Enhanced Degradation of EGF Receptors by a Sorting Nexin, SNX1

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The vectorial movement of proteins requires specific recognition by components of the vesicular trafficking machinery. A protein, sorting nexin–1 (SNX1), was identified in a human cell line that bound to a region of the epidermal growth factor receptor (EGFR) containing the lysosomal targeting code. SNX1 contains a region of homology to a yeast vacuolar sorting protein, and overexpression of SNX1 decreased the amount of EGFR on the cell surface as a result of enhanced rates of constitutive and ligand-induced degradation. Thus, SNX1 is likely to play a role in sorting EGFR to lysosomes.

Ligand-activated growth factor and nutrient receptors are internalized by coated pits and transported together to endosomes. The task of segregating receptors destined for lysosomal degradation (such as EGFR) from those that are recycled (such as transferrin receptors) is accomplished within the endosomal compartment. Because sorting the internalized EGFR from endosomes to lysosomes is saturable (*1*), specific recognition elements are thought to mediate this process. Lysosomal targeting sequences that contain either tyrosine- or di-leucine–based motifs (*2*, *3*) function to direct proteins to the lysosome after endocytosis from the cell surface and after release from the Golgi. In both yeast and mammals, receptor transport by these pathways is signal-mediated and saturable, implicating a class of recognition proteins, the sorting nexins. Here, we report the isolation and characterization of a sorting nexin, SNX1, that fulfills criteria for sorting EGFR to lysosomes.

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We isolated a cDNA encoding the COOH-terminal 58 amino acids of SNX1 from a HeLa cDNA library in a yeast two-hybrid screen (*4*) using the core tyrosine kinase domain of EGFR (*5*). A 1974–base pair SNX1 cDNA was assembled from full-length and partial cDNAs isolated from a λgt11 HeLa cDNA library (*6*). The SNX1 cDNA encoded a 522–amino acid hydrophilic protein with a calculated molecular mass of 59.2 kD (Fig. 1A). Sequence database searches with the SNX1 peptide sequence identified a region of 31% identity over 83 amino acids between SNX1 (residues 183 to 265) and yeast Mvp1p (Fig. 1B). Mvp1p was isolated as a multicopy suppressor of Vps1p mutants deficient in carboxypeptidase Y receptor trafficking, and it colocalizes with Vps1p to Golgi membranes (*7*), indicating that this homology defines a potential membrane-trafficking domain. The existence of a human expressed sequence tag 70% identical to SNX1, including part of the COOH-terminal EGFR-binding domain, suggests that SNX1 defines a family of molecules.

In vitro transcription and translation of the SNX1 cDNA produced a protein with an apparent molecular mass of 66 kD (Fig. 1C). In protein immunoblotting experiments (Fig. 1D) an antibody to the 15 COOH-terminal amino acids of SNX1 recognized a 66-kD protein in extracts from tissue culture cells of human, primate, and mouse origin that was distributed between the cytoplasm and perinuclear vesicles. After ligand-induced internalization from the cell surface, EGFR colocalized with SNX1 in the perinuclear vesicular compartment (Fig. 1E).

To determine the structural requirements in EGFR necessary for binding SNX1, we evaluated the interaction of portions of the EGFR intracellular domain with the SNX1 COOH-terminus using the yeast two-hybrid system (Table 1). In contrast to the EGFR core tyrosine kinase domain (residues 663 to 958) used in the screen, the entire intracellular domain (res-

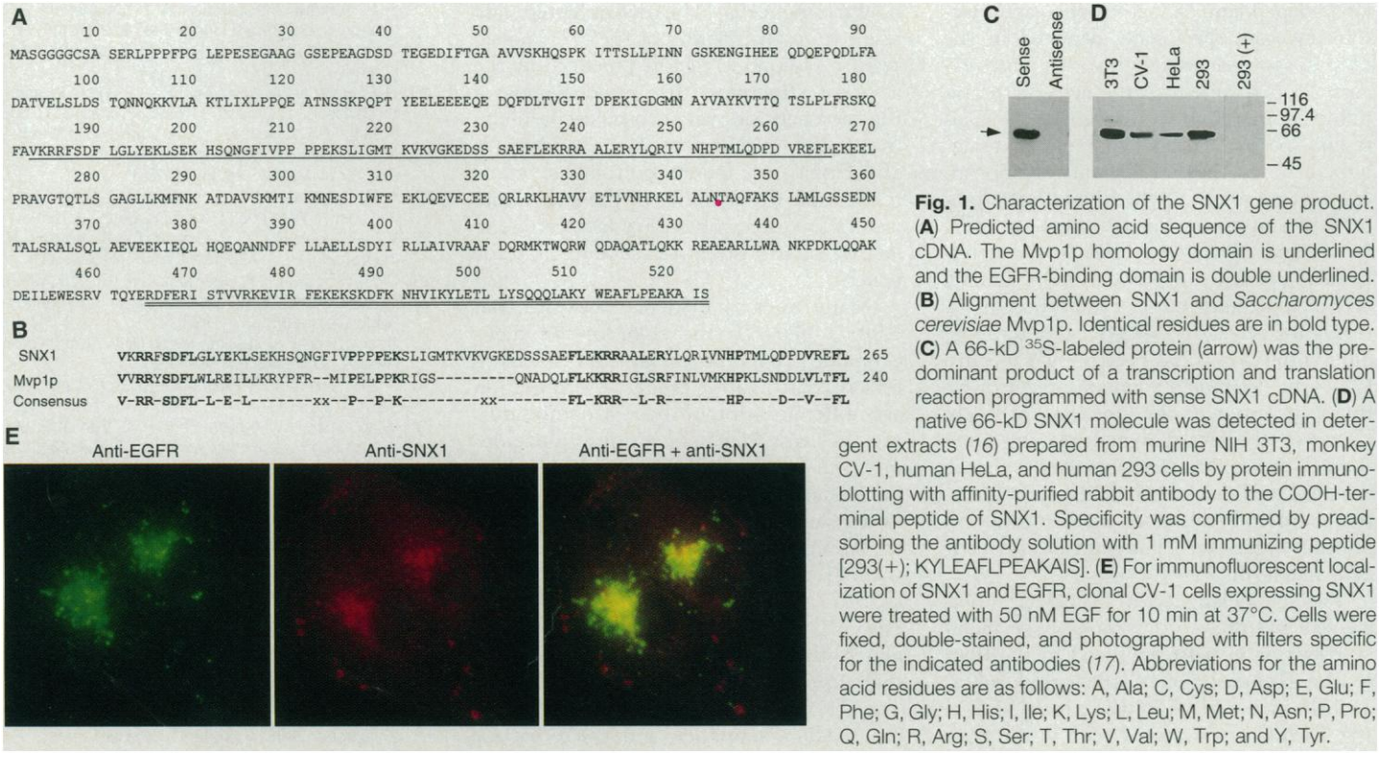

Fig. 1. Characterization of the SNX1 gene product. (**A**) Predicted amino acid sequence of the SNX1 cDNA. The Mvp1p homology domain is underlined and the EGFR-binding domain is double underlined. (**B**) Alignment between SNX1 and *Saccharomyces cerevisiae* Mvp1p. Identical residues are in bold type. (**C**) A 66-kD ^{35}S-labeled protein (arrow) was the predominant product of a transcription and translation reaction programmed with sense SNX1 cDNA. (**D**) A native 66-kD SNX1 molecule was detected in detergent extracts (*16*) prepared from murine NIH 3T3, monkey CV-1, human HeLa, and human 293 cells by protein immunoblotting with affinity-purified rabbit antibody to the COOH-terminal peptide of SNX1. Specificity was confirmed by preadsorbing the antibody solution with 1 mM immunizing peptide [293(+); KYLEAFLPEAKAIS]. (**E**) For immunofluorescent localization of SNX1 and EGFR, clonal CV-1 cells expressing SNX1 were treated with 50 nM EGF for 10 min at 37°C. Cells were fixed, double-stained, and photographed with filters specific for the indicated antibodies (*17*). Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

idues 647 to 1186) failed to interact with SNX1. Antibody to phosphotyrosine did not recognize EGFR(647–1186) in yeast (*8*), indicating that the autophosphorylation-dependent conformational changes (*9*) that expose cryptic binding sites in holo receptors (*10*) did not occur. Indeed, interaction was restored by deleting the COOH-terminal 229 amino acids. Further truncation (leaving residues 647 to 942) abolished interaction with SNX1, thereby defining a 15–amino acid domain (residues 943 to 957) adjacent to the tyrosine kinase core as essential for binding to SNX1. This domain contains the predicted EGFR lysosomal targeting code Tyr-Leu-Val-Ile (*11*) and differs between EGFR and ERBB2, but not between EGFR and ERBB3 or ERBB4. When tested in yeast, SNX1 did not bind the analogous core tyrosine kinase domain of rat ERBB2 (Table 1). Thus, SNX1 is likely to specifically recognize the lysosomal targeting code of EGFR.

To test the hypothesis that SNX1 functions in the lysosomal targeting of EGFR, clonal populations of monkey CV-1 cells overexpressing SNX1 40-fold were prepared (*12*). Of 15 lines analyzed quantitatively by flow cytometry for surface EGFR, eight were highly deficient in surface EGFR relative to cells transfected with empty vector. Scatchard analysis of the binding of ^{125}I-labeled EGF revealed a 75% reduction in the number of receptors at the cell surface from 52,200 receptors per cell [dissociation constant (K_D) = 0.73 nM] to 12,100 receptors per cell (K_D = 1.34 nM). In all EGFR-deficient lines analyzed, down-regulation of surface EGFR by SNX1 overexpression was paralleled by a decrease in the steady-state EGFR mass in cell lysates that was further diminished by treatment with 10 nM EGF for 30 min (Fig. 2A). Down-regulation of EGFR by SNX1 was remarkably specific; overexpression of SNX1 did not alter ERBB2 or platelet-derived growth factor receptor levels in CV-1 cells. In SNX1 clonal lines that retained higher concentrations of EGFR, the addition of EGF caused a more rapid degradation of EGFR compared with vector-transfected cells (Fig. 2B). Overexpression of SNX1 decreased the half-life of EGFR from 12 to 2 hours (*13*). After 1 hour of labeling with [^{35}S]methionine, similar amounts of full-length ^{35}S-labeled EGFR were observed in vector- and SNX1-transfected cells, indicating that the reduction in steady-state EGFR mass did not reflect a change in synthesis or Golgi processing but was a result of accelerated turnover.

Selective pressure to maintain a critical level of EGFR signaling could account for clonal variation in EGFR expression in CV-1 cells. A transient transfection assay was used to evaluate the ability of SNX1 to down-regulate cotransfected EGFR under nonselective conditions. In agreement with the down-regulation of endogenous EGFR in CV-1 cells, human 293 cells transfected with SNX1 had fewer EGFRs than vector-transfected cells (Fig. 3A). Ligand activation of the remaining EGFRs by EGF was not affected, as judged by blotting with antibody to phosphotyrosine. Deletion of the COOH-terminus of SNX1 containing the EGFR-binding domain blocked EGFR down-regulation (Fig. 3B). Similarly, in CV-1 cells transfected with this COOH-terminal truncation, no inhibition of EGFR expression was detected (*8*). In 293 cells, kinase-inactive EGFR (in which Lys has been mutated to Met at position 721) was down-regulated less efficiently by SNX1 than was kinase-active EGFR (Fig. 3C).

SNX1 possesses the binding specificity and functional properties appropriate for a sorting nexin. Overexpression of SNX1 down-regulated endogenous EGFR but not ERBB2 or PDGF receptors in stably transfected CV-1 cells. The turnover of cell surface receptors in cells overexpressing SNX1 was enhanced by EGF treatment and required an active kinase, establishing a function for SNX1 in the endocytic sorting pathway. The COOH-terminal domain of SNX1 that recognizes the EGFR lysosomal targeting signal adjacent to the tyrosine kinase domain is required for down-regula-

Table 1. EGFR residues 943 to 957 are required for interaction with SNX1. The indicated portions of EGFR or ERBB2 were fused to the DNA binding domain of LexA and tested for interaction with the COOH-terminal 58 amino acids of SNX1 fused to the B42 acid patch transcriptional activation domain in a yeast two-hybrid assay (*18*). The β-galactosidase activity of cells incubated with either dextrose or galactose was calculated from the absorbance (*A*) measurements at 420 and 600 nm and expressed as $A_{420}A_{600}^{-1}\ min^{-1} \times 1000$ ± SD (*n* = six samples). FI, fold induction.

LexA fusion	β-Galactosidase activity		FI
	Dextrose	Galactose	
EGFR(663–958)	1 ± 0.4	400 ± 70	400
EGFR(647–1186)	0.6 ± 0.3	4 ± 2	6.7
EGFR(647–957)	1 ± 0.5	500 ± 130	500
EGFR(647–942)	0.4 ± 0.3	6 ± 2	15
ERBB2*	3 ± 0.8	8 ± 3	2.7

*The ERBB2 kinase domain corresponds to EGFR residues 647 to 973.

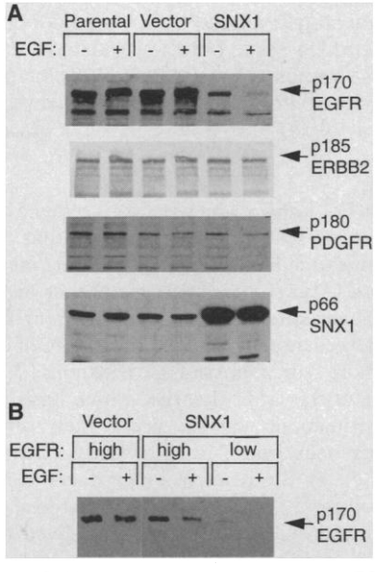

Fig. 2. SNX1 down-regulates endogenous EGFR in CV-1 cells. (**A** and **B**) Whole-cell lysates prepared from serum-starved CV-1 cell transfectants incubated in the absence or presence of 10 nM EGF for 30 min were protein immunoblotted with antibodies to EGFR (rabbit polyclonal antibody 1964), ERBB2 (rabbit polyclonal antibody 1917), PDGF receptor (Transduction Laboratories, Lexington, Kentucky), and SNX1 (affinity-purified rabbit polyclonal antibody).

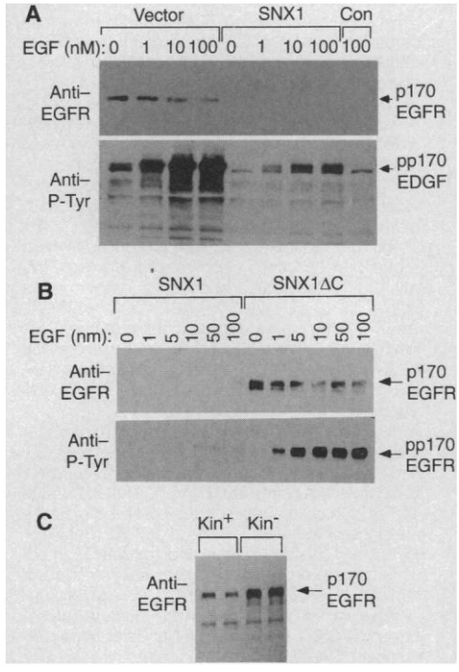

Fig. 3. The EGFR-binding domain of SNX1 is required for down-regulation. (**A**) CMV enhancer–and promoter–driven SNX1 and EGFR cDNAs were cotransfected into 293 cells. After 48 hours, transfected cells were treated with the indicated concentrations of EGF for 30 min. Lysates were protein immunoblotted with antibodies to EGFR (1964) and phosphotyrosine (anti–P-Tyr) (PY20, Transduction Laboratories), the latter of which recognizes autophosphorylated EGFR (pp170 EGFR). Con indicates nontransfected control cells. (**B**) Deletion of the COOH-terminal EGFR-binding domain of SNX1 (residues 456 to 522; SNX1ΔC) abolished the inhibitory activity of SNX1 on expression of cotransfected EGFR. (**C**) Comparison of the efficiency with which cotransfected wild-type (Kin$^+$) or kinase inactive (Kin$^-$) EGFR were down-regulated by SNX1 in 293 cells. Samples are from duplicate transfections.

tion. The Mvp1p-homology domain of SNX1 is a candidate for interacting with other components of the vesicular trafficking machinery to segregate EGFR into endosomal membranes targeted for degradation. These features define a family of proteins that confer specificity on receptor sorting in the endocytic pathway. As a negative regulator of EGFR on cell surfaces, SNX1 influences the efficiency with which EGF activates intracellular signal transduction pathways.

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5. The two-hybrid screening system consisted of yeast strain EGY48 (*MAT***a** *trp1 ura3 his3 LEU2::pLexAop6-LEU2*); plasmids pEG202, pJG4-5, pSH18-34, and pRFHM1; and a HeLa library in pJG4-5 (*14*). A cDNA encoding EGFR residues 663 to 958 was obtained by polymerase chain reaction (PCR) with Pfu polymerase (Stratagene, La Jolla, CA) by using a human EGFR cDNA and cloned into the yeast LexA fusion expression plasmid pEG202. Interactor clones were selected by leucine auxotrophy and galactose-inducible β-galactosidase activity. Library plasmids were recovered from positive colonies with *Escherichia coli* strain KC8 (*pyrF::*Tn*5, hsdr, leuB600, trpC9830, lacD74, strA, galK, hisB436*) and retransformed into EGY48 yeast carrying plasmid (pRFHM1) containing a LexA-bicoid fusion (*4*) to eliminate nonspecific binders. The 5′ ends of specific plasmids were sequenced by cycle sequencing (Epicentre Technologies, Madison, WI) with ^{35}S-labeled deoxyadenosine triphosphate and a primer (5′-GATGTTAACGATACCAGCCTCTTGCT-GAGT-3′) that bound upstream of the vector-cDNA fusion junction. The sequence was used to search the nucleotide sequence databases of the National Center for Biotechnology Information using the BLAST algorithm (*15*).
6. A HeLa λgt11 cDNA library (Clontech, Palo Alto, CA) was plated and screened with a ^{32}P-labeled oligonucleotide complementary to the 5′ end of the SNX1 library plasmid insert (5′-CTTTCTCAAACCTCACT-TCT-3′). Six phages were plaque-purified, the cDNAs subcloned into the Eco RI site of pMOBII (Gold Biotechnology, St. Louis, MO), and transposon insertions used to determine the complete sequence of two of the inserts on both strands. Areas of ambiguity were clarified with gene-specific primers. The sequence of SNX1 is deposited in GenBank (accession number U53225).
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12. An SNX1 (residues 1 to 522) expression vector was prepared in pCEP4 (Invitrogen, San Diego, CA). Transfections were performed by exposure of cells on 6-cm-diameter plates to calcium phosphate DNA coprecipitates (15 μg per 2 × 10^6 cells) for 4 to 6 hours. For transient assays, lysates from human embryonic kidney 293 cells were prepared 48 hours later for protein immunoblotting. Stable African green monkey kidney CV-1 cells were prepared by expanding the cells 48 hours after transfection onto 10-cm-diameter plates in the presence of hygromycin (320 μg/ml) and selected for 14 to 21 days. Clonal lines were prepared from isolated colonies with cloning rings.
13. For the determination of EGFR half-life, CV-1 cells were grown in the absence of methionine in Dulbecco's modified Eagle's medium containing 10% dialyzed calf serum for 2 hours and incubated with ^{35}S-methionine (0.65 mCi/ml) for 1 hour. Cells were incubated in complete medium, and radio immunoprecipitation assay extracts [1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 10 mM Hepes (pH 7.6), and 150 mM NaCl] were prepared after incubation for various times and immunoprecipitated with antibodies 528 and 13A9 to EGFR. After they were washed, the immunoprecipitates were solubilized in SDS sample buffer, electrophoresed, and visualized by fluorography, and the radioactivity was quantified by liquid scintillation counting.
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16. The detergent extraction buffer was 1% Triton X-100, 50 mM Hepes (pH 7.4), 10% glycerol, and 75 mM NaCl. Whole-cell lysate buffer was 1% SDS and 10 mM Hepes (pH 7.4). Buffers were supplemented with the following phosphatase and protease inhibitors: 10 mM NaF, 1 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, leupeptin (10 μg/ml), antipain (10 μg/ml), and aprotinin (10 μg/ml). Products of the coupled rabbit reticulocyte lysate transcription and translation reaction (Promega, Madison, WI) were separated by gel electrophoresis and visualized by fluorography.
17. Cells were fixed in paraformaldehyde; permeabilized with saponin; stained with antibody to SNX1 (anti-SNX1) and a mixture of antibodies to EGFR [immunoglobulin G's (IgGs) 528, 13A9, and 225], followed by Texas Red–conjugated goat anti-rabbit IgG and fluorescein isothiocynate–conjugated goat anti-mouse IgG; and visualized by epifluorescence illumination.
18. We generated the cDNAs encoding EGFR residues 647 to 1186, 647 to 957, and 647 to 942 by PCR with Pfu polymerase (Stratagene) using a human EGFR cDNA, and the products were cloned into the yeast LexA fusion expression plasmid pEG202. The rat ERBB2 core tyrosine kinase domain was generated by PCR. For β-galactosidase assay, extracts were prepared from logarithmic-phase yeast diluted into Ura$^-$His$^-$Trp$^-$ broth containing 2% dextrose or 2% galactose (to induce the library plasmid insert) and grown at 30°C for 18 to 22 hours (*14*).

19. We thank R. Brent for providing the components of the yeast two-hybrid system and A. Nesterov and H. S. Wiley for helpful discussions. Supported by NIH grants F32DK08666 (to R.C.K.) and CA58689 (to G.N.G.) and by funds provided by the Breast Cancer Fund of the State of California through the Breast Cancer Research Program of the University of California, grant numbers 1FB-0314 (to R.C.K.) and 1KB-0140 (to D.L.C.).

22 November 1995; accepted 29 February 1996

Determination of Life-Span in *Caenorhabditis elegans* by Four Clock Genes

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The nematode worm *Caenorhabditis elegans* is a model system for the study of the genetic basis of aging. Maternal-effect mutations in four genes—*clk-1, clk-2, clk-3,* and *gro-1*—interact genetically to determine both the duration of development and life-span. Analysis of the phenotypes of these mutants suggests the existence of a general physiological clock in the worm. Mutations in certain genes involved in dauer formation (an alternative larval stage induced by adverse conditions in which development is arrested) can also extend life-span, but the life extension of Clock mutants appears to be independent of these genes. The *daf-2(e1370) clk-1(e2519)* worms, which carry life-span–extending mutations from two different pathways, live nearly five times as long as wild-type worms.

It is not known why organisms senesce and die. One set of theories suggests that life-span is timed, much like puberty and menopause (*1*). Another set suggests that organisms accumulate damage throughout life, which eventually leads to the failure of one or more critical physiological systems (*2, 3*). One of the latter theories, which has some experimental support, posits that senescence may result from oxidative damage caused by the reactive by-products of metabolism (*3, 4*). One way to investigate the nature of aging is to study long-lived mutants. Here we describe four genes that interact genetically to determine both the duration of postembryonic development and the adult life-span of the nematode worm *Caenorhabditis elegans*.

In a screen for maternally rescued viable mutations in *C. elegans*, we recovered mutations in three genes—*clk-1*, *clk-2*, and *clk-3*—that show the Clock (Clk) phenotype, a pleiotropic alteration of developmental and behavioral timing (*5, 6*). The phenotype of *gro-1(e2400)* places it, too, in the Clk class of genes (*5*). Mutations in the best characterized of these genes, *clk-1*, lengthen early embryonic cell cycles, embryonic and postembryonic development, as well as the period of rhythmic adult behaviors, such as swimming, pharyngeal pumping, and defecation (*5*). Furthermore, *clk-1* mutants have a longer mean and maximum life-span than the standard wild-type strain N2 (*5*).

To characterize these genes further, we determined the life-span of the reference strain of these genes at 15°, 18°, 20°, and 25°C. All four mutants have longer mean and maximum life-spans than the wild type at all temperatures (Table 1). For example, at 18°C, all Clk mutants have a mean life-span at least 3 days longer than that of the

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