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ABSTRACT

The translation terminator Sup35p assembles into self-replicating fibrillar aggregates that are responsible for the [*PSI*$^+$] prion state. The Q/N-rich N-terminal domain together with the highly charged middle-domain (NM domain) drive the assembly of Sup35p into amyloid fibrils *in vitro*. NM domains are highly divergent among yeasts. The ability to convert to a prion form is however conserved among Sup35 orthologs. In particular, the *Yarrowia lipolytica* Sup35p stands out with an exceptionally high prion conversion rate. In the present work, we show that different *Yarrowia lipolytica* strains contain one of two Sup35p orthologs that differ by the number of repeats within their NM domain. The *Y. lipolytica* Sup35 proteins are able to assemble into amyloid fibrils. Contrary to *S. cerevisiae* Sup35p, fibrils made of full-length or NM domains of *Y. lipolytica* Sup35 proteins did not bind Thioflavin-T, a well-known marker of amyloid aggregates.

1. Introduction

In yeast, the eukaryotic release factor Sup35p, required for translation termination [1–3], has prion properties responsible for the [*PSI*$^+$] trait [4–6]. In [*PSI*$^+$] cells, soluble Sup35p is dramatically depleted by its aggregation into self-replicating fibrillar protein assemblies. As a consequence, defective translation termination leads to increased nonsense suppression events [4–10].

The domains responsible for the translation termination and prion propagation functions within Sup35p are well defined [11,12]. The N-terminal domain (spanning amino acid residues 1–123) is critical for prion propagation, driving the switch from the monomeric, functional [*psi*$^-$] state to the aggregated [*PSI*$^+$] prion state. This domain is unusually rich in glutamine and asparagine residues, and contains several imperfect repeats of the aggregation-prone sequence PQGGYQQ-YN [13]. The overexpression of the N-terminal domain in cells expressing full-length Sup35p triggers prion conversion [14], whereas replacing Sup35p by a variant containing only the C-terminal domain causes the irreversible loss of [*PSI*$^+$] [15–17]. Various mutations within the N-terminal Q/N-rich region or the oligopeptide repeats in the prion domain have been previously shown to affect the propagation, strength and stability of [*PSI*$^+$] [18,19]. The highly charged middle domain (M, amino acids 124–253) is dispensable for prion propagation, yet it increases the stability of the protein, ensures steady [*PSI*$^+$] propagation during cell division and is an interaction site for molecular chaperones [20]. The translation termination function of Sup35p requires the highly conserved and compactly folded C-terminal GTPase domain (amino acids 254–685) [11,21]. We previously showed that point mutations within the C-terminal domain of Sup35p affect [*PSI*$^+$] propagation [22].

Full-length Sup35p and its N and NM domains spontaneously assemble into protein fibrils *in vitro* [9,23–25]. Fibrils formed by Sup35 N or Sup35NM exhibit the characteristics of amyloids in that they are unbranched, have increased resistance to proteolysis, bind Congo red and Thioflavin T, exhibit a 4.7 Å reflection in X-ray fiber diffraction images and exhibit Fourier Transform Infrared (FT-IR) spectra dominated by a peak at 1620 cm^{-1} [9,23,26,27]. We previously showed that Sup35NM and full-length Sup35p assemblies are different [25].

Sup35p NM domains primary structure are highly divergent among yeasts. Nonetheless, the ability of Sup35p to convert to a prion form appears conserved among distantly related yeasts [28–31]. In particular, when expressed in *S. cerevisiae,* the *Yarrowia lipolytica* Sup35p stood out with an exceptionally high spontaneous

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prion conversion rate of 10^{-1} compared to 10^{-5}-10^{-7} for other yeast orthologs [31]. Thus, it is of interest to understand how the behavior of *Y. lipolytica* Sup35p observed in a heterologous cellular context translates *in vitro* in terms of fibrillar assembly properties.

Here, we show that different *Y.lipolytica* strains contain one of two Sup35p orthologs that differ by the number of repeats within their NM domain. The *Y. lipolytica* Sup35 proteins are able to assemble into protein fibrils that we show to be of amyloid nature by FT-IR spectroscopy. Interestingly, contrary to *S. cerevisiae* Sup35p, fibrils made of full-length or NM domains of *Y. lipolytica* Sup35 proteins did not bind Thioflavin-T, a well-known marker of amyloid aggregates.

2. Materials and methods

2.1. Nomenclature note

To avoid confusion between orthologs, the *Saccharomyces cerevisiae* Sup35p and Sup35NM proteins are hereafter named *Sc.*Sup35p and *Sc.*Sup35NM throughout the manuscript. The corresponding *Yarrowia lipolytica* orthologs are hereafter named *Yl.*Sup35p and *Yl.*Sup35NM.

2.2. Strains and plasmids

Plasmids pET15b-*SUP35* and pET15b-*SUP35*NM allowing the overexpression of hexahistidine-tagged *Sc.*Sup35p and *Sc.*Sup35NM in *Escherichia coli* were previously described [24,25]. The *Yl.SUP35-A* and *Yl.SUP35*NM-*A* coding sequences were obtained by PCR using genomic DNA from the *Yarrowia lipolytica* strains 136,463 and PO1d [32,33]. The *Yl.SUP35*–B and *Yl.SUP35*NM-*B* coding sequences were obtained by PCR using genomic DNA from the *Yarrowia lipolytica* strain IAM-4948 (a generous gift from Dr. Yoshikazu Nakamura, University of Tokyo) [31]. PCR fragments were cloned at the *Nde*I-*Bam*HI sites in pET15b, generating the pET15b-*Yl.SUP35-A,* pET15b-*Yl.SUP35*–B, pET15b-*Yl.SUP35*NM-*A* and pET15b-*Yl.SUP35*NM-*B* plasmids, allowing the overexpression of corresponding hexahistidine-tagged proteins. All constructs were verified by DNA sequencing.

2.3. Assembly of Sup35p into protein fibrils

The Sup35 proteins were overexpressed in *Escherichia coli* strain BL21-CodonPlus, purified by affinity chromatography as previously described [24,25], and stored at −80 °C. At least two independent preparations of each protein were used throughout the study. Sup35 proteins were dialyzed for 2 h at 4 °C against the assembly buffer (50 mM Tris.Cl, pH 8.0, 200 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, 2 mM EGTA) and centrifuged for 5 min at 15,000 g and at 4 °C. The supernatant was recovered, the protein concentration was adjusted to the desired concentration (typically 10 μM) in assembly buffer and the samples incubated at 6 °C under very gentle agitation (<100 rpm). At regular time intervals, aliquots were removed from the assembly reaction and mixed with Thioflavin T (10 μM). Fluorescence was recorded with a Quantamaster QM 2000-4 spectrofluorometer (Photon Technology International, NJ) using excitation and emission wavelengths set at 440 and 480 nm.

2.4. Electron microscopy

Fibril preparations were imaged in a Jeol 1400 transmission electron microscope on carbon-coated grids (200 mesh) following negative staining with 1% uranyl acetate. Images were recorded with a Gatan Orius CCD camera (Gatan Inc, Pleasanton, CA, USA) and processed with the ImageJ software (NIH).

2.5. Fourier transform InfraRed spectroscopy (FTIR)

FTIR spectra recording and analysis was performed as described previously [22]. Fibrils were centrifuged for 20 min at 16,000 g and at 4 °C then extensively washed with D_2O. The spectra were recorded on a JASCO 660 Plus FTIR spectrometer equipped with a nitrogen-cooled MTC detector. The background consisted of D_2O and water vapor. A total of 512 interferograms were collected with a resolution of 2 cm^{-1}. The sample chamber was continuously purged with CO_2-free dry air. All the spectra were baseline-corrected, smoothed and normalized prior to further data processing. The amide I (1600-1700 cm^{-1}) band of the spectra was fitted using a Gaussian species model centered at 1624, 1640, 1652, 1664, and 1678 cm^{-1} (during the fitting procedure, the peak positions were free to vary inside an interval width that was limited to 25 cm^{-1} while peak height was free).

3. Results

3.1. The Yarrowia lipolytica Sup35p A and B orthologs display different NM domains

We found that different *Y. lipolytica* strains encode one of two different *SUP35* orthologs. The *SUP35* ortholog we cloned from the 136,463 and PO1d strains, which are related to the W29 strain used for the *Y. lipolytica* genome sequencing [34], was named *Yl.SUP35* A and encodes a 728 amino acids long *Yl.*Sup35p A protein (Fig. 1A). The *SUP35* ortholog we cloned from the IAM-4948 strain previously used by Nakamura and colleagues [31] was named *Yl.SUP35* B and encodes a 742 amino acids long *Yl.*Sup35p B protein (Fig. 1A). The differences between the *Y. lipolytica* and *S. cerevisiae* Sup35 proteins were previously described [31] and lie essentially in their NM domains which contain very different repeated motifs (Fig. 1B and Table 1). *Yl.*Sup35p A and *Yl.*Sup35p B differ by the number of each of the four repeats found in their NM domains (Fig. 1B and Table 1). Conversely, as expected from the essential nature of the function it fulfills, the C-terminal eRF3 domains of *S. cerevisiae* and *Y. lipolytica* Sup35p orthologs are highly conserved (Fig. 1B).

3.2. Yarrowia lipolytica Sup35 proteins assemble into thioflavin T-negative amyloid fibrils

S. cerevisae and *Y. lipolytica* Sup35 proteins were overexpressed in *E. coli* and purified as previously described [24,25]. Next, we assessed the ability of these purified proteins to spontaneously assemble into fibrils under physiological pH and salt concentration conditions [22,24,25]. The assembly kinetics were followed by Thioflavin-T binding, as described [22,24,25,35]. As expected from our previous work, *Sc.*Sup35p formed Thioflavin T-positive fibrils after 2–3 days of incubation (Fig. 2A and Fig. 2B). No Thioflavin T fluorescence was detected upon incubation of *Yl.*Sup35p A and *Yl.*Sup35p B proteins under the same conditions for over 30 days, suggesting these proteins did not assemble into fibrils (Fig. 2A). Indeed, negative-stained electron microscopy of *Y. lipolytica* Sup35 proteins revealed only amorphous aggregates, oligomers and pre-fibrillar species during the first 7 days of incubation (Fig. 2C). However, longer incubations of 30 days allowed the *Yl.*Sup35p A and *Yl.*Sup35p B proteins to assemble into fibrils (Fig. 2C). *Sc.*Sup35p, *Yl.*Sup5p A and *Yl.*Sup35p B fibrils displayed very similar morphologies (Fig. 2B and C). Modifications in the experimental conditions of the assembly reactions (*e.g.* temperature, agitation speed) did not allow a faster assembly of *Yl.*Sup35p proteins nor did it result in the formation of Thioflavin T-positive fibrils (data not

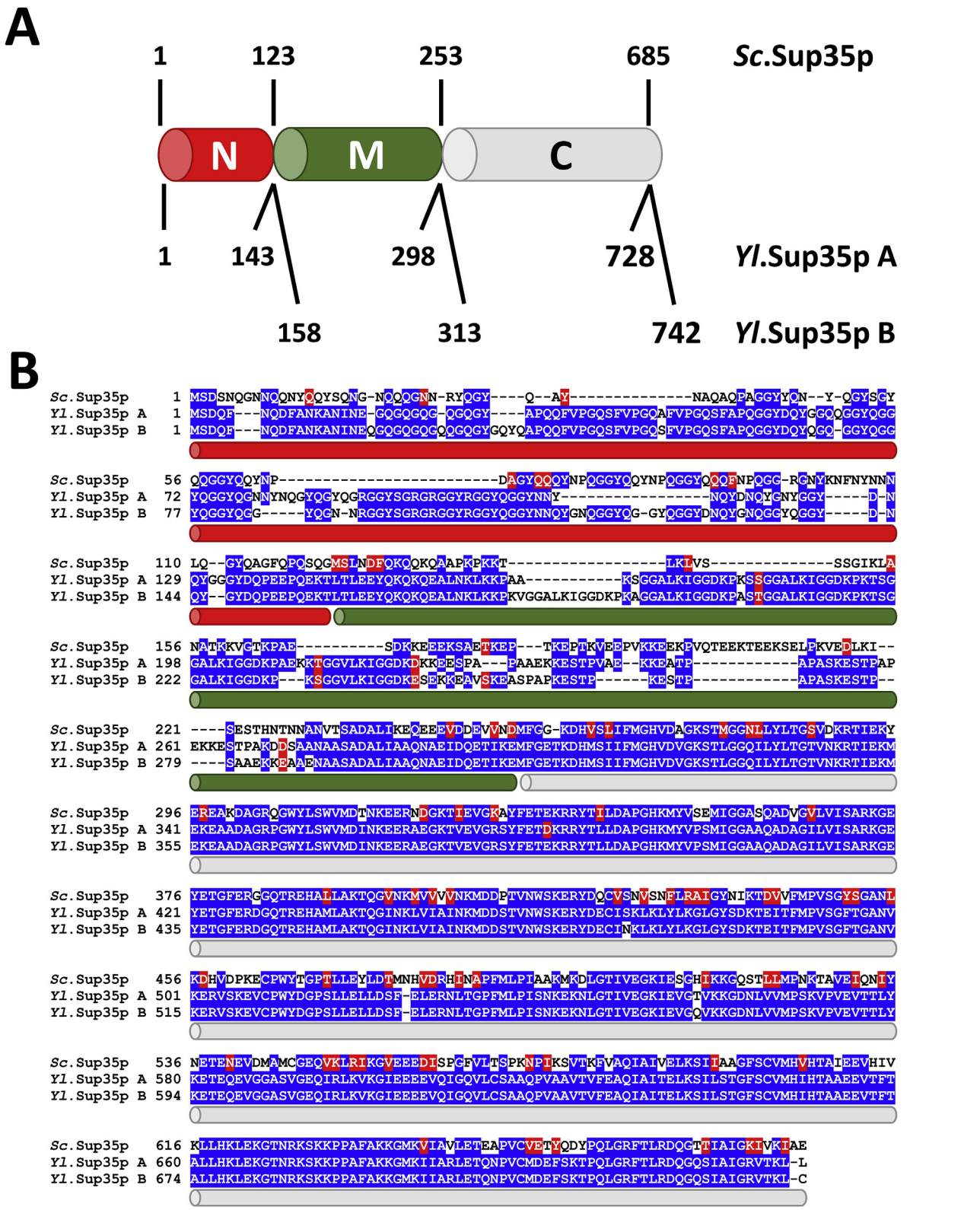


Fig. 1. Alignment of *Sc*.Sup35, *Yl*.Sup35p A and *Yl*.Sup35p B proteins. The *S. cerevisiae Sc*.Sup35p coding sequence (Uniprot P05453) was obtained from the 74-D694 strain. The *Yl*.Sup35p A coding sequence was obtained from *Y. lipolytica* strains 136,463 and PO1d (Uniprot Q6CFC9). The *Yl*.Sup35p B coding sequence was obtained from *Y. lipolytica* strain SS397A (accession number AB039752). **(A)** Primary structure and domain organization of *Sc*.Sup35p, *Yl*.Sup35p A and *Yl*.Sup35p B proteins (see text for details). **(B)** Sequence alignment of *Sc*.Sup35p, *Yl*.Sup35p A and *Yl*.Sup35p B proteins (the alignment was made using T-COFFEE and BOXSHADE).

Table 1
Consensus sequences and positions of the repeat motifs (R) in *S. cerevisiae* and *Y. lipolytica* Sup35 proteins.

protein	consensus motif	motif residues				
Sc.Sup35p	PQGGYQQ-YN	R1 [41–49]	R2 [56–64]	R3 [65–74]	R4 [75–83]	R5 [84–93]
Yl.Sup35p A	FVPGQS	R1 [34–39]	R2 [40–44]	R3 [46–51]		
	QGGYQGGYQGGY	R1 [65–76]]	R2 [77–94]	R3 [99–110]	R4 [118–130]	
	GGALKIGGDKP	R1 [169–179]	R2 [183–193]	R3 [197–207]	R4 [213–222]	
	KESTP	R1 [236–240]	R2 [245–249]	R3 [254–258]	R4 [263–267]	
Yl.Sup35p B	FVPGQS	R1 [40–45]	R2 [46–51]	R3 [52–57]		
	QGGYQGGYQGGY	R1 [70–81]	R2 [82–93]	R3 [98–109]	R4 [116–127]	R5 [134–145]
	GGALKIGGDKP	R1 [180_190]	R2 [193–203]	R3 [207–217]	R4 [221–231]	R5 [234–243]
	KESTP	R1 [260–264]	R2 [265–269]	R3 [274–278]		

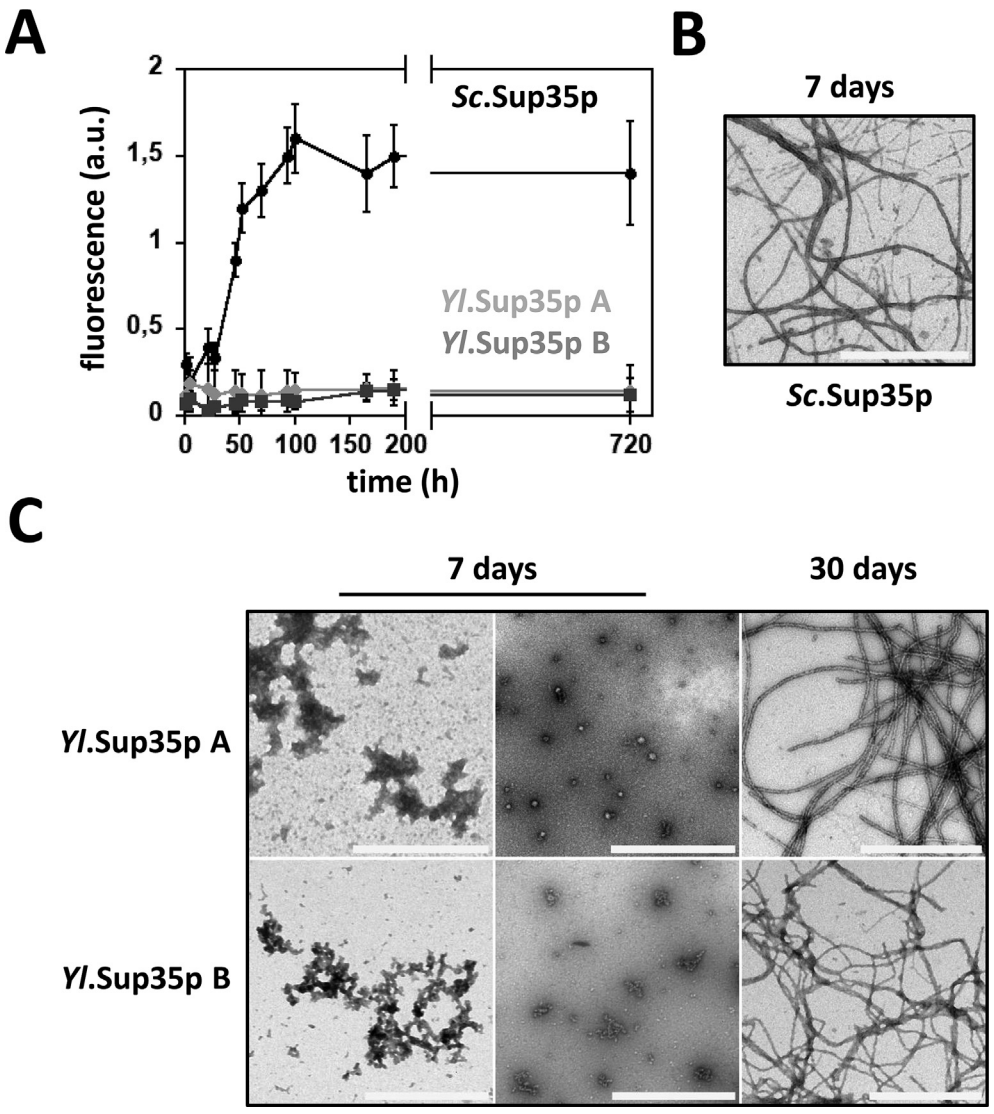


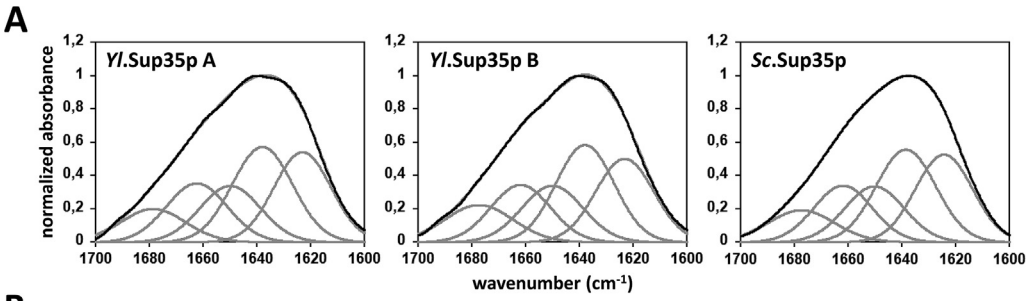
Fig. 2. Assembly of full-length *Y. lipolytica* and *S. cerevisiae* Sup35p proteins. (A) Full-length *Y. lipolytica* and *S. cerevisiae* Sup35 proteins were allowed to assemble at a final concentration of 10 μM and at 6 °C under very low agitation (<100 rpm). Aliquots were removed at regular time intervals and assayed using the Thioflavin T binding assay described in the Materials and Methods section. **(B, C)** Assembly reactions were imaged at the indicated time points using negative-stained electron microscopy (scale bars, 1 μm).

shown).

Next, we assessed the secondary structure content of fibrillar *Sc*.Sup35p, *Yl*.Sup35p A and *Yl*.Sup35p B by Fourier transform infrared (FTIR) spectroscopy (Fig. 3A). The FT-IR spectra showed very similar amide I bands for all fibrils, with a shoulder at 1624 cm^{-1} indicative of a cross-β-sheet amyloid structure (Fig. 3A). Fourier deconvolution and curve fitting of the spectra showed no significant differences in the secondary structure content of the three kinds of fibrils, with ~62% of β-sheet structures (Fig. 3B), in agreement with previous results [22].

The NM domains of *Yarrowia lipolytica* Sup35p A and B assemble into thioflavin T-negative fibrils.

We previously showed that the C-terminal domain of *Sc*.Sup35p affects its assembly properties into fibrils, and that fibrillar



B

protein	β-sheets	α-helix	others
Sc.Sup35p	62%	19%	19%
Yl.Sup35p A	62%	19%	19%
Yl.Sup35p B	62%	19%	19%

Fig. 3. FT-IR spectra of fibrillar *Y. lipolytica* and *S. cerevisiae* Sup35 proteins. **(A)** The recorded FT-IR spectra (black lines) and curve fit data (grey lines) are shown for fibrillar *Y. lipolytica* and *S. cerevisiae* Sup35 proteins. The spectra were fitted using a Gaussian species model centered at 1624, 1640, 1652, 1664 and 1678 cm-1. **(B)** Secondary structure content of *S. cerevisiae* and *Y. lipolytica* Sup35p fibrils derived from the deconvolution of the FTIR data presented in (A).

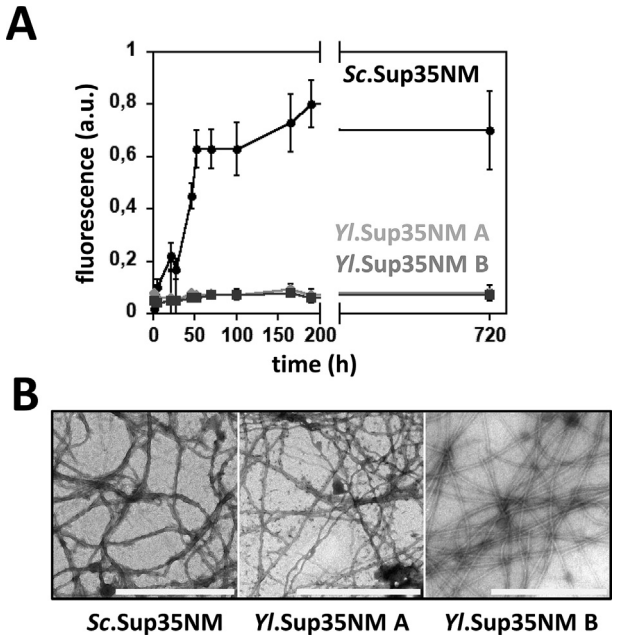


Fig. 4. Assembly of *Y. lipolytica* and *S. cerevisiae* Sup35NM proteins. **(A)** The indicated purified proteins were allowed to assemble at a final concentration of 10 μM at 6 °C under very low agitation (<100 rpm). Aliquots were removed at regular time intervals and assayed using the Thioflavin T binding assay described in the Materials and Methods section. **(B)** *Sc*.Sup35NM (7 days), *Yl*.Sup35NM A and *Yl*.Sup35NM B (30 days) fibril preparations were imaged using negative-stained electron microscopy (scale bars, 1 μm).

Sc.Sup35p and *Sc*.Sup35NM show significant structural differences [22,25,36]. We then asked whether the *Yl*.Sup35NM and B domains would behave differently than their full-length counterparts. The NM domains of *S. cerevisiae* and *Y. lipolytica* Sup35 proteins were overexpressed in *E. coli,* purified, and their ability to assemble into fibrils assessed by Thioflavin T binding and negative-stain electron microscopy (Fig. 4). While the *Sc*.Sup35NM domain readily assembled into thioflavin T-positive fibrils [25], *Yl*.Sup35NM A and *Yl*.Sup35NM B, like their full-length counterparts, were only able to assemble into fibrils that remained Thioflavin T-negative after 30 days of incubation (Fig. 4).

4. Discussion

In the present paper, we show that two phylogenetically divergent *Y. lipolytica* Sup35p orthologs assemble into amyloid fibrils devoid of thioflavin T binding capacity. *Y. lipolytica* IAM-4948 strain expresses the *Yl*.Sup35p B ortholog [31], while the W29 reference strain (and its derivatives) expresses the *Yl*.Sup35p A ortholog [34] (Fig. 1 and Table 1). Despite the slight differences observed between these two orthologs, mostly within their NM domains (Table 1), purified *Yl*.Sup35p A and *Yl*.Sup35p B behaved similarly in our test tube assays (Figs. 2–4).

In a *S. cerevisiae* reporter system, *Y. lipolytica* Sup35p showed an unusually high propensity to convert to the prion form [31]. From these data, we expected the assembly properties of *Yl*.Sup35p *in vitro* to mirror its behavior *in vivo*, meaning fast assembly kinetics. Contrary to this initial assumption, the assembly of *Yl*.Sup35p and *Yl*.Sup35NM, realized under the same experimental conditions as those used for *Sc*.Sup35p and *Sc*.Sup35NM [22,24], was very slow and it took up to 30 days to obtain homogenous fibrillar preparations (Fig. 2A, C and 4). Oligomers and amorphous or prefibrillar aggregates, which could correspond to intermediates *en route* to assembly, were observed before *Yl*.Sup35p fibrils formed (Fig. 2C).

Thioflavin T, as well as other fluorescent dyes (*e.g.* Congo red, primuline), are often used as markers of amyloids. *Yl*.Sup35p and *Yl*.Sup35NM fibrils did not bind Thioflavin T in our experimental conditions (Figs. 2A and 4A) despite their amyloid nature, as demonstrated by FT-IR spectroscopy (Fig. 3). We recently reported that amyloid fibrils made of dipeptide-repeat proteins do not bind Thioflavin T albeit their amyloid nature [37]. Similarly, human and fish islet amyloid polypeptide (IAPP), which differ by 11 non conservative substitutions, form thioflavin T-positive and thioflavin T-negative amyloid fibrils, respectively [38]. We also showed in the past not only that non-amyloid and amyloid Ure2p yeast prion fibrils bind Thioflavin T [39] but also that non fibrillar Ure2p assemblies obtained in the presence of Hsp104p bind Thioflavin T to a higher extent than fibrillar assemblies [40]. Finally, we demonstrated that Congo red binding to fibrillar assemblies and the associated yellow-green birefringence in polarized light are not indicative of an amyloid nature [41]. Thus, cautions need be taken when using fluorescent dyes to assess the assembly and structural characteristics of prions and prion-like proteins.

Several hypotheses can be made to account for the marked

differences we report for the behavior of *Yl*.Sup35p *in vitro* compared to what was previously known from *in vivo* assays [31]. These could lie in fundamental differences between our experimental assembly conditions and the unique environment of the cytosol. The unusually high prion conversion rate of *Yl*.Sup35p could also result from its specific or stochastic interactions with partner proteins within the *S. cerevisiae* reporter cells [31], such as components of the translation machinery, molecular chaperones or the cytoskeleton, that are likely to affect its assembly properties. The importance of the cellular context will need to be determined by assessing whether *Yl*.Sup35p can form prions, and at which frequency, in *Yarrowia lipolytica*.

Author contributions

MK designed and performed the experiments. MK and RM analyzed the data and wrote the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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