Lack of Peroxisomal Catalase Causes a Progeric Phenotype in Caenorhabditis elegans

Oleh I. Petriv‡ and Richard A. Rachubinski§

From the Department of Cell Biology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Studies using the nematode Caenorhabditis elegans as a model system to investigate the aging process have implicated the insulin/insulin-like growth factor-1 signaling pathway in the regulation of organismal longevity through its action on a subset of target genes. These targets can be classified into genes that shorten or extend life-span upon their induction. Genes that shorten life-span include a variety of stress response genes, among them genes encoding catalases; however, no evidence directly implicates catalases in the aging process of nematodes or other organisms. Using genetic mutants, we show that lack of peroxisosomal catalase CT-2 causes a progeric phenotype in C. elegans. Lack of peroxisomal catalase also affects the developmental program of C. elegans, since Δctl-2 mutants exhibit decreased egg laying capacity. In contrast, lack of cytosolic catalase CTL-1 has no effect on either nematode aging or egg laying capacity. The Δctl-2 mutation also shortens the maximum life-span of the long lived Δctl-1 mutant and accelerates the onset of its egg laying period. The more rapid aging of Δctl-2 worms is apparently not due to increased carbonylation of the major C. elegans proteins, although altered peroxisome morphology in the Δctl-2 mutant suggests that changes in peroxisomal function, including increased production of reactive oxygen species, underlie the progeric phenotype of the Δctl-2 mutant. Our findings support an important role for peroxisomal catalase in both the development and aging of C. elegans and suggest the utility of the Δctl-2 mutant as a convenient model for the study of aging and the human diseases acatalasemia and hypocatalasemia.

A subset of genes of the nematode Caenorhabditis elegans are direct targets (1, 2) of the FOXO family transcription factor DAF-16 (3), a key regulator of the insulin/insulin-like growth factor I signaling pathway implicated in the aging process (1, 4). These target genes usually contain specific nucleotide sequences in their upstream regulatory regions capable of binding DAF-16 and can be directly repressed or activated upon binding of DAF-16 (5). Specific transcriptional regulation of target genes by DAF-16 is purported to lead to an extended or shortened life-span for the nematode. Among the target genes regulated by DAF-16 are those encoding heat shock proteins and cytochrome p450s involved in the cell stress response and genes encoding proteins responsible for antioxidant defense, such as the mitochondrial superoxide dismutase SOD-3, the metallothionein homolog MTL-1, and the catalases CTL-1 and CTL-2 (5).

Superoxide dismutases (SODs) and catalases are scavengers of reactive oxygen species (ROS) and H2O2, respectively, and have been suggested to play important roles in the aging process in C. elegans (6). Catalase levels are increased in the long-lived C. elegans mutants age-1 (6–8), eat-2 (9), and daf-2 (10, 11), whereas catalase gene expression is decreased in a short lived strain mutated for DAF-16 (12). The daf-16 mutation also largely suppresses the increases in catalase activity observed in age mutants (10).

In addition to the insulin/insulin-like growth factor-1 signaling pathway, dietary restriction and oxidative stress are also thought to be major determinants of the aging process in C. elegans and other model organisms (10, 13). Under conditions of dietary restriction that extend monoxenic, and especially axenic, worm life-span, catalase and SOD levels have been found to be increased dramatically in a DAF-16-independent manner (10). A correlation between increases in SOD and catalase activity and hyperresistance to oxidative stress has also been observed in some long lived mutants (8). During the dauer larval stage when the nematode is developmentally arrested, nonfeeding, and able to survive several times the normal life-span, catalase and/or SOD activities are substantially up-regulated (8, 14). Moreover, treatment of worms with SOD/catalase mimetics extends their life-spans (15, 16). These and other findings from different model organisms (13, 17–20) suggest that catalases play an important role in the aging process; however, a definitive statement on the role of individual catalases in the aging process in C. elegans requires investigation of this process in mutant strains deleted for the individual catalase genes.

Two forms of catalase, the cytosolic CTL-1 and the peroxisomal CTL-2, have been reported for C. elegans (21, 22). A previous attempt to engage CTL-1 directly in the control of aging in C. elegans was unsuccessful (21). Molecular features of the catalase gene locus in C. elegans, namely the presence of different catalase genes exhibiting a high level of sequence identity, make it difficult, if not impossible, to use the results of methods such as RNA interference (4, 5, 23) to permit definitive statements on the roles of individual catalases in the aging process in C. elegans. Here, using C. elegans strains mutated for individual catalase genes, we present evidence directly imp-

---

* This work was supported by grants from the Canadian Institutes of Health Research (to R. A. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at http://www.jbc.org) contains four additional figures.

‡ Supported by a Studentship from the Alberta Heritage Foundation for Medical Research.

§ Canada Research Chair in Cell Biology and an International Research Scholar of the Howard Hughes Medical Institute. To whom correspondence should be addressed: Dept. of Cell Biology, University of Alberta, Medical Sciences Bldg, 5-14, Edmonton, Alberta T6G 2H7, Canada. Tel.: 780-492-9868; Fax: 780-492-9278; E-mail: rick.rachubinski@ualberta.ca.

The abbreviations used are: SOD, superoxide dismutase; ROS, reactive oxygen species.
Fig. 1. Structure of the ctl locus in the C. elegans genome. A genomic DNA fragment 11,931 bp in length and encompassing the three catalase genes of C. elegans is presented. This region contains several binding sites for the transcription factor DAF-16 and a large number of inverted DNA repeats. The adenyl residue of the initiating codon of each catalase gene is designated "0" and serves as the point of reference for numbering the nucleotide sequence upstream (negative values) and downstream (positive values) of a particular catalase gene.

RESULTS

Characterization of the Catalase Genes of C. elegans and Analysis of Catalase Enzymatic Activity in the Nematode—The C. elegans genome contains three catalase genes in tandem: ctl-3, ctl-1, and ctl-2 (Figs. 1 and S1). 2 DAF-16 binding motifs (1) are present upstream and downstream of all three genes (Fig. 1). The catalase genes exhibit extensive sequence identity. ctl-1 is identical to ctl-2 between nucleotides 1 and 304 and to ctl-3 from nucleotide 304 until its end (Figs. 1 and S1). The coding region of ctl-2 exhibits 76.7% sequence identity to the coding region of ctl-3 (Figs. 1, S1, and S2). The intervening region between the ctl-1 and ctl-3 genes is 100% identical in sequence to the intervening region between the ctl-1 and ctl-2 genes (Figs. 1 and S1). Extensive sequence identity among the catalase genes makes it difficult to analyze their individual expression by techniques such as Northern blotting and suggests that the results of RNA interference and gene microarray analysis should be interpreted with caution.

The catalases themselves can be distinguished from one another because of differences in their biochemical properties. Because the pl values of the three catalases differ significantly (Table I), they can be readily separated by native gel electrophoresis and detected by staining for enzymatic activity (Fig. 2A). We isolated a worm strain, LB90 (Δctl-2), harboring a deletion of the ctl-2 gene (Figs. 1 and S1, Δctl-2). The Δctl-2 mutant strain exhibits no detectable CTL-2 enzymatic activity (Fig. 2A). DNA sequencing of the strain ctl-1(Δu800)II, which had been claimed to exhibit decreased catalase activity due to a premature termination codon in the ctl-1 gene (21), showed that the entire ctl-1 gene and extensive sequence upstream were missing in this mutant (Figs. 1 and S1, Δctl-1). Native gel electrophoresis confirmed the absence of CTL-1 enzymatic activity in the Δctl-1 strain (Fig. 2A). Attempts to isolate mutants of the ctl-3 gene were unsuccessful. In dauer larvae, a developmental stage during which animals do not feed and fat metabolism is shifted to fat storage (30), the level of CTL-1 activity is increased, whereas the level of peroxisomal CTL-2 activity appears to be similar to that found in wild-type worms (Fig. 2A). Δctl-2 and Δctl-1 mutants do not fail to form the dauer stage (data not shown). Quantitative colorimetric analysis of catalase activity showed that deletion of the ctl-2 gene (Fig. 2B, Δctl-2, and

\[2\] NCBI database entry NP_496979; Wormbase, open reading frames Y54G11A.6, Y54G11A.5a, Y54G11A.5b, and Y54G11A.13.
Table II) reduced catalase activity to ~20% of the total catalase activity observed in wild-type worms, whereas deletion of the ctl-1 gene (Fig. 2B, Δctl-1, and Table II) led to a much smaller reduction in catalase activity to ~75% of the total catalase activity observed in wild-type worms. Therefore, peroxisomal catalase CTL-2 contributes most of the total catalase activity in C. elegans.

Lack of Peroxisomal, but Not Cytosolic, Catalase Causes Accelerated Aging of C. elegans—We compared the mean and maximum life-span and egg laying capacity of catalase mutant worms versus wild-type worms to determine whether the different catalases in C. elegans could have a role in the development and aging of the nematode. Δctl-1 mutants showed no difference in life-span or egg laying capacity compared with wild-type worms, whereas Δctl-2 mutants had a significantly shortened (16%) life-span and decreased egg laying capacity (Fig. 3 and Table II). Introduction of the Δctl-2 mutation into long lived Δclk-1 mutant worms did not result in a shortening of their mean life-span (Fig. 3 and Table II), consistent with a previous finding that the extended life-span of Δclk-1 mutant worms is not related directly to the antioxidant action of catalase (34). Nevertheless, we observed a significantly shortened

![Fig. 2. Activity of catalases in C. elegans.](image)

**Table II**

| Strain          | Mean life-span | Maximum life-span | Catalase activity in vitro | Eggs laid/worm |
|-----------------|----------------|-------------------|---------------------------|----------------|
|                 | days from hatching | days from hatching | % of wild type |                  |
| Wild type       | 14.6 ± 1.4 (n = 717) | 20.8 ± 3.1 (n = 6) | 100 | 310 ± 31.3 |
| Δctl-1          | 15.3 ± 1.2 (n = 364) | 21.7 ± 0.5 (n = 4) | 74.6 ± 6.7 (n = 8) | 332.5 ± 25.9 |
| Δctl-2          | 12.1 ± 0.9 (n = 365) | 16.6 ± 1.1 (n = 5) | 19.6 ± 6.1 (n = 11) | 231.1 ± 22.5 |
| Δclk-1          | 24.7 ± 2.7 (n = 277) | 32.7 ± 1.5 (n = 3) | 171.5 ± 19.8 (n = 4) | 160.6 ± 20.6 |
| Δclk-1;Δctl-2   | 24.3 ± 1.6 (n = 161) | 29.3 ± 1.2 (n = 5) | ND | 130.7 ± 24.7 |

* Based on unpaired t test for two populations.
* Comparison with wild-type strain.
* Significantly different at $p < 0.01$.
* Significantly different at $0.01 < p < 0.05$.
* Comparison with clk-1 strain.
* Not determined.

![Fig. 3. Effects of mutation of the ctl-1 and ctl-2 genes on life-span and egg laying capacity of wild-type and Δclk-1 mutant worms.](image)
(14%) maximum life-span and an acceleration of ~12 h in the onset of the egg laying period in Δclk-1;Δctl-2 double mutant worms as compared with Δctl-1 mutant worms (Fig. 3 and Table II).

Increased protein carbonylation has been observed during cell aging (31). Carbonylation of major worm proteins such as vitellogenin-6 (32) increases from 5 days to 10 days in both wild-type worms and Δctl-1 and Δctl-2 mutant worms, but the overall increase in carbonylation is less for both mutants than for wild-type worms (Fig. 4). This smaller increase in carbonylation observed for the Δctl-1 and Δctl-2 mutants may result from a compensatory up-regulation of other antioxidant enzymes in these mutants. Evidence for such compensatory changes in the synthesis of unknown enzymes with catalase activity could be seen for the Δctl-1 and Δctl-2 mutants (Fig. 2A, arrowheads). The carbonylation of major protein species therefore appears not to be the cause of the progeric phenotype of the Δctl-2 mutant.

**Cells of the Δctl-2 Mutant Exhibit Abnormal Peroxisome Morphology**—A deficiency in the peroxisomal β-oxidation enzyme acyl-CoA oxidase leads to increased levels of intraperoxisomal H₂O₂ and alters the morphology of peroxisomes (33). A lack of peroxisomal catalase CTL-2 might be expected to exert a similar effect on peroxisome morphology due to decreased breakdown of intraperoxisomal H₂O₂. We therefore examined the morphology of peroxisomes in wild-type and Δctl-2 mutant worms by fluorescent confocal microscopy and electron microscopy. The mean size of peroxisomes is increased in Δctl-2 mutant worms versus wild-type worms (Fig. 5A), and peroxisomes tend to cluster in the Δctl-2 mutant (Fig. 6A). Electron microscopy revealed that these clustered peroxisomes are often associated with lipid vesicles (Fig. 6B, left panel) or with specific multivesicular structures of unknown origin and function (Fig. 6B, right panel). Fat metabolism is apparently normal in Δctl-2 mutant worms, since they accumulate lipid droplets in their cells of the same size accumulated by cells of wild-type worms (Fig. 5B).

**DISCUSSION**

The *C. elegans* genome contains three genes encoding different catalases. To our knowledge, no other metazoan organism has been reported to have multiple catalase genes. The most active form of catalase in *C. elegans* is peroxisomal catalase CTL-2, which contributes ~80% of total catalase activity in the nematode. The remaining catalase activity is contributed by cytosolic catalase CTL-1, a previously unreported catalase CTL-3 encoded by the open reading frame Y54G11A.13, and unknown enzymes exhibiting catalase activity (cf. Fig. 2A). Attempts to obtain a strain mutant for the cti-3 gene were unsuccessful; however, expression of a green fluorescent protein reporter under the control of a promoter fragment extending 657 bp upstream from the cti-3 initiating codon was localized to pharyngeal muscle cells and neurons (Supplemental Data, Fig. S3).

Previous results from studies employing RNA interference had suggested the importance of CTL-2 activity for longevity in *C. elegans* (5). However, because of the high degree of nucleotide identity among the catalase genes, the results of RNA interference studies must be interpreted with caution. Using worms deleted for the cti-2 gene, we have demonstrated directly for the first time that a lack of peroxisomal catalase accelerates aging in *C. elegans*. A lack of CTL-2 resulted in a slightly delayed and extended egg laying period. Importantly, mutation of the cti-1 gene did not affect either nematode lifespan or egg laying capacity, demonstrating that the effects of cti-2 mutation on these characteristics were not simply the result of reduced overall catalase activity. Introduction of the Δctl-2 mutation into the long lived Δclk-1 mutant increased the
Fig. 6. Peroxisomes exhibit altered morphology in \textit{ctl-2} mutant worms. 
\textbf{A}, peroxisomes were detected by fluorescence confocal microscopy of green fluorescent protein tagged with the tripeptide peroxisome targeting signal 1, Ser-Lys-Leu (23). Peroxisomes are larger and form aggregates in cells of the \textit{ctl-2} mutant as compared with peroxisomes in cells of wild-type worms. \textit{Bar}, 5 μm. \textbf{B}, electron microscopy of gut cells (left panel) or hypodermal cells (right panel) of \textit{ctl-2} worms showing that peroxisomes (:white arrowheads) are often associated with lipid droplets (LD) or vesicular structures of unknown origin (arrowhead). \textit{M}, mitochondrion. \textit{Bar}, 1 μm.

The effects of a lack of peroxisomal catalase on organismal longevity are not restricted to \textit{C. elegans}. In the yeast \textit{S. cerevisiae}, loss of cytosolic catalase \textit{T} has little effect on chronological life-span, although its levels are highly induced during the stationary phase of growth (18). We measured the chronological life-spans of wild-type \textit{S. cerevisiae} and of the mutants \textit{Δcta1} and \textit{Δctt1}, respectively. We found that a lack of peroxisomal catalase, but not cytosolic catalase, decreased the viability of yeast by −15-fold (Supplemental Data, Fig. S4). Therefore, a shorter life-span may be a general consequence of a lack of peroxisomal catalase.

In closing, a lack of peroxisomal catalase \textit{CTL-2}, but not cytosolic catalase \textit{CTL-1}, causes a progeric phenotype in the nematode \textit{C. elegans}. The \textit{ctl-2} mutant of \textit{C. elegans} represents a convenient model not only for the study of aging but possibly also for the study of the human diseases acatalasemia and hypocatalasemia (23, 43, 44). Our results support and extend recent findings that peroxisomes not only have important roles in cell metabolism but also are involved in the developmental processes of eukaryotic organisms (23, 43, 25).

Acknowledgments—We thank Bernard Lemire for the gift of a library of \textit{C. elegans} mutant worms and for helpful discussion; Honey Chan for help with confocal microscopy and electron microscopy; David Pilgrim, Vladimir Titorenko, and Paul Melançon for helpful scientific discourse; and the Caenorhabditis Genetic Center for providing mutant nematode strains.

REFERENCES
1. McElwee, J., Bubb, K., and Thomas, J. H. (2003) \textit{Aging Cell} \textbf{2}, 111–121
2. Lee, S. S., Kennedy, S., Tolonen, A. C., and Ruvkun, G. (2003) \textit{Science} \textbf{300}, 644–647
3. Ogg, S., Paradis, S., Gotlieb, S., Patterson, G. I., Lee, L., Tissenbaum, H. A., and Ruvkun, G. (1997) \textit{Nature} \textbf{389}, 994–999
4. Hsu, A. L., Murphy, C. T., and Kenyon, C. (2003) \textit{Science} \textbf{300}, 1142–1145
5. Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Ramath, R. S., Ahiringer, J. L., and Kenyon, C. (2003) \textit{Nature} \textbf{424}, 277–284
6. Vanfleteren, J. R. (1993) \textit{Biochem. J.} \textbf{292}, 655–668
7. Yaman, S., Yashima, K., and Ishii, N. (2002) \textit{Mech. Aging Dev.} \textbf{132}, 1579–1587
8. Larsen, P. L. (1993) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{90}, 8905–8909
9. Houchoff, K., Braeckman, B. P., Lenaerts, I., Bys, K., De Vreese, A., Van Eygen, S., and Vanfleteren, J. R. (2002) \textit{Exp. Gerontol.} \textbf{37}, 1357–1369
10. Houchoff, K., Braeckman, B. P., Johnson, T. E., and Vanfleteren, J. R. (2003) \textit{Exp. Gerontol.} \textbf{38}, 947–954
11. Hekimi, S., Burgess, J., Bussiere, F., Meng, Y., and Benard, C. (2001) \textit{Trends Genet.} \textbf{17}, 712–718
12. Wallkop, C. A., Kimura, K. D., Lee, M. S., and Ruvkun, G. (2000) \textit{Science} \textbf{290}, 147–150
13. Jaworski, S. M. (1998) \textit{Exp. Gerontol.} \textbf{33}, 723–783
14. Houchoff, K., Braeckman, B. P., Lenaerts, I., Bys, K., De Vreese, A., Van Eygen, S., and Vanfleteren, J. R. (2002) \textit{Exp. Gerontol.} \textbf{37}, 1015–1021
15. Gill, M. S., Olsen, A., Sampayo, J. N., and Lithgow, G. J. (2003) \textit{Free Radic. Biol. Med.} \textbf{35}, 558–565
16. Meier, S., Ravenscroft, J., Malik, S., Gill, M. S., Walker, D. W., Clayton, P. E., Wallace, D. C., Maflory, D., Doctorow, S. R., and Lithgow, G. J. (2000) \textit{Science} \textbf{289}, 1567–1569
17. van Zanduyk, S. M., Schier, P. J., and Smart, K. A. (2002) \textit{Mech. Aging Dev.} \textbf{123}, 365–373
18. Longo, V. D., Gralla, E. B., and Valentine, J. S. (1996) *J. Biol. Chem.* **271**, 12275–12280
19. Boulianne, G. L. (2001) *Mech. Ageing Dev.* **122**, 883–894
20. Mackay, W. J., and Bewley, G. C. (1989) *Genetics* **122**, 643–652
21. Tsuh, J., Lau, J. F., Ma, C., Hahn, J. H., Hoque, R., Rothblatt, J., and Chalfie, M. (1999) *Nature* **399**, 162–166
22. Togo, S. H., Maebuchi, M., Yokota, S., Bun-Ya, M., Kawahara, A., and Kamiryo, T. (2000) *Eur. J. Biochem.* **267**, 1307–1312
23. Petriv, O. I., Pilgrim, D. B., Rachubinski, R. A., and Titorenko, V. I. (2002) *Physiol. Genomics* **10**, 79–91
24. Lewis, J. A., and Fleming, J. T. (1995) *Methods Cell Biol.* **48**, 3–29
25. Woodbury, W., Spencer, A. K., and Stahman, M. A. (1971) *Anal. Biochem.* **44**, 301–305
26. Hope, I. A. (1999) *C. elegans: A Practical Approach*, pp. 62–63 Oxford University Press, Oxford, UK
27. Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S., and de Duve, C. (1968) *J. Cell Biol.* **37**, 482–513
28. Woodbury, W., Spencer, A. K., and Stahman, M. A. (1971) *Anal. Biochem.* **44**, 301–305
29. Page dimensions: 621.0x801.0
30. Guarente, L., Ruvkun, G., and Amasino, R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 11034–11036
31. Goto, S., Nakamura, A., Radak, Z., Nakamoto, H., Takahashi, R., Yasuda, K., Sakurai, Y., and Ishii, N. (1999) *Mech. Ageing Dev.* **107**, 245–253
32. Nakamura, A., Yasuda, K., Adachi, H., Sakurai, Y., Ishii, N., and Goto, S. (1999) *Biochem. Biophys. Res. Commun.* **264**, 580–583
33. Pan, C. Y., Pan, J., Usuda, N., Yeldandi, A. V., Rao, M. S., and Reddy, J. K. (1998) *J. Biol. Chem.* **273**, 15639–15645
34. Braeckman, B. P., Houthooft, K., Brys, K., Lenaerts, I., De Vreeze, A., Van Eygen, S., Raes, H., and Vanfleteren, J. R. (2002) *Mech. Ageing Dev.* **123**, 1447–1456
35. Wang, A., Boutis, P., and Hekimi, S. (1995) *Genetics* **139**, 1247–1259
36. Shibata, Y., Branicky, R., Landaverde, I. O., and Hekimi, S. (2003) *Science* **302**, 1779–1782
37. Germain, V., Rylott, E. L., Larson, T. R., Sherson, S. M., Bechtold, N., Carde, J. P., Bryce, J. H., Graham, I. A., and Smith, S. M. (2001) *Plant J.* **26**, 1–12
38. Smith, J. J., Brown, T. W., Zitzen, G. A., and Rachubinski, R. A. (2000) *J. Biol. Chem.* **275**, 20168–20178
39. Chang, C. C., South, S., Warren, D., Jones, J., Moser, A. B., Moser, H. W., and Gould, S. J. (1999) *J. Cell Sci.* **112**, 1579–1590
40. van Roermund, C. W. T., Tabak, H. F., van den Berg, M., Wanders, R. J. A., and Hettema, E. H. (2000) *J. Cell Biol.* **150**, 489–498
41. Wetterau, J. R., Aggerbeck, L. P., Bouma, M. E., Eisenberg, C., Munck, A., Hermier, M., Schmitz, J., Guy, G., Rader, D. J., and Gregg, R. E. (1992) *Science* **258**, 999–1001
42. Segrest, J. P., Jones, M. K., De Loof, H., and Dashti, N. (2001) *J. Lipid Res.* **42**, 1346–1367
43. Thiessinger, H., Moellers, B., Dodt, G., Kanau, W.-H., and Driscoll, M. (2003) *J. Cell Sci.* **116**, 1797–1804
44. Ogata, M. (1991) *Hum. Genet.* **86**, 331–340
Lack of Peroxisomal Catalase Causes a Progeric Phenotype in *Caenorhabditis elegans*

Oleh I. Petriv and Richard A. Rachubinski

*J. Biol. Chem. 2004, 279:19996-20001.*

doi: 10.1074/jbc.M400207200 originally published online March 2, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400207200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/03/10/M400207200.DC1

This article cites 42 references, 19 of which can be accessed free at
http://www.jbc.org/content/279/19/19996.full.html#ref-list-1