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# Life span extension by dietary restriction is reduced but not abolished by loss of both SIR2 and HST2 in *Podospora anserina*

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ABSTRACT

Dietary restriction (DR) extends life span of many organisms, from yeast to mammals. The question of whether or not the SIR2 protein functions to mediate life span extension in response to DR remains debated. In this paper, we studied the relationship between SIR2 and DR in the filamentous fungus *Podospora anserina*. We show that the loss of *PaSir2*, *PaHst2* or *PaPnc1* does not alter life span under standard conditions. *PaHst2* is the closest paralog of *PaSir2* and the ortholog of yeast *HST2* and *PaPnc1* is the ortholog of the yeast *PNC1* which encodes a nicotinamidase that deaminates nicotinamide, a natural inhibitor of SIR2. As observed for other organisms, overexpression of *PaSir2* weakly increases life span under standard condition. Under DR conditions, deletion of the *PaSir2* or *PaHst2* genes induce a significant reduction in life span extension, while the double mutant strongly reduces life span extension. However, a clear response to DR subsists in the double mutant, demonstrating that DR acts through a SIR2/HST2 independent pathway.



## 1. Introduction

Dietary restriction (DR) is the most robust non-genetic intervention that increases life span in numerous species (Masoro, 2005). However, mechanisms responsible for the anti-aging effects of DR remain uncertain (Kennedy et al., 2007). In the yeast *Saccharomyces cerevisiae* as in *Drosophila* it has been shown that the extension of life span by DR requires *SIR2*, a protein encoding an $NAD^+$-dependent histone deacetylase (Anderson et al., 2003; Lin et al., 2000, 2002; Rogina and Helfand, 2004). The *Caenorhabditis elegans sir-2.1* homolog was also thought to act through a DR pathway (Wang and Tissenbaum, 2006). In addition, it has been shown that overexpression of *SIR2* extends the replicative life span of the yeast *S. cerevisiae* (Kaeberlein et al., 1999) and life span of post-mitotic animals such as *C. elegans* and *Drosophila melanogaster* (Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). This result has been strengthened in yeast by the overexpression of *PNC1* that also leads to an increase of life span (Anderson et al., 2003). *PNC1* encodes a nicotinamidase that deaminates nicotinamide, which is a natural inhibitor of Sir2p (Bitterman et al., 2002). Thus, either the increase in copy number of the *SIR2* gene or the direct increase in Sir2p activity can lead to an increase in life span. In yeast, this effect is thought to result from a reduced formation of extra-chromosomal circles of rDNA, known as ERCs, which accumulate at lower levels in the presence of increased activity of SIR2 (Sinclair and Guarente, 1997). Since the accumulation of ERCs is not known to be correlated with aging of other systems than yeast, the mechanism whereby *SIR2* is implicated in the control of life span in other organisms is currently unclear. Recently, the mediation of the DR effect via *SIR2* has been strongly challenged in yeast by the observation that DR can extend life span in the absence of *SIR2* (Kaeberlein et al., 2004) and in *C. elegans*, where it has been shown that the lack of SIR2 has no effect on the life span extension by DR due to the 2-deoxy-D-glucose (Schulz et al., 2007), complete removal of food (Hansen et al., 2007; T.L. Kaeberlein et al., 2006; Lee et al., 2006), or mutation in the *eat-2* gene (Hansen et al., 2007). The debate over this question remains very active at the moment: some authors propose that HST2, one of the four SIR2 paralogs, is responsible for the SIR2-independent DR effect while opponents favor a parallel pathway that is indepen-

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dent of all SIR2 paralogs (Guarente, 2005; M. Kaeberlein et al., 2006; Kennedy et al., 2005; Lamming et al., 2005, 2006; Longo and Kennedy, 2006).

To further understand the role of *SIR2* in the control of aging across species, we addressed two major questions to the filamentous fungus *Podospora anserina* which presents a limited life span (Rizet, 1953) and is sensitive to DR (Maas et al., 2004): does the overexpression of *SIR2* extend life span in *P. anserina* and does its absence inhibit the response to DR? We show here that the overexpression of *PaSir2* weakly increases life span under standard conditions. Moreover, loss of *PaSir2*, *PaHst2* or *PaPnc1* does not alter life span under standard conditions. Under DR conditions, single *PaSir2* or *PaHst2* deletions slightly reduce life span extension, while the double mutant strongly reduces life span extension. However, even the double mutant remains sensitive to DR suggesting that at least one other pathway is involved in the DR response.

## 2. Materials and methods

### 2.1. P. anserina strains and growth conditions

All strains used in this study were derived from the *s* wild type strain (Rizet, 1952). Life span analyses were performed on standard synthetic medium (M2, containing 10 g per liter of dextrin) as described (Bauer et al., 1994). For dietary restriction assays, the concentration of dextrin has been chosen according to the results of different tests performed across a range of dextrin concentrations. On the one hand, 5 g per liter is not sufficient to induce a strong life span extension in the wild type and in the *PaSir2Δ* or *PaHst2Δ* mutant strains. On the other hand, 0.1 g per liter induces a strong DR response (both phenotypically and for the life span extension) but growth rate is reduced, suggesting that mycelia begin to suffer from a lack in nutrients. Therefore, dietary restriction was realized by lowering down the amount of dextrin ten times to 1 g per liter.

### 2.2. Phylogenetic analysis

Protein alignments were carried out using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). The result was inspected by eye and manually edited using BioEdit v7.0.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Five domains encompassing a total of 87 amino acids were retained (Fig. 1A). An unrooted cladogram was constructed by maximum likelihood using on-line PHYML (Guindon and Gascuel, 2003; Guindon et al., 2005) (Fig. 1B). The reliability of branches was evaluated by bootstraping with 500 replicates. Identical groups of orthologs were obtained using Neighbor-Joining method (Thompson et al., 1997 and data not shown).

### 2.3. Making of strains carrying loss of function or overexpressing alleles

The construction of 'knock-out' strains of *PaSir2*, *PaHst2* and *PaPnc1* has been described (El-Khoury et al., 2008). A pcdna2-1 vector containing the *PaSir2* gene (#GA0AA12AG05) was obtained from a shotgun library made for the *P. anserina* genome project in collaboration with the Genoscope (Evry, France). A 680 bp-long

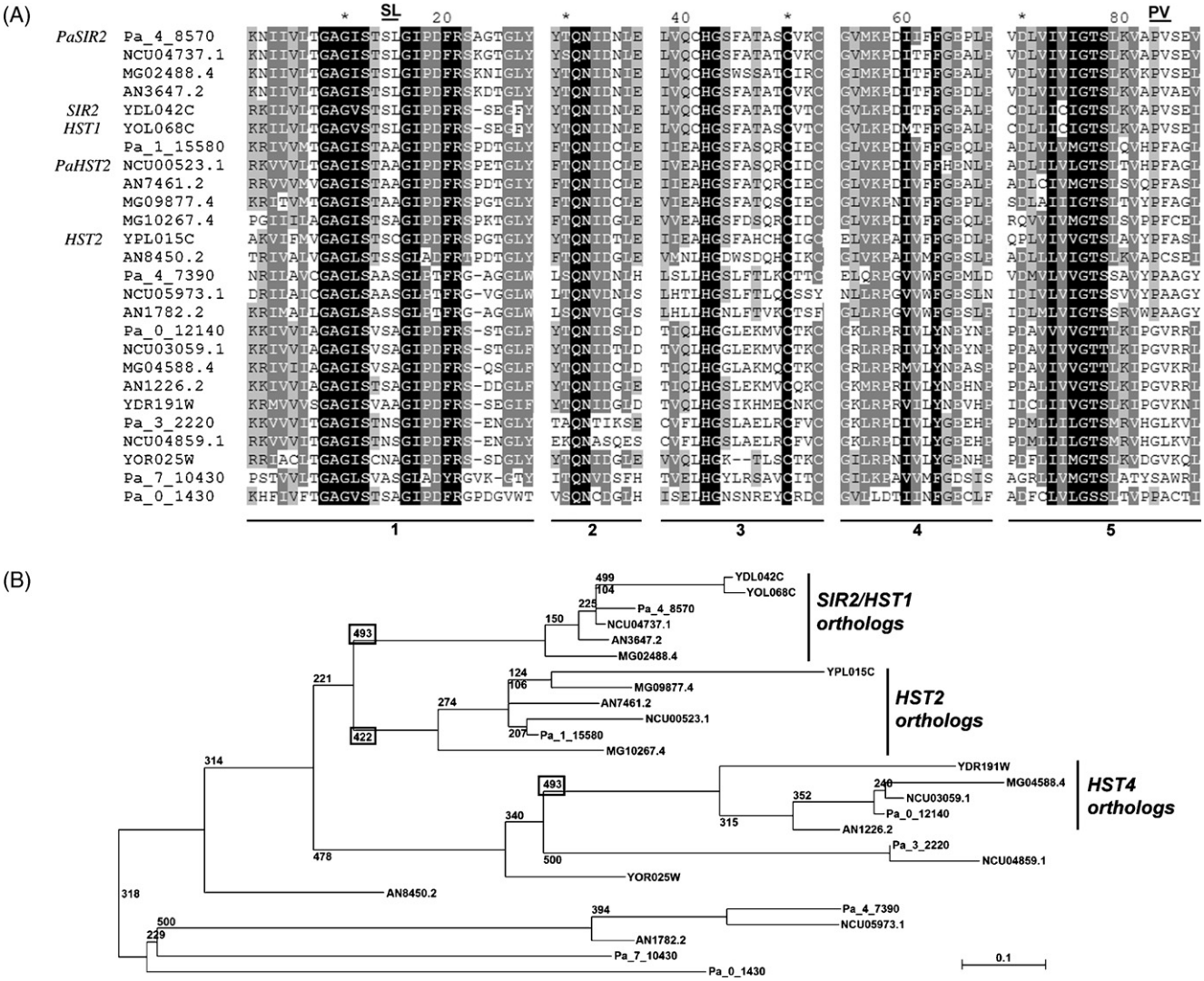


**Fig. 1.** Phylogeny of Sirtuins in fungi. (A) Alignment of five conserved domain peptide sequences (underlined) of the Sirtuins from *S. cerevisiae* (Y), *P. anserina* (Pa), *M. grisea* (MG), *A. nidulans* (AN) and *N. crassa* (NC). Two specific motives of the group of orthologs SIR2/HST1 are indicated (SL and PV). (B) Maximum likelihood tree reveals the Sirtuins orthologs groups. Bootstrap values are given at branch points. Nodes defining orthologs groups are outlined.