atg8-PE/LC3-II form. Atg8/LC3-II is membrane bound, whereas Atg8-PE/LC3-I is cytosolic. By associating with both the inner and outer membranes of the autophagosome, Atg8-PE/LC3-II is the only autophagy reporter that is reliably associated with completed autophagosomes. In the nematode *Caenorhabditis elegans*, the ortholog of Atg8/LC3 is LGG-1. Here, we discuss how changes in the levels of LGG-1-II (and the paralog LGG-2) protein can, with appropriate controls, be used to monitor autophagy activity in nematodes and present a protocol for monitoring changes in the protein levels of different forms of LGG-1 by western blotting.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution’s Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPE: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

- Bleach solution <R>
- Enhanced chemiluminescence (ECL) detection kit
- Feeding plates containing *Caenorhabditis elegans*
- Isopropanol
- M9 minimal medium buffer <R>
- Ponceau S solution (diluted to 1× with distilled water before use) <R>
- Primary antibodies against green fluorescent protein (GFP) and/or LGG-1
- Protease inhibitor cocktail
- SDS gel-loading buffer (2×; diluted to 1× with distilled water before use) <R>
- SDS-polyacrylamide gel <R>

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Secondary antibody
Stacking gel (5%) <R>
TBST blocking buffer (TBST containing 5% w/v nonfat dry milk)
Tris-buffered saline (TBS; 10×, pH 7.5) <R>
Tris-buffered saline containing 0.1% Tween-20 (TBST)
Tris-glycine buffer (diluted to 1× with distilled water before use) <R>
Western transfer buffer (1×) <R>

**Equipment**

Eppendorf 5417C table top centrifuge (or equivalent)
Erlenmeyer flask
Fiber pads
Filter paper
Gel electrophoresis cassette and power supply
Gel transfer cell and sandwich cassette
Microcentrifuge tubes
Nitrocellulose (or PVDF) membrane
Plastic bag (or Tupperware dish)
Rocker
Sponge pad
Whatman filter paper

**METHOD**

For a summary of the whole procedure, see Figure 1.

Collect L4 animals in M9 buffer and prepare samples for SDS gel electrophoresis
Carry out SDS polyacrylamide gel electrophoresis using prepared samples
Transfer proteins from the polyacrylamide gel to a nitrocellulose membrane using a protein transfer apparatus
Incubate the nitrocellulose membrane in blocking solution (containing 5% non-fat dry milk)
Wash the nitrocellulose membrane 3× in TBST at 10-min intervals and incubate the membrane with primary antibodies and then secondary antibodies
Use an ECL detection kit to visualize protein bands

**FIGURE 1.** Flowchart summarizing a protocol for quantitating the levels of autophagy marker proteins by western blotting in the nematode *Caenorhabditis elegans*. ECL, enhanced chemiluminescence; L4, larval stage 4; TBST, Tris-buffered saline containing 0.1% Tween-20.
Sample Preparation

1. Collect worms of the appropriate stage as follows:

   **For larvae preparation**
   
   i. Wash all L4 larvae or young adult hermaphrodites from feeding plates using 1 mL of M9 minimal medium buffer, and transfer animals to a 1.5-mL microcentrifuge tube.
   
   ii. Rinse worms several times by adding 100–300 μL of M9 buffer and centrifuging for 1 min at 2000 rpm. Between washes, discard the supernatants, leaving a pellet of animals.

       *Proceed to Step 2.*

   **For embryo preparation**
   
   i. Collect all gravid adults by washing plates with 1 mL of sterile water. Transfer worms to a 15 mL conical tube containing 500 μL of freshly prepared bleach solution.
   
   ii. Shake well or vortex tube for a few seconds, and repeat a few times, for no longer than 10 min. *The adults dissolve, whereas the embryos remain intact.*
   
   iii. Remove the bleach solution by centrifuging the 15 mL conical tube at 2000 rpm and carefully pipette out the supernatant.
   
   iv. Rinse worms several times by adding 1 mL of M9 buffer and centrifuging for 1 min at 2000 rpm. Between washes, discard the supernatants, leaving a pellet of embryos.

       *Proceed to Step 2.*

2. Snap-freeze the animals in liquid nitrogen and add an equal volume of 1× SDS gel-loading buffer containing a protease inhibitor cocktail to the sample and boil for 3–10 min at 100°C. *It is important to also prepare marker proteins of known molecular mass for control purposes.*

3. After boiling, centrifuge the samples at 2000 rpm for 1 min at room temperature, cool on ice for 5 min, and then load the samples onto an SDS-PAGE gel (see Step 7).

SDS-PAGE

This procedure was modified from Sambrook and Russell (2001).

4. Assemble the polyacrylamide gel apparatus by inserting two glass plates into the gel caster, as described by the manufacturer.

5. Prepare a 12%–20% polyacrylamide resolving gel.
   
   i. Immediately after preparing the gel mixture in an Erlenmeyer flask, swirl it and rapidly pour it into the gap between the glass plates.

       *Be sure to leave enough space for the 5% stacking gel.*
   
   ii. Overlay the polyacrylamide gel with isopropanol.
   
   iii. Allow the gel to polymerize for ~30 min.
   
   iv. Once polymerization is complete, pour off the isopropanol and rinse the top of the resolving gel a few times with deionized water. Remove all residual isopropanol and water.

6. Prepare a 5% stacking gel.
   
   i. Immediately after preparing the gel mixture, rapidly pour it onto the polymerized resolving gel.
   
   ii. Carefully insert a clean gel comb into the stacking gel solution by avoiding air bubbles.
   
   iii. Allow the stacking gel to polymerize at room temperature.
   
   iv. After polymerization is complete, mount the gel in the electrophoresis apparatus and add the Tris-glycine buffer to the top and bottom reservoirs of the apparatus. Remove the gel comb and wash all wells with Tris-glycine buffer to remove any unpolymerized acrylamide.
7. Load \( \sim 15 \) µL of sample into each well.
8. Attach the electrophoresis apparatus to the power supply, as described by the manufacturer’s instructions. Run the gel at \( \sim 180 \) to 200 V or until the bromophenol blue dye in the sample buffer reaches the bottom of the gel.
9. Once electrophoresis is complete, gently remove the glass plates from the gel apparatus and carefully release the polyacrylamide gel.

**Membrane Transfer**

*This procedure was modified from Bio-Rad Laboratories Mini-Trans-Blot instruction manual.*

10. Cool the 1× western transfer buffer to 4°C. Cut the nitrocellulose membrane (or PVDF membrane) and filter paper to the dimensions of the polyacrylamide gel. Soak the membrane, filter paper, and fiber pad in western transfer buffer until ready to use.
11. Prepare the western blot sandwich in the following order: start with the clear side of the case, followed by the sponge pad, Whatman filter paper, membrane, gel, Whatman filter paper, sponge pad, and the black side of the cassette. Close the cassette firmly and ensure that all bubbles are removed from the sandwich.
12. Place the transfer cassette in the transfer cell with the black side facing black, and fill the cell with cooled western transfer buffer. Transfer for 1 h at 4°C and \( \sim 90 \) V.

*Make sure that the cassette is positioned in the correct direction so that the proteins in the gel transfer to the membrane. The voltage required may vary according to the manufacturer’s instructions. The membrane can be stored at 4°C for a few weeks after proteins have been transferred.*

**Immunoblotting**

*This procedure was modified from You et al. (2006).*

13. *(Optional)* Check the membrane for successful transfer of proteins before blocking (Step 14) using Ponceau S stain.
   1. Incubate the blot for 1–5 min in 1× Ponceau S solution (1×) on a rocker.
   2. Rinse the blot with distilled water to rid the blot of stain, until protein bands are clearly visible.
   3. Wrap blot with plastic wrap and take a picture or a photocopy before proceeding to Step 14. *Keep the membrane from drying out.*
14. Incubate the membrane in TBST blocking buffer for \( \sim 1 \) h at room temperature on a rocker.
15. Dilute the primary antibody (i.e., anti-LGG-1 [Tian et al. 2010] or anti-GFP [Kang et al. 2007; Alberti et al. 2010]), in 2 mL of TBST blocking buffer at the appropriate concentration and incubate the membrane overnight at 4°C on a rocker.

*For primary antibodies, use the dilution factor suggested by the manufacturer; anti-GFP from Roche has been commonly used at a 1:500 dilution (Alberti et al. 2010; Djeddi et al. 2012). As antibodies vary, and the protocols can vary, determine the concentration of the primary antibody empirically before the start of the experiment.*
16. Wash the membrane three times with \( \sim 50–100 \) mL of TBST for 5 min each on a rocker.
17. Incubate the membrane with the secondary antibody at the correct concentration (as directed by the antibody manufacturer, or as previously described (You et al. 2006; Alberti et al. 2010), for 1 h at room temperature on a rocker.
18. Rinse the membrane three times with \( \sim 50–100 \) mL of TBST for 10 min each on a rocker.
19. Rinse the membrane once with distilled water.
20. Use an ECL detection kit, as instructed by the manufacturer, to visualize the protein bands.

*For valuable troubleshooting advice, see the Abcam website (http://www.abcam.com/protocols/western-blot-troubleshooting-tips).*
Establishing LGG-1 as a Marker of Autophagy

A common way to measure the induction of autophagy is to compare the amount of Atg8/LC3-I with that of Atg8-PE/LC3-II using western blot analysis (Kabeya et al. 2000; Mizushima and Yoshimori 2007). This is because changes in the amount of LC3-II are closely associated with changes in the number of autophagosomes present in a cell (Kabeya et al. 2000). Atg8/LC3 is initially synthesized as an unprocessed form, which is proteolytically processed by Atg4 to form Atg8/LC3-I, and then modified into the phosphatidylethanolamine (PE)-conjugated Atg8-PE/LC3-II form (Kabeya et al. 2000; Kirisako et al. 2000). Atg8/LC3-II is the membrane-bound form of Atg8/LC3, whereas Atg8-PE/LC3-I is cytosolic (Kabeya et al. 2000). Atg8-PE/LC3-II associates with both the inner and outer membrane of the autophagosome, thus Atg8-PE/LC3-II is the only autophagy reporter that is reliably associated with completed autophagosomes (Klionsky 2012).

As with mammalian LC3 and yeast Atg8, the carboxyl terminus of LGG-1 appears to be conjugated to PE, with LGG-1-I and LGG-1-II being visible on a western blot as one major band and minor band, respectively (Kang et al. 2007; Alberti et al. 2010; Tian et al. 2010). In contrast, LGG-2 contains two minor bands as opposed to one; yet it is not clearly defined which minor band represents the lipidated form of LGG-2 (Alberti et al. 2010). Under normal nonstress conditions, the protein levels of LGG-1-I are higher than that of LGG-1-II. Under conditions that induce autophagy, LGG-1-I levels still appear higher than that of LGG-1-II; however, an overall increase in the amount of LGG-1-II is apparent (Kang et al. 2007; Alberti et al. 2010). A similar increase is also found for LGG-2 protein levels (Alberti et al. 2010). Therefore, changes in LGG-1-I and LGG-2 protein levels can be used to monitor autophagy activity in C. elegans.

Although Atg8-PE/LC3-II is reliably associated with the autophagosome, its protein levels might not change in a predictable manner upon autophagy induction (Mizushima and Yoshimori 2007; Klionsky 2012). For example, upon induction of autophagy in mammalian cells, the total levels of LC3 might not change; instead an increase in the conversion of LC3-I to LC3-II, or a decrease in the level of LC3-II relative to that of LC3-I, might result. The decrease in LC3-II can be due to the rapid lysosomal degradation of LC3-II (Huang et al. 2000; Klionsky 2012). Therefore, although changes in the protein levels of LGG-1 can be used to monitor autophagy, caution should be used when evaluating such changes.

Issues Arising from LGG-1 Western Data Analysis

Western blot analysis provides a convenient way to measure any changes in the levels of the different forms of LGG-1; however, caution is advised when interpreting the quantity of LGG-1-II using western blot analysis. A direct way to measure changes in overall LGG-1-II levels is through quantification—by comparing the protein levels of LGG-1-II with the protein levels of a housekeeping gene product (i.e., tubulin) or with those of LGG-1-I (Kang et al. 2007; Michelet et al. 2009; Alberti et al. 2010; Barth et al. 2010). In addition, it is important to use appropriate standardization controls to ensure equal loading between samples as this can change the amount of LGG-1-I and LGG-1-II protein between samples (Klionsky 2012). Furthermore, the stress condition of the animals before experimental manipulation should be at a minimum to ensure unaltered levels of LGG-1-I and LGG-1-II protein at the start of an experiment.

Increased levels of LGG-1-II relative to LGG-1-I can reflect autophagosome accumulation, owing to increased autophagy, or an accumulation of autophagosomes, as a result of defective lysosomal degradation (Michelet et al. 2009; Alberti et al. 2010; Barth et al. 2010; Lu et al. 2011; Klionsky 2012). Alternatively, based on mammalian studies, lower levels of LGG-1-II compared with LGG-1-I can represent defective autophagy, as a result of poor LGG-1-I to LGG-1-II conversion, or increased autophagic flux, resulting in the rapid degradation of LGG-1-II (Mizushima and Yoshimori 2007). The use of lysosomal inhibitors is one way to distinguish between all these possibilities (Oka and Futai 2000; Ji et al. 2006; Mizushima and Yoshimori 2007; Pivtoraiko et al. 2010).
Alternatively, protein extracts isolated from mutant animals previously shown to alter the lipidation of LGG-1 can also be useful as positive and/or negative controls in blots that measure changes in LGG-1 protein levels. Guanine nucleotide-binding protein subunit beta-2 (GPB-2) is a G-protein β subunit involved in the muscarinic signaling pathway, and gpb-2 mutants, following starvation, have elevated levels of autophagy in pharyngeal muscles, visualized by the expression of GFP::LGG-1, and also have a higher ratio of lipidated LGG-1 to nonlipidated LGG-1, when compared with wild-type controls (Kang et al. 2007). lgg-2 mutants, which have defects in the acidification and degradation of autophagosomes, produce elevated levels of both lipidated and nonlipidated forms of LGG-1 (Manil-Segalen et al. 2014). In addition, protein extracts isolated from animals fed double-stranded RNA (dsRNA) against the C. elegans ortholog of the target of rapamycin (TOR), let-363 (Long et al. 2002), and rab-7, the small GTPase involved in endosome–lysosomal fusion events (Bucci et al. 2000), can also be used as controls to monitor changes in the levels of the different forms of LGG-1 protein (Alberti et al. 2010). RNA interference (RNAi) against let-363 induces autophagy, observed by elevated levels of GFP::LGG-1 in the hypodermis and intestine, and has been reported to cause a decrease in the levels of nonlipidated LGG-1, but an increase in the levels of lipidated LGG-1, when compared with empty-vector controls. In contrast, RNAi against rab-7, which leads to increased levels of GFP::LGG-1 as a result of defective lysosomal fusion, results in elevated levels of both lipidated and nonlipidated LGG-1, compared with controls (Alberti et al. 2010).

It is important to note that the loss of certain autophagy genes can inhibit autophagy without affecting LC3-II/Atg8-PE formation (Klionsky 2012), which appears to be true also for LGG-1-II in C. elegans (Tian et al. 2010; Lu et al. 2011; Liang et al. 2012). Therefore, additional methods might be required to determine whether autophagy is functional when observing changes in the protein levels of LGG-1.

Additionally, LGG-1-II levels can be influenced by the type of antibodies against LGG-1 used, as well as the type of membrane used during protein transfer (Barth et al. 2010; Klionsky 2012). In C. elegans, experiments that visualize the lipidated and nonlipidated forms of LGG-1 by western blotting can use primary antibodies against LGG-1, such as antibodies specific to GFP, for strains expressing GFP::LGG-1 (Kang et al. 2007; Alberti et al. 2010; Tian et al. 2010).

In mammalian cells, during the initial periods of starvation, the amount of LC3-I can be inversely proportional to that of LC3-II; however, as the starvation period is prolonged, the levels of both LC3-I and LC3-II have been shown to decrease (Mizushima and Yoshimori 2007). Although this has not been fully examined for LGG-1 in C. elegans, one should consider the appropriate length of time that animals are exposed to starvation. Finally, LGG-1 has been shown to localize to phagosomes in cells that engulf and degrade apoptotic cells in C. elegans (Li et al. 2012). Thus, conditions that induce apoptosis should be at a minimum when evaluating the levels of LGG-1-II during autophagy.

In summary, a way to measure autophagy induction is to analyze the levels of LGG-1-II and compare them with the levels of LGG-1-I; however, several considerations should be made to ensure the proper interpretation of results.

Concluding Remarks

This protocol has discussed how western blot analysis can be used to monitor autophagy by evaluating the levels of lipidated and nonlipidated Atg8/LC3/LGG-1. However, an increase in the lipidated form of Atg8/LC3/LGG-1-II can reflect the induction of autophagy and/or inhibition of autophagy, and is therefore not a direct measure of autophagic flux without the use of additional methods (Klionsky 2012). In mammalian cells, the accumulation of LC3-II can result from an increase in autophagy activity or defective lysosomal degradation (Mizushima and Yoshimori 2007). The addition of lysosomal protease inhibitors, the introduction of a mutation, or RNAi treatment that results in defective lysosomal degradation, can differentiate between an increase in autophagy or the reduction of autophagic function. As described by Mizushima and Yoshimori, an additional increase in LGG-1-II levels, observed under conditions that block the fusion between autophagosomes and lysosomes, would be...
indicative of autophagy induction (Mizushima and Yoshimori 2007). In contrast, no change in LGG-1-II levels after treatment with agents or RNAi that decrease lysosomal degradation is indicative of a block in the autophagic pathway. Thus, in summary, with careful interpretation of results, changes in the protein levels of LGG-1 can provide a way to measure changes in autophagy activity.

**Recipes**

**Bleach Solution**

3.5 mL sterile H2O
0.5 mL NaOH (5 N)
1 mL household bleach (sodium hypochlorite)

Make the solution fresh each time it is needed.

**M9 Minimal Medium Buffer**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>KH2PO4</td>
<td>22 mM</td>
</tr>
<tr>
<td>Na2HPO4</td>
<td>22 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>85 mM</td>
</tr>
<tr>
<td>MgSO4</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

Autoclave for 15 min on liquid cycle. Allow medium to cool and store at room temperature.

**Ponceau S Solution**

Ponceau S (0.2%)
Trichloroacetic acid (3%)
Sulfosalicylic acid (3%)

For a 10× stock, make a solution of 2% Ponceau S, 30% Trichloroacetic acid, and 30% Sulfosalicylic acid. Bring up to volume with H2O. The solution is stable at room temperature for over 1 year.

**SDS Gel-Loading Buffer (2×)**

100 mM Tris-Cl (pH 6.8)
4% (w/v) SDS (sodium dodecyl sulfate; electrophoresis grade)
0.2% (w/v) bromophenol blue
20% (v/v) glycerol
200 mM DTT (dithiothreitol)

Store the SDS gel-loading buffer without DTT at room temperature. Add DTT from a 1 M stock just before the buffer is used. 200 mM β-mercaptoethanol can be used instead of DTT.

**SDS-Polyacrylamide Gel**

30% Acrylamide mix (Sigma-Aldrich A3574)
1.5 M Tris (pH 8.8)
10% SDS
10% ammonium sulfate
0.04% TEMED (N,N,N′,N′-tetramethylethlenediamine)

Combine ingredients immediately before use.
Stacking Gel (5%)
To prepare 5% stacking gel mixture, combine in the following order:
2 mL of 30% acrylamide mix
3 mL of 0.5 M Tris-HCl (pH 6.8)
0.12 mL of 10% (w/v) SDS
6.76 mL of H2O
0.12 mL of 10% ammonium persulfate
0.006 mL of N,N′,N′-tetramethylethlenediamine (TEMED)

Tris-Buffered Saline (TBS; 10×, pH 7.5)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>24.2 g</td>
<td>200 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>87.7 g</td>
<td>1.5 M</td>
</tr>
</tbody>
</table>

Combine ingredients in ~800 mL of H2O. Adjust pH to 7.5 and bring final volume to 1 L. Sterilize by autoclaving.

Tris-Glycine Buffer
Prepare a 5× stock solution in 1 liter of H2O.
15.1 g Tris base
94 g glycine (electrophoresis grade)
50 mL 10% SDS (electrophoresis grade)
The 1× working solution is 25 mM Tris-Cl/250 mM glycine/0.1% SDS. Use Tris-glycine buffers for SDS-polyacrylamide gels.

Western Transfer Buffer
25 mM Tris-Cl
192 mM Glycine
20% (v/v) methanol, pH 8.3
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