The prohibitin family of mitochondrial proteins regulate replicative lifespan

P.J. Coates*, D.J. Jamieson†‡, K. Smart§, A.R. Prescott# and P.A. Hall*

Cellular senescence is determined by multiple factors, including the genetic regulation of metabolism and responses to endogenous and exogenous stresses [1–4]. Recent studies implicate a limited number of gene products in elongating lifespan in yeast and *Caenorhabditis elegans* [2–4]; these include the *C. elegans* gene *clk-1*, a central regulator of metabolism [5], and yeast *RAS2,* which controls the response to ultraviolet irradiation and other stresses [3]. Another gene postulated to affect senescence is *PHB1*, the yeast homologue of prohibitin [3], a rodent gene initially identified as a potential regulator of growth arrest and tumour suppressor [6–8]. Highly conserved prohibitin homologues have been identified in mammals [9], *Drosophila* [10], *C. elegans* [9], plants [11] and yeast. A second mammalian gene, encoding BAP37, a protein with sequence similarity to prohibitin, is thought to be involved in lymphocyte function [9]. Here, we show that the nuclear-encoded mammalian prohibitin and BAP37 proteins are present in mitochondria, are co-expressed, and interact physically with each other. Deletion of the *Saccharomyces cerevisiae* homologues, *PHB1* and *PHB2,* results in a decreased replicative lifespan and a defect in mitochondrial membrane potential. Our observations highlight the relationship between the metabolic efficiency of cells and the ageing process, and provide evidence for its evolutionary conservation.

Addresses: *Department of Molecular and Cellular Pathology; †Biomedical Research Centre, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK. §School of Biological and Molecular Sciences, Oxford-Brooks University, Oxford OX3 OBP, UK. #Department of Biochemistry, University of Dundee, Dundee DD1 4HN, UK.

‡Present address: Department of Biological Sciences, Herriot Watt University, Edinburgh EH14 4AS, UK.

Correspondence: Philip Coates
E-mail: pjcoates@ninewells.dundee.ac.uk

Received: **21 April 1997**
Revised: **28 May 1997**
Accepted: **10 June 1997**

Current Biology 1997, 7:R607–R610
http://biomednet.com/elecref/0960982200700607

Results and discussion

Searching current databases revealed that, like prohibitin, BAP37 is highly conserved, with homologues in humans, rodents, *C. elegans* and *S. cerevisiae*. The mid-regions of the prohibitin and BAP37 proteins are remarkably similar (Figure 1), and all of the sequences contain a series of repeated leucine residues and a predicted coiled-coil region. In contrast, the amino and carboxyl termini of prohibitin show no homology to BAP37. The high degree of conservation of the two proteins suggests that they have important cellular functions, and the structural similarities suggest that their functions might be related. In addition, the cyanobacterium *Synechocystis* open reading frames GenBank D90900 and D90903 have homology to prohibitin and BAP37. Hence, prohibitin and BAP37 appear to have been present during evolution for approximately 2 billion years [12].

Prohibitin has been suggested to lie in the inner mitochondrial membrane of mammalian cells [8,13], although other observations indicate that both prohibitin and BAP37 are associated with the plasma membrane [9]. Using two different monospecific antipeptide antisera that recognise either the 32 kDa prohibitin protein or the 37 kDa BAP37 protein, we found a clear colocalisation of both proteins with mitochondria in human, rodent and *Xenopus laevis* cell lines (Figure 2). The localisation of both proteins was distinct from that of the trans-Golgi network or endosomes, and no immunoreactivity for either protein was found on the plasma membrane of any cell type examined. Western immunoblot analysis of murine tissue extracts showed that the levels of prohibitin and BAP37 vary in different organs, but their ratio is constant (Figure 3a).

In view of the apparent colocalisation and co-expression of prohibitin and BAP37, we investigated whether they are physically associated. Co-immunoprecipitation of detergent lysates from HeLa cells was used to show a specific interaction of prohibitin and BAP37 (Figure 3b). The mouse, rat and *Xenopus* homologues of prohibitin and BAP37 also co-immunoprecipitated, illustrating the evolutionary conservation of the interaction (Figure 3c), and the interaction was seen in all mammalian cell lines tested.

In order to gain an insight into function, we turned to the *S. cerevisiae* homologues of prohibitin (*PHB1*) and BAP37 (*PHB2*). Deletion of either gene was performed in haploid cells. Cells without a *PHB1* coding sequence subsequently had their *PHB2* deleted, and the converse second recombination was also performed; in all cases, two separate clones were propagated and analysed. None of the mutants showed any detectable abnormality in

Figure 1

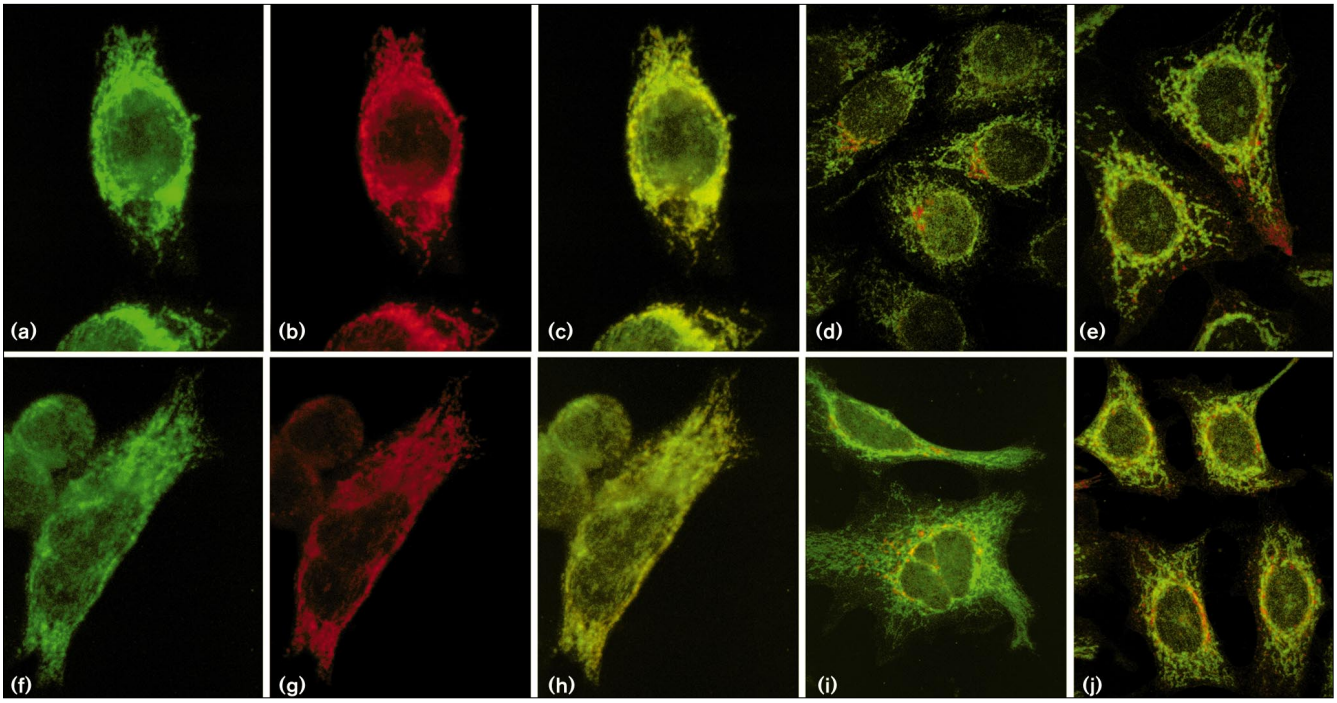
```
                ·· |·· | |··|· ·||·|||| ··|···· ·||| ·|· ··   |·· ||  |· ··|·····
Human phb       HFLIPWVQKPIIFDCRSRPRNVPVITGSKDLQNVNITLRILFRPVASQLPRIFTSIGEDYDERVLPS
Mouse phb       HFLIPWVQKPIIFDCRSRPRNVPVITGSKDLQNVNITLRILFRPVASQLPRIYTSIGEDYDERVLPS
Yeast phb1      HFLVPWLQKAIIYDVRTKPKSIATNTGTKDLQMVSLTLRVLHRPEVLQLPAIYQNLGLDYDERVLPS
Human BAP37     HFRIPWFQYPIIYDIRARPRKISSPTGSKDLQMVNISLRVLSRPNAQELPSMYQRLGLDYEERVLPS
Mouse BAP37     HFRIPWFQYPIIYDIRARPRKISSPTGSKDLQMVNISLRVLSRPNAQELPSMYQRLGLDYEERVLPS
Yeast phb2      HFIFPWLDTPIIYDVRAKPRNVASLTGTKDLQMVNITCRVLSRPDVVQLPTIYRTLGQDYDERVLPS

                ·  ·|··||··|·|·||····· |·  |  |·  ··  · | ·|··||· ||·| ·|· ··· ··|
Human phb       ITTEILKSVVARFDAGELITQRELVSRQVSDDLTERAATFGLILDDVSLTHLTFGKEFTEAVEAKQV
Mouse phb       ITTEILKSVVARFDAGELITQRELVSRQVSDDLTERAATFGLILDDVSLTHLTFGKEFTEAVEAKQV
Yeast phb1      IGNEVLKSIVAQFDAAELITQREIISQKIRKELSTRANEFGIKLEDVSITHMTFGPEFTKAVEQKQI
Human BAP37     IVNEVLKSVVAKFNASQLITQRAQVSLLIRRELTERAKDFSLILDDVAITELSFSREYTAAVEAKQV
Mouse BAP37     IVNEVLKSVVAKFNASQLITQRAQVSLLIRRELTERAKDFSLILDDVAITELSFSREYTAAVEAKQV
Yeast phb2      IVNEVLKAVVAQFNASQLITQREKVSRLIRENLVRRASKFNILLDDVSITYMTFSPEFTNAVEAKQI

                ···|·|·· ·|·|·· ·||| || ·|·||
Human phb       AQQEAERARFVVEKAEQQKKAAIISAEGDS
Mouse phb       AQQEAERARFVVEKAEQQKKAAIISAEGDS
Yeast phb1      AQQDAERAKFLVEKAEQERQASVIRAEGEA
Human BAP37     AQQEAQRAQFLVEKAKQEQRQKIVQAEGEA
Mouse BAP37     AQQEAQRAQFLVEKAKQEQRQKIVQAEGEA
Yeast phb2      AQQDAQRAAFVVDKARQEKQGMVVRAQGEA
```

Sequence homology of the central region of prohibitin and BAP37. Identities are marked by a dot above the sequences, and a line represents similarity. The conserved leucines referred to in the text are in bold type. Human phb represents the human prohibitin sequence from residues 55 to 218 [7]. Human BAP37 is shown from amino acids 69 to 232 of the sequence contained in GenBank (accession number U47924). Mouse phb [9] is from residues 55 to 218 and BAP37 is from residues 68 to 231. Yeast sequences are from *S. cerevisiae*. Yeast phb1 is from GenBank (U16737), residues 57–220; yeast phb2 is from EMBL (X87941), amino acids 86–249.

growth on rich or defined medium, and the cells were able to grow with glucose, glycerol, galactose or ethanol as the sole carbon source.

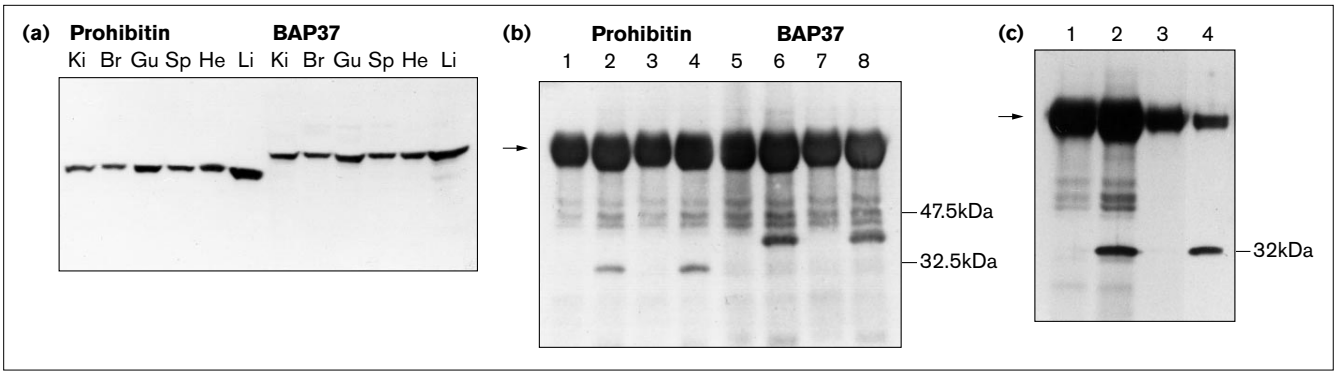
Because *PHB1* expression has been suggested to change with cellular senescence [3], we examined the replicative lifespan of the mutant cells. Cells lacking both *PHB1* and

Figure 2



Mitochondrial localisation of prohibitin and BAP37. HeLa cells were fixed in cold methanol/acetone and stained by immunofluorescence using FITC-labelled anti-rabbit immunoglobulins for prohibitin **(a–e)** or BAP37 **(f–j)**, seen in green. Mitochondria, the trans-Golgi network, or endosomes were stained with murine antibodies and detected with Texas Red-conjugated secondary antibodies (red). Single images are shown for prohibitin (a) or BAP37 (f) and for the mitochondrial marker MAB1273 (b,g). The colocalisation is apparent from the yellow colour seen in the merged images (c,h). In contrast, merged images for the trans-Golgi network with prohibitin (d) or BAP37 (i) show dissimilar distributions, as does the endosomal marker with prohibitin (e) or BAP37 (j)

Figure 3

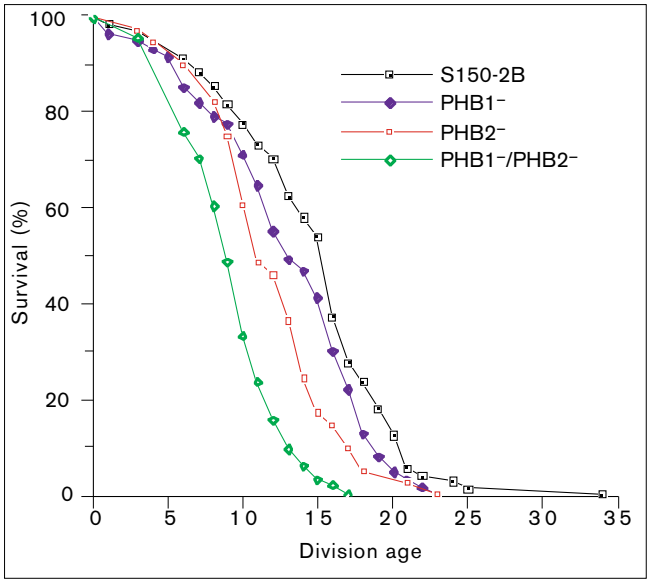


Co-expression and association of prohibitin and BAP37. **(a)** Expression of prohibitin and BAP37. Total protein (20 μg) from mouse tissues were western blotted for prohibitin or BAP37. Ki, kidney; Br, brain; Gu, gut; Sp, spleen; He, heart; Li, liver. **(b)** Interaction between prohibitin and BAP37. HeLa cells were lysed in either 1% NP40 (lanes 1, 2, 5 and 6) or in 1% Triton-X-100 (lanes 3, 4, 7 and 8). The lysates were immunoprecipitated with pre-immune serum (lanes 1, 3, 5 and 7), or with BAP37 antiserum (lanes 2, 4, 6 and 8). Equivalent aliquots of the immune precipitates were separated by SDS–PAGE, blotted onto nitrocellulose and probed for either prohibitin (lanes 1–4) or BAP37 (lanes 5–8). The presence of prohibitin in the BAP37 immunoprecipitates is clearly identified (lanes 2 and 4), and is not seen when pre-immune sera are used for the immunoprecipitation (lanes 1 and 3). The arrow denotes the presence of immunoglobulin heavy chain from the immunoprecipitating antisera. **(c)** BAP37 and prohibitin interact in *Xenopus* cells. XIK2 cells were lysed in 1% Triton-X-100 and immunoprecipitated with pre-immune BAP37 serum (lane 1), BAP37 antiserum (lane 2), pre-immune prohibitin serum (lane 3), or affinity-purified prohibitin antiserum (lane 4). After western blotting, all the precipitates were probed for the presence of prohibitin. Lane 2 shows the presence of prohibitin in the BAP37 immunoprecipitate.

PHB2 showed a significant decrease in mean lifespan compared with wild-type cells ($p = 1.8053E^{-10}$ using two sample *t*-test assuming unequal variances). Estimations of the maximum lifespan were derived by calculation of the 10% upper limit of survival and were again significantly different between the wild-type and the double-mutant strains. Deletion of either *PHB1* or *PHB2* exerted an intermediate effect on both the mean and maximum replicative lifespan (Figure 4). During these experiments, we noticed a lengthening of cell division time, as well as characteristic morphological changes as the yeast cells progressed through their lifespan. These changes were seen after relatively few generations in the double-mutant strains. Such alterations are characteristic of ageing cells [14] and help to show that the double-mutant strains undergo premature ageing, rather than simply an early death. We also assayed the four strains for resistance to a variety of stresses which correlate with lifespan in yeast [2]. We found no difference in the response of *PHB1* and *PHB2* single or double mutants to heat shock, ultraviolet irradiation, oxidative stress (hydrogen peroxide, paraquat and tertiary-butyl hydroperoxide), or starvation, compared with wild-type strains. The mutants grew equally rapidly in bulk culture and showed no increased sensitivity to stresses, again suggesting that the replicative lifespan differences constitute a specific phenotype.

In view of the localisation of the two proteins, we measured the mitochondrial membrane potential by incorporation of the lipophilic cationic cyanine dye $DiOC_6(3)$ followed by fluorescence-activated cell sorting [15]. Deletion of *PHB1* and *PHB2* in the same cell caused an approximately 80% reduction in average mitochondrial membrane potential compared with wild-type cells, and slight decreases were

Figure 4



Replicative lifespan analysis in *S. cerevisiae*. The graph shows the proportion of cells surviving (%) after each replicative cycle for the wild-type strain (S150-2B) and for cells lacking *PHB1*, *PHB2* or both (*PHB1*$^-$/*PHB2*$^-$). The mean lifespans are 14.8, 13.1, 12.0 and 9.3 divisions, and the number of divisions at which 90% of the cells have died are 23.6, 20.6, 19.4 and 14.8, respectively.

seen in single-mutant strains. Taken together, these data show that *PHB1* and *PHB2* have a functional as well as a physical interaction which influences mitochondrial function and replicative lifespan. Prohibitin and BAP37 seem to represent a novel class of highly conserved 'longevity assurance proteins'. It is noteworthy that the level of *PHB1* mRNA has been reported to change during the normal lifespan of yeast [3], so the lack of Phb1p protein is associated with aged cells under normal conditions and, as we have shown, *PHB* deficiency causes premature ageing when experimentally repressed. Interestingly, the expression of both prohibitin and BAP37 is relatively low in normal mammalian cells when compared with immortal cell lines and tumours [9,16].

The mechanism by which the levels of the *PHB* genes are regulated during the lifespan is unknown. Previous data from yeast have shown that the patterns of genes regulated by the Sir silencing proteins changes progressively during ageing, and this is responsible for the appearance of the ageing-associated phenotype of sterility [17]. Most recently, proteins of the Sir complex have been shown to redistribute in the nucleus during ageing [18], so one obvious possibility is that the *PHB* genes are subject to differential silencing during the lifespan. Our observations on the properties of the *PHB* genes highlight the notion that regulation of mitochondrial function is a primary determinant of cellular ageing. The clear effect of caloric restriction on longevity of mammals [1] is consonant with these observations. Such complex functions are likely to be controlled by both positive and negative regulators that facilitate the cellular responses to environmental factors. In the case of the nematode *clk-1* gene, loss-of-function mutations lead to prolongation of lifespan through a generalised slowing down of metabolism [5], whereas *PHB1* and *PHB2* have opposing cellular consequences. These observations place the mitochondrion at the centre of cellular lifespan determination.

Materials and methods

Polyclonal antisera to prohibitin or BAP37 were raised in rabbits by repeated injections of the carboxy-terminal 25 amino acids of the murine proteins, conjugated to keyhole limpet haemocyanin (Sigma) with glutaraldehyde [19]. Murine antibodies specific for mitochondria (MAB1273, Chemicon International), for the trans-Golgi network (TGN46), or for endosomal compartments (OKT9) were used for immunofluorescence. Immunochemical methods were performed according to Harlow and Lane [19]. The S150-2B haploid strain of *S. cerevisiae* (*leu2-3,112; ura3-52; trp1-289; his3-Δ1*) was used. Initial disruptions of the *PHB1* and *PHB2* open reading frames utilised the pFA6-KanMX4 plasmid [20]. The technique of double fusion polymerase chain reaction was used and transformed cells were selected with 200 mg/l G418 (Life Technologies). PCR analysis confirmed that the relevant open reading frame had been replaced with the *Kan*^{r} gene. For disruption of both genes in the same cell, G418 resistant cells were transformed using PCR products prepared using the pFA6-HIS3MX6 plasmid, which contains the *S. pombe HIS3* coding region. Verification of correct open reading frame replacement was performed using PCR (all oligonucleotide sequences are available on request). Lifespan determinations were made for at least 40 cells from each strain, which were followed from the time of their birth as virgin cells until their death using standard methods [2]. For measurement of mitochondrial membrane potential, cells were grown to late log phase in YP medium containing glucose before resuspension in YP containing 2% ethanol and growth for 2 h. The cyanine dye $DiOC_6(3)$ (Molecular Probes) was diluted to 0.1 μM and cells stained for 30 minutes before washing and analysis by FACS [15].

Acknowledgements

We thank the Association for International Cancer Research for financial support, Sreenivasan Ponnambalam for TGN46 and OKT9 antibodies, David Lane, Frances Fuller-Pace and David Meek for critical reading of the manuscript, and Emma Warbrick and David Nicholls for valuable discussion.

References

1. Sohal RS, Weindruch R: **Oxidative stress, caloric restriction and aging.** *Science* 1996, **273:**59-63.
2. Kennedy BK, Austriaco NR, Zhang J, Guarente L: **Mutation in the silencing gene *SIR4* can delay aging in *S. cerevisiae*.** *Cell* 1996, **80:**485-496.
3. Jazwinski SM: **Longevity, genes, and aging.** *Science* 1996, **273:**54-59.
4. Lithgow GJ: **Invertebrate gerontology: the age mutations of *Caenorhabditis elegans*.** *Bioessays* 1996, **18:**809-811.
5. Ewbank JJ, Barnes TM, Lakowski B, Lussier M, Bussey H, Hekimi S: **Structural and functional conservation of the *Caenorhabditis elegans* timing gene *clk-1*.** *Science* 1997, **275:**980-983.
6. Nuell MJ, Stewart DA, Walker L, Friedman V, Wood CM, Owens GA, *et al.*: **Prohibitin, an evolutionarily conserved intracellular protein that blocks DNA synthesis in normal fibroblasts and HeLa cells.** *Mol Cell Biol* 1991, **11:**1372-1381.
7. Sato T, Saito H, Swensen J, Olifant A, Wood C, Danner D, *et al.*: **The human prohibitin gene located on chromosome 17q21 is mutated in sporadic breast cancer.** *Cancer Res* 1992, **52:**2643-1646.
8. Dell'Orco RT, McClung JK, Jupe ER, Liu XT: **Prohibitin and the senescent phenotype.** *Exp Gerontol* 1996, **31:**245-252.
9. Terashima M, Kim K-M, Adachi T, Nielsen PJ, Reth M, Kohler G, Lamers MC: **The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein.** *EMBO J* 1994, **13:**3782-3792.
10. Eveleth DD, Marsh JL: **Sequence and expression of the Cc gene, a member of the dopa decarboxylase gene cluster of *Drosophila*: possible transcriptional regulation.** *Nucleic Acids Res* 1986, **14:**6169-6183.
11. Snedden WA, Fromm H: **Characterization of the plant homologue of prohibitin, a gene associated with antiproliferative activity in mammalian cells.** *Plant Mol Biol* 1997, **33:**753-756.
12. Doolittle RF, Feng D-F, Tsang S, Cho G, Little E: **Determining divergence times of the major kingdoms of living organisms with a protein clock.** *Science* 1996, **271:**470-477.
13. Ikonen E, Fiedler K Parton RG, Simons K: **Prohibitin, an antiproliferative protein, is localized to mitochondria.** *FEBS Lett* 1995, **358:**273-277.
14. Mortimer RK, Johnston JR: **Lifespan of individual yeast cells.** *Nature* 1959, **183:**1751-1752.
15. Petit PX, Glab N, Kieffer MH, Metezeau P: **Discrimination of respiratory dysfunction in yeast mutants by confocal microscopy, image, and flow cytometry.** *Cytometry* 1996, **23:**28-38.
16. Asamoto M, Cohen SM: **Prohibitin gene is overexpressed but not mutated in rat bladder carcinomas and cell lines.** *Cancer Lett* 1994, **83:**201-207.
17. Smeal T, Claus J, Kennedy B, Cole F, Guarente L**: Loss of transcriptional silencing in old mother cells of *S. cerevisiae*.** *Cell* 1996, **84:**633-642.
18. Kennedy BK, Gotta M, Sinclair DA, Mills K, McNabb DS, Murthy M, *et al.*: **Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in *S. cerevisiae*.** *Cell* 1997, **89:**381-391.
19. Harlow E, Lane DP: *Antibodies. A Laboratory Manual.* New York: Cold Spring Harbor Laboratory Press; 1988:1-726.
20. Wach A: **PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*.** *Yeast* 1996, **12:**259-265.