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Céline Martineau

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THÈSE

pour obtenir le grade de

Docteur

de

**l'Institut des Sciences et Industries du Vivant et de l'Environnement
(AgroParisTech)**

Spécialité : Sciences de la vie et de la santé

*présentée et soutenue publiquement
par*

Céline MARTINEAU

le 28 Janvier 2010

**SPECIFICITES FONCTIONNELLES DES Hsp70 CYTOPLASMIQUES
CHEZ LA LEVURE**

Directeur de thèse : Mehdi KABANI

CNRS, UPR 3082 Laboratoire d'Enzymologie et Biochimie Structurales, F-91198 Gif-sur-Yvette

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M Mehdi KABANI, Docteur, **CNRS UPR 3082**
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Résumé - Abstract

Les Hsp70 constituent une famille de chaperons moléculaires ubiquitaires qui jouent des rôles essentiels dans le repliement, le transport ou la dégradation des protéines. Le cytoplasme des cellules eucaryotes contient plusieurs paralogues de Hsp70 fortement conservés qui diffèrent essentiellement par leur expression spatio-temporelle. Plusieurs travaux suggèrent que ces paralogues ont des spécificités fonctionnelles que nous avons cherché à mettre en lumière et caractériser par des approches génétiques.

Dans une première étude, nous avons comparé les activités des Hsp70 des levures *Saccharomyces cerevisiae* (Ssa1-4) et *Yarrowia lipolytica* (Ssa5-8) lorsqu'elles sont exprimées comme unique Hsp70 chez *S. cerevisiae*. Nous avons montré que ces Hsp70 : 1) assurent la viabilité des cellules mais avec des taux de croissance très différents ; 2) ont des effets variables sur la propagation et la stabilité des prions [*URE3*] et [*PSI+*] ; et 3) permettent la dégradation protéasomale de CFTR avec des cinétiques comparables. Dans une seconde étude, nous avons montré que la formation de biofilms chez la levure dépend de la machinerie Hsp70 qui contrôle, *via* des voies distinctes, l'expression, la maturation et le recyclage d'une adhésine de surface (Flo11) requise pour ce processus. Enfin, nous avons construit et caractérisé des mutants de *Y. lipolytica* dans lesquels un ou plusieurs gènes codant des chaperons moléculaires et autres acteurs de la protéostase (*e.g.* Hsp70, Hsp104, CHIP) ont été invalidés.

Malgré une forte homologie et une redondance fonctionnelle, les Hsp70 possèdent des propriétés distinctes permettant aux cellules de faire face à différents types de substrats et de conditions de stress.

Mots clés : Hsp70 ; Hsp40 ; levure ; prion ; biofilm

Functional specificities among yeast cytosolic Hsp70s

Hsp70s are a highly conserved family of ubiquitous molecular chaperones that play essential roles in protein folding, transport or degradation. The cytosol of most eukaryotic cells contains multiple highly conserved Hsp70 orthologs that differ mainly by their spatio-temporal expression patterns. While several reports suggest that specialized functions of Hsp70 orthologs were selected through evolution, few studies addressed systematically this issue.

First, we compared the ability of Ssa1-Ssa4 from *Saccharomyces cerevisiae* and Ssa5-Ssa8 from the evolutionary distant yeast *Yarrowia lipolytica* to perform Hsp70-dependent tasks when expressed as the sole Hsp70 for *S. cerevisiae* *in vivo*. We showed that Hsp70 isoforms: 1) supported yeast viability yet with markedly different growth rates; 2) influenced the propagation and stability of the [*PSI+*] and [*URE3*] prions; but 3) did not significantly affect the proteasomal degradation rate of CFTR. Second, we showed that biofilm formation in yeast depends on the Hsp70 machinery that controls, through distinct pathways, the expression, maturation and recycling of a cell-surface adhesin (Flo11) required for this process. Finally, we constructed and analyzed *Y. lipolytica* mutants bearing one or multiple deletion(s) in genes encoding molecular chaperones and others proteostasis modulators (*e.g.* Hsp70, Hsp104, CHIP).

Despite very high homology and overlapping functions, the different Hsp70 orthologs have evolved to possess distinct activities that are required to cope with different types of substrates or stress situations.

Keywords: Hsp70; Hsp40; yeast; prion; biofilm

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... A la douceur toujours vivante du sorbet à la framboise.

« Les vérités polyphoniques de la complexité exaltent [...]. Il est tonique de s'arracher à jamais au maître mot qui explique tout, à la litanie qui prétend tout résoudre. Il est tonique enfin de considérer le monde, la vie, l'homme, la connaissance, l'action comme systèmes ouverts. L'ouverture, brèche sur l'insondable et le néant, blessure originaire de notre esprit et de notre vie, est aussi la bouche assoiffée et affamée par quoi notre esprit et notre vie désirent, respirent, s'abreuvent, mangent, baisent. »

Edgar Morin - Le paradigme perdu : la nature humaine, page 233, Editions du Seuil 1973

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Liste des abréviations

5-FOA : *5-FluoroOrotic Acid* ; acide 5-fluoroorotique

Bag1 : *Bcl-2-associated athanogene 1* ; athanogène 1 associé à Bcl-2

BAP : *BiP-Associated Proteins* ; protéines associées à BiP

CFTR : *Cystic Fibrosis Transmembrane conductance Regulator* ; régulateur transmembranaire associé à la mucoviscidose

CHIP : *Carboxyl terminus of Hsp70-Interacting Protein* ; protéine interagissant avec la région C-terminale des Hsp70

DTT : DiThioThreitol

ERAD : *Endoplasmic Reticulum-Associated Degradation* ; dégradation associée au réticulum endoplasmique

GPI : GlycosylPhosphatidylInsositol

HA : HémAgglutinine

HSE : *Heat Shock Element* ; élément du choc thermique

HSF : *Heat Shock transcription Factor* ; facteur de transcription du choc thermique

Hsp : *Heat Shock Proteins* ; protéines de choc thermique

MAPK : *Mitogen-Activated Protein Kinase* ; protéine kinase activée par les mitogènes

NAC : *Nascent chain-Associated Complex* ; complexe associé aux chaînes naissantes

NBD : *Nucleotide Binding Domain* ; domaine de liaison au nucléotide

NEF : *Nucleotide Exchange Factor* ; facteur d'échange

ORF : *Open Reading Frame* ; phase ouverte de lecture

PKA : *Protein Kinase A* ; protéine kinase A

RAC : *Ribosome-Associated Complex* ; complexe associé au ribosome

RE : Réticulum Endoplasmique

SBD : *Substrate Binding Domain* ; domaine de liaison au substrat

SDD-AGE : *Semi-Denaturing Detergent-Agarose Gel Electrophoresis* ; électrophorèse en gel d'agarose en conditions semi-dénaturantes

TPR : *Tetratricopeptide Repeats* ; répétitions de tétratricopeptides

UBA : *UBiquitin Association* ; domaine d'association à l'ubiquitine

UPR : *Unfolded Protein Response* ; réponse aux protéines mal repliées

ZFLR : *Zinc-Finger-Like Region* ; domaine de type doigt de zinc

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Avant-Propos

Cette thèse présente l'étude des spécificités fonctionnelles des orthologues de Hsp70 cytoplasmiques de deux levures, *Saccharomyces cerevisiae* et *Yarrowia lipolytica*.

Les résultats présentés sont articulés en trois parties. Ils décrivent premièrement la comparaison systématique *in vivo*, intra- et inter-espèce(s), des orthologues de Hsp70 dans leur capacité à assurer divers processus cellulaires, notamment la thermotolérance, le repliement d'un substrat dénaturé, la propagation et la stabilité des prions, et la dégradation d'une protéine par le protéasome. Ils ont également révélé l'implication des Hsp70 et de leurs cofacteurs dans la formation de biofilms chez *Saccharomyces cerevisiae*, qui contrôlent ce processus *via* des voies distinctes. Enfin, la caractérisation de la machinerie Hsp70 de la levure distante *Yarrowia lipolytica* a conforté la forte robustesse de ce système.

Au cours de ma thèse, nous nous sommes intéressés à des processus cellulaires très divers, dont la description exhaustive aurait considérablement alourdi l'introduction générale. J'ai ainsi privilégié la description de la machinerie Hsp70, replacée dans le contexte de la protéostase, qui est l'élément structurant de ma thèse, et qui favorise l'ouverture sur sa problématique générale. Les différents processus cellulaires et méthodologies abordés seront donc décrits dans les parties correspondantes.

L'introduction générale présentera dans un premier temps le rôle des chaperons moléculaires ainsi que leur coopération pour le maintien de la protéostase afin de situer les fonctions exercées par les Hsp70. Le système Hsp70, comprenant les Hsp70, les protéines à domaine J et les facteurs d'échange, sera ensuite décrit afin de présenter les causes de la redondance fonctionnelle et les origines probables des spécificités fonctionnelles entre paralogues de Hsp70 cytoplasmiques. Enfin, les spécificités fonctionnelles connues des Hsp70 seront présentées par le biais d'une revue dont je suis co-auteur.

Les différents résultats obtenus lors de ce travail seront présentés, souvent sous forme de publications précédées d'une introduction en français, puis discutés dans une conclusion générale. Enfin, des perspectives à ce travail seront proposées.

INTRODUCTION GENERALE

I. Rôles et organisation des chaperons moléculaires dans la cellule

Qu'il s'agisse d'organismes uni- ou pluricellulaires, les cellules interagissent avec leur environnement, ont un impact sur celui-ci et inversement. En réponse aux variations permanentes au sein du système auquel elles appartiennent, elles possèdent la capacité de maintenir un équilibre dynamique de fonctionnement, ou homéostasie. Cette capacité leur permet de tendre vers l'état physiologique le plus favorable à leur survie ou à celle de l'organisme dont elles dépendent.

I.A. Introduction : la protéostase

La protéostase est une composante de l'homéostasie relative au maintien de l'équilibre dynamique de fonctionnement du protéome. Elle gouverne le repliement, l'assemblage, la localisation et la concentration des protéines cellulaires et extracellulaires. Lorsque la protéostase est déficiente, l'équilibre n'est plus maintenu et la cellule tend vers un événement catastrophique, lié à des pertes de fonctions cellulaires et/ou à l'apparition d'espèces protéiques aux propriétés toxiques. Par exemple chez l'Homme, la mucoviscidose est liée à une perte de fonction, due à des mutations héréditaires engendrant un repliement inefficace et la dégradation de la protéine CFTR (*Cystic Fibrosis Transmembrane conductance Regulator* ; régulateur transmembranaire associé à la mucoviscidose) (Cheng et al., 1990 ; Dean et al., 1990 ; Ward and Kopito, 1994; White et al., 1990). Les maladies neurodégénératives comme les maladies de Parkinson, de Huntington ou encore d'Alzheimer sont dues à la fois à une perte de fonction liée à l'agrégation des protéines ou fragments protéolytiques correspondants (α -synucléine, huntingtine, amyloïde β et tau, respectivement) et à la toxicité de certains intermédiaires d'assemblage (Chiti and Dobson, 2006 ; Sideras and Gertz, 2009).

La protéostase consiste en un réseau de régulations qui s'étend de la transcription à la synthèse, au repliement, à l'assemblage, au trafic, à la désagrégation et à la dégradation des protéines. Ce réseau interagit avec l'ensemble des autres systèmes de régulation et il est soumis aux contraintes spécifiques à la cellule et à son environnement. Ainsi, il dépend du maintien de l'intégrité du génome, des régulations génétiques, épigénétiques, métaboliques, et des conditions environnementales. La protéostase est étroitement liée au développement et au vieillissement de l'organisme. Le maintien de la protéostase nécessite donc une régulation fine et une coordination de chacune des voies impliquées (Figure 1, page suivante) (Balch et al., 2008 ; Powers et al., 2009).

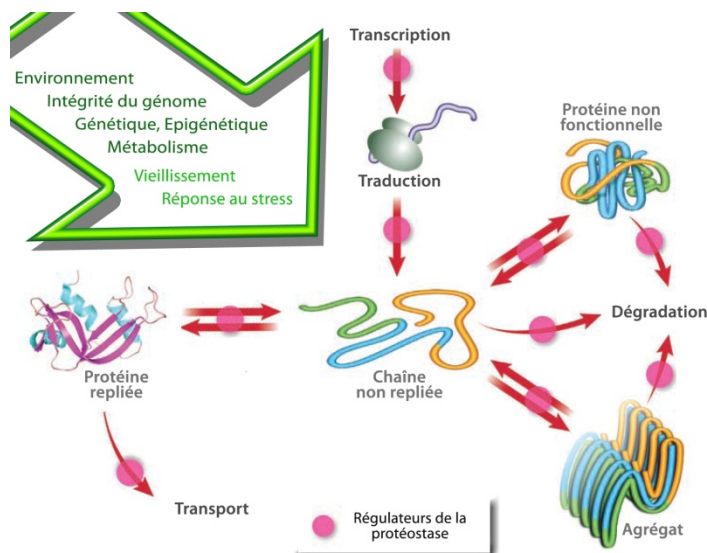


Figure 1 : Réseau de régulation de la protéostase

Les régulateurs de la protéostase (ronds roses) interviennent à tous les niveaux du réseau régulant le repliement correct des protéines et la dégradation des espèces non natives. Ce réseau est influencé à une échelle supérieure par la réponse au stress et le vieillissement par exemple, eux-mêmes dépendants des propriétés intrinsèques de la cellule ou de l'organisme, et de son environnement extérieur.

Figure adaptée d'après (Balch et al., 2008).

L'observation de ce réseau révèle une balance entre d'une part le repliement des chaînes polypeptidiques en protéines natives et d'autre part la formation de protéines mal repliées et d'agrégats protéiques.

I.B. Repliement et agrégation des protéines

I.B.1. De l'hypothèse thermodynamique aux paysages énergétiques de repliement

Au début des années 1960, les travaux de Christian Anfinsen et de ses collaborateurs sur la renaturation de la ribonucléase *in vitro* aboutissent à la formulation de l'hypothèse thermodynamique pour expliquer le repliement des protéines sous leur forme fonctionnelle (Anfinsen et al., 1961 ; Haber and Anfinsen, 1961 ; 1962; Sela et al., 1957). Cette hypothèse propose que la structure tridimensionnelle d'une protéine native dans son environnement physiologique normal est celle pour laquelle l'énergie libre de la totalité du système est la plus basse. Cela signifie que la séquence primaire en acides aminés comporte toute l'information nécessaire à l'établissement des interactions inter-atomiques aboutissant à une structure tertiaire native dans un environnement donné (Anfinsen, 1973). Les théories sur le processus de repliement ont par la suite exclu la possibilité d'un mécanisme de repliement aléatoire, et le principe d'un cheminement passant par des intermédiaires stables a été introduit.

Aujourd'hui, le repliement des protéines est décrit sous forme de paysage énergétique en entonnoir représentant l'énergie libre de la chaîne polypeptidique en fonction de l'entropie, donc des propriétés conformationnelles de la protéine (Figure 2). La rugosité de l'entonnoir reflète les interactions intramoléculaires au sein de la protéine en cours de repliement, qui

peuvent être natives ou non. Dans le cas d'une protéine au repliement très rapide, l'entonnoir aura une apparence faiblement rugueuse (Figure 2.a). Il a été montré que le repliement, même des petites protéines (de composition inférieure à 100 acides aminés), passe par un ou plusieurs états de repliement partiel (Brockwell and Radford, 2007). En général, les entonnoirs de repliement ont une apparence vallonnée. Les chaînes polypeptidiques forment des intermédiaires de repliement stables (vallées) puis passent par des états de transition (collines) pour atteindre leur conformation native, état où l'énergie libre est la plus faible (Figure 2.b).

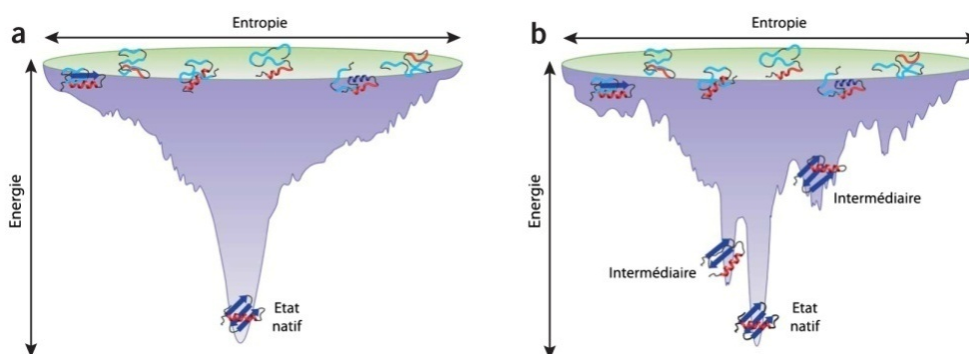


Figure 2 : Représentation schématique des paysages énergétiques en entonnoir

Les chaînes polypeptidiques dénaturées représentent un large ensemble de structures issues d'interactions natives et non natives.

a. Exemple d'un paysage énergétique relativement lisse dans lequel les chaînes polypeptidiques tendent vers une réduction de l'énergie libre interne pour atteindre leur conformation native.

b. Exemple d'un paysage énergétique plus rugueux dans lequel les chaînes polypeptidiques passent par un ou plusieurs intermédiaires de repliement avant d'atteindre leur conformation native.

Figure adaptée d'après (Bartlett and Radford, 2009).

Ces modèles, bien qu'ils participent à la compréhension du processus de repliement, ne décrivent que le comportement d'une chaîne polypeptidique en solution diluée. Dans la cellule, le repliement des protéines doit être efficace tout en étant soumis à de nombreuses contraintes décrites ci-après.

I.B.2. Contraintes liées au repliement des protéines et agrégation

Les macromolécules occupant de 20 à 30 % du volume cellulaire, la première contrainte qui s'applique sur les chaînes polypeptidiques est donc l'encombrement macromoléculaire, ou effet d'exclusion de volume (Ellis, 2001 ; Ellis and Minton, 2003). Il provoque une limitation stérique qui est à l'origine de compactions, de collisions et d'interactions intermoléculaires attractives et répulsives. Ce phénomène a pour conséquence de favoriser le repliement par des

confinements locaux (Zhou, 2008 ; Zhou et al., 2008), mais aussi d'accentuer la formation d'interactions intramoléculaires non natives. Il augmente également le taux d'agrégation des chaînes polypeptidiques qui ont tendance à s'associer pour former des structures plus compactes, plus mobiles, donc de plus faible énergie libre pour le système (Ellis, 2006; Minton, 2005). Ces structures peuvent être plus stables que la conformation native de la protéine, ce qui rend leur repliement irréversible (Figure 3).

Une autre contrainte est la lenteur du processus de traduction. Les chaînes polypeptidiques naissantes sont exposées à cet environnement encombré durant des périodes allant de quelques secondes à plusieurs minutes jusqu'à ce que la protéine, ou un domaine de la protéine, ait été synthétisé. Durant ce laps de temps, les chaînes polypeptidiques peuvent être sujettes au mauvais repliement et à l'agrégation si elles n'acquièrent pas une conformation stable (Hartl and Hayer-Hartl, 2009 ; Kramer et al., 2009). Enfin, les modifications post-traductionnelles des protéines peuvent affecter leur repliement, tout comme leur transport et notamment leur translocation à travers les membranes.

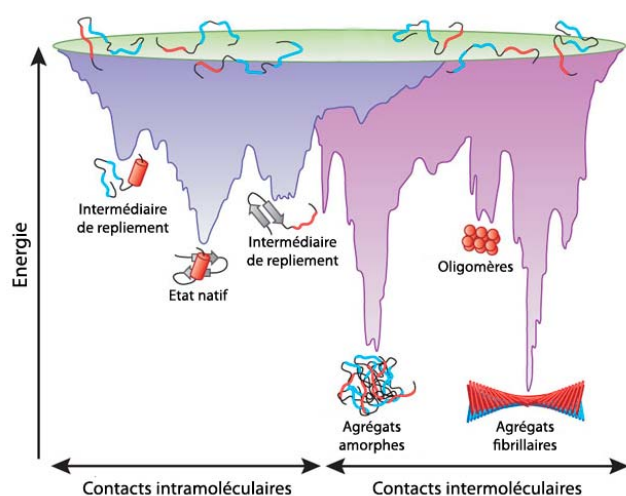


Figure 3 : Représentation schématique d'un paysage énergétique prenant en compte le repliement et l'assemblage des protéines

La région bleue représente le paysage énergétique du repliement des chaînes polypeptidiques vers leur conformation native. La région violette représente le paysage énergétique menant vers l'assemblage et l'agrégation des protéines. Les deux régions sont chevauchantes, les paysages énergétiques du repliement et de l'assemblage ne sont pas cloisonnés. Ainsi, un intermédiaire de repliement peut

intervenir dans un assemblage intermoléculaire, et inversement.

Figure adaptée d'après (Hartl and Hayer-Hartl, 2009).

L'assemblage de protéines en complexes est une partie intégrante du fonctionnement des cellules. Ces assemblages peuvent être des homopolymères (filaments d'actine) ou des hétéro-complexes de composition plus ou moins variable (polymérase, ribosomes). En revanche, l'agrégation des protéines déséquilibre la protéostase, conduisant à des situations souvent délétères pour l'organisme. C'est le cas par exemple des maladies neurodégénératives comme les maladies d'Alzheimer, de Parkinson, de Huntington, et de l'encéphalopathie spongiforme.

Lorsqu'ils sont correctement repliés, les protéines/peptides responsables de ces différentes maladies ont des fonctions distinctes. Cependant elles ont toutes une forte propension à former des agrégats aux caractéristiques structurales communes (*e.g.* amyloïdes), qui conduisent à des effets cytotoxiques comparables.

Les cellules ont évolué dans le sens d'une minimisation du phénomène d'agrégation des protéines. L'environnement intracellulaire est stabilisé dans des conditions physicochimiques favorables au repliement (température, pH, force ionique, conditions redox). La propension à l'agrégation est naturellement faible pour une majorité des séquences polypeptidiques. Différents systèmes de dégradation des protéines, notamment des espèces mal repliées et/ou toxiques, se sont mis en place, comme l'autophagie et le système protéasomal. Enfin, un arsenal de chaperons moléculaires est au cœur du dispositif permettant le repliement natif des protéines et le maintien de la protéostase.

I.C. Les chaperons moléculaires

I.C.1. Concept de chaperon moléculaire et mode d'action

Le concept de chaperon moléculaire émerge des travaux de Ronald Laskey *et al.* qui utilisent le terme pour la première fois en 1978 (Laskey *et al.*, 1978). Ils décrivent une fonction cellulaire permettant à la fois de favoriser l'assemblage d'un complexe et de prévenir et/ou désassembler les interactions non productives dues à l'encombrement macromoléculaire. De plus, la protéine responsable de cette fonction n'est pas un composant final de la structure native, et n'apporte pas d'information conformationnelle (Earnshaw *et al.*, 1980; Laskey and Earnshaw, 1980 ; Laskey *et al.*, 1978). Le terme est repris en 1986 par Hugh Pelham et John Ellis pour désigner l'ensemble des protéines possédant cette fonction. Ils y ajoutent les protéines qui assistent le repliement et le dépliement des chaînes polypeptidiques (Ellis, 1987 ; Pelham, 1986).

Un même principe général de fonctionnement réunit l'ensemble des chaperons moléculaires. Ils se lient de manière transitoire à des segments hydrophobes des chaînes polypeptidiques et augmentent indirectement l'efficacité de repliement ou d'assemblage en limitant les interactions non natives (Ellis, 2006). Ils n'accélèrent pas les étapes limitantes, fonction exercée par des catalyseurs comme les isomérases.

Les chaperons moléculaires sont présents dans tous les organismes et tous les compartiments cellulaires. Ils sont exprimés de manière constitutive et/ou inductible par un stress (à l'origine de leur nom de *Heat Shock Proteins* ; protéines de choc thermique) afin d'assurer des « fonctions de ménage » (*e.g.* repliement, transport des protéines) et de réponse au stress (*e.g.* désagrégation, dégradation des protéines mal repliées).

Chez les procaryotes, il est considéré que les mêmes chaperons moléculaires participent à la fois au repliement et à l'assemblage des protéines nouvellement synthétisées ou dénaturées suite à un stress cellulaire (Bukau et al., 1996). Il a été montré que chez *Escherichia coli*, les chaperons moléculaires majoritaires (l'Hsp70 DnaK et la chaperonine GroEL/GroES), ainsi que leurs co-chaperons, interagissent avec un grand nombre de protéines néo-synthétisées (Deuerling et al., 1999 ; Ewalt et al., 1997 ; Teter et al., 1999), mais que leur expression est également induite par des stress et qu'elles sont requises pour la prise en charge des protéines dénaturées par un choc thermique (Parsell and Lindquist, 1993). Chez les eucaryotes, les travaux de Judith Frydman et collaborateurs ont mis en évidence l'existence de deux réseaux de chaperons moléculaires distincts (Albanese et al., 2006). L'un est inductible par les stress, et regroupe les chaperons moléculaires connus pour participer à la tolérance aux stress et la prévention de l'agrégation, tandis que le deuxième est réprimé par les stress et co-régulé avec la machinerie traductionnelle. Ainsi, les deux réseaux interviendraient respectivement dans le repliement et l'assemblage des protéines dénaturées et dans la biogenèse des protéines, avec une zone de chevauchement dénotant une redondance fonctionnelle (Albanese et al., 2006). En plus de ces travaux menés par une approche globale, des spécificités fonctionnelles au sein des différents paralogues de chaperons moléculaires eucaryotes ont été décrites (Daugaard et al., 2007 ; Kabani and Martineau, 2008b). Une spécialisation de fonctions a également été décrite au sein des Hsp70 procaryotes (Genevaux et al., 2007).

I.C.2. Fonctions et organisation des chaperons moléculaires dans la cellule

Différentes familles de chaperons moléculaires coexistent dans la cellule. Elles n'ont pas forcément de relation structurale et sont traditionnellement classées selon leur poids moléculaire : petites Hsp, Hsp40, Hsp70, Hsp90 et Hsp100. Leur action est concertée dans la prise en charge des protéines de leur repliement *de novo* à leur dégradation, pour former un réseau indispensable au contrôle qualité des protéines et au maintien de la protéostase (Figure 4).

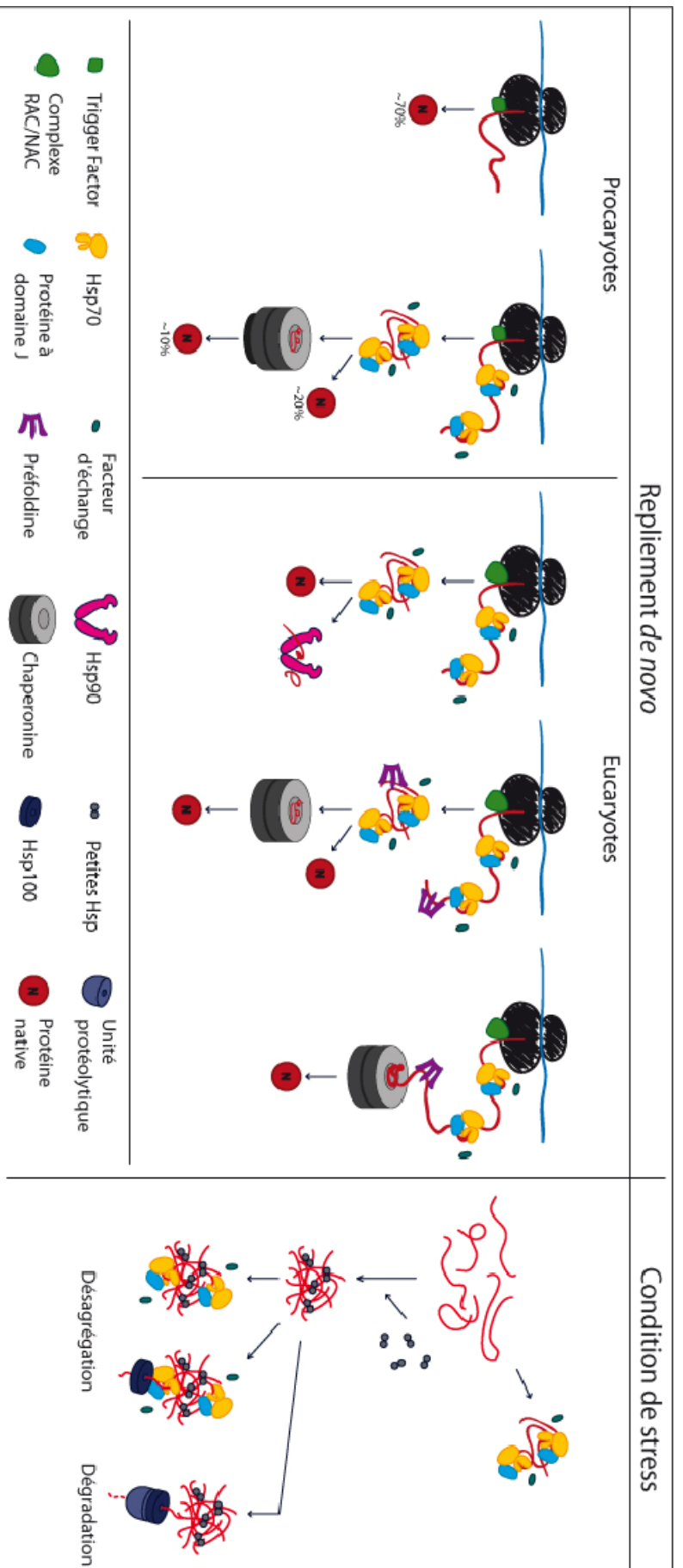


Figure 4 : Coordination des chaperons moléculaires cytosoliques

Replieement *de novo*. Chez les procaryotes, les chaînes naissantes interagissent avec le Trigger Factor à la sortie du ribosome. Environ 70% des protéines pourraient adopter leur conformation native sans assistance supplémentaire. Le système Hsp70 prend également en charge les chaînes naissantes. Il permet le replieement de 20% des protéines, les 10% restant nécessitant l'action des chaperonines GroEL/GroES. Chez les eucaryotes, les complexes RAC et NAC remplacent le Trigger factor. Le système Hsp70 permet le replieement d'une fraction des protéines, et peut transférer certaines chaînes aux Hsp90. Le replieement et le transfert des substrats aux chaperonines peuvent être assistés par la prefoldine.

Condition de stress. Les chaînes polypeptidiques dénaturées sont prises en charge par le système Hsp70 et par les petites Hsp. Chez certains organismes, les Hsp100 peuvent assister la solubilisation et la dégradation des agrégats.

Figure inspirée de (Hartl and Hayer-Hartl, 2009) et (Liberek et al., 2008).

1.C.2.a. Synthèse des protéines et repliement de novo

Les chaînes polypeptidiques sont prises en charge dès le début de leur synthèse par des chaperons moléculaires liés au ribosome (Hartl and Hayer-Hartl, 2009 ; Kramer et al., 2009). Chez les procaryotes le Trigger Factor protège les régions hydrophobes de la chaîne naissante dès leur sortie du ribosome pour maintenir la solubilité de la protéine (Hesterkamp et al., 1996 ; Kaiser et al., 2006 ; Merz et al., 2008; Stoller et al., 1995 ; Valent et al., 1995). Le système Hsp70 intervient ensuite pour les chaînes polypeptidiques plus longues (Deuerling et al., 1999; Teter et al., 1999). Il consiste en une Hsp70 assistée de manière coopérative par une Hsp40 et un facteur d'échange (ce système sera détaillé dans la partie II). Ce système favorise les repliements co- et post-traductionnels. Lorsque le repliement est inefficace (pour environ 10 % des protéines totales) les Hsp70 recrutent la chaperonine GroEL/GroES qui poursuit le processus (Langer et al., 1992). Les chaperonines sont de larges complexes cylindriques qui encapsulent au moins une portion des chaînes polypeptidiques pour faciliter leur repliement dans un environnement confiné.

Chez les eucaryotes, le complexe RAC (*Ribosome-Associated Complex* ; complexe associé au ribosome), remplace le Trigger Factor. C'est un hétérodimère stable composé chez la levure des protéines Ssz1 (Hsp70) et Zuo1 (Hsp40) (Gautschi et al., 2001). RAC stimule l'activité ATPase d'une autre Hsp70 liée au ribosome, Ssb1/2, et favorise ainsi la liaison de la chaîne polypeptidique naissante par Ssb1/2 (Conz et al., 2007; Gautschi et al., 2001 ; Huang et al., 2005). Ces trois chaperons sont génétiquement liés, la délétion de chaque membre de la triade aboutissant à des phénotypes similaires (Gautschi et al., 2001 ; Gautschi et al., 2002; Hundley et al., 2002 ; Yan et al., 1998). La zutine Zuo1 est nécessaire à la fixation de RAC au ribosome (Gautschi et al., 2001). Ssz1 est une Hsp70 dont la fonction est peu connue. Son domaine de liaison aux substrats n'est pas requis pour sa fonction, et elle ne possède pas d'activité ATPase en complexe avec Zuo1 (Conz et al., 2007; Hundley et al., 2002). Cependant, elle module l'activité de Zuo1, et la formation du complexe RAC en solution stabilise la dynamique conformationnelle globale de Zuo1 et Ssz1, tout en augmentant la dynamique du domaine J de Zuo1, nécessaire à la stimulation de l'activité ATPase de Ssb1/2 (Fiaux et al., 2009; Huang et al., 2005). Chez l'Homme, l'orthologue de Zuo1, MPP11, est associé aux ribosomes et forme un complexe avec Hsp70L1 (Hundley et al., 2005 ; Otto et al., 2005).

Le complexe NAC (*Nascent chain-Associated Complex* ; complexe associé aux chaînes naissantes) peut également s'associer aux ribosomes et aux chaînes naissantes (Funfschilling and Rospert, 1999; Wang et al., 1995 ; Wiedmann et al., 1994). Chez les eucaryotes, ce complexe est constitué de deux sous-unités, α et β (Beatrix et al., 2000; Reimann et al., 1999). La liaison de NAC au ribosome se fait uniquement par la sous-unité β , et les deux sous-unités interagissent avec la chaîne polypeptidique naissante (Beatrix et al., 2000). Cependant le rôle de NAC dans le repliement des protéines est mal connu. Les Hsp70, ainsi qu'un autre chaperon moléculaire, la préfoldine, peuvent délivrer la protéine cliente aux chaperonines (TRiC/CCT) (Frydman et al., 1994 ; Vainberg et al., 1998). Ce couplage des chaperons moléculaires facilite le repliement co-translationnel, et évite la libération de protéines non natives dans le cytosol (Siegers et al., 1999; Thulasiraman et al., 1999). Les Hsp70 peuvent également transférer leur protéine cliente aux Hsp90 *via* le cofacteur Hop (Sti1 chez la levure), notamment pour le repliement de protéines de régulation comme les kinases et les facteurs de transcription (Chen et al., 1996 ; Chen and Smith, 1998 ; McClellan et al., 2007; Smith et al., 1993 ; Wegele et al., 2006).

Chez les eucaryotes, environ 30-50 % des protéines empruntent la voie sécrétoire (Buck et al., 2007 ; Ghaemmaghami et al., 2003 ; Kanapin et al., 2003). Elles sont transloquées dans le réticulum endoplasmique (RE), point d'entrée de la voie sécrétoire, où elles subissent des modifications co- et post-translationnelles (*e.g.* formation de ponts disulfure, glycosylation) (Buck et al., 2007; van Anken and Braakman, 2005). Le repliement des protéines y suit le même principe général mais la machinerie n'est pas strictement identique à celle du cytosol. On retrouve le système Hsp70 comprenant la protéine BiP, des facteurs d'échange et des protéines à domaine J (Hsp40), une Hsp90, des petites Hsp et des chaperons moléculaires spécifiques du réticulum endoplasmique, tels que des protéines de la famille des lectines (*e.g.* calnexine, calréticuline) (Buck et al., 2007; Kleizen and Braakman, 2004 ; van Anken and Braakman, 2005). Le réticulum possède également des enzymes impliquées dans le repliement comme les peptidyl-prolyl *cis-trans* isomérases (Cpr5 et Kkb2 chez la levure) ou les protéines disulfures isomérases (*e.g.* Pdi1, Ero1 chez la levure) (van Anken and Braakman, 2005).

1.C.2.b. Contrôle qualité des protéines

Chez les eucaryotes, l'expression des chaperons moléculaires est induite en condition de stress par les facteurs de transcription HSF (*Heat Shock transcription Factors* ; facteurs de

transcription du choc thermique) qui se lient aux éléments HSE (*Heat Shock Elements* ; éléments du choc thermique) présents en amont des gènes codant les Hsp (Morimoto, 1998 ; Shamovsky and Nudler, 2008). Un seul HSF est exprimé chez la levure (Sorger and Pelham, 1988; Wiederrecht et al., 1988) tandis que trois ont été identifiés chez l'Homme (Nakai et al., 1997; Rabindran et al., 1991; Schuetz et al., 1991). HSF1, le plus étudié, est directement régulé par les chaperons moléculaires (Morimoto, 2002; Voellmy and Boellmann, 2007). Hors condition de stress, HSF1 est localisé dans le cytoplasme et maintenu dans un état inactif par les chaperons moléculaires. En condition de stress, l'équilibre est rapidement déplacé vers un état trimérique et hyper-phosphorylé compétant pour l'activité transcriptionnelle. Lorsque le pool de chaperons moléculaires devient excédentaire, HSF1 est de nouveau inhibé, permettant un retour à l'état basal.

Le réticulum endoplasmique, siège du repliement et de l'assemblage des protéines sécrétaires, possède un système de contrôle qualité des protéines qui lui est propre. Seules les protéines correctement repliées ou assemblées quittent le réticulum endoplasmique, les autres étant retenues dans le RE et dégradées (Bukau et al., 2006 ; Schroder, 2008). Deux systèmes principaux empêchent l'accumulation des protéines mal repliées, la réponse aux protéines mal repliées ou UPR (*Unfolded Protein Response*) et la dégradation associée au réticulum endoplasmique ou ERAD (*Endoplasmic Reticulum-Associated Degradation*).

Différents systèmes de rétention existent dans le RE pour assurer le contrôle qualité de la maturation des protéines sécrétées, et retenir les protéines non matures ou n'ayant pas acquit leur conformation native (van Anken and Braakman, 2005). Seul le rôle de l'Hsp70 BiP dans la voie UPR sera détaillé ci-après. Les protéines mal repliées sont reconnues par BiP et séquestrées jusqu'à ce qu'elles acquièrent leur conformation native. Lorsque la capacité de repliement du réticulum endoplasmique est dépassée, l'accumulation des espèces mal repliées provoque indirectement l'induction de l'UPR. L'UPR fut premièrement caractérisée chez *Saccharomyces cerevisiae*, où elle implique une voie de signalisation médiée par la protéine transmembranaire Ire1 (Hetz and Glimcher, 2009). Il a été initialement proposé que BiP/Kar2 interagit avec Ire1 dans la lumière du RE pour maintenir ce senseur dans un état monomérique inactif (Bertolotti et al., 2000 ; Kimata et al., 2003). Dans le cas d'une accumulation des espèces mal repliées, la libération de BiP/Kar2 constituerait le signal inducteur de l'UPR en permettant la dimérisation de Ire1 (Kimata et al., 2007). Dans une seconde étape, l'interaction directe des protéines mal repliées avec Ire1 provoquerait l'activation de son activité ribonucléase au niveau de son domaine cytoplasmique (Kimata et al., 2007). L'activation de

Ire1 permet l'épissage non conventionnel de l'ARN messager *HAC1* (Cox and Walter, 1996 ; Mori et al., 1996). Le facteur de transcription Hac1 est ainsi synthétisé et induit l'expression des gènes cibles de l'UPR dont les chaperons moléculaires du RE, la machinerie de l'ERAD, ainsi que les composants de la voie sécrétoire et de la synthèse du RE (Travers et al., 2000). Chez les eucaryotes supérieurs, l'UPR est plus complexe. Elle fait intervenir deux senseurs supplémentaires, PERK et ATF6 (Harding et al., 1999 ; Haze et al., 1999; Hetz and Glimcher, 2009). Le domaine luminal de PERK et ATF6 interagit avec BiP, et cette interaction est également perdue lors d'un stress du RE (Bertolotti et al., 2000 ; Chen et al., 2002 ; Shen et al., 2002). La réponse UPR provoque une répression de la synthèse des protéines et la stimulation de l'ERAD pour favoriser l'élimination des protéines mal repliées (Harding et al., 1999 ; Schroder and Kaufman, 2005; Travers et al., 2000). La capacité de repliement du réticulum endoplasmique est également augmentée, par la surexpression des chaperons moléculaires et des enzymes liées au repliement, ainsi que par l'augmentation de son volume qui permet de diminuer la concentration interne en protéines mal repliées (Dorner et al., 1989 ; Kozutsumi et al., 1988 ; Schroder and Kaufman, 2005).

Les protéines mal repliées du réticulum endoplasmique, qu'elles soient solubles ou membranaires, sont dégradées par l'ERAD (Vembar and Brodsky, 2008). Leur reconnaissance est assurée à la fois par les chaperons moléculaires du réticulum endoplasmique et du cytosol (pour les protéines membranaires). Concernant le système Hsp70, BiP interagit avec un certain nombre de substrats dans la lumière du réticulum endoplasmique (Knittler et al., 1995 ; Nishikawa et al., 2001; Schmitz et al., 1995). Les Hsp70 cytosoliques interagissent avec les régions exposées des protéines membranaires comme par exemple la protéine CFTR et favorisent leur dégradation (Meacham et al., 1999 ; Rubenstein and Zeitlin, 2000). La machinerie utilisée pour la dégradation des protéines semble dépendre de la localisation du mépliection au sein de la protéine à dégrader. Ainsi, un mépliection dans une région cytoplasmique, luminale, ou membranaire, fera intervenir respectivement la voie ERAD-C, ERAD-L ou ERAD-M (Carvalho et al., 2006; Denic et al., 2006 ; Vashist and Ng, 2004). Ces voies, identifiées chez la levure, peuvent être chevauchantes. De manière générale, les protéines solubles mal repliées reconnues dans la lumière du réticulum endoplasmique doivent être rétro-transloquées vers le cytoplasme, où résident les machineries d'ubiquitination et de dégradation. Les protéines membranaires peuvent être ubiquitinylées avant ou pendant leur rétro-translocation. La plupart des substrats de l'ERAD sont ubiquitinylés avant leur dégradation protéasomale (Vembar and Brodsky, 2008). Ce processus nécessite l'action d'une enzyme E1 d'activation de l'ubiquitine et d'une

enzyme E2 conjuguant l'ubiquitine sur une ubiquitine ligase E3. Les ubiquitine ligases greffent l'ubiquitine sur la protéine à dégrader au niveau d'un résidu lysine. Dans certains cas, les enzymes E4 d'extension de la chaîne d'ubiquitine favorisent l'ERAD (Kohlmann et al., 2008 ; Nakatsukasa et al., 2008; Richly et al., 2005).

Les ubiquitine ligases interagissent avec, peuvent être régulées par et peuvent réguler le système Hsp70. C'est notamment le cas de l'ubiquitine ligase CHIP (*Carboxyl terminus of Hsp70-Interacting Protein* ; protéine interagissant avec la région C-terminale des Hsp70) qui participe à la balance entre repliement et dégradation des protéines (Ballinger et al., 1999 ; Esser et al., 2004). Par exemple, CHIP et le facteur d'échange Bag1, en complexe avec Hsp70, inhibent son activité ATPase et orientent sa fonction vers le processus de dégradation par le protéasome (Ballinger et al., 1999 ; Demand et al., 2001; Meacham et al., 2001). *A contrario*, l'activité ubiquitine ligase de CHIP est inhibée lorsqu'elle est en complexe avec une Hsp70 et le facteur d'échange HspBP1, et la dégradation par le protéasome est atténuée (Alberti et al., 2004). De plus, CHIP participe à la fois à l'activation de HSF1 et à la dégradation des Hsp70 après une réponse au stress (Dai et al., 2003 ; Kim et al., 2005 ; Kundrat and Regan, 2009; Qian et al., 2006).

1.C.2.c. Agrégation des protéines

Le bon repliement des protéines ne garantit pas pour autant leur stabilité à long-terme, qui peut être affectée par un ensemble de contraintes cellulaires et de conditions environnementales. *In vitro*, Zylicz et collaborateurs ont montré que le système Hsp70 seul était suffisant pour la désagrégation de certains substrats (Skowyra et al., 1990 ; Ziemienowicz et al., 1993). *In vivo*, dans des conditions de stress cellulaire, les petites Hsp se lient aux protéines dénaturées pour prévenir leur agrégation et aux agrégats pour favoriser leur solubilisation en modifiant leurs propriétés physicochimiques (Haslbeck et al., 2005 ; Liberek et al., 2008). Chez les procaryotes et les eucaryotes inférieurs, une coopération entre les Hsp70 et les désagrégases Hsp100 permet de solubiliser les agrégats. Ces derniers sont également éliminés par protéolyse lorsque les Hsp100 sont couplées à une unité protéolytique (protéasome chez les eucaryotes, famille des peptidases ClpP chez les procaryotes) (Liberek et al., 2008). Le génome des eucaryotes supérieurs ne code pas d'Hsp100. La désagrégation est assurée par le système Hsp70 et par les petites Hsp (*e.g.* α -cristallines). Les protéines extraites des agrégats sont prises en charge par la machinerie de contrôle qualité pour être repliées ou dégradées (Liberek et al., 2008).

I.C.3. Acteurs majeurs du repliement et du dépliement des protéines

I.C.3.a. Famille des chaperonines

Les chaperonines sont des complexes protéiques cylindriques d'environ 800 kDa composés d'un double anneau formant chacun une cavité permettant d'encapsuler les protéines à replier (Hartl and Hayer-Hartl, 2009; Horwich et al., 2007). L'encapsulation d'une protéine permet son isolement et donc la prévention de son agrégation, son dépliement quand elle est sous forme d'intermédiaire piégé dans une trappe énergétique, et son confinement qui favorise le repliement (Brinker et al., 2001 ; Lin and Rye, 2004 ; Tang et al., 2006). Selon A.L. Horwich et ses collaborateurs, les chaperonines faciliteraient le repliement des protéines par un mécanisme passif de prévention de l'agrégation et n'apporteraient pas d'information conformationnelle (Apetri and Horwich, 2008 ; Horwich et al., 2009). Elles serviraient de *cage d'Anfinsen* comme proposé par le groupe de R.J. Ellis en 1993 (Saibil et al., 1993). Le fonctionnement de cette nano-machine est ATP-dépendant et peut être itératif si la protéine n'acquiert pas sa conformation native.

Les chaperonines sont séparées en deux groupes (Horwich et al., 2007). Les membres du groupe I (appelés Hsp60) sont présents chez les bactéries (GroEL) et dans les organites d'origine procaryote (mitochondries et chloroplastes). Chaque anneau est similaire et constitué de sept sous-unités identiques (Hendrix, 1979 ; Hohn et al., 1979 ; Hutchinson et al., 1989; Pushkin, 1982), et coopère de manière ATP-dépendante avec la protéine Hsp10 (GroES chez les bactéries) qui se fixe au-dessus de la cavité d'encapsulation, formant ainsi un couvercle (Xu et al., 1997). GroES forme également un anneau de sept sous-unités identiques (Chandrasekhar et al., 1986 ; Hartman et al., 1992; Lubben et al., 1990) (Figure 5.a, page suivante). Les membres du groupe II sont retrouvés chez les *Archeae* (thermosome) et le cytosol des cellules eucaryotes (TRiC/CCT). TRiC/CCT est constituée de deux anneaux identiques comprenant chacun huit sous-unités différentes (CCT $\alpha, \eta, \delta, \theta, \gamma, \beta, \zeta, \epsilon$) (Liou and Willison, 1997 ; Martin-Benito et al., 2007). C'est une extension émanant de la structure qui fait office de couvercle (Meyer et al., 2003). Le thermosome, selon les espèces, est constitué de une (Andra et al., 1996) ou deux (Waldmann et al., 1995) protéines différentes assemblées en anneaux identiques de huit (Andra et al., 1996 ; Ditzel et al., 1998; Phipps et al., 1993) ou neuf sous-unités (Knapp et al., 1994 ; Marco et al., 1994) (Figure 5.a, page suivante).

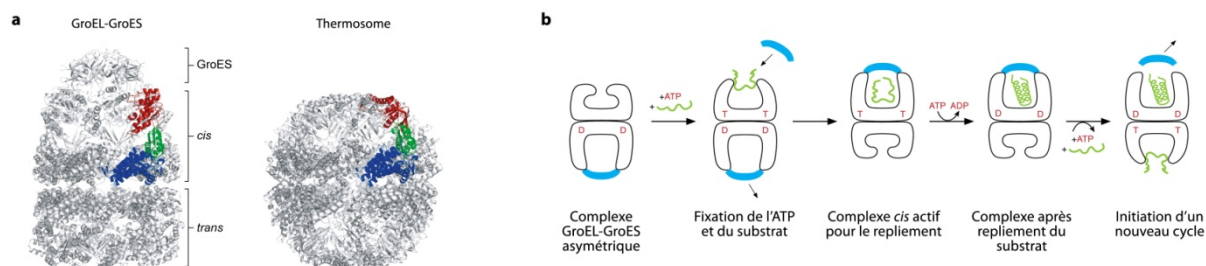


Figure 5 : Structure des chaperonines de types I et II et cycle réactionnel de GroEL-GroES

a. Modèles cristallographiques du couple GroEL-GroES d'*Escherichia coli* (codes PDB : 1OEL et 1AON, respectivement) et du thermosome de l'*Archae Thermoplasma acidophilum* (code PDB : 1A6D) (Braig et al., 1995 ; Ditzel et al., 1998; Xu et al., 1997). L'une des sous-unités est colorée ; son domaine apical en rouge, son domaine intermédiaire en vert, et son domaine équatorial en bleu.

b. Mécanisme de repliement d'un polypeptide par GroEL-GroES. Les nucléotides sont notés en rouge (T : ATP et D : ADP), GroES est dessinée en bleu et le substrat polypeptidique en vert. Ce mécanisme est détaillé ci-après.

Figure adaptée d'après (Horwich et al., 2007) et (Horwich et al., 2009).

Les chaperonines fonctionnent selon un cycle réactionnel ATP-dépendant (Figure 5.b) (Horwich et al., 2009; Horwich et al., 2007). Le cycle de GroEL-GroES débute par la fixation d'une protéine non native au niveau de l'anneau en *trans* du complexe GroEL-GroES-ATP *via* des interactions hydrophobes. La fixation de GroES induit un changement de conformation de l'anneau qui permet l'encapsulation du substrat dans la cavité hydrophile. Elle provoque également la libération de l'ADP et de l'autre molécule de GroES en *trans*. Le repliement de la protéine substrat a lieu dans cette cavité appelée *cis*. Après hydrolyse de l'ATP, l'affinité de GroEL pour GroES diminue. L'ATP et un nouveau substrat se fixent en *trans*, provoquant la libération de GroES et du premier substrat en *cis*. D'après Booth *et al.*, les changements conformationnels induits par les nucléotides au niveau des domaines intermédiaires et équatoriaux sont conservés entre les chaperonines de type I et de type II (Booth et al., 2008). Les mouvements du domaine apical seraient différents, provoquant des mécanismes de fermeture du couvercle différents. Cependant, le fonctionnement global de ce chaperon moléculaire vis-à-vis du repliement des protéines semble conservé (Booth et al., 2008).

Il existe une coopération entre le système Hsp70 et les chaperonines qui favorise un repliement efficace des protéines (Frydman et al., 1994; Langer et al., 1992). Chez les procaryotes, cette coopération semble se faire sans interaction stable entre les deux chaperons moléculaires. En revanche, chez les eucaryotes, TRiC/CCT interagit avec les Hsp70 qui pourraient livrer le substrat à replier directement dans la cavité de la chaperonine (Cuellar et al., 2008). Cuellar *et al.* proposent que le domaine NBD d'Hsc70 interagisse avec CCT

uniquement au niveau de la sous-unité CCT β . Cette interaction serait spécifique, aucune interaction entre DnaK (ou son domaine NBD) et GroEL, ou entre Hsc70 et GroEL n'ayant pu être mise en évidence. De plus, les Hsp110 ne semblent pas non plus interagir avec la CCT. L'interaction entre les deux chaperons serait ATP-dépendante et une modélisation moléculaire place le domaine SBD de l'Hsp70 face à la cavité de la CCT. Ainsi, même si la résolution de leur structure ne permet pas de confirmer leur hypothèse, Cuellar *et al.* évoquent la possibilité de changements conformationnels au sein de l'Hsp70 et/ou de la CCT en complexe, qui provoqueraient le transfert de la protéine cliente de l'Hsp70 vers la CCT (Cuellar et al., 2008). Cette hypothèse s'appuie sur le fait que la sous-unité CCT β et les sous-unités adjacentes jouent un rôle important dans la liaison de protéines non repliées comme l'actine et la tubuline (Llorca et al., 2000; Llorca et al., 1999).

I.C.3.b. Hsp90

Les Hsp90 sont des chaperons moléculaires présents chez les bactéries et dans le cytoplasme, le réticulum endoplasmique et les mitochondries eucaryotes. Ils sont essentiels chez les eucaryotes, naturellement abondants, et leur niveau d'expression est fortement induit par les stress environnementaux. Cependant, les Hsp90 semblent avoir une spécificité pour un nombre de substrats plus restreint que les autres chaperons moléculaires, notamment pour des protéines clientes impliquées dans des voies de signalisation (facteurs de transcription, protéines kinases). Leur fonction serait d'assurer les étapes tardives du repliement et de stabiliser des protéines ayant déjà acquis une conformation native. Ainsi, les Hsp90 stabiliseraient des conformations instables de protéines nécessitant la fixation de leur ligand pour obtenir leur conformation finale, et participeraient à l'assemblage et au désassemblage de complexes. Cette stabilisation des protéines clientes permettrait également de prendre en charge des protéines partiellement dénaturées suite à un stress cellulaire (McClellan et al., 2007 ; Wandinger et al., 2008).

Les Hsp90 sont des dimères flexibles dont chaque monomère est constitué d'un domaine N-terminal de liaison aux nucléotides (N), d'un domaine intermédiaire (M) et d'un domaine de dimérisation C-terminal (C). Le fonctionnement de ces chaperons est soumis à une régulation allostérique par l'ATP (Figure 6, page suivante).

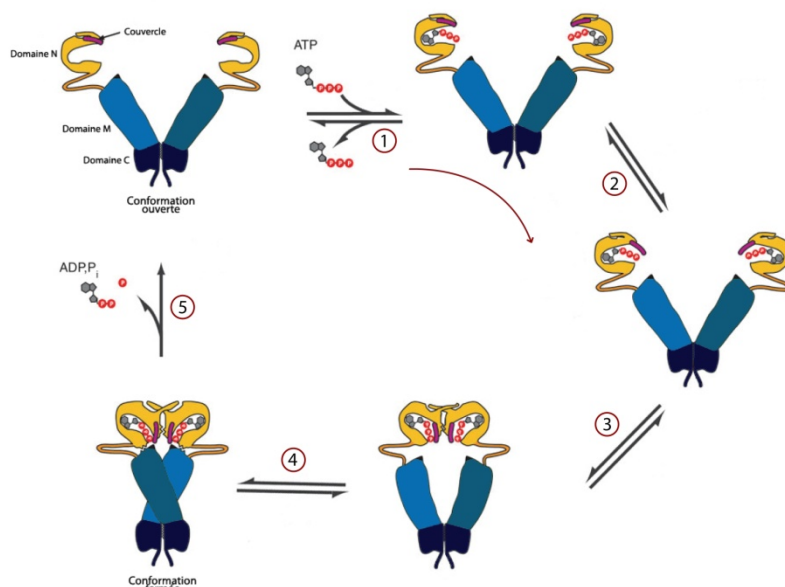


Figure 6 : Schéma du cycle ATPase des Hsp90

La fixation du nucléotide induit la fermeture et la rotation du dimère pour obtenir une forme compacte et enroulée. Le détail du cycle est détaillé ci-après.

Figure adaptée d'après (Hessling et al., 2009).

Après fixation de l'ATP dans chaque domaine N (1), ces derniers se referment sur eux-mêmes (2) puis dimérisent (3) pour former une structure compacte dans laquelle chaque monomère s'enroule autour de l'autre (4). Des interactions sont générées entre les domaines M et N, et le dimère est dans une conformation permettant l'hydrolyse des molécules d'ATP. Après hydrolyse, les domaines N se dissocient, libèrent les ADP et les Pi produits, et le dimère revient à sa conformation initiale (5). Plusieurs cofacteurs régulent ce cycle ATPase, en interagissant préférentiellement avec des conformations spécifiques du dimère d'Hsp90. C'est notamment le cas de la protéine Sti1 (Hop) de *S. cerevisiae* qui inhibe la dimérisation des Hsp90 au niveau des domaines N-terminaux (Hessling et al., 2009; Wandinger et al., 2008). Malgré la caractérisation de ce cycle, la fixation des substrats par les Hsp90 est encore mal connue. Dans le cas de la kinase Cdk4, elle est liée de manière asymétrique au domaine M et probablement au domaine N d'un des deux monomères (Vaughan et al., 2006). Il reste à déterminer si ce mode d'association peut être généralisé aux autres substrats d'Hsp90 ou non.

Comme pour les chaperonines, il existe une coopération entre les systèmes Hsp70 et Hsp90 qui favorise le repliement des protéines clientes. Cette coopération est médiée par le cofacteur Hop qui interagit principalement *via* ses domaines de répétitions de tétratricopeptides (*TPR : Tetratricopeptides Repeats*) avec le motif C-terminal EEVD des Hsp70 et des Hsp90 (Onuoha et al., 2008; Scheufler et al., 2000). La communication entre les deux systèmes chaperons permettrait aux Hsp70 de livrer des substrats particuliers aux Hsp90 (Wegele et al., 2004; Wegele et al., 2006).

I.C.3.c. Hsp100

Les Hsp100 appartiennent à la superfamille des AAA+ (ATPases Associées à des Activités cellulaires diverses). Elles ne sont présentes que chez les procaryotes, les eucaryotes inférieurs, et les chloroplastes chez les plantes. Leur structure en anneau hexamérique est conférée par le(s) domaine(s) AAA des monomères. Les Hsp100 sont divisées en deux groupes selon la composition de leurs monomères ; le groupe I possède des monomères à deux domaines AAA, le groupe II des monomères à un seul domaine AAA. Leur fonction dans la dégradation ou le repliement des protéines dépend quant à elle de leur association avec un système protéolytique ou un système chaperon, respectivement (Figure 4) (Kirstein et al., 2009; Liberek et al., 2008 ; Mogk et al., 2008 ; Saibil, 2008).

Lorsque les Hsp100 sont associées à une chambre protéolytique (protéasome chez les eucaryotes, famille des peptidases ClpP chez les procaryotes), elles se lient aux protéines étiquetées pour la dégradation et les déplient par enfilage dans leur canal central. La dégradation est ainsi favorisée par transfert direct à la chambre protéolytique (Kirstein et al., 2009). Lorsqu'elles sont associées aux Hsp70, les Hsp100 interviennent dans la désagrégation des protéines par le même mécanisme d'enfilage. Les Hsp70 contrôlent et initient l'interaction des Hsp100 avec les protéines à désagréger (Glover and Lindquist, 1998 ; Weibezahn et al., 2004 ; Zietkiewicz et al., 2004). Les conditions d'agrégation et les propriétés des agrégats semblent déterminer la présence d'Hsp100 dans le processus de désagrégation (Ben-Zvi and Goloubinoff, 2002 ; Lewandowska et al., 2007). Initialement, il a été proposé que la taille des agrégats est déterminante pour l'action des Hsp100 (Diamant et al., 2000; Goloubinoff et al., 1999). Au-delà de ce paramètre, les propriétés conformationnelles des protéines au sein des agrégats semblent décisives quant à l'action des Hsp100 (Lewandowska et al., 2007). Le système Hsp70 (ainsi que les autres chaperons moléculaires) participent également au repliement des protéines après leur extrusion du canal.

La protéine Hsp104 de *Saccharomyces cerevisiae* appartient au groupe I des Hsp100. Cette protéine de 102 kDa est présente en équilibre dynamique entre un état inactif et sa forme active hexamérique (Hattendorf and Lindquist, 2002; Schirmer et al., 2001). L'activité de désagrégation de Hsp104 est couplée au système Hsp70 (Glover and Lindquist, 1998). Hsp104 est impliquée dans la thermotolérance acquise, sa quantité dans les cellules est faible en conditions normales de croissance, et fortement augmentée en conditions de stress (Lindquist and Kim, 1996; Sanchez and Lindquist, 1990). Elle permet la solubilisation des agrégats mais n'a pas d'action sur leur prévention (Glover and Lindquist, 1998). De plus, un

mutant $\Delta hsp104$ ne permet pas la propagation des prions de levure [*PSI+*] et [*URE3*] (Chernoff et al., 1995 ; Moriyama et al., 2000).

I.C.3.d. Petites Hsp

La famille des petites Hsp est la plus variée et la moins conservée des familles de chaperons moléculaires. Les petites Hsp sont retrouvées chez tous les règnes et sont caractérisées par la faible masse moléculaire de leurs monomères (de 15 à 43 kDa), leur assemblage en oligomères, et la présence d'un domaine « α -cristalline » conservé (Haslbeck et al., 2005 ; Liberek et al., 2008). Les petites Hsp se lient aux protéines dénaturées pour éviter leur agrégation et participent à la renaturation des agrégats (Horwitz, 1992 ; Jakob et al., 1993). De plus, chaque oligomère peut fixer plusieurs protéines clientes (Haslbeck et al., 1999; Lee et al., 1997). Leur spectre de substrats est large, des études montrant qu'elles sont capables de maintenir un tiers des protéines cytosoliques sous forme soluble en condition de stress thermique (Basha et al., 2004; Haslbeck et al., 2004). Cependant, le spectre de substrats des petites Hsp est chevauchant mais pas strictement identique (Haslbeck et al., 2004).

Les petites Hsp interviennent notamment en réponse aux stress thermique et oxydatif (Haslbeck et al., 1999 ; Jakob et al., 1999). Dans ces conditions, les petites Hsp sont activées et leur affinité pour les substrats augmente (Barbirz et al., 2000; Haslbeck et al., 1999). L'activation des petites Hsp semble différer en fonction des membres, mais régulée par un ensemble d'états d'oligomérisation. Par exemple, l' α -cristalline forme une gamme d'oligomères de tailles variables, avec cependant une prépondérance de certains oligomères (Aquilina et al., 2004 ; 2005). La structure dimérique de l' α -cristalline semble caractéristique des petites Hsp, et a été proposée comme étant l'oligomère actif de ces chaperons moléculaires (Benesch et al., 2008; Van Montfort et al., 2001). Même si elles semblent capables d'interagir avec l'ATP (Biswas and Das, 2004; Muchowski and Clark, 1998), il est communément considéré que l'ATP ne joue pas un rôle direct sur la fonction chaperon des petites Hsp (Haslbeck et al., 2005). La libération des substrats n'est pas spontanée mais servirait de réservoir aux autres chaperons moléculaires (Ehrnsperger et al., 1997; Lee et al., 1997). Enfin, les petites Hsp forment des complexes dynamiques avec un échange de sous-unités constant entre certains membres de cette famille (Bova et al., 2000 ; Sobott et al., 2002; Studer and Narberhaus, 2000).

I.D. Conclusion : les Hsp70 au cœur de la protéostase

Les Hsp70 jouent un rôle central dans le repliement des protéines. En plus de leur fonction de chaperon moléculaire, elles interagissent avec les autres systèmes chaperons pour leur transférer des substrats (chaperonines, Hsp90, Hsp100). Elles interagissent également avec d'autres systèmes cellulaires, comme les systèmes de dégradation par le protéasome, de transport et de translocation. De plus, les Hsp70 sont directement impliquées dans le contrôle qualité des protéines à travers la régulation de leur propre expression, la voie UPR et la dégradation associée au réticulum endoplasmique. Les Hsp70 agissent donc en tant que médiateurs au sein de la protéostase, favorisant à la fois le bon repliement des protéines et l'élimination des espèces néfastes pour la cellule ou l'organisme.

II. Le système Hsp70

La découverte du système Hsp70 est issue de travaux sur le bactériophage λ d'*Escherichia coli* (Georgopoulos, 2006). A la fin des années 1970, il a été montré que des mutations aux loci *dnaK*, *dnaJ* et *grpE* bloquent la réplication du bactériophage λ (Georgopoulos, 1977 ; Saito and Uchida, 1977; Sunshine et al., 1977). A la fin des années 1980, il a été montré *in vitro* que les protéines correspondantes déstabilisent un complexe entre les protéines λ P du phage et la protéine de l'hôte DnaB, une hélicase nécessaire à la réplication du phage (Alfano and McMacken, 1989 ; Zylicz et al., 1989). Parallèlement, DnaK, DnaJ et GrpE ont été identifiées comme des Hsp (Ang et al., 1986; Bardwell et al., 1986 ; Georgopoulos et al., 1982) avant de devenir des modèles historiques pour l'étude du système Hsp70. DnaK et DnaJ correspondent respectivement aux protéines de stress thermique Hsp70 et Hsp40. GrpE a été identifiée comme étant un facteur d'échange, et il a été montré que l'action combinée de GrpE et de DnaJ stimule l'activité ATPase de DnaK de manière synergique (Liberek et al., 1991).

Aujourd'hui, nous savons que les Hsp70 sont des chaperons moléculaires ubiquitaires, présents dans tous les organismes et tous les organites. Cette famille de protéines est l'une des plus conservées au cours de l'évolution (Boorstein et al., 1994 ; Brocchieri et al., 2008; Gupta and Singh, 1994). Les Hsp70 sont impliquées dans un large spectre de fonctions cellulaires et sont généralement différenciées en fonction de leur expression spatio-temporelle.

II.A. Structure, cycle, fonction

II.A.1. Structure

Tous les membres de la famille Hsp70 possèdent une architecture commune. Elle consiste en un domaine N-terminal de liaison au nucléotide (NBD : *Nucleotide Binding Domain*) de 44 kDa, d'un domaine de liaison au substrat (SBD : *Substrate Binding Domain*) de 18 kDa et d'un domaine C-terminal de 10 kDa formant un couvercle au-dessus du domaine de liaison au substrat. Une région flexible permet la communication entre le domaine de liaison au nucléotide et l'ensemble domaine de liaison au substrat/couvercle (Figure 7, page suivante). Les Hsp70 cytoplasmiques possèdent également une région riche en glycines et phénylalanines qui contient le motif EEVD à l'extrémité C-terminale. Ce motif n'est pas présent dans les Hsp70 liées aux ribosomes (Ssb1/2) ou les Hsp70 du RE (BiP). Il permet l'interaction des Hsp70 avec les protéines à domaine J et avec des protéines contenant un domaine TPR (Freeman et al., 1995 ; Liu et al., 1999).

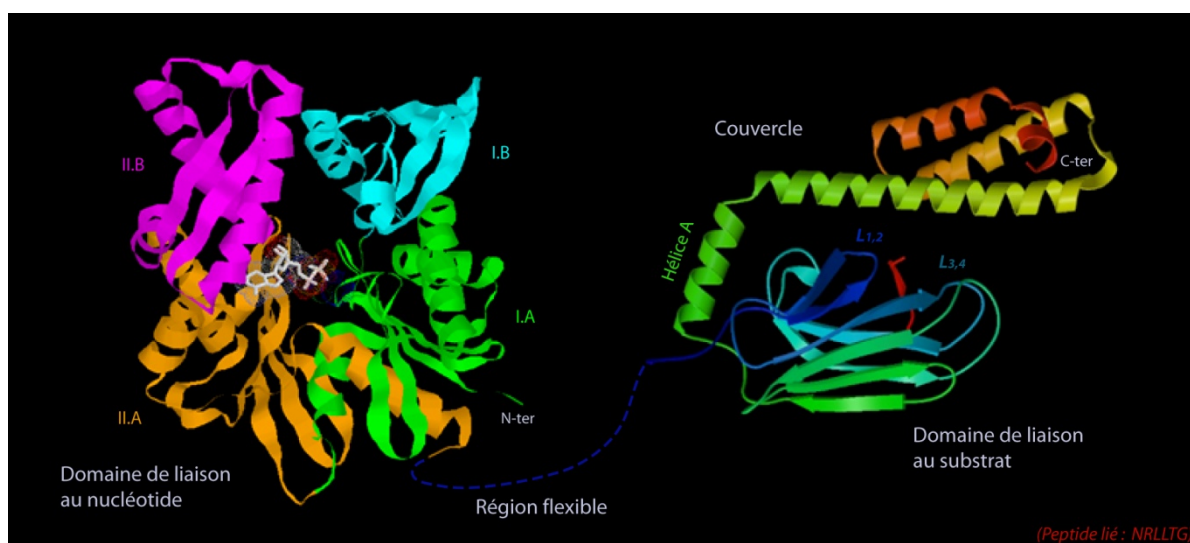


Figure 7 : Structure des domaines des Hsp70

Structure du domaine de liaison au nucléotide de l'Hsc70 bovine (code PDB 3HSC) (Flaherty et al., 1990) et du domaine de liaison au substrat de DnaK d'*Escherichia coli* (code PDB 1DKZ) (Zhu et al., 1996). La structure du domaine de liaison au substrat est présentée en liaison avec le peptide NRLLTG.

Figure adaptée d'après (Kabani et al., 2003).

Le domaine de liaison au nucléotide, ou domaine ATPase, est extrêmement conservé (alignement en page 66). Il est constitué de deux lobes (I et II) eux-mêmes divisés en deux sous-domaines (A et B). Les lobes sont séparés par une large crevasse au fond de laquelle se lie le nucléotide en complexe avec un ion Mg^{2+} et deux ions K^{+} (Flaherty et al., 1990). La flexibilité de ce domaine permet des réorientations de chaque sous-domaine. Cette propriété engendre l'ouverture et la fermeture du site de liaison au nucléotide (Zhang and Zwietering, 2004). En fonction du nucléotide fixé (ADP ou ATP) les sous-domaines subissent une rotation modulant l'accès à la crevasse (Bhattacharya et al., 2009).

Le domaine de liaison au substrat est constitué d'un sandwich de deux feuillets β à quatre brins. La zone d'interaction avec le substrat forme un canal recouvert en partie par une arche constituée des boucles $L_{1,2}$ et $L_{3,4}$ (Figure 7). Le domaine de liaison au substrat est fermé par un couvercle mobile composé de trois hélices α (Zhu et al., 1996). La flexibilité de la structure permet la séquestration et la libération du substrat par fermeture et ouverture du couvercle α -hélicale. L'interaction avec le substrat a lieu au niveau du canal, par la reconnaissance de segments hydrophobes exposés des protéines clientes. DnaK reconnaît préférentiellement des segments d'environ une douzaine de résidus composés de quatre ou cinq acides aminés hydrophobes encadrés par deux régions enrichies en acides aminés basiques (Rudiger et al., 1997). Les caractéristiques structurales du domaine de liaison au

substrat et de son couvercle peuvent participer à la spécificité des Hsp70 vis-à-vis de certains substrats et à leur spécialisation fonctionnelle. La séquence du domaine de liaison au substrat, bien que très conservée au sein des Hsp70, présente une variabilité au niveau du canal entre les boucles L_{1,2} et L_{3,4}. De plus, le couvercle, assurant le maintien du substrat dans le domaine de liaison, est la région la plus variable des Hsp70.

Les domaines NBD et SBD sont reliés par une région flexible conservée (Vogel et al., 2006b). Cette région joue un rôle critique dans la communication inter-domaines. La liaison de l'ATP au niveau du NBD provoque la courbure et l'insertion de la région flexible entre le NBD et le SBD. La région flexible est de nouveau exposée lorsque l'ATP est hydrolysé (Rist et al., 2006 ; Swain et al., 2007; Vogel et al., 2006b).

II.A.2. Cycle ATPase

L'interaction des Hsp70 avec leurs substrats est régulée de manière allostérique par leur cycle ATPase. La fixation du substrat par les Hsp70 est couplée à l'hydrolyse de l'ATP, et sa libération est couplée à la dissociation de l'ADP (Figure 8).

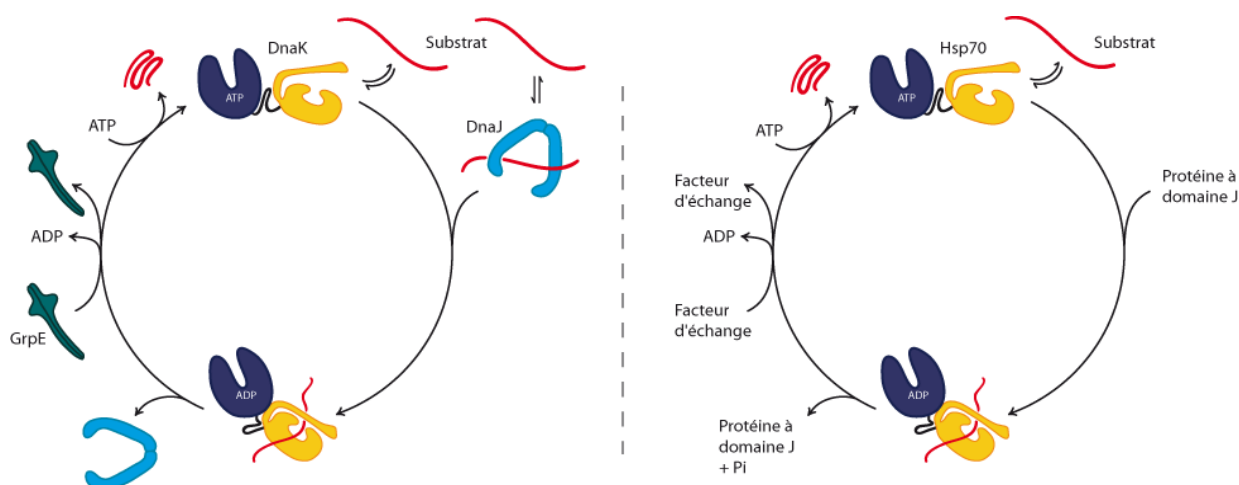


Figure 8 : Modèles du cycle ATPase des Hsp70

A gauche. Cycle de DnaK avec la protéine à domaine J DnaJ et le facteur d'échange GrpE.

A droite. Modèle général du cycle ATPase des Hsp70.

Lorsque les Hsp70 sont sous forme ATP, la fréquence d'ouverture et de fermeture du couvercle sur le domaine de liaison au substrat est élevée, et l'affinité pour le substrat est faible. Après hydrolyse de l'ATP, le changement de conformation de l'Hsp70 stabilise la fermeture du couvercle et l'interaction avec le substrat (McCarty et al., 1995 ; Schmid et al., 1994 ; Zhu et al., 1996). Dans le modèle canonique tripartite DnaK/DnaJ/GrpE d'*Escherichia coli*, cette étape limitante est stimulée par la protéine à domaine J DnaJ qui livre le substrat à DnaK (Liberek et al., 1991 ; McCarty et al., 1995; Schroder et al., 1993 ; Szabo et al., 1994).

L'activité ATPase de DnaK est alors stimulée de manière synergique par la fixation du substrat et par DnaJ (Laufen et al., 1999). La dissociation de l'ADP est stimulée par le facteur d'échange GrpE (Liberek et al., 1991). Elle permet la fixation d'une nouvelle molécule d'ATP et la libération du substrat. Chez les eucaryotes, la régulation allostérique des Hsp70 est conservée. Cependant, ils possèdent plusieurs facteurs d'échange ainsi qu'un arsenal plus important de protéines à domaine J. Ces dernières, contrairement à DnaJ, ne possèdent pas toutes de site de liaison au substrat.

D'un point de vue mécanistique, la liaison d'un substrat est couplée à la réorganisation conformationnelle de l'Hsp70. Sous la forme ATP, le domaine de liaison au nucléotide aurait une conformation écartée, la région intermédiaire flexible serait repliée entre le NBD et le SBD, et le couvercle serait ouvert au-dessus du SBD. La fixation du substrat et l'interaction avec une protéine à domaine J favoriseraient le déplacement de la région intermédiaire. Après hydrolyse de l'ATP, le NBD aurait une conformation plus fermée suite à une rotation de ses sous-domaines, la région intermédiaire serait plus exposée, et l'hélice A du couvercle interagirait au niveau d'un sillon présent à la base du NBD. Cette interaction, ainsi que le rapprochement du SBD vers le NBD, stabiliseraient la conformation fermée de l'Hsp70 (Bertelsen et al., 2009; Bhattacharya et al., 2009 ; Chang et al., 2008 ; Jiang et al., 2005 ; Rist et al., 2006 ; Swain et al., 2007 ; Vogel et al., 2006a ; Vogel et al., 2006b).

II.A.3. Mécanismes d'action des Hsp70

La fonction de base des Hsp70 est de lier les segments hydrophobes exposés des protéines dont la conformation est non native. Leur rôle est de prévenir l'agrégation des protéines, promouvoir leur repliement natif, et solubiliser les agrégats.

Le mécanisme par lequel les Hsp70 favorisent le repliement des protéines est cependant mal compris. Une première hypothèse leur attribue un rôle passif. Elles offriraient aux protéines clientes une opportunité supplémentaire d'atteindre leur conformation native à chaque cycle de liaison/libération. Conjointement ces cycles maintiendraient une concentration faible en substrats libres ce qui préviendrait l'agrégation des protéines (Chiti et al., 2002). Une seconde hypothèse propose que la répétition des cycles provoque un dépliement local des protéines mal repliées, agrégées, et des intermédiaires de repliement. Ces déploiements permettraient de diminuer l'énergie d'activation nécessaire pour atteindre la conformation native (Ben-Zvi et al., 2004 ; Ben-Zvi and Goloubinoff, 2001 ; Goloubinoff and De Los Rios, 2007; Slepnev and Witt, 2002).

Les Hsp70 semblent capables de solubiliser certains agrégats protéiques sans l'assistance des Hsp100 (Skowyra et al., 1990). Leur mécanisme de désagrégation pourrait être expliqué par le modèle de *traction entropique* (De Los Rios et al., 2006 ; Goloubinoff and De Los Rios, 2007). Cependant, cette activité serait faible et marginale, et est sujette à controverses.

II.B. Co-chaperons des Hsp70

II.B.1. Protéines à domaine J

L'hydrolyse de l'ATP est une étape limitante du cycle pour la plupart des Hsp70. Elle est stimulée par l'action synergique de la fixation du substrat et de la stimulation par les protéines à domaine J (Karzai and McMacken, 1996 ; Laufen et al., 1999; Liberek et al., 1991). Les protéines à domaine J permettent également de recruter les Hsp70 pour assurer des fonctions particulières comme la dissociation des manteaux de clathrine lors de l'endocytose (voir partie II.B.1.b. Fonctions).

Ce sont des co-chaperons ubiquitaires dont la diversité augmente avec la complexité de l'organisme duquel elles sont issues. Pour exemple, six gènes codant des protéines à domaine J ont été identifiés chez *Escherichia coli* (Genevaux et al., 2007), 22 chez *Saccharomyces cerevisiae* (Walsh et al., 2004), et au moins 41 dans le génome humain (Qiu et al., 2006). Elles sont présentes dans tous les organites et diffèrent selon leur localisation cellulaire, leur distribution tissulaire, et leur fonction.

II.B.1.a. Structure et classification

Toutes les protéines appartenant à cette famille possèdent un domaine J qui permet l'interaction avec le domaine NBD des Hsp70. Le domaine J de DnaJ est constitué de quatre hélices α et d'une boucle entre les hélices II et III qui forment un motif *coil-coiled* (Figure 9.b, page suivante) (Pellecchia et al., 1996). Cette boucle présente le motif conservé HPD (Histidine, Proline, Acide aspartique) responsable de la stimulation de l'activité ATPase des Hsp70. Le domaine J seul est capable d'assurer les fonctions essentielles des protéines à domaine J, en stimulant l'activité intrinsèque des Hsp70 (Sahi and Craig, 2007). Cependant, les protéines à domaines J peuvent également interagir avec le domaine SBD des Hsp70, et certaines sont capables de leur transférer un substrat (Summers et al., 2009).

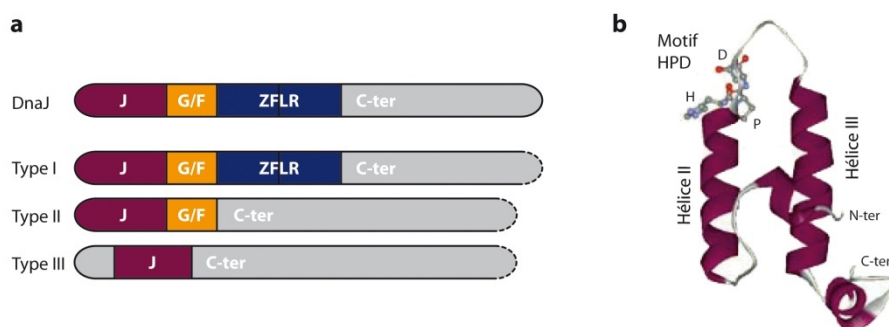


Figure 9 : Classification structurale des protéines à domaine J

a. Illustration schématique de la classification des protéines J selon leur degré de similarité avec DnaJ. J : domaine J ; G/F : région riche en glycines et phénylalanines ; ZFLR : domaine de type doigt de zinc ; C-ter : région C-terminale dont la longueur varie en fonction des protéines à domaine J.

b. Structure du domaine J de DnaJ d'*Escherichia coli* (code PDB 1XBL) d'après (Pellecchia et al., 1996). Figure adaptée d'après (Mayer and Bukau, 2005).

La classification des protéines à domaine J est basée sur leur composition en différents domaines et leur degré de similarité avec DnaJ (Figure 9.a) (Qiu et al., 2006 ; Summers et al., 2009). Les protéines à domaine J de type I possèdent les domaines caractéristiques de DnaJ, soit un domaine J en N-terminal, une région flexible riche en glycines et phénylalanines (région G/F), un domaine de type doigt de zinc (ZFLR : *Zinc-Finger-Like Region*) riche en cystéines et un domaine C-terminal moins conservé. Les protéines à domaine J de type II présentent la même architecture sans domaine ZFLR. Les protéines à domaine J de type III possèdent un domaine J dont la position est variable, et des domaines fonctionnels divers permettant d'assurer des fonctions spécifiques. La région G/F semble essentielle au fonctionnement de certaines protéines à domaine J (Lopez et al., 2003). Elle pourrait intervenir dans la coordination entre les domaines J et C-terminal des protéines de types I et II mais son rôle précis reste incompris (Tzankov et al., 2008). Le domaine ZFLR est impliqué dans la liaison des substrats et leur transfert aux Hsp70 (Fan et al., 2005; Linke et al., 2003 ; Szabo et al., 1996). Dans le cas des protéines à domaine J de classes I et II, le domaine C-terminal est un domaine de liaison aux substrats et d'homodimérisation. Les protéines à domaine J de classe III possèdent elles des domaines variés comme des hélices transmembranaires, des domaines TPR ou des régions riches en cystéines. Ces domaines définissent leur localisation cellulaire et leur spécificité fonctionnelle.

II.B.1.b. Fonctions

La variabilité et le nombre important de protéines à domaine J, en plus de leurs différences de localisation, permettent d'apporter une diversité fonctionnelle aux Hsp70. Certaines protéines à domaine J sont spécifiques d'un orthologue de Hsp70, tandis que

d'autres ont un spectre plus large. Réciproquement, les orthologues de Hsp70 n'interagissent pas avec toutes les protéines à domaine J d'un même compartiment cellulaire (Figure 10).

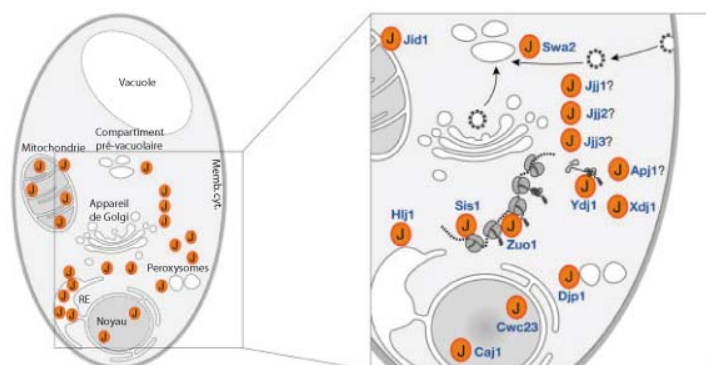


Figure 10 : Localisation cellulaire et organisation des protéines à domaine J de *Saccharomyces cerevisiae*

La localisation des 22 protéines à domaine J est montrée sur la gauche. La section de droite détaille l'organisation des protéines à domaine J cytosoliques et nucléaires. Figure adaptée d'après (Walsh et al., 2004).

Les protéines à domaine J de types I et II ont une fonction similaire de reconnaissance et de transfert des substrats non natifs aux Hsp70 (Kelley, 1998). Leur affinité pour les substrats peut dépendre de la région G/F, du domaine ZFLR et du domaine C-terminal, ce qui leur confère des spectres d'action différents. Ces protéines n'ont pas toutes une fonction généraliste. Chez *Saccharomyces cerevisiae*, certaines sont impliquées dans des fonctions spécifiques comme Djp1 dans la biogenèse des peroxysomes (Hetteema et al., 1998) ou Sis1 dans l'initiation de la traduction (Zhong and Arndt, 1993).

Les protéines à domaine J de type III possèdent des fonctions spécifiques. Elles ne sont pas impliquées dans le repliement mais plutôt dans des processus d'assemblage et de désassemblage de complexes. Par exemple, chez *Saccharomyces cerevisiae*, Cwc23 est impliquée dans l'épissage des ARN messagers (Sahi et al., 2009), Zuo1 fait partie du complexe RAC (Gautschi et al., 2001; Yan et al., 1998), Jjj1 est impliquée dans la biogénèse des ribosomes (Meyer et al., 2007), et Swa2 dans le désassemblage des manteaux de clathrine (Gall et al., 2000).

II.B.2. Facteurs d'échange

Les constantes de dissociation de l'ADP varient en fonction des Hsp70. Cette variation dépend de différences subtiles dans la structure du NBD, notamment à l'interface entre les deux lobes formant la crevasse de liaison au nucléotide. Les Hsp70 ont donc des dépendances différentes vis-à-vis des facteurs d'échange (NEF : *Nucleotide Exchange Factor*). Les orthologues d'HscA, une Hsp70 d'*Escherichia coli*, semblent compléter leur cycle indépendamment des facteurs d'échange. Les orthologues d'Hsc70 humaines, aux constantes de dissociation intermédiaires, semblent nécessiter un facteur d'échange uniquement lorsque le taux d'hydrolyse de l'ATP est fortement stimulé. D'autres orthologues comme DnaK, aux

constantes de dissociation faibles, dépendent obligatoirement des facteurs d'échange pour compléter leur cycle (Mayer and Bukau, 2005).

Plusieurs familles de facteurs d'échange ont été identifiées, GrpE chez les procaryotes, et Bag1, Fes1/HspBP1 et Hsp110 chez les eucaryotes. Elles diffèrent dans leur structure et dans leur mécanisme d'action (Kabani, 2009).

II.B.2.a. GrpE

Historiquement, GrpE a été le premier facteur d'échange à être découvert. C'est un facteur d'échange essentiel d'*Escherichia coli* dont l'expression est inductible par un stress. Chez les eucaryotes, les orthologues de GrpE ne sont retrouvés que dans les mitochondries et les chloroplastes.

GrpE est un homodimère asymétrique de forme allongée (Harrison, 2003; Harrison et al., 1997). Les monomères sont constitués d'une région N-terminale non structurée, d'une longue puis d'une courte hélice α et d'un domaine en feuillet β en C-terminal. L'interaction de GrpE avec le domaine NBD de DnaK se fait essentiellement avec le monomère proximal. La moitié supérieure de la longue hélice α et la courte hélice α du monomère proximal sont courbées le long de la surface du NBD. Son domaine en feuillet β pénètre le NBD pour contacter l'entrée de la crevasse de liaison au nucléotide (Figure 11). Cette interaction provoque une rotation du sous-domaine II.B de 14° et l'ouverture de la crevasse de liaison au nucléotide. Les interactions entre le sous-domaine II.B et le nucléotide sont rompues, permettant la dissociation du nucléotide. La fixation d'une nouvelle molécule d'ATP provoque la dissociation de DnaK et GrpE. Une boucle étendue au sommet du sous-domaine II.B de DnaK semble nécessaire à la fixation de GrpE qui ne stimule pas la dissociation du nucléotide des Hsp70 ne la possédant pas (Brehmer et al., 2001).



Figure 11 : Structure et représentation schématique du complexe GrpE–NBD de DnaK

GrpE provoque l'ouverture de la crevasse de liaison au nucléotide par une rotation du domaine II.B du NBD de DnaK.

Figure adaptée d'après (Bukau et al., 2006).

GrpE est capable de stimuler la dissociation de l'ADP et de l'ATP de manière non discriminante. Il stimule également la dissociation du substrat de DnaK *via* son domaine N-

terminal non structuré (Han and Christen, 2003; Mally and Witt, 2001 ; Mehl et al., 2001). Les résultats obtenus par ces groupes, et ceux obtenus par Brehmer *et al.* (Brehmer et al., 2004) sont plus ou moins contradictoires mais soulignent tous le rôle de GrpE dans la dissociation des substrats. De plus, il a été proposé que le domaine N-terminal de GrpE pourrait servir de leurre pour le domaine SBD de DnaK (Brehmer et al., 2004; Chesnokova et al., 2003). Cette hypothèse est appuyée par Moro *et al.* qui ont montré que le fragment 1-33 de GrpE servirait de pseudo-substrat, et que la moitié N-terminale de la longue hélice α interagirait avec la région flexible de DnaK (Moro et al., 2007).

Les longues hélices α de GrpE lui permettent de fonctionner comme un thermo-senseur. A des températures élevées, elles adoptent une conformation réversible en spirale qui diminue l'activité facteur d'échange de GrpE (Grimshaw et al., 2001). Cette caractéristique semble conservée chez les différents homologues de GrpE (Groemping and Reinstein, 2001 ; Moro and Muga, 2006).

Une vingtaine d'années après la découverte de GrpE, et malgré la découverte de nombreux membres des familles Hsp70 et Hsp40, aucun homologue de ce facteur d'échange n'était identifié dans le cytosol eucaryote. Il a longtemps été proposé que les Hsp70 eucaryotes aient évolué de façon à ce que la dissociation de l'ADP ne soit pas une étape limitante de leur cycle ATPase (Hohfeld et al., 1995). Suite à de nombreuses années de débat, il s'est avéré que non seulement les facteurs d'échange eucaryotes existent, mais que cette fonction est assurée par trois familles de protéines distinctes, sans lien avec GrpE ou entre elles.

II.B.2.b. Protéines à domaine BAG

La protéine Bag1 (*Bcl-2-associated athanogen 1* ; athanogène 1 associé à Bcl-2) fut le premier facteur d'échange eucaryote identifié. D'abord isolée comme partenaire de la protéine anti-apoptotique Bcl-2 (Takayama et al., 1995), il a été montré par la suite qu'il interagit avec les Hsp70 pour catalyser la libération de l'ADP (Hohfeld and Jentsch, 1997).

La protéine Bag1 est exprimée dans les cellules humaines sous quatre isoformes et appartient à la famille hétérogène des protéines à domaine BAG. Les membres de cette famille présentent la caractéristique commune de posséder un domaine BAG impliqué dans l'interaction avec le domaine de liaison au nucléotide des Hsp70. Chez l'Homme, il existe six protéines Bag qui interagissent toutes avec les Hsp70 (Gehrmann et al., 2005 ; Hohfeld and Jentsch, 1997 ; Kalia et al., 2004 ; Takayama et al., 1997 ; Takayama et al., 1999 ; Thress et

al., 2001). Cinq d'entre elles possèdent un unique domaine BAG à leur extrémité C-terminale, alors que Bag5 possède quatre domaines BAG (Doong et al., 2002 ; Kabbage and Dickman, 2008; Takayama and Reed, 2001). La composition en domaines des protéines Bag est hétérogène ce qui leur procure des spécificités de substrats et des localisations cellulaires différentes (Doong et al., 2002 ; Kabbage and Dickman, 2008; Takayama and Reed, 2001). En ce sens, les protéines à domaine BAG sont comparées aux protéines à domaine J dont le rôle est également de recruter les Hsp70 pour assurer des fonctions spécifiques. Bag1 est par exemple impliquée dans la régulation de nombreuses fonctions comme la protection contre l'apoptose, la dégradation protéasomale, ou encore l'activation de la protéine kinase Raf-1 (Alberti et al., 2003). Certaines protéines à domaine BAG ont des rôles antagonistes, comme Bag1 et Bag2 vis-à-vis de l'ubiquitine ligase CHIP. Alors que Bag1 stimule la dégradation protéasomale associée à CHIP (Demand et al., 2001), Bag2 semble inhiber son activité ubiquitine ligase (Arndt et al., 2005; Dai et al., 2005).

La structure du domaine BAG de Bag1, seule ou en complexe avec le domaine NBD de Hsc70, met en évidence une structure totalement différente de celle de GrpE (Briknarova et al., 2001 ; Sondermann et al., 2001). Le domaine BAG est replié en trois hélices antiparallèles. Les hélices H2 et H3 interagissent avec les sous-domaines I.B et II.B du NBD, provoquant son ouverture par une rotation du sous-domaine II.B de 14°. Ainsi, GrpE et Bag1 induisent une ouverture comparable du NBD avec des mécanismes d'action différents (Harrison et al., 1997 ; Sondermann et al., 2001) (Figure 12). De plus, Bag1 est incapable de catalyser la dissociation de l'ATP, et la dissociation de l'ADP est affectée par la présence du phosphate inorganique (Gassler et al., 2001). Contrairement au cas de GrpE, les résidus du NBD impliqués dans l'interaction avec le domaine BAG sont conservés chez toutes les Hsp70 cytosoliques humaines. GrpE et Bag1 montrent une sélectivité pour leurs partenaires respectifs en raison des différences structurales des sites de liaison au nucléotide entre les Hsc70 eucaryotes et DnaK (Brehmer et al., 2001).

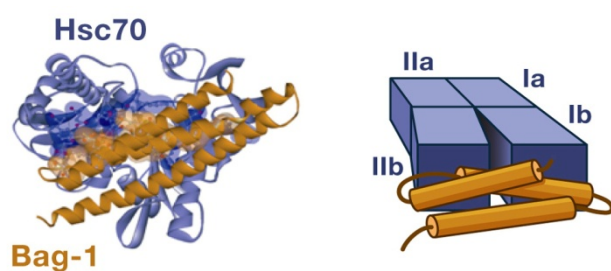


Figure 12 : Structure et représentation schématique du complexe Bag1-NBD de DnaK

Bag1 provoque l'ouverture de la crevasse de liaison au nucléotide par une rotation du domaine II.B du NBD de DnaK.

Figure adaptée d'après (Bukau et al., 2006).

Des orthologues de protéines Bag ont également été identifiés chez les levures *Schizosaccharomyces pombe* et *Yarrowia lipolytica*. Chez *Saccharomyces cerevisiae*, la protéine Snl1 participerait à l'assemblage des complexes multiprotéiques au niveau des membranes nucléaire et du réticulum endoplasmique.

II.B.2.c. HspBP1 et protéines BAP

Cette famille de facteurs d'échange regroupe les homologues de HspBP1 cytosoliques et dans le réticulum endoplasmique, les protéines BAP (*BiP-Associated Proteins* ; protéines associées à BiP) (Kabani et al., 2003).

Sls1 fut le premier membre de cette famille à être identifié à partir d'études sur la voie sécrétoire de la levure *Yarrowia lipolytica*. Il a été montré qu'elle favorise la translocation cotraductionnelle des protéines à travers la membrane du réticulum endoplasmique *via* son interaction avec l'Hsp70 Kar2/BiP, Hsp70 de la lumière du réticulum (Boisrame et al., 1996 ; Boisrame et al., 1998). Il a ensuite été démontré que les homologues de Sls1 chez *Saccharomyces cerevisiae* et chez les mammifères, Sls1/Sil1 et BAP respectivement, interagissent avec la forme ADP de BiP, stimulent son activité ATPase conjointement aux protéines à domaine J, et catalysent la libération de l'ADP (Chung et al., 2002; Kabani et al., 2000a ; Tyson and Stirling, 2000).

La recherche d'orthologues cytosoliques de Sls1 dans le génome de *Saccharomyces cerevisiae* a conduit à l'identification de Fes1 et à sa caractérisation en tant que facteur d'échange de l'Hsp70 cytoplasmique Ssa1 (Kabani et al., 2002a). Fes1 agit également en tant que facteur d'échange des Hsp70 associées aux ribosomes, Ssb1 et Ssb2 (Dragovic et al., 2006b). L'homologue humain de Fes1, HspBP1, était initialement connu comme répresseur des Hsp70 (Raynes and Guerriero, 1998). Il a été identifié par homologie de séquence avec Fes1 et Sls1, et également caractérisé comme étant un facteur d'échange (Kabani et al., 2002b). HspBP1 et Fes1 ne peuvent assurer leur fonction de facteur d'échange vis-à-vis de DnaK, suggérant une spécificité vis-à-vis des homologues de Hsp70, comme pour GrpE et Bag1 (Brehmer et al., 2001 ; Kabani et al., 2002b).

HspBP1 est constituée d'un domaine N-terminal non structuré et d'un large domaine en hélices α qui interagit avec le domaine NBD des Hsp70 (McLellan et al., 2003). Le domaine non structuré (BP1c) a une forme allongée et est constitué uniquement d'un enchaînement d'hélices α . Quatre répétitions centrales d'Armado (ARM1-4, α 3-14) sont encadrées par quatre hélices légèrement plus longues (α 1-2 et α 15-16). La structure du complexe HspBP1-Hsp70 a été réalisée à partir de BP1c et du lobe II du domaine NBD de l'Hsp70 humaine

(Shomura et al., 2005). Elle révèle un mécanisme encore différent de ceux de GrpE et Bag1. BP1c enveloppe le sous-domaine II.B du NBD. Cette interaction causerait le déplacement du sous-domaine IB en raison de l'encombrement stérique généré, et donc une distorsion des deux lobes du NBD permettant la libération du nucléotide (Figure 13).

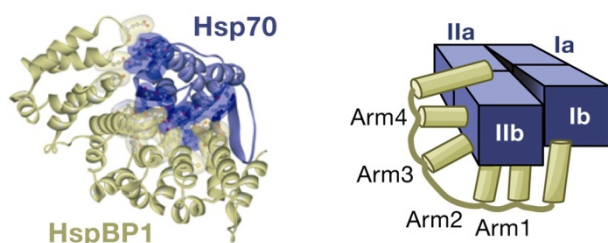


Figure 13 : Structure et représentation schématique du complexe HspBP1 NBD de DnaK

HspBP1 provoque un déplacement du domaine I.B du NBD de DnaK.

Figure adaptée d'après (Bukau et al., 2006).

II.B.2.d. Famille Hsp110

La famille Hsp110 regroupe les Hsp110 cytosoliques et leur homologues dans le réticulum endoplasmique, les Grp170. Ces chaperons moléculaires ont des séquences et des structures similaires aux Hsp70, et appartiennent à la superfamille des Hsp70 (Liu and Hendrickson, 2007; Shaner and Morano, 2007). L'architecture en domaines des Hsp110 est globalement semblable à celle des Hsp70. Les différences majeures résident dans une large boucle acide d'environ 100 à 140 acides aminés insérée entre le domaine de liaison au peptide et le couvercle et dans des extensions à l'extrémité C-terminale (Shaner and Morano, 2007).

Les Hsp110 sont des chaperons moléculaires abondants exprimés dans tous les tissus. Ils peuvent être exprimés de manière constitutive mais aussi inductible par un stress selon les homologues (Easton et al., 2000). Les Hsp110 n'ont pas une fonction de repliement proprement dite mais de séquestration (Oh et al., 1997 ; Oh et al., 1999). Elles pourraient également coopérer avec le système Hsp90 pour replier des substrats particuliers (Goekeler et al., 2002). Contrairement aux Hsp70, l'activité ATPase des Hsp110 ne semble pas essentielle à leur fonction, qui dépendrait uniquement de la fixation de l'ATP mais pas de son hydrolyse (Raviol et al., 2006; Shaner et al., 2004). Les Hsp110 cytosoliques humaines et l'homologue Sse1 de *Saccharomyces cerevisiae* interagissent fonctionnellement et physiquement avec les Hsp70 (Shaner et al., 2005 ; Yam et al., 2005 ; Yamagishi et al., 2004), et ont été caractérisées comme étant des facteurs d'échange (Dragovic et al., 2006a ; Raviol et al., 2006 ; Shaner et al., 2006).

Sse1 a été cristallisée seule (Liu and Hendrickson, 2007), en complexe avec le domaine de liaison au nucléotide d'une Hsp70 humaine (Polier et al., 2008) et d'une Hsc70 bovine (Schuermann et al., 2008). La structure de Sse1 en complexe avec l'ATP apparaît comme un

homodimère avec une orientation en tête-à-queue de chaque protomère (Liu and Hendrickson, 2007). Sse1 a une structure globalement comparable à celle des Hsp70, les domaines sont relativement conservés, mais leur organisation diffère. Les domaines NBD et SBD sont intimement associés dans les protomères et le long couvercle ne recouvre pas le SBD mais s'étend le long du NBD (Liu and Hendrickson, 2007). En complexe avec le domaine NBD des Hsp70, ce dernier est embrassé à la fois par le domaine NBD et par le couvercle de Sse1. Cet arrangement provoque une rotation de 27° du sous-domaine II.B du NBD de l'Hsp70 et ainsi l'ouverture de la crevasse de liaison au peptide de l'Hsp70 et la libération de l'ADP (Polier et al., 2008 ; Schuermann et al., 2008) (Figure 14).

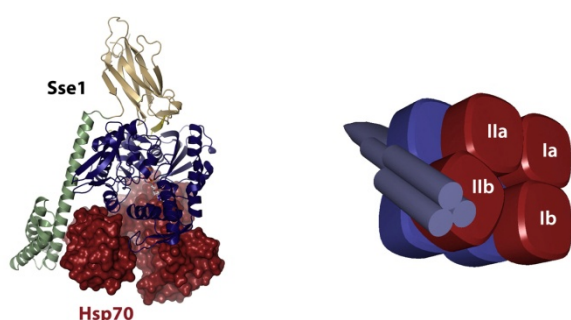


Figure 14 : Structure et représentation schématique du complexe Sse1-Hsp70 humaine (NBD)

Sse1 provoque une rotation du sous-domaine II.B du NBD de Hsp70.

Figure adaptée d'après (Polier et al., 2008).

Un modèle de coopération des Hsp70 et des Hsp110 dans le repliement des protéines a été proposé (Polier et al., 2008). Hsp70 fixerait une protéine cliente après hydrolyse de l'ATP stimulée par une protéine à domaine J. Hsp110 interagirait alors avec Hsp70 pour induire la libération de l'ADP de cette dernière. Hsp110, *via* sa liaison avec Hsp70, pourrait interagir avec le substrat, stabilisant le complexe ternaire. La formation de tels complexes pourrait faciliter la prise en charge de protéines clientes de taille importante pour limiter leur agrégation et faciliter leur repliement. La fixation d'une nouvelle molécule d'ATP par l'Hsp70 dissocierait le complexe.

III. Spécificités fonctionnelles des Hsp70

III.A. Le système Hsp70 d'*Escherichia coli* ne se limite pas à la triade DnaK/DnaJ/GrpE

Chez *Escherichia coli*, deux Hsp70 ont été identifiées en plus de DnaK, HscA (Kawula and Lelivelt, 1994 ; Seaton and Vickery, 1994) et HscC (Kluck et al., 2002; Yoshimune et al., 1998). Son génome code également six protéines à domaine J, et un seul facteur d'échange, GrpE. Des études génétiques montrent que les gènes *dnaK*, *hscA* et *hscC* ne sont pas essentiels, sous certaines conditions de laboratoire (Hesterkamp and Bukau, 1998 ; Kluck et al., 2002). La coexistence de ces chaperons moléculaire amène donc la question de leur redondance et/ou de leurs spécificités fonctionnelles (Genevaux et al., 2007). Les réponses se trouvent dans leurs différences d'expression, leurs différences intrinsèques, leurs interactions avec leurs co-chaperons, et leur spécificité pour certains substrats.

DnaK, HscA et HscC sont exprimées de manière constitutive. Cependant, seule l'expression de DnaK est induite par une augmentation de la température (Kluck et al., 2002; Lelivelt and Kawula, 1995 ; Neidhardt and VanBogelen, 1981 ; Seaton and Vickery, 1994). Le gène *hscA* et le gène *hscB* (codant une protéine à domaine J) sont cotranscrits et surexprimés en condition de stress froid, mais leur expression ne semble pas induite lors d'un stress chaud (Lelivelt and Kawula, 1995). DnaK est également l'Hsp70 la plus abondante (approximativement 10000 copies par cellule, et le double en condition de stress). En comparaison, l'abondance de HscA est d'environ 2500 copies par cellule, et celle de HscC de 200 à 500 copies par cellule (Genevaux et al., 2007).

De plus, le fonctionnement de ces Hsp70 ne requiert pas les mêmes co-chaperons. L'interaction de chaque Hsp70 est restreinte à des protéines à domaine J spécifiques. L'activité ATPase de DnaK est stimulée par DnaJ (Liberek et al., 1991) et DjlA (Genevaux et al., 2001), et CbpA stimule l'activité chaperon de DnaK (Chae et al., 2004). En revanche, DnaK ne semble pas interagir avec HscB, DjlB et DjlC (Genevaux et al., 2007). HscA semble interagir spécifiquement avec HscB (Silberg et al., 1998). Enfin, l'activité ATPase de HscC est stimulée uniquement par DjlC (Kluck et al., 2002). La forte homologie de séquence entre DjlC et DjlB suggère que ces deux protéines à domaine J agiraient comme co-chaperon de HscC (Genevaux et al., 2007).

GrpE stimule l'activité ATPase de DnaK et HscC (Liberek et al., 1991 ; Yoshimune et al., 2002) en présence d'une protéine à domaine J appropriée mais ne semble pas interagir avec HscA (Brehmer et al., 2001; Silberg et al., 1998).

D'un point de vue fonctionnel, DnaK jouerait un rôle général dans la prise en charge des protéines néo-synthétisées et des protéines dénaturées. Les fonctions de HscA et HscC ne peuvent se substituer totalement à celle de DnaK (Hesterkamp and Bukau, 1998 ; Kluck et al., 2002). HscA et la protéine à domaine J HscB seraient spécialisées dans l'assemblage des centres [Fe-S] (Seaton and Vickery, 1994 ; Takahashi and Nakamura, 1999 ; Vickery and Cupp-Vickery, 2007) et la réponse aux stress froids (Lelivelt and Kawula, 1995). Les fonctions de HscC sont mal connues. Son invalidation n'est pas létale pour la souche, et les phénotypes associés sont une hypersensibilité aux radiations UV et aux ions cadmium (Kluck et al., 2002).

III.B. La complexité du système augmente avec celle de l'organisme

La levure *Saccharomyces cerevisiae* possède neuf Hsp70. Leur première différence réside dans leur localisation cellulaire. Kar2/BiP est spécifique au réticulum endoplasmique (Normington et al., 1989) et Ssc1 est spécifique à la mitochondrie (Craig et al., 1989). Les Hsp70 cytosoliques comprennent Ssa1, Ssa2, Ssa3 et Ssa4, ainsi que Ssz1, Ssb1 et Ssb2 qui sont associées aux ribosomes (Gautschi et al., 2001 ; Nelson et al., 1992). Dès 1987, Elizabeth Craig et ses collaborateurs cherchent à définir les interactions fonctionnelles au sein des Ssa (Werner-Washburne et al., 1987). Ils montrent que les gènes *SSA1-4* ne sont pas essentiels mais que l'expression d'au moins l'un de ces gènes est essentielle à la survie de la souche. Ils montrent également que l'expression de *SSA3*, comme *SSA4*, est inductible par un choc thermique. L'analyse d'un ensemble de mutants *ssa* (simples, doubles, triples) ainsi que des tests de thermotolérance à partir de ces mêmes souches les ont amenés à la conclusion que Ssa1, Ssa2, Ssa3 et Ssa4 sont fonctionnellement redondants. L'hypothèse développée alors était que la présence de paralogues inductibles par un stress permet l'augmentation de la quantité globale de chaperons pour répondre à ce stress. Elle fut d'autant plus admise que les différences de séquence entre les Ssa sont minimes. Le pourcentage d'identité des séquences en acides-aminés s'échelonne de 79 % entre Ssa2 et Ssa3 à 97 % entre Ssa1 et Ssa2 (Kabani and Martineau, 2008b).

En 1991, Gao *et al.* mettent en évidence la première spécificité fonctionnelle entre les paralogues constitutifs Ssa1 et Ssa2 (Gao et al., 1991). Ils montrent que l'activité de Ssa2 dans le désassemblage des manteaux de clathrine est significativement plus élevée que celle de Ssa1. Judith Frydman et collaborateurs démontrent ensuite l'existence de deux réseaux de chaperons moléculaires aux fonctions distinctes, l'un intervenant dans le repliement *de novo*

et l'autre dans la réponse au stress. Ces réseaux possèderaient une zone de recouvrement des deux types de fonctions, dans laquelle se situerait Ssa1 (Albanese et al., 2006). Il a également été montré que les formes inductibles des Hsp70 possèdent des propriétés spécifiques à la prise en charge des protéines dénaturées (Sharma et al., 2009).

Les Hsp70 possèdent donc des fonctions à la fois redondantes et spécifiques. Le génome de *Saccharomyces cerevisiae* code 22 protéines à domaine J ainsi que deux types de facteurs d'échange (Fes1 et Sse1/2). Chez l'Homme, 17 gènes codant des Hsp70 ont été identifiés, ainsi que 30 pseudogènes (Brocchieri et al., 2008). Ces Hsp70 sont différenciées par leur expression spatio-temporelle, mais possèdent également des spécificités fonctionnelles décrites dans la revue ci-après (Daugaard et al., 2007). La régulation du système Hsp70 est subtile, et dépend très certainement de la coopération entre les différents acteurs du système.

III.C. Revue

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MULTIPLE HSP70 ISOFORMS IN THE EUKARYOTIC CYTOSOL: MERE REDUNDANCY OR FUNCTIONAL SPECIFICITIES?

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La Figure 1 (page 339 de la revue) a été involontairement rognée lors de sa publication. Le format de la Table 1 (pages 344-345 de la revue) a également été modifié. L'alignement constituant la Figure 1 et la Table 1 au format original ont donc été ajoutés à la suite de la revue pour une meilleure lisibilité.

Multiple Hsp70 Isoforms in the Eukaryotic Cytosol: Mere Redundancy or Functional Specificity?

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Abstract: Hsp70 molecular chaperones play a variety of functions in every organism, cell type and organelle, and their activities have been implicated in a number of human pathologies, ranging from cancer to neurodegenerative diseases. The functions, regulations and structure of Hsp70s were intensively studied for about three decades, yet much still remains to be learned about these essential folding enzymes. Genome sequencing efforts revealed that most genomes contain multiple members of the Hsp70 family, some of which co-exist in the same cellular compartment. For example, the human cytosol and nucleus contain six highly homologous Hsp70 proteins while the yeast *Saccharomyces cerevisiae* contains four canonical Hsp70s and three fungal-specific ribosome-associated and specialized Hsp70s. The reasons and significance of the requirement for multiple Hsp70s is still a subject of debate. It has been postulated for a long time that these Hsp70 isoforms are functionally redundant and differ only by their spatio-temporal expression patterns. However, several studies in yeast and higher eukaryotic organisms challenged this widely accepted idea by demonstrating functional specificity among Hsp70 isoforms. Another element of complexity is brought about by specific cofactors, such as Hsp40s or nucleotide exchange factors that modulate the activity of Hsp70s and their binding to client proteins. Hence, a dynamic network of chaperone/co-chaperone interactions has evolved in each organism to efficiently take advantage of the multiple cellular roles Hsp70s can play. We summarize here our current knowledge of the functions and regulations of these molecular chaperones, and shed light on the known functional specificities among isoforms.

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Key Words: Hsp70, Ssa1, chaperone network, functional specificity.

INTRODUCTION

The 70-kDa heat-shock proteins (Hsp70s) are a ubiquitous family of molecular chaperones found in all organisms and sub-cellular compartments where they play essential housekeeping functions in protein folding, synthesis, assembly, transport across biological membranes and degradation. They are also involved in quality control processes, such as protein refolding after a stress injury, and control the activity of regulatory proteins in signal transduction pathways [1]. Hsp70s constitute one of the most conserved protein families in evolution (Fig. 1), and members of this family may be constitutively expressed or stress-inducible (hence their classification as heat-shock proteins) [2-6].

The functional pleiotropy of Hsp70s is achieved through the evolutionary amplification and diversification of *HSP70* genes, cofactors that recruit and regulate Hsp70s for specific cellular functions, and cooperation of Hsp70s with other chaperone systems such as TRiC/CCT or Hsp90 [1]. All of these cellular activities depend on the ability of Hsp70s to interact with hydrophobic stretches of proteins in an ATP-dependent manner. Hsp70s are highly conserved and are composed of an N-terminal 44-kDa ATPase domain (also named adenine nucleotide-binding domain or NBD), an 18-

kDa peptide-binding domain (PBD) and a C-terminal 10-kDa "lid" domain. The NBD has a bilobal structure and each globular lobe (I and II) is further conventionally divided into two subdomains (A and B) [7]. The interdomain linker connecting the NBD to the PBD is highly conserved and plays a critical role in the allosteric regulation of Hsp70s [8, 9]. Cytosolic Hsp70s also contain a G/P-rich C-terminal region containing an EEVD-motif that mediates their binding to Tetra-Trico-Peptide repeat (TPR)-domain containing co-chaperones such as the C-terminus-Hsp70-Interacting Protein CHIP. The EEVD motif is absent from specialized Hsp70s such as the ribosome-bound Ssb1/2p in yeast (see below).

Hsp70s cycle between two stable conformations with different affinities for substrates: in the ATP-bound state, Hsp70s display fast on-and-off rates of peptide binding, whereas in the ADP-bound state these constants are slowed, resulting in tighter association of the substrate within the PBD [10, 11]. The modulation of the affinity for polypeptide substrates is triggered by a conformational change in the lid that, upon ATP hydrolysis, closes on the substrate that is then trapped within the PBD [12]. It appears that ADP-ATP exchange is critical for substrate release and for the recycling of the molecular chaperone. This allosteric regulation of Hsp70s activity is tightly controlled by specific co-chaperones (Fig. 1A).

The well characterized Hsp40/DnaJ family members share a common ~70-amino-acid J-domain that mediates

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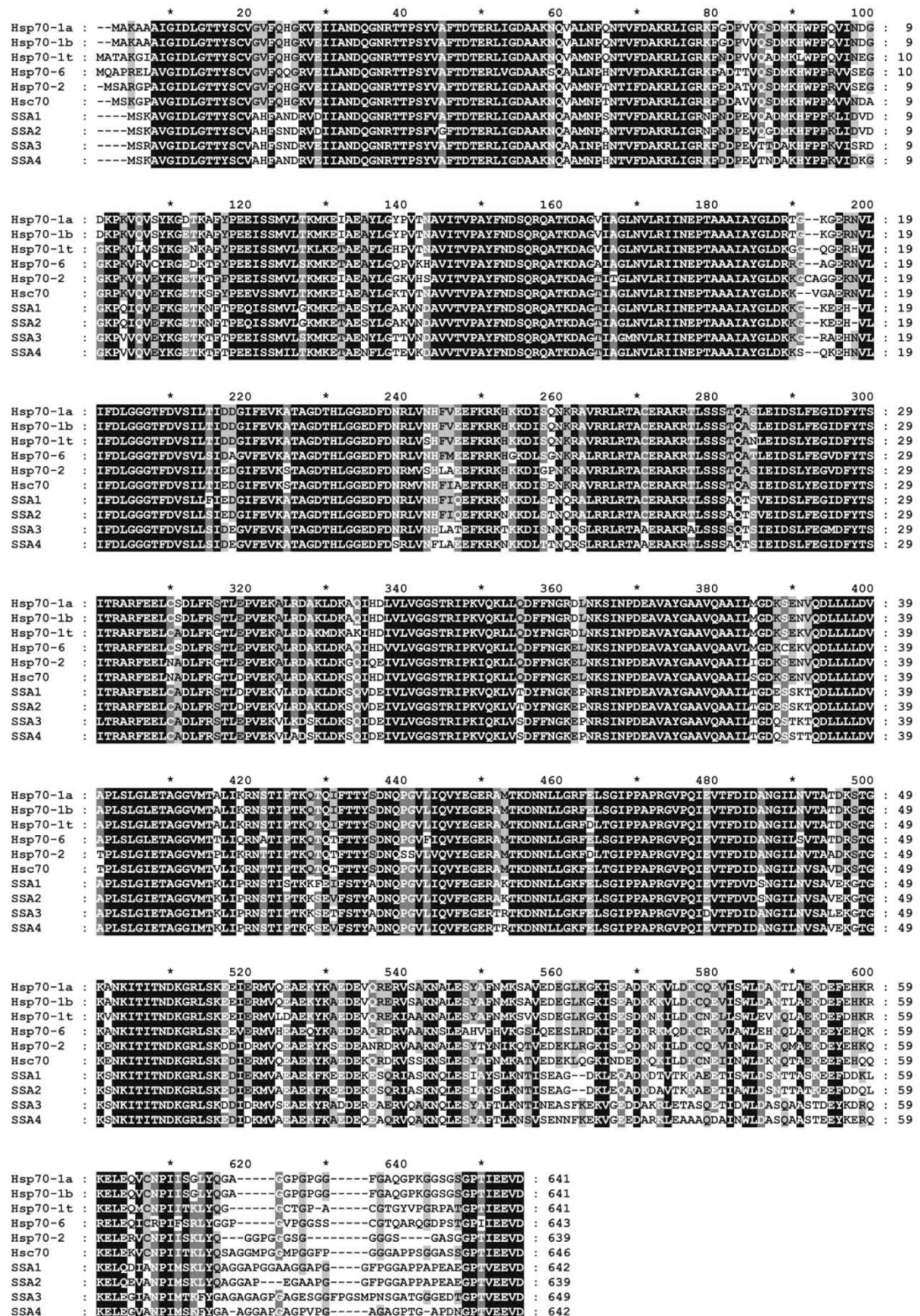


Fig. (1). The alignment of yeast and human cytosolic Hsp70s shows their remarkable evolutionary conservation and highlights the variability of the C-terminal lid domain (this figure was made using clustalX 1.83 and GeneDoc v2.6).

binding to Hsp70s and activation of their weak intrinsic ATPase activity (Fig. 2). These cofactors, by virtue of their cellular localization and/or association to specific complexes, recruit Hsp70s to specific sites of the cell and therefore greatly contribute to the wide range of functions assigned to Hsp70s (reviewed in [13-17]).

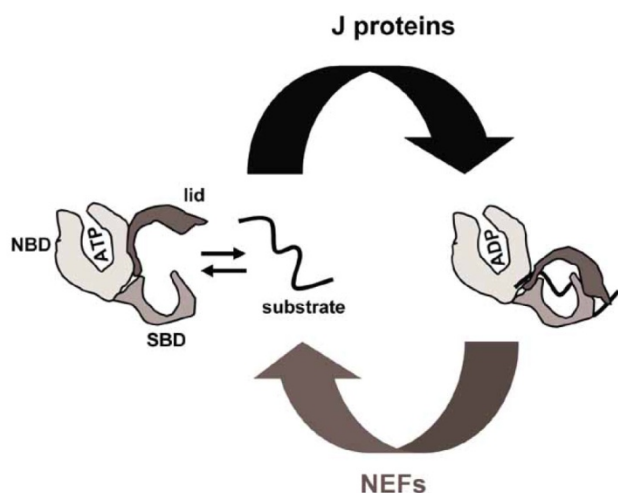


Fig. (2). The Hsp70 ATPase cycle. In the ATP-bound state, Hsp70 have low affinity for substrates. Upon J-protein-stimulated ATP hydrolysis, a conformational switch of the lid tightly locks the substrate within the substrate binding domain. NEF catalyzed-ADP release and subsequent ATP re-binding trigger substrate release.

Whereas ATP hydrolysis is stimulated by members of a common Hsp40/DnaJ family, ADP-ATP exchange is catalyzed by several evolutionary unrelated classes of nucleotide exchange factors (NEFs) (Fig. 2); these include GrpE homologs in prokaryotes and the Bag1, HspBP1/Fes1p and Hsp110 families in eukaryotes (reviewed in [1, 18]). It should be noted that Hsp110s are molecular chaperones homologous in sequence and structure to Hsp70s and together these proteins form the super-family of Hsp70s [19, 20]. The domain architecture of Hsp110 is therefore very similar to that of Hsp70s; the main noticeable difference lies in large (~100-140 amino acids) acidic insertions between the PBD and the lid and also at the C-terminus [20]. Hsp110s are not protein folding chaperones, yet they act as efficient “holdases” by binding to and preventing aggregation of denatured proteins [21, 22]. Mammalian Hsp110 and yeast Sse1p were shown to functionally and physically interact with counterpart Hsp70s [23-25], and to be potent nucleotide-activated NEFs [26-28].

THE EUKARYOTIC CYTOSOL CONTAINS MULTIPLE MEMBERS OF THE HSP70 SUPER-FAMILY

Genome sequencing efforts confirmed early observations that eukaryotes have several Hsp70 encoding genes [4]. Evidently, some of these Hsp70s are compartment specific and fulfil unique and essential functions in the endoplasmic reticulum, mitochondria or plastids. These organelle-specific Hsp70s are generally encoded by a single gene in most organisms. Strikingly, multiple Hsp70 isoforms very often coexist in the same cytosol and are encoded by different ho-

mologous genes. In Table (1) we describe the cytosolic Hsp70 machinery of various fully sequenced eukaryotic organisms, ranging from yeast to human. For most of these selected species, the Hsp70 system has been recently described in details at the genomic and/or functional levels [6, 29-34].

With the exception of ribosome-associated specialized Hsp70s (that do not contain the C-terminal EEVD motif), canonical Hsp70s are generally believed to be functionally redundant, the main differences between isoforms lying in their spatio-temporal expression. This is exemplified by studies in yeast that showed that only one member of the Ssa group of Hsp70s can support yeast growth if expressed at sufficient levels [29]. Additionally, heterologously expressed Hsp70s were shown to protect mammalian cells and transgenic animals from various stresses, demonstrating that the protective role of these molecular chaperones is highly conserved [35-38].

However, many studies also raised the possibility of functional specificity among Hsp70 isoforms, meaning that a particular Hsp70 will for instance preferentially bind to a subset of client proteins and/or co-chaperones to perform a unique task. Such a specialization among Hsp70s may explain the need for several Hsp70-encoding genes and their different tissue-expression patterns in complex multicellular organisms. In this review, we will discuss examples of functional specificities among Hsp70s in yeast and humans, hypothesize on the molecular basis that may govern this specificity, and suggest directions for future research in this field.

REDUNDANT AND SPECIALIZED FUNCTIONS AMONG HSP70S IN YEAST

The Cytosolic Hsp70 System in Yeast

The yeast *Saccharomyces cerevisiae* contains seven cytosolic Hsp70s that fall into two major groups: 1) the canonical Ssa1, Ssa2p, Ssa3p and Ssa4p proteins and 2) the ribosome-associated Ssb1p, Ssb2p and Ssz1p proteins (Table 1) [4].

The highly homologous Ssa proteins differ by their expression pattern: the *SSA2* gene is constitutively expressed at high levels; the *SSA1* gene is constitutively expressed, yet at lower levels than *SSA2*, and is also induced by stress; the *SSA3* and *SSA4* genes are not expressed during normal vegetative growth, but their expression is dramatically induced upon stress; additionally, Ssa3p levels are increased upon entry into stationary phase [29, 39-42]. The simultaneous deletion of these four genes is lethal, but can be complemented by the over-expression of either one of them, suggesting redundant functions for these molecular chaperones [29]. It should be noted that a *Δssa1Δssa2* mutant is viable and constitutively thermotolerant because of the up-regulation of *SSA3*, *SSA4* and other components of the general stress response [29, 39, 40]. However, the *Δssa1Δssa2* mutant forms small colonies at 23°C and is thermosensitive for growth at 37°C, suggesting that the functions carried by Ssa1p and/or Ssa2p cannot be fully complemented by the induction of *SSA3* and *SSA4* [29, 39, 40].

The nearly identical Ssb1p and Ssb2p proteins interact with nascent chains at the yeast ribosome and provide a dispensable function in the process of translation and in early

folding events [43, 44]. These Hsp70s are unable to complement the lack of Ssa proteins, indicating that these two groups of Hsp70s clearly have distinct non overlapping functions [4]. Ssb1p and Ssb2p are recruited to the ribosome through their interaction with the stable heterodimeric Ribosome-Associated Complex (RAC) that is composed of the J-domain protein Zuo1p (or zootin) and the atypical Hsp70 Ssz1p [45, 46]. The simultaneous deletion of *SSB1* and *SSB2* results in slow growth, cold sensitivity and hypersensitivity to translation-inhibiting drugs such as hygromycin B or paromomycin. Identical phenotypes are observed upon deletion of *SSZ1* or *ZUO1*, suggesting that Ssb1/2p and RAC form a functional triad *in vivo* [45-48]. The Ssb1/2p proteins are not stimulated by other J-proteins than Zuo1p and require their PBD for function [49, 50]. The Ssz1p protein diverges markedly from other Hsp70s: it contains an unusually short PBD and lacks the C-terminal lid domain; in fact, truncated Ssz1p lacking nearly the entire PBD is fully functional *in vivo* [51]; Ssz1p is not an ATPase *in vitro* and its nucleotide binding ability is not strictly required *in vivo* [52]. Ssz1p has been proposed to serve as a structural scaffold between Ssb1/2p and Zuo1p [53], although this may not be its only function [52]. While Ssb proteins are generally considered fungal-specific, we have identified an uncharacterized Ssb homolog in *C. elegans* (Table 1). Moreover, a mammalian RAC complex has been recently described, and is comprised of the Zuo1 homolog MPP11 and the Ssz1 homolog Hsp70L1 (or Hsp70-14; Table 1) [54].

In *S. cerevisiae*, the Ssa proteins are recruited and activated by 11 cytosolic J-proteins [55], and both Ssa and Ssb proteins can be regulated by the Fes1p and Sse1/2p NEFs [23, 24, 26, 27, 56, 57]. Whether the different Ssa isoforms have preferences and/or different affinities for distinct J-proteins or NEFs has not been systematically tested, but remains an attractive possibility that could partly explain the functional specificities observed among Hsp70 isoforms. Indeed, a functional distinction between cytosolic, ER or mitochondrial Hsp70s has been greatly documented and was attributed to specific interactions between these Hsp70s and their partner J-proteins. In particular, cytosolic and ER luminal Hsp70s could not replace one another in *in vitro* reconstitution assays of protein translocation across the ER membrane [58, 59]. Moreover, mitochondrial Hsp70 was unable to replace the ER luminal Hsp70 in similar assays [60]. In both cases, this has been attributed to impaired interactions of the “extraneous” Hsp70 with the “endogenous” compartment-specific J-proteins, suggesting convergent evolution of chaperone and co-chaperones for proper interaction [16, 58, 60].

As shown in Table (1), Ssa, Ssb and Ssz homologues are present in most yeasts that mainly differ with respect to the number of Ssa-encoding genes: only two in *Schizosaccharomyces pombe* and *Candida albicans*, and four in *Y. lipolytica*. We are currently investigating the regulations and functions of the four *Y. lipolytica* Ssa proteins that we have named Ssa5-8 to avoid confusion with the *S. cerevisiae* Hsp70 system (Martineau C.N. and Kabani M., unpublished data). A comparison of the Hsp70 systems in these two distantly related yeasts will provide useful information on the plasticity and dynamics of Hsp70-containing chaperone networks.

Examples of Functional Specificity Among the Ssa Group of Hsp70s

Early evidence for functional specificity among Ssa proteins was brought by a study from Eisenberg and collaborators that investigated the ability of yeast cytoplasmic Hsp70s to dissociate clathrin from bovine brain coated vesicles [61]. Using purified Hsp70 preparations from wild-type yeast, they first showed that the *in vitro* clathrin uncoating activity is associated with Ssa but not Ssb proteins. They then showed that higher uncoating activities could be achieved using Hsp70 preparations from a Δ *Ssa1* mutant than from a Δ *Ssa2* mutant. Because Ssa3p and Ssa4p are nearly undetectable in these preparations, they concluded that Ssa2p has a markedly higher uncoating activity than Ssa1p. Moreover, they showed that Ssa1p inhibits the uncoating activity of Ssa2p, probably by blocking Hsp70-binding sites on clathrin baskets [61]. The molecular basis of this functional difference in clathrin uncoating between these highly homologous Hsp70s remains to be directly addressed. One possible explanation is that Ssa2p and Ssa1p could have markedly different affinities for clathrin and/or the J-protein auxilin (Swa2p) which is specifically required to activate Hsc70 during endocytosis [62]. In humans and fruit fly, clathrin uncoating was similarly shown to depend on Hsc70 and Hsc70-4, respectively, which are the most abundant and constitutive Hsp70s in these organisms [63, 64]. This is not surprising given that Hsp70 acts stoichiometrically in clathrin uncoating, and therefore fair amounts of the molecular chaperone are required to fulfil this vital cellular process [65].

Another functional distinction between Ssa1p and Ssa2p has been reported with respect to their roles in prion propagation. The yeast epigenetic factors [PSI+], [URE3] and [PIN+] are the prion forms of the Sup35p, Ure2p and Rnq1p proteins, respectively [66-68]. These infectious proteins self-propagate as amyloids [66, 67, 69-72] and therefore constitute a powerful and safe model to investigate mammalian prion-related diseases such as sheep scrapie or transmissible spongiform encephalopathies [73]. Molecular chaperones of the Hsp104, Hsp70 and Hsp40 families play critical roles in prion formation and propagation [74, 75]. The fungal specific Hsp104 molecular chaperone promotes the ATP-dependent solubilization of aggregated misfolded proteins with the help of Hsp70 and Hsp40 [76]. Hsp104 is strictly required for yeast prion propagation, as the deletion of the *HSP104* gene or the inactivation of Hsp104 by growth in the presence of 5 mM guanidium chloride cure cells from [PSI+], [URE3] and [PIN+]. In contrast, the overexpression of Hsp104 cures [PSI+] but not [URE3] [67, 77-80]. Hsp104 was proposed to mediate protein-only inheritance by remodelling large prion aggregates into new self-replicating particles (or seeds) [69, 81-83]. The effects of Hsp70s and Hsp40s on prion propagation are complex and prion-dependent [75]. Mutation of the cytosolic Hsp70 Ssa1p impairs [PSI+] propagation while its overexpression was shown to inhibit [PSI+] curing by overexpressed Hsp104 [84, 85]. The overproduction of Ssa1p, but not Ssa2p, cures [URE3] [86], whereas mutations in *SSA2*, but not *SSA1*, impair [URE3] propagation [87]. The overexpression of Ydj1p, the most abundant yeast Hsp40, cures cells from [URE3] [78], whereas mutations in Sis1p cure [PIN+] [88]. Similarly, NEFs have been shown to influence prion propagation. Dan-

iel Masison and collaborators have isolated an *SSA1-21* mutation that weakens [PSI⁺] propagation [84]. They then showed that in the *SSA1-21* background the depletion of Fes1p further destabilized [PSI⁺], whereas its overexpression restored almost normal [PSI⁺] propagation [89]. However, contrasting results have been reported for [PSI⁺] propagation when *FES1* is deleted in an otherwise wild-type background [89, 90]. The deletion of *SSE1* and *FES1* completely blocked [URE3] propagation, but only the overexpression of Sse1p cured it [90-92]. In addition, the deletion of *SSE1* severely inhibited [PSI⁺] formation while the overexpression of Sse1p improved it [92]. The Hsp70 machinery appears as an important modulator of amyloid formation and protein-only inheritance, and the complex phenotypes obtained upon mutations in its components indicates that a dynamic network of specific chaperone/co-chaperone/substrate interactions occur *in vivo*. A better understanding of the role of the Hsp70 system in the process of amyloidogenesis will benefit from *in vitro* approaches where the effects of purified chaperones and co-chaperones on the assembly of amyloid fibrils can be precisely tested [93, 94].

We have recently showed that mutations in components of the cytosolic Hsp70 machinery (*e.g.* *FES1*, *SSE1*, *YDJ1*, *SSA1*) dramatically impair biofilm (or ‘mat’) formation in yeast [95]. We showed that the overexpression of *FES1* rescues the defect in biofilm formation of a Δ *Sse1* strain, but the opposite is not true: the Δ *fes1* mutant is unable to form ‘mats’ on semi-solid medium with or without *SSE1/2* overexpression [95]. Thus, Fes1p and Sse1/2p play both overlapping and distinct functions as suggested by earlier studies [27, 96]. Remarkably, we showed that a Δ *Ssa1* mutant, in an otherwise wild-type background for the other SSA genes, is severely affected in biofilm formation [95]. The deletion of *SSA2* had more subtle effects suggesting that Ssa1p is specifically required for ‘mat’ formation [95]. Moreover, we showed that the additional deletion of *SSA3* and *SSA4* aggravated the phenotypes of the Δ *Ssa1* and Δ *Ssa2* mutants, indicating a possible functional cooperation between the constitutive and inducible Hsp70s [95].

THE HUMAN HSP70 SYSTEM

The Cytosolic Hsp70 System in Humans

Several reviews and studies attempted to provide an accurate description of the human Hsp70 family, a task that has been complicated by confusing and conflicting nomenclature in the literature [6, 34, 97]. However, extensive literature and database search, facilitated by the availability of the human genome, recently allowed Alberto Macario and collaborators to provide a detailed description of human Hsp70 encoding genes [34]. They identified up to 17 Hsp70-encoding genes that fall into evolutionary and functionally distinct groups, as well as 30 pseudogenes [34]. For each of these genes, an impressively high number of mRNA variants and isoforms were identified through EST analysis, although their functional relevance is still unknown [34]. In Table (1), we chose to present only the *bona fide* human cytosolic Hsp70s for which expression has been proved and that correspond to Ssa or Sse-like proteins [6, 34, 97].

The human cytosolic Hsp70s are comprised of six canonical members, named Hsp70-1a, Hsp70-1b, Hsp70-1t,

Hsp70-2, Hsp70-6 and Hsc70, and of an Ssz-like protein named Hsp70-14 (or Hsp70L1) [6, 34, 97] (Table 1). These proteins were also described with different acronyms that are indicated in Table (1). While the expression of Hsp70-1a, Hsp70-1b and Hsp70-6 is strongly induced by stress [6, 34], all these cytosolic Hsp70s differ considerably with respect to their expression patterns in the different tissues and developmental stages, as predicted by assessing the relative number of ESTs per tissue type [34].

The heat-shock cognate Hsc70 is by far the most expressed Hsp70 in all tissues with particularly high levels in the vascular tissue [34], and is believed to play essential house-keeping functions in protein folding, transport across biological membranes, prevention of protein aggregation and uncoating of clathrin vesicles [6, 98]. The intron-less genes encoding Hsp70-1A, Hsp70-1B and Hsp70-1t are all closely linked and located within the same MHC class III region on chromosome 6 [99, 100], yet their expression patterns differ [6, 34, 101]. Hsp70-1a and Hsp70-1b are expressed in most tissues, and in most cases Hsp70-1a levels are much higher than Hsp70-1b levels [6, 34]. Moreover, very high expression levels were observed for Hsp70-1a in the spleen [34]. On the other hand, Hsp70-1t is expressed at almost undetectable levels in most tissues, except in testis, adipose tissue and pituitary glands where its levels are much higher [6, 34, 101]. In contrast to Hsp70-1a and Hsp70-1b, the levels of Hsp70-1t are not induced by heat stress [101]. Hsp70-2 is expressed at low to undetectable levels in most tissues, and at high levels in testis, brain and umbilical cord [6, 34]. The expression of Hsp70-6 is only induced after severe stress insults [102, 103], although moderate levels of this protein have been reported in some blood cells as well as in the bladder, trachea and esophagus [6, 34].

Thus, all cytosolic Hsp70s are constitutively expressed at varying levels making each tissue and developmental stage unique in its Hsp70 content [6, 34]. Important efforts will be needed to define the specific roles of each Hsp70 in each cell and tissue type, taking into account the possibility that these Hsp70s may functionally interact with one another, either positively or negatively, depending on their respective abundance. Whether the Hsp70 co-chaperones content also varies among cell and tissue types has yet to be determined, but could also be a major determinant of the functional specialization of Hsp70s.

Examples of Functional Specificity Among the Human Cytosolic Hsp70s

Understanding the need for up to six Hsp70 family members in human cells and sorting out their redundant from their specialized functions have proven difficult and technically challenging. Most of the published data on human Hsp70s were obtained by studying the constitutive Hsc70 or the major heat-inducible Hsp70 (which in fact refers to both Hsp70-1a and Hsp70-1b) that were often considered as equivalent and functionally interchangeable. [6, 104]. However, a clear distinction between constitutive and inducible Hsp70 isoforms emerged from several studies, including the characterization of mouse knock-out models [6]. It appeared very logically that constitutive Hsp70s mostly play important housekeeping functions while inducible Hsp70s are required to cope with stress situations [6].

Hence, Hsc70 was shown to be essential for viability in vertebrates, including fruit fly [105], mouse [106] and human cell lines [107, 108], whereas mice deficient for the homologues of human Hsp70-1a and Hsp70-1b (Hsp70.1 and Hsp70.3) develop normally in standard conditions but show significantly decreased resistance to stress situations such as heat shock, cardiac ischemia or radiation [6, 109-117].

Additionally, Hsp70 and Hsc70 were shown to have differential and antagonistic effects with regard to the intracellular trafficking of ENaC, an epithelial sodium chloride channel: Hsp70 promoted the maturation and functional cell surface expression of ENaC in *Xenopus* oocytes, whereas Hsc70 disfavoured it [104]. Similarly, both the overexpression of Hsp70 and the decrease of Hsc70 levels *via* the use of sodium 4-phenylbutyrate were shown to promote the intracellular trafficking of the Δ F508 mutant of the cystic fibrosis transmembrane conductance regulator (CFTR) [118-120].

Hsp70 levels are abnormally high in a wide variety of tumor cell types and contribute to tumorigenesis and resistance to chemotherapy by directly interfering with several key components of the apoptotic signalling pathway [121]. Similarly, an up-regulation of the HspBP1 NEF was observed in various tumor cell types [122, 123]. HspBP1 has been shown to modulate Hsp70s activity either negatively [124, 125] (at high HspBP1/Hsp70 molar ratios) or positively (at low HspBP1/Hsp70 molar ratios) [126]. Interestingly, HspBP1 was shown *in vivo* to bind with a much higher affinity to the stress-inducible Hsp70 than to the abundant and constitutively expressed Hsc70. Hence, HspBP1 is present in great excess (~10 fold) compared to its preferential Hsp70 partner in normal cells. However, the HspBP1/Hsp70 molar ratio may be lowered considerably in tumor cells (from ~10 to ~2) because the up-regulation of Hsp70 is greater than that of HspBP1 in these cells [123]. This may contribute to the Hsp70-mediated resistance to chemotherapy, as tumor cells with high HspBP1/Hsp70 molar ratios were much more susceptible to anticancer drugs than were those with a low ratio [123]. In addition, anticancer drugs up-regulated HspBP1 in various cancer cells, while no effect was observed on Hsp70 levels [123]. These findings suggest that HspBP1 antagonizes the prosurvival function of Hsp70 [123]. In support of this hypothesis, RNAi-mediated depletion of HspBP1 markedly reduced the susceptibility of tumor cells to anticancer drugs [123]. Conversely, transient ectopic expression of HspBP1 in tumor cells enhanced the cathepsin-mediated cell death induced by anticancer drugs [123]. This pro-apoptotic function of HspBP1 appeared to be dependent on its ability to bind to Hsp70 and was inhibited by heat-shock induced up-regulation of Hsp70 [123]. These findings exemplify that part of the functional distinction among Hsp70 isoforms may lie in differential interaction with their co-chaperones, thereby forming specific functional networks in each cell or tissue type. Remarkably, both primate and plant Hsc70 were able to support growth of a *Dssal-4* yeast mutant, whereas the corresponding inducible Hsp70s did not [127], suggesting an evolutionary conserved functional distinction among these molecular chaperones.

The Hsp70-2 protein appears to play an important role in spermatogenesis in humans and mice *via* its essential chap-

eroning functions for the cyclinB/cdc2 complex during meiosis, and for spermatid DNA-packaging proteins involved in post-meiotic genome reorganization [6, 128-132]. Additionally, Hsp70-2 is upregulated in some primary and metastatic breast cancers, and was shown to be required for the growth and survival of several human cancer cells [6, 107, 108, 133]. The depletion of Hsp70 or Hsp70-2 from cancer cells resulted in markedly different phenotypes and gene expression profiles, whereas their concomitant depletion resulted in synergistic antiproliferative effects, suggesting separate non-overlapping functions for these Hsp70s [108].

By virtue of their cellular protective role, Hsp70s are important candidates in gene-longevity association studies [134, 135]. Indeed, the ability to cope with cellular stress by the induction of Hsp70 was shown to decline with age in numerous *in vivo* and *in vitro* models [134, 136-139]. Moreover, the presence of extra-copies of a heat-inducible Hsp70-encoding gene increased the lifespan of transgenic fly models after a transient mild heat-shock [140]. Specifically, polymorphisms positively or negatively associated with longevity were identified in the genes encoding the MHC-III-linked Hsp70-1a, Hsp70-1b and Hsp70-1t proteins, suggesting that unique anti-ageing functions are fulfilled by these isoforms (reviewed in [134, 135]).

CONCLUSIONS AND FUTURE DIRECTIONS: MOLECULAR BASIS OF HSP70 SPECIALIZATION

Despite a high degree of sequence conservation and overlapping chaperoning functions, members of the Hsp70 superfamily appear to have evolved specialized functions for which they can not replace each other. This functional specificity is most apparent and widely accepted for compartment-specific and ribosome-associated Hsp70s, and also for the Hsp110 family members that evolved to act as Hsp70 NEFs [6, 98]. However, less attention has been paid to the possibility that a similar specialization might occur among the highly conserved cytosolic Hsp70s, although it may provide an answer to why some organisms such as humans or *Drosophila melanogaster* need up to 6 or 10 cytosolic Hsp70s, respectively (Table 1). A recent study demonstrated functional differences among highly conserved ribosomal protein paralogs in yeast, indicating that the co-existence of multiple members of a given protein family is not only the result of gene duplication and mere redundancy, but also significantly contributes to cellular homeostasis [141].

Future efforts will have to identify and characterize the unique functions played by individual Hsp70s in particular cell or tissue types. Several non-mutually exclusive hypotheses can be made, according to our current knowledge of Hsp70 function, to understand the molecular basis of such specialization. A first model would be that Hsp70 isoforms differentially interact with the various cytosolic Hsp70 co-chaperones, as observed for the preferred interaction of HspBP1 with Hsp70 and not with Hsc70 [123]. A second model would be that Hsp70 isoforms bind to different sets of client proteins or to different types of substrates (*e. g.* native client proteins, nascent proteins, misfolded proteins, protein aggregates, etc.). In support of this hypothesis, different peptide binding specificities were described for bacterial DnaK,

Table 1. The Cytosolic Hsp70s of Various Eukaryotes are Shown Together with Some Important Co-Chaperones (Because of their High Number, J-Proteins are not Shown). UniProt Accession Numbers are Provided in Most Cases, Along with the Alternative Acronyms by which these Proteins were Described when Appropriate

	Yeasts				Nematode	Green Algae	Ascidian	Plant	Fruit fly	Human
	<i>S. cerevisiae</i>	<i>S. pombe</i>	<i>C. albicans</i>	<i>Y. lipolytica</i>	<i>C. elegans</i>	<i>C. reinhardtii</i>	<i>C. intestinalis</i>	<i>A. thaliana</i>	<i>D. melanogaster</i>	<i>H. sapiens</i>
Canonical Hsp70's	Ssa1p (P10591)	<i>Sp.</i> Ssa1p (Q10265)	<i>Ca.</i> Ssa1p (P41797)	Ssa5p (Q6C0E9)	Hsp70-1 (P09446)	<i>Cr.</i> Hsp70-3 (P25840)	<i>Ci.</i> HSPA1/6/7-like (P91874)	<i>At.</i> Hsp70-1 (P22953)	Hsp70Aa (P82910)	Hsp70-1a (Hsp70, Hsp72, Hsp70-1) (P08107)
	Ssa2p (P10592)	<i>Sp.</i> Ssa2p (O59855)	<i>Ca.</i> Ssa2p (P46587)	Ssa6p (Q6C3G5)	Hsp70-7 (Q9XTL8)		<i>Ci.</i> HSPA2/8	<i>At.</i> Hsp70-2 (P22954)	Hsp70Ab (P02825)	Hsp70-1b (Hsp70, Hsp72, Hsp70-1) (P08107)
	Ssa3p (P09435)			Ssa7p (Q6C9V0)	Hsp70-8 (Q9XTL8)			<i>At.</i> Hsp70-3 (O65719)	Hsp70Ba (Q8INI8)	Hsp70-1t (Hsp70-hom) (P34931)
	Ssa4p (P22202)			Ssa8p (Q6C864)	Hsp70-9 (Q45246)			<i>At.</i> Hsp70-4 (Q9LHA8)	Hsp70Bbb (Q9VG58)	Hsp70-2 (Hsp70-3) (P54652)
								<i>At.</i> Hsp70-5 (Q959N1)	Hsp70Bb (Q9BIS2)	Hsp70-6 (Hsp70B') (P17066)
									Hsp70Bc (Q9BIR7)	Hsc70 (Hsp70-8, Hsp73) (P11142)
									Hsc70-1 (P29843)	
									Hsc70-2 (P11146)	
									Hsc70-4 (P11147)	
									Hsp68 (097125)	
Other Hsp70's	Ssb1p (P11484)	<i>Sp.</i> Ssb1p (Sks2) (Q10284)	<i>Ca.</i> Ssb1p (P87222)	<i>Yl.</i> Ssb1p (Q6CIA7)	Hsp70-10 (Q9TW52)	-	-	-	-	-
	Ssb2p (P40150)									
	Ssz1p (P38788)	<i>Sp.</i> Ssz1p (P87142)	<i>Ca.</i> Ssz1p (Q5A678)	<i>Yl.</i> Ssz1p (Q6CEW0)	-	-	-	-	-	Hsp70-14 (Hsp70L1) (Q0VDF9)
Hsp110's	Sse1p (P32589)	<i>Sp.</i> Sse1p (Pss1) (O59838)	<i>Ca.</i> Sse1p (Q96VB9)	<i>Yl.</i> Sse1p (Q6C618)	Hsp110-1 (Q05036)	-	<i>Ci.</i> HSPA4/4L/HSPH1	<i>At.</i> Hsp70-14 (Q957C0)	Hsc70Cb (Q9XZT5)	Hsp110 (Hsp70-4) (P34932)
	Sse2p (P32590)							<i>At.</i> Hsp70-15 (Q9CA95)		Hsp110 (Hsp70-4L) (O95757)
								<i>At.</i> Hsp70-16 (A8MRM9)		Hsp105 (Q92598)
HspBP1	Fes1p (P38260)	<i>Sp.</i> Fes1p (O43030)	<i>Ca.</i> Fes1p (Q59NN8)	<i>Yl.</i> Fes1p (Q6C239)	-	-	-	<i>At.</i> HspBP1-1 (Q9M346)	?	HspBP1 (Q9NZL4)
								<i>At.</i> HspBP1-2 (Q84J81)		
								<i>At.</i> HspBP1-3 (Q9LZL7)		
Bag proteins	Snl1p (P40548)	Bag-1A (O59739)	<i>Ca.</i> Snl1p (Q59NB3)	<i>Yl.</i> Bag1 (Q6C9J3)	<i>Ce.</i> Bag-1 (O44739)	-	<i>Ci.</i> BAG1	<i>At.</i> Bag-1 (Q0WUQ1)	starvin, isoform A (Q9VU81)	Bag-1L, Bag-1M, Bag-1S (Q99933)

(Table 1) contd....

	Yeasts				Nematode	Green Algae	Ascidian	Plant	Fruit fly	Human
	<i>S. cerevisiae</i>	<i>S. pombe</i>	<i>C. albicans</i>	<i>Y. lipolytica</i>	<i>C. elegans</i>	<i>C. reinhardtii</i>	<i>C. intestinalis</i>	<i>A. thaliana</i>	<i>D. melanogaster</i>	<i>H. sapiens</i>
		Bag-1B (O59739)			Unc-23a (O61980)		<i>CI</i> BAG3	<i>At</i> .Bag-2 (Q8LEP8)	starvin, isoform B (Q9VU83)	Bag-2 (O95816)
					Unc-23b (Q86S24)		<i>CI</i> .BAT3	<i>At</i> .Bag-3 (Q9LYP4)	starvin, isoform C (Q9VU82)	Bag-3 (O95817)
					Unc-23c (Q5TKA9)			<i>At</i> .Bag-4 (O65021)		Bag-4 (O95429)
								<i>At</i> .Bag-5 (O65373)		Bag-5 (Q9UL15)
								<i>At</i> .Bag-6 (O82345)		Bag-6, BAT3 (P46379)
								<i>At</i> .Bag-7 (Q9LVA0)		
CHIP	-	-	-	<i>Yl</i> .Chn1p (Q6C1F4)	Chn-1 (Q9BMU2)	<i>Cr</i> .CHIP (A8J756)	-	<i>At</i> .CHIP (Q9SRS9)	<i>Dm</i> .CHIP (Q9XYW6)	CHIP (Q9UNE7)

mammalian Hsc70 and BiP [142, 143]. Similarly, the DnaK protein from the archaea *Methanosarcina mazei* failed to fully complement a *dnak* mutant in *Escherichia coli*, and was shown to have markedly different peptide binding properties [144, 145]. Importantly, the 10-kDa C-terminal domain of Hsp70s is the less conserved region and could play an important role in modulating Hsp70-substrate interactions (Fig. 1). Additionally, different Hsp70 isoforms could cooperate in the process of protein folding by binding to different exposed sites on a given substrate. To our knowledge, the side-by-side and thorough biochemical characterization of Hsp70 isoforms from a single model organism has not been reported but would certainly help clarifying the functions and regulations of these essential folding enzymes. Because Hsp70s are relevant targets for drug-based treatments for cancers or protein folding disorders, the identification of the molecular determinants that govern their functional specificities in particular cell or tissue types will greatly improve the efficacy of these approaches without unwillingly affecting vital cellular processes.

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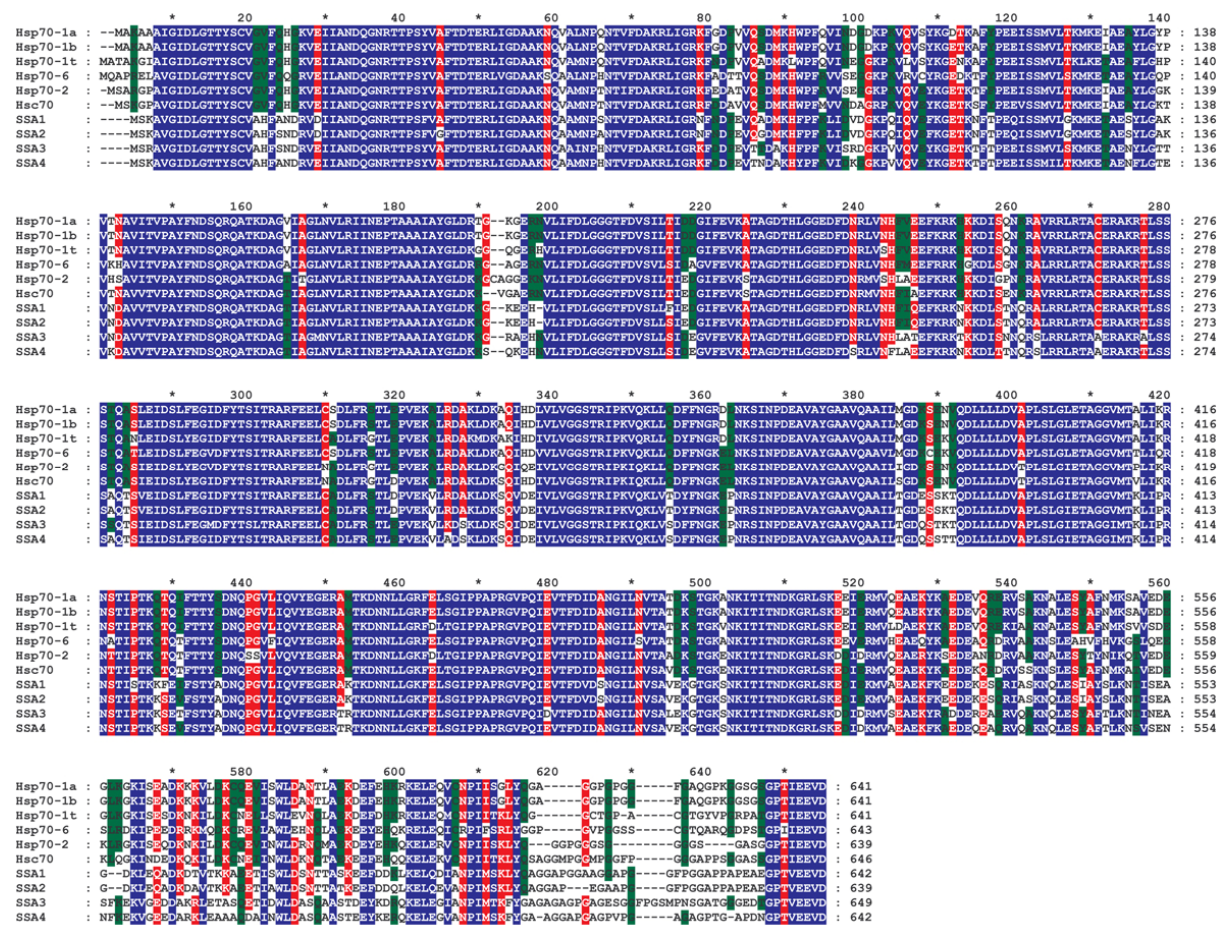
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Correction apportée à la Figure 1 (page 339) de la revue précédente

The alignment of yeast and human cytosolic Hsp70s shows their remarkable evolutionary conservation and highlights the variability of the C-terminal lid domain (this figure was made using ClustalX 1.83 and GeneDoc v2.6).

	Yeasts		Nematode	green algae	ascidian	Plant	Fruit fly	Human
	<i>S. pombe</i>	<i>C. albicans</i>	<i>Y. lipolytica</i>	<i>C. reinhardtii</i>	<i>C. intestinalis</i>	<i>A. thaliana</i>	<i>D. melanogaster</i>	<i>H. sapiens</i>
Canonical Hsp70's	<i>Sp. Ssa1p</i> (P10591) <i>Sp. Ssa2p</i> (P10592) <i>Sp. Ssa3p</i> (P109435) <i>Sp. Ssa4p</i> (P22202)	<i>Ca. Ssa1p</i> (P41797) <i>Ca. Ssa2p</i> (P46587)	<i>Ssa5p</i> (Q6C0E9) <i>Ssa6p</i> (Q6C3G5) <i>Ssa7p</i> (Q6C9V0) <i>Ssa8p</i> (Q6C864)	<i>Cr. Hsp70-3</i> (P25640)	<i>Ci. HSPA1/67-like</i> (P91874) <i>Ci. HSPA2/8</i>	<i>At. Hsp70-1</i> (P22953) <i>At. Hsp70-2</i> (P22954) <i>At. Hsp70-3</i> (Q65719) <i>At. Hsp70-4</i> (Q8LH48) <i>At. Hsp70-5</i> (Q659N1)	<i>Hsp70Aa</i> (P82910) <i>Hsp70Ab</i> (P02825) <i>Hsp70Ba</i> (Q8IN18) <i>Hsp70Bb</i> (Q9V358) <i>Hsp70Bc</i> (Q9BIS2) <i>Hsp70Bc</i> (Q9BIR7) <i>Hsc70-1</i> (P28643) <i>Hsc70-2</i> (P11146) <i>Hsc70-4</i> (P11147) <i>Hsp88</i> (Q97125)	<i>Hsp70-1a</i> (Hsp70, Hsp72, Hsp70-1) (P08107) <i>Hsp70-1b</i> (Hsp70, Hsp72, Hsp70-1) (P08107) <i>Hsp70-1l</i> (Hsp70-hom) (P34931) <i>Hsp70-2</i> (Hsp70-3) (P54652) <i>Hsp70-6</i> (Hsp70E) (P17066) <i>Hsc70</i> (Hsp70-6, Hsp73) (P11142)
Other Hsp70's	<i>Sab1p</i> (P11484) <i>Sab2p</i> (P40150)	<i>Ca. Sab1p</i> (P91722)	<i>Yi. Ssb1p</i> (Q6CIA7)	<i>Hsp70-10</i> (Q9TW52)				
	<i>Sp. Ssz1p</i> (P38788)	<i>Ca. Ssz1p</i> (Q5AG78)	<i>Yi. Ssz1p</i> (Q6CEW0)					<i>Hsp70-14</i> (Hsp70L-1) (Q0VDF9)
Hsp110's	<i>Sse1p</i> (P32589) <i>Sse2p</i> (P32590)	<i>Ca. Sse1p</i> (Q6VVB9)	<i>Yi. Sse1p</i> (Q6C618)		<i>Ci. HSPA4/4L/HSPH1</i>	<i>At. Hsp70-14</i> (Q657C0) <i>At. Hsp70-15</i> (Q8CA95) <i>At. Hsp70-16</i> (A8MRM9)	<i>Hsc70Cb</i> (Q9XZT5)	<i>Hsp110</i> (Hsp70-4) (P34932) <i>Hsp110</i> (Hsp70-4L) (Q85757) <i>Hsp105</i> (Q92588)
HspBP1	<i>Fes1p</i> (P38260)	<i>Ca. Fes1p</i> (Q59NN8)	<i>Yi. Fes1p</i> (Q6C239)					<i>HspBP1</i> (Q9NZL4)
Bag proteins	<i>Bag-1A</i> <i>Bag-1B</i> (Q59739)	<i>Ca. Sni1p</i> (Q59NB3)	<i>Yi. Bag1</i> (Q6C9J3)	<i>Ca. Bag-1</i> (Q44739) <i>Unc-23a</i> (Q61980) <i>Unc-23b</i> (Q86S24) <i>Unc-23c</i> (Q5TKA9)	<i>Ci. BAG1</i> <i>Ci. BAG3</i> <i>Ci. BAT3</i>	<i>At. Bag-1</i> (Q0WUQ1) <i>At. Bag-2</i> (Q8LEP8) <i>At. Bag-3</i> (Q8LYP4) <i>At. Bag-4</i> (Q65021) <i>At. Bag-5</i> (Q65373) <i>At. Bag-6</i> (Q82345) <i>At. Bag-7</i> (Q8LV40)	<i>starvin, isoform A</i> (Q5VUJ81) <i>starvin, isoform B</i> (Q5VUJ83) <i>starvin, isoform C</i> (Q5VUJ82)	<i>Bag-1L, Bag-1M, Bag-1S</i> (Q98933) <i>Bag-2</i> (Q95816) <i>Bag-3</i> (Q95817) <i>Bag-4</i> (Q95429) <i>Bag-5</i> (Q9UL15) <i>Bag-6, BAT3</i> (P46379)
CHIP			<i>Yi. Chn1p</i> (Q6C1F4)	<i>Cr. CHIP</i> (A8J756)		<i>At. CHIP</i> (Q8SR59)	<i>Dm. CHIP</i> (Q8XYW6)	<i>CHIP</i> (Q9UNET)

Correction apportée à la Table 1 (pages 344-345) de la revue précédente

The cytosolic Hsp70s of various eukaryotes are shown together with some important co-chaperones (because of their high number, J-proteins are not shown). UniProt accession numbers are provided in most cases, along with the alternative acronyms by which these proteins were described when appropriate.

IV. Problématique et objectifs

Le cytoplasme de la plupart des cellules eucaryotes contient plusieurs paralogues de Hsp70 hautement conservés qui diffèrent essentiellement par leur expression spatio-temporelle. Cependant, ils possèdent également des spécificités fonctionnelles, qui sont peu décrites, et généralement pas de manière systématique. De plus, les différents paramètres régissant ces spécificités sont peu étudiés.

Les objectifs de cette thèse ont été :

Premièrement de décrire les spécificités fonctionnelles des Hsp70 le plus exhaustivement possible.

Deuxièmement de comprendre leur origine. (Qui peut être liée aux différents types de substrats et/ou à la coopération des Hsp70 avec les co-chaperons, d'autres chaperons, ou d'autres protéines impliquées dans la protéostase.)

La caractérisation fonctionnelle du système Hsp70 est d'autant plus importante que ce système est au cœur de la protéostase. Le système Hsp70 est directement lié aux maladies conformationnelles, mais est également impliqué dans plusieurs maladies dont les cancers (Broadley and Hartl, 2009 ; Dudek et al., 2009; Macario and De Macario, 2007 ; Powers et al., 2009). Il est une cible privilégiée pour des futurs traitements de l'ensemble de ces pathologies, pour rétablir la protéostase, ou au contraire déclencher la mort spécifique des cellules cancéreuses (Galluzzi et al., 2009; Powers et al., 2009). Dans le cas des cancers, le système Hsp70 inhibe l'apoptose à plusieurs niveaux (Morano, 2007). Par exemple, il participe au pouvoir anti-apoptotique de Bcl-2 (Takayama et al., 1995), il inhibe la formation de l'apoptosome (Saleh et al., 2000), ou inhibe des effecteurs de la voie extrinsèque (Gurbuxani et al., 2003 ; Ruchalski et al., 2006). De plus, les formes inductibles des Hsp70 sont surexprimées dans les cellules cancéreuses, provoquant une résistance à la chimiothérapie (Galluzzi et al., 2009). Une stratégie de traitement est l'inactivation des Hsp70 pour provoquer une mort sélective des cellules cancéreuses. En ce sens, Leu *et al.* présentent un inhibiteur spécifique des orthologues inductibles, qui provoque la mort des cellules cancéreuses en ayant une toxicité moindre sur des cellules saines (Leu et al., 2009).

Cependant, dans le développement de stratégies thérapeutiques, il semble indispensable de cibler des fonctions spécifiques des Hsp70 afin de limiter au maximum les effets délétères du traitement sur l'organisme. La complexité du système Hsp70 et du système chaperon en

général, ainsi que leur pléiotropie, implique une régulation fine qui demande à être caractérisée.

Pour cela, nous avons mis en place trois approches.

La première est une comparaison systématique de la capacité des paralogues de Hsp70 cytoplasmiques à assurer différents processus cellulaires *in vivo*.

La deuxième est l'identification de nouvelles fonctions des Hsp70.

La troisième est la caractérisation de la machinerie Hsp70 de la levure *Yarrowia lipolytica*.

RESULTATS

I. Comparaison systématique intra- et inter-espèce(s) des spécificités fonctionnelles des Hsp70 cytoplasmiques de type Ssa

I.A. Introduction à l'Article 1

La première stratégie que nous avons adoptée pour mettre en évidence et caractériser des spécificités fonctionnelles au sein des Hsp70 cytoplasmiques a été de réaliser une comparaison intra- et inter-espèce(s) de huit membres de cette famille (Sharma et al., 2009).

Cette étude a été réalisée en collaboration avec le groupe de Daniel C. Masison (NIH, Bethesda, USA) qui a mis au point le système expérimental que nous avons utilisé (Tutar et al., 2006). Ce modèle permet de comparer individuellement chaque Hsp70 d'intérêt en utilisant une souche $\Delta ssa1-4$ de *Saccharomyces cerevisiae*, dans laquelle les gènes codant les quatre protéines Ssa ont été invalidés (Tutar et al., 2006). La présence d'au moins un membre de la sous-famille des SSA étant essentielle à la croissance cellulaire (Werner-Washburne et al., 1987), ce mutant survit grâce à la présence d'un plasmide *URA3* portant le gène *SSA1* (Tutar et al., 2006). Un second plasmide *LEU2* permettant l'expression d'une Hsp70 d'intérêt est ensuite introduit dans cette souche et le plasmide *URA3* contre-sélectionné sur un milieu contenant du 5-FOA (*5-FluoroOrotic Acid* ; acide 5-fluoroorotique) (Figure 15).

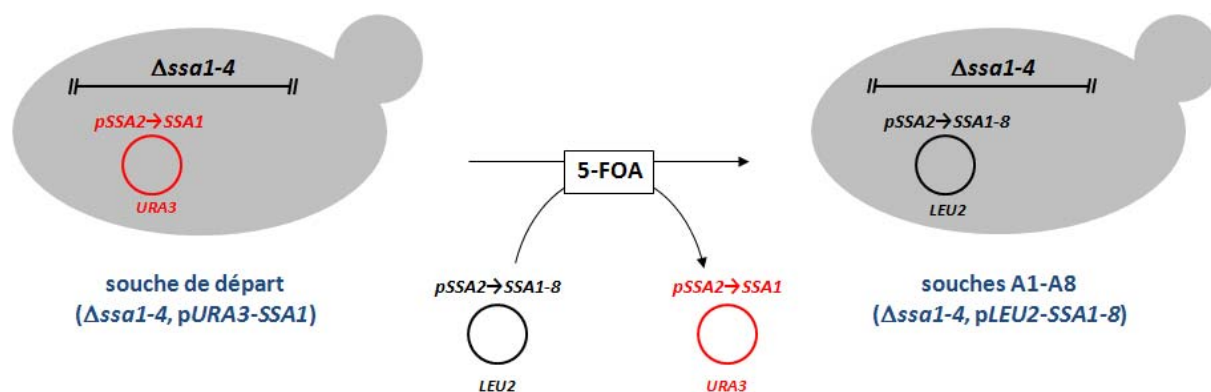


Figure 15 : Modèle permettant de comparer les Hsp70 individuellement

La souche $\Delta ssa1-4$ survit grâce à la présence du plasmide *pURA3-SSA1*. Un plasmide *pLEU2-SSA1-8* est introduit dans la souche et le premier plasmide est contre-sélectionné sur un milieu contenant du 5-FOA.

Ainsi, nous obtenons une collection de souches n'exprimant qu'une seule protéine Ssa et dont nous pouvons comparer les propriétés vis-à-vis de divers processus cellulaires. Afin que la comparaison ne soit pas biaisée par des niveaux d'expression différents, toutes les Hsp70 étudiées ont été exprimées sous le contrôle du même promoteur constitutif du gène *SSA2* (Tutar et al., 2006).

Dans notre cas, nous avons choisi de comparer les protéines Ssa1-4 de *Saccharomyces cerevisiae* et les protéines Ssa5-8 de *Yarrowia lipolytica* (Kabani and Martineau, 2008b), et ce pour deux raisons principales. D'après les études décrites dans la littérature (Kabani and Martineau, 2008b), les différences entre les protéines Ssa se révéleraient probablement subtiles et nous avons donc prédit qu'elles seraient exacerbées entre les protéines de *Y. lipolytica* exprimées dans un contexte hétérologue. Par ailleurs, nous avons initié une caractérisation de la machinerie Hsp70 chez *Y. lipolytica* (voir III. Caractérisation du système Hsp70 de *Yarrowia lipolytica*), ce qui devait nous permettre de valider dans un contexte natif les observations faites chez *S. cerevisiae*.

Cette étude a fait l'objet d'une publication (Sharma et al., 2009) dont je suis co-premier auteur et qui est articulée comme suit.

Dans un premier temps, nous avons caractérisé le profil d'expression des Hsp70 de *Y. lipolytica* et avons montré que l'expression de *SSA6* et *SSA7* est inductible par un stress thermique, à l'instar de *SSA3* et *SSA4* chez *S. cerevisiae* (Werner-Washburne et al., 1987), alors que l'expression de *SSA5* et *SSA8* est constitutive (Sharma et al., 2009). Nous avons ensuite construit huit souches (A1-8) exprimant chacune individuellement l'une des huit protéines Ssa, selon le système expérimental décrit ci-dessus (Figure 15).

Ces huit souches étant viables, nous en avons conclu que Ssa1-8 sont toutes capables d'assurer les fonctions cellulaires essentielles à la croissance de la levure. Cependant, des différences significatives de croissance ont été observées entre les huit souches, aussi bien à température permissive qu'à haute température, indiquant que Ssa1-8 n'accomplissent pas ces fonctions cellulaires avec la même efficacité.

Nous avons ensuite estimé la thermotolérance des souches en mesurant leur taux de viabilité après un choc thermique à 52°C. Ces expériences ont montré que les formes inductibles (Ssa3, Ssa4, Ssa6 et Ssa7) confèrent une thermorésistance environ 10 à 25 fois plus élevée que les formes constitutives (Ssa1, Ssa2, Ssa5 et Ssa8). De plus, cette capacité à protéger la cellule d'un choc thermique est indépendante de la bonne ou mauvaise croissance des

souches ; ainsi la souche A6 qui pousse le plus lentement est néanmoins la souche la plus thermorésistante.

Nos collaborateurs ont mesuré la réactivation de la luciférase *in vivo* dans les souches A1-8 après inactivation thermique. Là encore, des différences significatives ont été observées entre les souches, ce qui montre que Ssa1-8 n'ont pas la même capacité à interagir avec ce substrat et/ou à coopérer avec Hsp104 ou d'autres systèmes luttant contre l'agrégation des protéines.

Faisant référence à leurs précédents travaux (Sharma and Masison, 2008), ils ont également montré que la propagation des prions [*PSI+*] et [*URE3*] varie en fonction de la protéine Ssa exprimée. Ces effets ne sont pas corrélés avec la nature constitutive ou inductible de l'Hsp70 en question, et des effets antagonistes ont été observés selon le prion considéré. Par exemple, dans la souche A3, [*PSI+*] se propage parfaitement avec un phénotype associé « fort », alors que [*URE3*] est instable avec un phénotype associé « faible ».

Nous avons ensuite montré au laboratoire par des expériences de transformations de sphéroplastes avec des extraits cellulaires de souches [*PSI+*] (technique permettant d'induire la conversion de cellules [*psi-*] en [*PSI+*]) que les différents phénotypes [*PSI+*] obtenus dans les souches A1-8 ne correspondent pas à la sélection de variants « forts » ou « faibles ». Il s'agit bien d'un même variant dont la propagation est affectée différemment selon l'Hsp70 exprimée dans la cellule.

Par ailleurs, Daniel C. Masison et collaborateurs ont analysé les agrégats intracellulaires formés par la protéine Sup35 (et qui sont à l'origine du prion [*PSI+*]) dans les souches A1-8 par l'intermédiaire d'une fusion Sup35-GFP et en utilisant la microscopie ou l'électrophorèse en gel d'agarose en conditions semi-dénaturantes (SDD-AGE : *Semi-Denaturing Detergent-Agarose Gel Electrophoresis*). Cette analyse n'a pas permis de corréler les différences observées entre la taille ou la morphologie des agrégats et le phénotype prion correspondant. Nous concluons de ces expériences que les résultats obtenus par ces méthodes, fréquemment utilisées par les laboratoires s'intéressant aux prions, doivent être considérés avec beaucoup de précautions et ne suffisent pas à prédire la propagation sous forme prion d'une protéine donnée. Nos collaborateurs ont également montré que la surexpression de *YDJI* permet de curer efficacement le prion [*URE3*] dans les souches A1, A2, A7 et A8 mais beaucoup moins efficacement dans les autres souches. Ceci indique que les différentes Hsp70 ne coopèrent pas de manière similaire avec leurs cofacteurs.

Enfin, dans une dernière partie, nous avons étudié la dégradation protéasomale de la protéine humaine CFTR, dont des mutations sont à l'origine de la mucoviscidose, exprimée dans les souches A1-8. Chez la levure, CFTR est entièrement retenue au niveau du réticulum

endoplasmique puis dégradée par la voie ERAD (Zhang et al., 2001). Nous avons pu montrer que la cinétique de dégradation de CFTR varie peu entre les souches A1-8 ; elle est légèrement plus rapide dans les souches A1, A4 et A8. De manière intéressante, la dégradation de CFTR est ralentie dans certaines souches (A1, A4 et A5) en présence du prion [*PSI+*]. Ceci indique que dans ces souches, l'activité de Hsp70 et son recyclage ne sont pas suffisants pour interagir avec les polymères de Sup35 tout en empêchant l'agrégation de CFTR. Une autre hypothèse est que le système ubiquitine-protéasome est inhibé par la présence des agrégats de Sup35 et/ou de CFTR, comme cela a été montré dans les cellules de mammifères (Bence et al., 2001).

I.B. Article 1

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**FUNCTION OF SSA SUBFAMILY OF HSP70 WITHIN AND ACROSS SPECIES VARIES
WIDELY IN COMPLEMENTING *SACCHAROMYCES CEREVISIAE* CELL GROWTH AND
PRION PROPAGATION**

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Function of SSA Subfamily of Hsp70 Within and Across Species Varies Widely in Complementing *Saccharomyces cerevisiae* Cell Growth and Prion Propagation

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Abstract

Background: The cytosol of most eukaryotic cells contains multiple highly conserved Hsp70 orthologs that differ mainly by their spatio-temporal expression patterns. Hsp70s play essential roles in protein folding, transport or degradation, and are major players of cellular quality control processes. However, while several reports suggest that specialized functions of Hsp70 orthologs were selected through evolution, few studies addressed systematically this issue.

Methodology/Principal Findings: We compared the ability of Ssa1p-Ssa4p from *Saccharomyces cerevisiae* and Ssa5p-Ssa8p from the evolutionary distant yeast *Yarrowia lipolytica* to perform Hsp70-dependent tasks when expressed as the sole Hsp70 for *S. cerevisiae* *in vivo*. We show that Hsp70 isoforms (i) supported yeast viability yet with markedly different growth rates, (ii) influenced the propagation and stability of the [PSI⁺] and [URE3] prions, but (iii) did not significantly affect the proteasomal degradation rate of CFTR. Additionally, we show that individual Hsp70 orthologs did not induce the formation of different prion strains, but rather influenced the aggregation properties of Sup35 *in vivo*. Finally, we show that [URE3] curing by the overexpression of Ydj1p is Hsp70-isoform dependent.

Conclusion/Significance: Despite very high homology and overlapping functions, the different Hsp70 orthologs have evolved to possess distinct activities that are required to cope with different types of substrates or stress situations. Yeast prions provide a very sensitive model to uncover this functional specialization and to explore the intricate network of chaperone/co-chaperone/substrates interactions.

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Introduction

Seventy-kilodalton heat-shock proteins (Hsp70) are ubiquitous molecular chaperones that play essential housekeeping functions in protein folding, synthesis, assembly, transport across biological membranes and degradation. They are also involved in quality control processes, such as protein refolding after a stress injury, and control the activity of regulatory proteins in signal transduction and cell proliferation pathways [1]. All of these cellular activities depend on the ATP-regulated ability of Hsp70 s to interact with exposed hydrophobic surfaces of proteins. Hsp70 family members share a highly conserved modular structure consisting in an N-terminal 44-kDa ATPase domain (also named adenine nucleotide binding domain or NBD), an 18-kDa substrate-binding domain (SBD) and a C-terminal 10-kDa lid domain. The interaction of Hsp70 s with

exposed hydrophobic stretches of protein substrates is regulated by adenine nucleotides. In the ATP-bound state, the SBD is in an open conformation resulting in low affinity for substrates. ATP hydrolysis triggers a conformational change of the lid that closes on the substrate and traps it within the SBD [2–4]. The exchange of ADP for ATP allows the return to an open-state and facilitates substrate release. The allosteric regulation of Hsp70 s is tightly regulated by various cochaperones. Members of the universal Hsp40/DnaJ-family stimulate the weak intrinsic ATPase activity of Hsp70 s and coordinate it with substrate binding, whereas ADP/ATP exchange is catalyzed by the unrelated GrpE, Bag1, Fes1/HspBP1 and Sse1/Hsp110 families of nucleotide exchange factors (NEFs) [1,5,6].

Hsp70 s constitute one of the most conserved protein families, and members of this family may be constitutively expressed or stress-inducible [7–12]. Hsp70 s of bacteria and humans are structurally

superimposable and retain 50% amino acid identity [7,9,10]. Hsp70 s from different species share functional redundancy as shown by replacing endogenous Hsp70s with those from other species. For example, *Drosophila* Hsp70 efficiently protected mammalian cells from heat stress [13] and human Hsp70 was able to complement the cytoprotective function of rodent Hsp70 both *in vitro* [14–16] and in transgenic animals [17–19]. Nevertheless, such complementation is often incomplete, which probably is explained by differences in functional interactions of Hsp70s with co-chaperones and other components of chaperone machines among the different species [20]. Most organisms contain multiple members of the Hsp70 family in virtually all cellular compartments [11]. Intriguingly, in eukaryotes, while organelle specific Hsp70s are generally encoded by a single gene, multiple highly homologous Hsp70 paralogs coexist in the cytosol [11]. For example, yeast contains seven cytosolic Hsp70s, among which four SSA-subfamily members (Ssa1–4) and three ribosome-associated Hsp70s (Ssb1–2p and Ssz1p). Similarly, fruit fly and human cells contain ten and seven bona fide Hsp70s, respectively [11,21].

Because of their high degree of conservation [7] and because only one Ssa protein is sufficient to allow yeast viability if expressed at sufficiently high levels [22], it has been long postulated that these Hsp70s were functionally redundant, only differing by their spatiotemporal expression patterns [11]. However, several recent studies (reviewed in [11]) pinpointed exquisite differences among Hsp70 orthologs suggesting that while they share redundant house-keeping functions, they may exhibit functional specificities that have yet to be fully deciphered.

Evidence for such functional specialization among Hsp70 paralogs from our laboratories emerged from studies on the propagation of the [URE3] and [PSI⁺] prions and biofilm formation in yeast. Yeast prions are self-replicating infectious aggregates of cellular proteins that cause distinct non-Mendelian phenotypes [23,24]. The [PSI⁺] and [URE3] traits are caused by the prion properties of the Sup35p and Ure2p proteins, respectively [25–30]. Molecular chaperones of the Hsp40 and Hsp70 families ([31,32] for review), as well as the Sse1/Hsp110 and Fes1/HspBP1 NEFs [33–35] play critical roles in prion formation and propagation. The overexpression of Ssa1p, but not Ssa2p, cures [URE3] [36], whereas mutations in SSA2, but not SSA1, impair [URE3] propagation [37]. Remarkably, yeast strains expressing individual Ssa proteins have markedly different prion phenotypes [38]. We have recently reported that the Hsp70 machinery is required for biofilm (or ‘mat’) formation in yeast [39]. Specifically, an *ssa1Δ* mutant, in an otherwise wild-type background for the other Ssa-encoding genes, is more affected in mat formation than an *ssa2Δ* mutant that has more subtle defects in this process [39]. Moreover, we showed that the additional deletion of SSA3 or SSA4 enhances the mat formation defects of the *ssa1Δ* and *ssa2Δ* mutants, suggesting functional cooperation between constitutive and inducible Hsp70s [39].

Here we used a previously described yeast reporter strain [38,40] to systematically compare the ability of eight Ssa proteins from the yeasts *Saccharomyces cerevisiae* and *Yarrowia lipolytica* to allow yeast growth, prion propagation and proteasomal degradation of a model protein. Our data suggest that functional differences among cytosolic Hsp70s provide yet another way to control and fine tune their cellular activity that may be required to cope with various substrates, functions and adverse conditions.

Results

Nomenclature and conditional expression of *Yarrowia lipolytica* Hsp70 orthologs

We have recently reported that the yeast *Yarrowia lipolytica* contains four orthologs of the Ssa subfamily of Hsp70 molecular

chaperones in the cytosol [11]. These proteins are highly conserved (Table 1 and Supplemental Figure S1) and it was therefore impossible to identify which of them corresponded to *S. cerevisiae* Ssa1p-Ssa4p solely based on reciprocal Blast analysis (data not shown). To avoid nomenclature confusion and for the sake of clarity, we arbitrarily chose to name the *Y. lipolytica* Ssa-type proteins Ssa5p to Ssa8p [11]. It is noteworthy that the Ssa5p-Ssa8p proteins share a higher degree of conservation with each other (92–95% identity) than the Ssa1p-Ssa4p proteins (79–97% identity) (Table 1). The *Yarrowia lipolytica* cytosol also contains orthologs of the ribosome-associated Ssb1/2p and Ssz1p proteins that we named *Yl.Ssb1p* (locus: YALI0A00132g; UniProt ID: Q6CIA7) and *Yl.Ssz1p* (locus: YALI0B12474g; UniProt ID: Q6CEW0) [11]. While a detailed molecular analysis of the *Y. lipolytica* Hsp70 machinery will be described elsewhere (Martineau C.N., LeDall M-T., Beckerich J-M. and Kabani M., unpublished data), it was essential for this study to characterize the expression patterns of Ssa5p-Ssa8p, as functional differences have been previously pointed out between constitutive and inducible Hsp70s [40]. To this aim, we documented the expression of each isoform in different growth conditions by RT-PCR analysis.

First, a wild-type *Y. lipolytica* strain was grown at 28°C and RNA was prepared from samples taken at early exponential phases. RT-PCR was performed to detect each Hsp70 isoform or the actin-encoding gene *ACT1* as a control. As shown in Figure 1, SSA5, SSA7 and *Yl.SSB1* were expressed in exponentially growing cells, whereas SSA6 and SSA8 were only modestly detected. As the cells progress in stationary phase, we barely detected SSA5 and SSA8 mRNAs, SSA6 levels remained very low, whereas SSA7 and *Yl.SSB1* mRNA levels were still easily detectable at the lowest RNA concentration used for the assay (Figure 1). It should be noted that the *ACT1* mRNA levels substantially decreased in stationary phase compared to exponential phase, which is consistent with earlier observations in *S. cerevisiae* or *Candida albicans* [41–43].

To assess stress inducibility, the same 28°C cultures were heat-shocked for one hour at 42°C, before RNA was extracted. In these conditions, SSA6 and SSA7 were up-regulated ~4–5 fold and ~1.5–2 fold, respectively, whereas the levels of SSA5, SSA8, and *Yl.SSB1* were not significantly affected (Figure 1 and data not shown). Similar results were obtained when the cells were shifted to 37°C, a temperature at which *Y. lipolytica* cells are unable to grow but can nevertheless survive for several hours (data not shown). Thus, from these data, we expect Ssa6/7p to be functionally similar to the inducible Ssa3/4p, whereas Ssa5/8p would be more similar to the constitutive Ssa1/2p. Finally, we can not exclude that the expression of the *Y. lipolytica* Hsp70 encoding genes is also modulated by yet unidentified environmental cues and stress injuries.

Table 1. Percent amino acid identity of Ssa proteins.

	Ssa1	Ssa2	Ssa3	Ssa4	Ssa5	Ssa6	Ssa7
Ssa2	97						
Ssa3	79	79					
Ssa4	81	81	87				
Ssa5	82	81	80	82			
Ssa6	81	81	80	82	94		
Ssa7	83	82	80	81	95	95	
Ssa8	81	81	81	82	93	92	92

doi:10.1371/journal.pone.0006644.t001

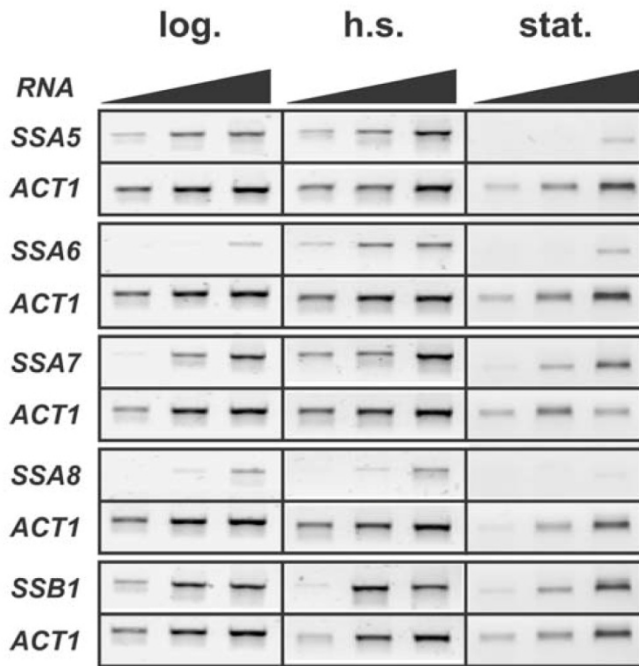


Figure 1. RT-PCR analysis of Hsp70 isoforms expression in *Yarrowia lipolytica*. Total RNA was isolated from a wild-type *Y. lipolytica* strain grown to early exponential phase at 28°C (left panel), followed by one hour shift at 42°C (middle panel) or left to grow to stationary phase (right panel). The expression of each Hsp70 ortholog was assessed by RT-PCR analysis (using 1, 10 or 100ng of RNA) as described in Materials and Methods using the *ACT1* gene as a loading control.
doi:10.1371/journal.pone.0006644.g001

Growth of *S. cerevisiae* cells expressing individual Ssa proteins varies widely

The *SSA* subfamily of Hsp70 molecular chaperones is essential for growth of *S. cerevisiae* [22]. Our earlier work with strains lacking all four chromosomal *SSA* genes and expressing individual Ssa proteins from plasmids under control of the *SSA2* promoter confirmed that Ssa1p, Ssa2p, Ssa3p and Ssa4p each support cell growth [22,38]. To extend this study we systematically compared the ability of individual Hsp70s from the yeasts *S. cerevisiae* (Ssa1p-Ssa4p) and *Y. lipolytica* (Ssa5p-Ssa8p) to handle known Hsp70-dependent tasks *in vivo*. We expected functional differences among Hsp70 isoforms to be subtle and mainly depend on different substrate preferences and/or different affinities for cochaperones. Because of their evolutionary distance, we expected that the *Y. lipolytica* Hsp70 proteins may interact less efficiently with *S. cerevisiae* cochaperones and that any functional differences between isoforms will therefore be exacerbated in this heterologous context.

The *LEU2*-based plasmids pA1, pA2, pA3 and pA4 that allow the expression of *SSA1*, *SSA2*, *SSA3*, and *SSA4* under the control of the strong and constitutive *SSA2* promoter, respectively, were described previously [38,40]. The pA5, pA6, pA7 and pA8 vectors that allow the expression of *SSA5*, *SSA6*, *SSA7* and *SSA8* under the control of the *SSA2* promoter, respectively, were similarly constructed (see Materials and Methods). These plasmids were used to transform strain G402, which lacks all chromosomal *SSA* genes and expresses *SSA1* from the *URA3*-based plasmid pRDW10 [44]. The ability of the pA1-A8 plasmids to support growth of G402 was assessed by challenging transformants on medium containing 5-fluoro-orotic acid (5-FOA) to counter-select against plasmid pRDW10. The resulting *Ura*⁻, *Leu*⁺ strains expressing

each individual Ssa protein were hereafter referred to as A1 to A8. All *SSA* genes supported yeast growth, albeit with markedly different growth rates indicating significant functional differences among these Hsp70 orthologs (Table 2 and Figure 2). As expected, *Yl.Ssb1p* was not able to support yeast growth, nor was an Ssa4p mutant with a D393G mutation in the conserved linker that is predicted to impair the inter-domain communication between the NBD and the SBD (Table 2). In rich liquid medium at optimal temperature A1 and A2 grew at near wild type rate, A3 cells grew somewhat more slowly and A4 cells took roughly 30% longer to divide (see Table 2). All of the *Y. lipolytica* Hsp70s supported cell growth but none as well as the *S. cerevisiae* Ssa proteins and there was more variability in growth rates. For example, A5 cells grew best, yet slightly more slowly than those expressing Ssa4p, and A6 cells took three times longer than wild type cells to double in numbers. Importantly, strains expressing inducible Hsp70s (A3, A4, A6 and A7) grew more slowly than their constitutive counterparts (A1, A2, A5 and A8), respectively. These results agree with our previously published data showing inability of primate inducible Hsp70 to confer yeast viability [40], and indicate that stress-inducible Hsp70s may be partly or totally unable to perform important house-keeping functions that require more efficient activities possessed by the constitutive isoforms.

Differences in growth were more pronounced when strains were grown on solid medium at different temperatures (see Figure 2). All strains expressing *Y. lipolytica* Hsp70s were temperature sensitive at 37°C, which might reflect reduced ability of these Hsp70s to protect important cellular factors or possibly a lower thermal stability of Hsp70s that evolved with this organisms’s lower optimal and maximal growth temperatures. Strain A6 was unable to grow while A7 and A8 were viable but did not form noticeable colonies after 2 days. Growth of A6 was also most sensitive to reduced temperature. Thus, although sequence identity of Ssa5p-Ssa8p is very similar to Ssa1p-Ssa4p, they functioned differently than all four *S. cerevisiae* Hsp70s in supporting growth. Additionally, although Ssa5p-Ssa8p are very highly homologous to each other, the considerable differences in

Table 2. Growth rates and prion phenotypes of strains expressing individual Ssa proteins.

Hsp70 isoform	Growth rate ^a (min/cell div)	Prion phenotype ^b	
		[<i>PSI</i> ⁺]	[<i>URE3</i>]
Ssa1p	110	++++	++
Ssa2p	107	+++	++++
Ssa3p	117	+++++	+/-
Ssa4p	141	++	+
Ssa5p	153	+++	+++
Ssa6p	306	+/-	-
Ssa7p	179	+/-	+
Ssa8p	164	+++	+++
<i>Yl.Ssb1</i>	inviabile	na	na
<i>Ssa4</i> ^{D393G}	inviabile	na	na

^aCells were grown in 15 ml of YPAD in 50 ml flasks shaking at 220 rpm at 30°. Our isogenic wild type strains 779-6A and 1075 have cell division time of 100 minutes.

^bBased on data from Figures 5 and 9 and shown relative to those of typical wild type cells (++++, exemplified by cells expressing Ssa1p for [*PSI*⁺] and Ssa2p for [*URE3*]); na, not applicable (Hsp70 did not support growth).

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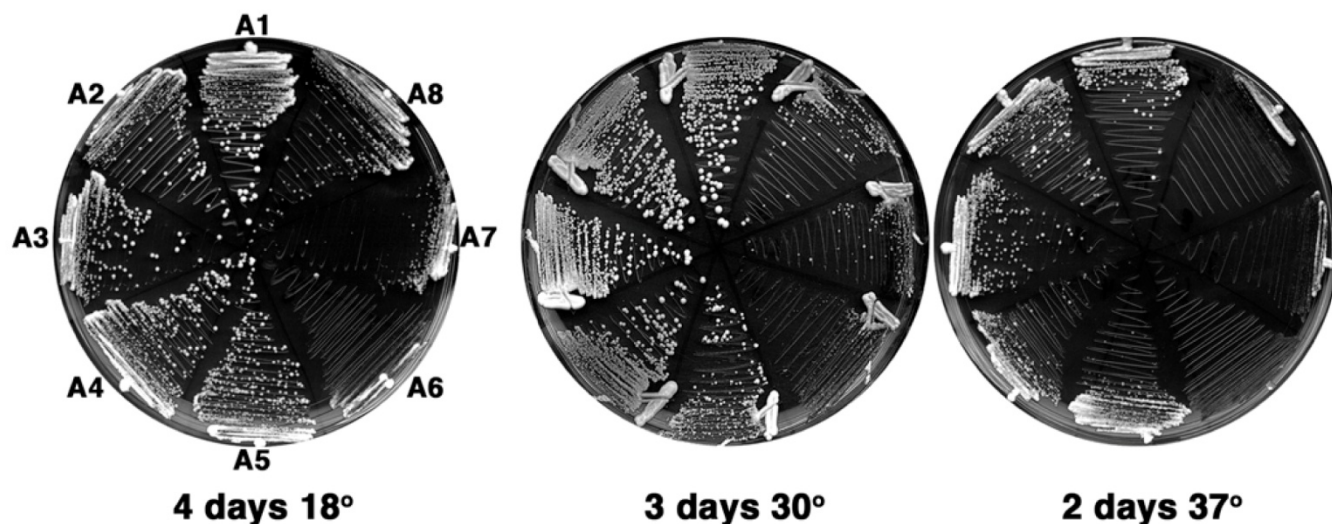


Figure 2. Growth of *S. cerevisiae* expressing individual Ssa proteins. G402 strains A1–A8 [*psi*⁻] cells were streaked onto YPAD plates and incubated at the indicated temperature for 2–4 days as indicated. Strains are as indicated on first panel, pattern of strains is the same on each plate. doi:10.1371/journal.pone.0006644.g002

their abilities to support growth of *S. cerevisiae* demonstrates clear functional distinctions among them.

Inducible Hsp70s provide increased thermoresistance

A major role played by Hsp70 molecular chaperones is to protect cells from stress injuries by preventing aggregation of partially unfolded proteins. Additionally, Hsp70 and Hsp40 cooperate with Hsp104, a molecular chaperone found in microorganisms and plants that acts by resolubilizing aggregated proteins [20,45] and is also required for [*PSI*⁺] propagation [46]. The survival rate of yeast cells to a lethal heat-shock can be greatly improved by pre-treating the cells with a mild non-lethal heat-shock, which stimulates the heat-shock and stress response pathways leading to elevated expression of heat-shock proteins (including Hsp70s and Hsp104) [12,47].

We compared the thermoresistance of the A1–A8 strains exposed to a lethal heat-shock at 52°C [40] in the absence of a mild heat-shock pre-treatment to avoid any differences between Hsp70 isoforms being masked by the up-regulation of other chaperones. As shown in Figure 3, the thermoresistance of all strains was comparable, except that the strains expressing inducible Hsp70 isoforms (A3, A4, A6, and A7) were significantly more resistant than those expressing constitutive Hsp70s. After 16 min at 52°C, ~10 to 14-fold more heat-resistant cells were recovered for the A3, A4 and A7 strains than for A2, which was the least resistant strain; the A6 strain showed the greatest survival rate with a 25-fold increase in the number of surviving cells compared to A2 (Figure 3C). This result indicates that inducible Hsp70s may be more efficient in cooperating with Hsp104 during the process of protein disaggregation and/or have more affinity with heat-denatured substrates than their constitutive counterparts. It is noteworthy that the A6 and A7 strains, which grow very slowly, were nevertheless as thermoresistant as the A3 and A4 strains (Figure 3C). This result indicates that the role of Ssa6p - and Ssa7p in protecting cells from stress is fully conserved in this heterologous context and that the reason why it poorly supports cell viability is probably due to its inability to replace constitutive Hsp70s in one or more house-keeping pathways.

Individual Ssaps vary in their ability to help proteins refold in vivo

The ability of chaperones to help proteins refold in vivo can be assessed by monitoring reactivation of a thermolabile bacterial luciferase after exposing cells to elevated temperature. When elevated temperature causes widespread protein aggregation, Hsp104 becomes critical for survival by disaggregating proteins in a reaction dependent upon Hsp70 and Hsp40 [20]. We monitored luciferase reactivation in strains A1–A8 by shifting exponentially growing cells from 30°C to 37°C for 30 minutes to induce expression of heat shock proteins, including Hsp104, and then to 42°C to cause extensive aggregation of luciferase. After this treatment the cells were allowed to recover at 25°C for 30 minutes. We measured luciferase after this recovery period and compared it with the activity in cells before the heat treatment (see Material and Methods). In wild type cells, over 40% of luciferase activity was restored, while in cells lacking Hsp104 only 15% was reactivated (Figure 4). Cells expressing Ssa6p were like cells lacking Hsp104, which would explain why A6 cells did not stably propagate prions (see below). Cells expressing Ssa1p had reduced reactivation function. Luciferase reactivation in the A2, A4, A7 and A8 strains was at or near wild type levels, while A3 and A5 consistently performed better than all the others (Figure 4).

[*PSI*⁺] propagation is highly variable in cells expressing individual Hsp70 orthologs

Strains G402 and 1161 allow us to monitor the prions [*PSI*⁺] (this section) and [*URE3*] (see below), respectively, by a white/red color phenotype and a requirement of adenine for growth. Cells with prions are white, those without prions are red (see Materials and Methods). The strength of prion phenotype is proportional to the extent that Sup35p or Ure2p is depleted into insoluble prion aggregates [48], which is reflected in the extent of pigment accumulation and growth without adenine. Weaker prion propagation is seen as intermediate (pink) colony color and/or slower growth without adenine. The stability of prions in a growing population requires continuous replication of prions in order to keep pace with cell divisions. Reduced prion replication can be seen as spontaneous appearance of red colonies in a population of cells spread on solid medium.

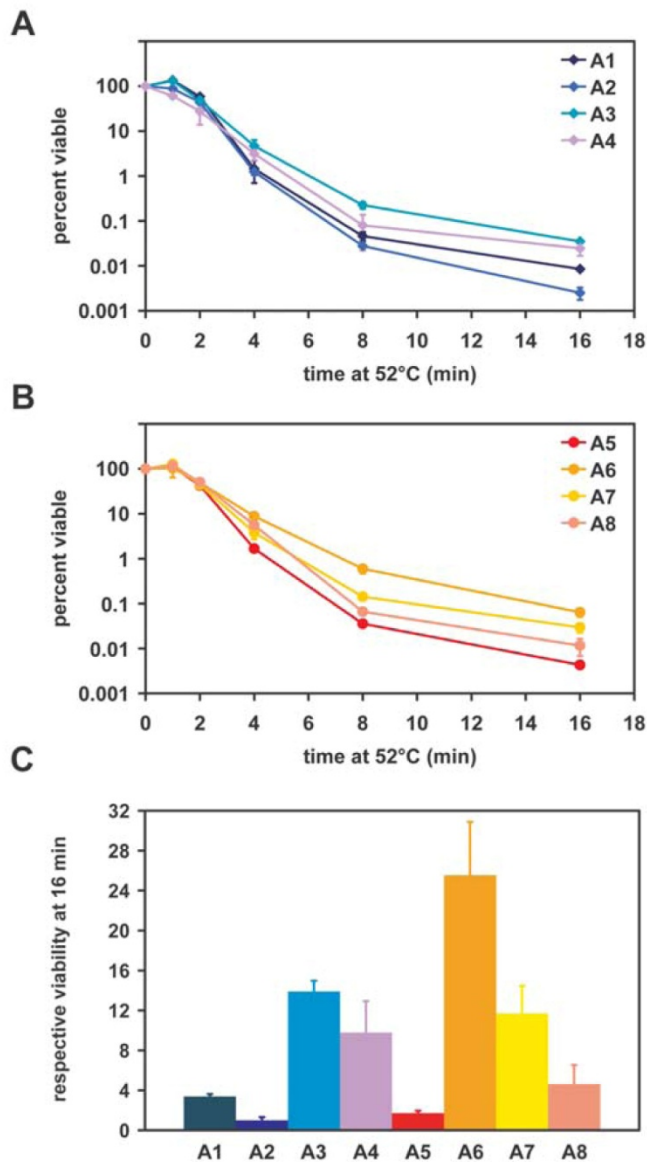


Figure 3. Thermoresistance properties of strains expressing individual Ssaps. The indicated strains were grown to early exponential phase at 28°C then shifted at 52°C. (A, B) Aliquots were periodically removed and cell viability was assessed by plating tenfold serial dilutions onto YPAD plates. (C) The viability of each strain at the 16 min time point was expressed as a fold enrichment over that of A2 which was arbitrarily set to 1. doi:10.1371/journal.pone.0006644.g003

Earlier we showed that $[PSI^+]$ strength varied in cells expressing Ssa1p-Ssa4p proteins individually [38]. Here we confirm these results (see Figure 5) showing $[PSI^+]$ was normal in cells expressing Ssa1p, as seen by white color on 1/2YPD and good growth on –Ade at 30°C. $[PSI^+]$ was slightly weaker in cells expressing Ssa2p, as seen by pink color on all media and weaker growth on –Ade, especially with increasing temperature, which weakens prions independently of other factors [38]. $[PSI^+]$ was stronger than normal in cells expressing Ssa3p, which remained ade+ and white even at 37°C, and weakest in cells expressing Ssa4p, which were pink and slow growing on –Ade even at 25°C. We find that strength of $[PSI^+]$ phenotype in strains A5-A8 also varied (see Figure 5). $[PSI^+]$ phenotype of strain A5 was similar to that of A2, and $[PSI^+]$ in strain A8 was somewhat weaker still but stronger

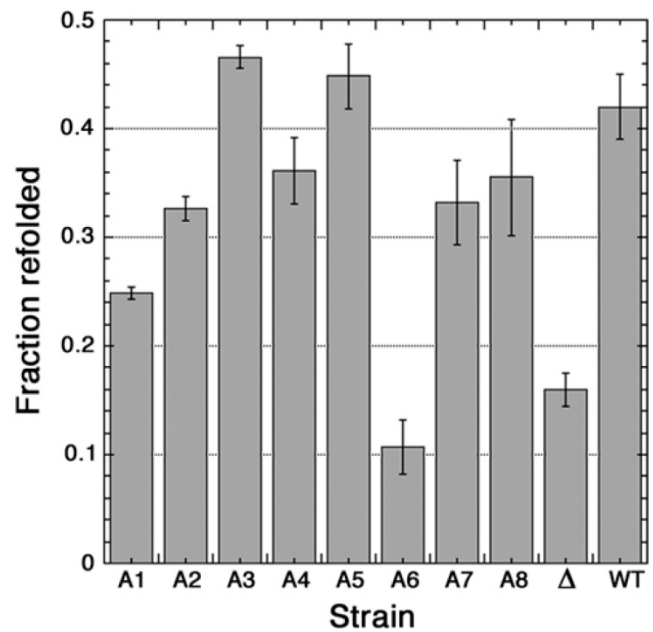


Figure 4. Influence of Ssa proteins on refolding of luciferase. Cultures of strains expressing a thermolabile luciferase and individual Ssa proteins (indicated at bottom) were shifted from 30°C to 37°C for 30 minutes and then to 42°C for one hour. Luciferase activity, expressed as a fraction of pre-heat shock activity, was measured after allowing cells to recover for 30 minutes at 25°C. Cycloheximide was added 50 minutes after shifting to 42°C to prevent synthesis of luciferase during the recovery period. Wild-type and *hsp104Δ* (Δ) strains (with intact *SSA1-4* genotype) were used as controls. Data are averages \pm standard deviation from at least two cultures for each strain measured in triplicate. doi:10.1371/journal.pone.0006644.g004

than in strain A4. Although we were able to isolate $[PSI^+]$ variants of strains A6 and A7, the prion was very weak and unstable. Combined with slower growth, especially of strain A6, it was difficult to characterize $[PSI^+]$ phenotype in these strains.

Mitotic transmission of $[PSI^+]$ was uniformly stable (see Figure 5B) in most of the strains with minor exceptions. Although freshly subcultured A5 and A8 strains displayed normal $[PSI^+]$ stability, when they were retrieved from 15% glycerol stocks stored at –80°C, $[psi^-]$ cells appeared at frequencies of 5–20%. Currently we have no explanation for this instability. It is possible the weakened state of the prion is more susceptible to changes in chaperone levels during freezing and thawing. If so, then the mechanisms causing the weakening of $[PSI^+]$ under normal growth conditions in these strains would be qualitatively different than the weakening in A2 or A4 cells, in which $[PSI^+]$ is stable from storage.

Individual Ssa proteins do not induce formation of different $[PSI^+]$ prion variants

A remarkable property of prion-prone proteins is their ability to generate structurally distinct prion strains, or variants, from the same polypeptide sequence that lead to distinct heritable phenotypes [32,49]. Purified Sup35p spontaneously assembles into self-seeding, β -sheet-rich amyloid fibers *in vitro* [50,51]. When introduced in $[psi^-]$ cells, these fibers efficiently convert them to the $[PSI^+]$ state [52–54]. Sup35p fibers adopt various conformations *in vitro* that trigger the formation of phenotypically distinct $[PSI^+]$ cells upon transformation into $[psi^-]$ cells [53–55]. Similarly, when cell extracts prepared from weak or strong $[PSI^+]$ variants are introduced into $[psi^-]$ cells, the resulting $[PSI^+]$

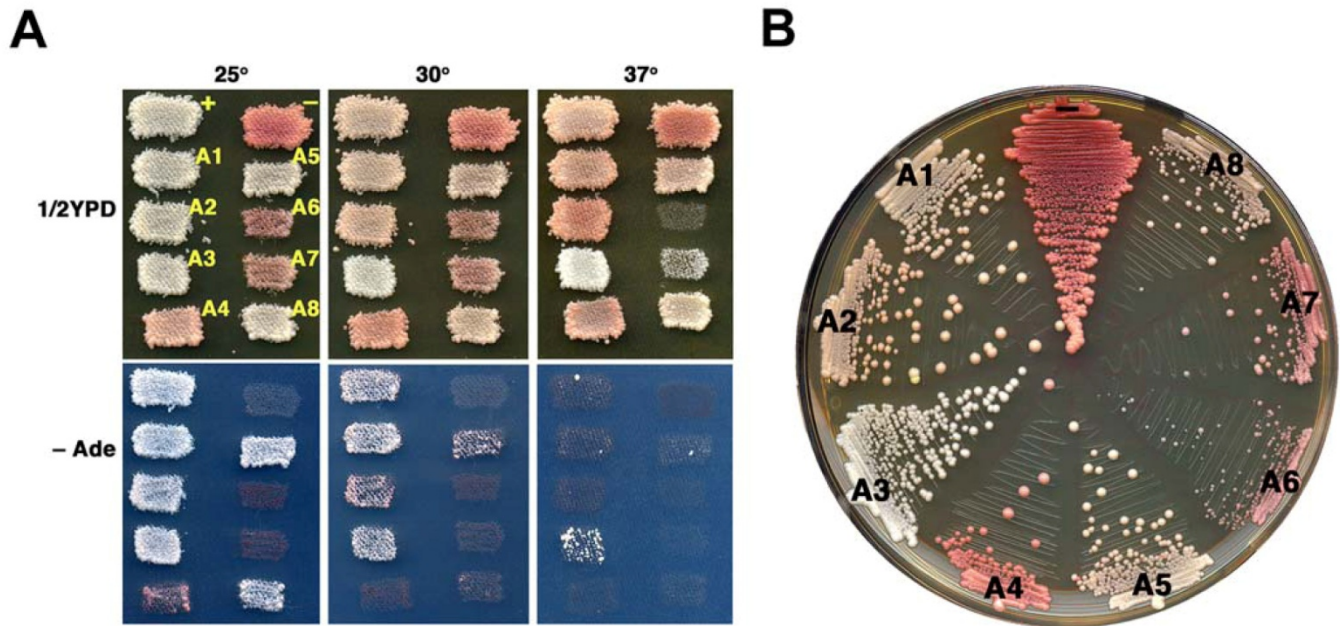


Figure 5. [PSI⁺] prion phenotype of cells expressing different Ssa proteins. (A) Patches of G402 strains A1-A8, as indicated, were grown over night at 30° on 1/2YPD and then replica-plated onto 1/2YPD plates and SD plates without adenine (-Ade), which were then incubated at the indicated temperatures. Plates at 25° were incubated three days. Plates at 30° were incubated two days. At 37° the 1/2YPD plate was incubated two days and the -Ade plate for three days. The whiter appearance of cells at 37° for strains A7 and A8 is due to reduced growth, which has not depleted enough adenine from the medium to cause pigment accumulation. **(B)** Streaks of cells from the same cultures used in **(A)** were incubated two days at 30° followed by three days at 25°. Red colonies in streaks arise from cells that lost [PSI⁺] before forming the colony. Red sectors in pink or white colonies are progeny of cells that lost [PSI⁺] during growth of the colony. In both **(A)** and **(B)** all strains are [PSI⁺] except A6 and A7, in which [PSI⁺] is very unstable (see text). [PSI⁺] (+) and [psi⁻] (-) variants of wild type strain 779-6A are included for comparison.
doi:10.1371/journal.pone.0006644.g005

cells retain the original prion characteristics [53,54]. Earlier work showed that altered chaperones can change the way the same prion variant propagates [48,56] or cause prions to evolve into variants with different propagation properties [57]. The weak [PSI⁺] phenotypes of some strains (e.g. A4) could be caused by weakened propagation of the same prion variant or by the formation of a different prion variant

To test the possibility that different, structurally distinct, prion variants are generated in the presence of different Hsp70 orthologs, we focused on A1 and A4 that have normal and weak prion phenotypes, respectively (Figure 5A). We prepared crude cell extracts from the A1 [PSI⁺] and A4 [PSI⁺] strains and used them, together with the *URA3*-based pRS316 plasmid, to transfect spheroplasts derived from the [psi⁻][pin⁻] 74D-694 strain (see Material and Methods section; [58]). [PSI⁺] cells were then selected among Ura⁺ transformants (see Material and Methods). If the strong and weak [PSI⁺] of the A1 and A4 strains correspond to structurally different prions, then we expect these prion characteristics to be transmitted to a wild-type strain [53,54]. In turn, if A1 and A4 have structurally similar prions, then we expect the prion phenotypes of a wild-type strain induced by the corresponding cell extracts to be similar [53,54]. As shown in Figure 6A, the prion phenotypes of 74D-694 [PSI⁺] cells obtained after transformation with the A1 [PSI⁺] or the A4 [PSI⁺] extracts were very similar, indicating that A1 and A4 do not contain structurally different prion strains. It should be noted that [PSI⁺] is slightly stronger in A1 than in the converted 74D-694 [PSI⁺] strains (Figure 6A). As expected, no [PSI⁺] colonies were obtained when 74D-694 was transfected with crude cell extracts prepared from the A1 [psi⁻] or the A4 [psi⁻] strains (data not shown).

The [PSI⁺] variant in all our strains originated in the parental G402 strain prior to the replacement of pRDW10 by pA1 or pA4.

Since the different Ssa proteins did not induce formation of new prion variants, the 74D-694 transfectants should carry this original prion variant and we should be able to reproduce the prion phenotypes of A1 and A4 using seeds from these 74D-694 [PSI⁺] cells. To confirm this conclusion, we used crude cell extracts from a strong [PSI⁺][pin⁻] derivative of strain 74D-694 to transfect [psi⁻] derivatives of A1 and A4 as described above. As shown in Figure 6B, the prion phenotypes of the transfected strains were very similar to those observed for the original [PSI⁺] strains: the A1 and A4 strains retained their strong and weak prion phenotypes, respectively. These data show that the prions in A1 and A4 are the same and the differences in [PSI⁺] phenotype are due to different effects of Hsp70 orthologs on the way this prion propagates.

Ssa Hsp70s variously affect size of prion polymers

The ways the different Ssa proteins affected prion strength and stability could be due to effects on prion growth or replication (polymer fragmentation). For example, Ssa2p and Ssa4p reduced [PSI⁺] strength but did not affect stability, suggesting they reduce incorporation of Sup35p into [PSI⁺] polymers but did not affect prion replication. By monitoring migration of prion polymers on semi-denaturing agarose gels (SDD-AGE), earlier work showed that inhibiting processes required for polymer fragmentation caused the average length of polymers to increase [59]. The size of polymers could also increase, however, if addition of monomers to polymers is enhanced while the rate of fragmentation remains unchanged. Thus, increased polymer size reflects either reduced fragmentation or enhanced growth. We used SDD-AGE to determine if the size of prion polymers in strains expressing the various Ssa proteins would provide an indication of whether prion growth or replication was being affected (Figure 7). We grew cells in rich medium at 30°C and used a previously described plasmid-

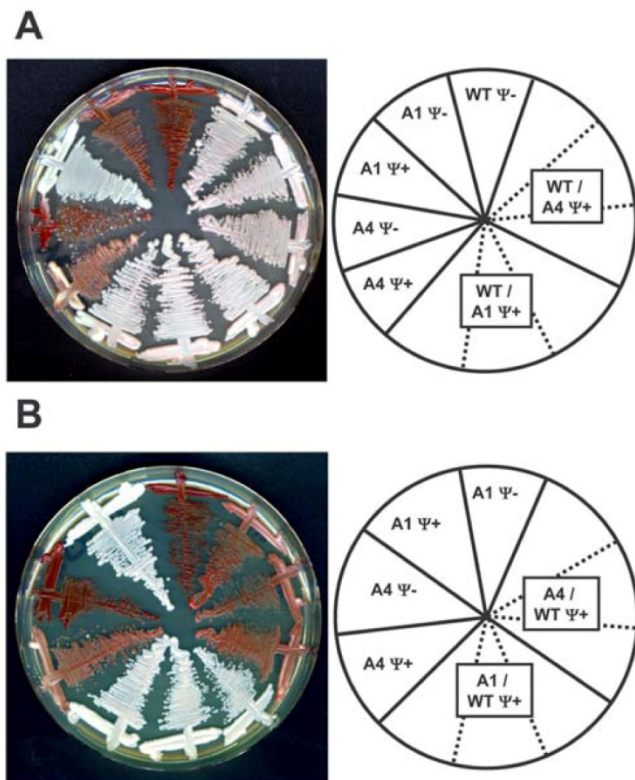


Figure 6. Strains expressing individual Ssaps do not generate different prion strains. (A) The wild-type 74D-694 [*psi*⁻][*pin*⁻] strain was transformed with crude extracts from the A1 [*PSI*⁺] and A4 [*PSI*⁺] strains as described in Materials and Methods. Induced 74D-694 [*PSI*⁺] colonies were isolated and their prion phenotype assessed on 1/2 YPD plates along with the [*psi*⁻] and [*PSI*⁺] derivatives of the A1 and A4 strains. For each transformation, three independent [*PSI*⁺] transformants are shown (their positions are indicated by dashed lines on the right panel) (B) The A1 [*psi*⁻] and A4 [*psi*⁻] strains were transformed with a crude cell extract from the 74D-694 [*PSI*⁺][*pin*⁻] strain. The prion phenotypes of representative [*PSI*⁺] transformants were assessed on 1/2 YPD plates and compared to those of the original A1 and A4 strains. For each transformation, three independent [*PSI*⁺] transformants are shown (their positions are indicated by dashed lines on the right panel). In each case, the plates were incubated for 3 days at 28°C and 4 days at 23°C.
doi:10.1371/journal.pone.0006644.g006

borne Sup35-GFP fusion protein (NGMC, [60]) so we could simultaneously monitor prion aggregation status in live cells (see below). Although we do not know whether this fusion protein incorporates within polymers of the endogenous Sup35p, the fusion protein is induced to form prions by the endogenous [*PSI*⁺] and retains the propagation characteristics of the prions that induce it [60].

All the Sup35-GFP from our wild type [*psi*⁻] strain with the intact *SSA* genotype migrated as monomer while the [*PSI*⁺] control showed less monomeric Sup35-GFP and a significant amount of Sup35-GFP migrating as a smear of high molecular weight material (Figure 7). Surprisingly, the strains with patterns most like wild type were A2, A5, and A8, all of which had weaker phenotypes. The apparent relative amount of monomer, however, correlated with phenotype. For example, A8 had the weakest phenotype and, by visual estimate, the highest relative amount of monomer. One possible explanation for these data is that A2, A5 and A8 modestly inhibit both fragmentation and growth of polymers to levels that produce fewer polymers but with a typical

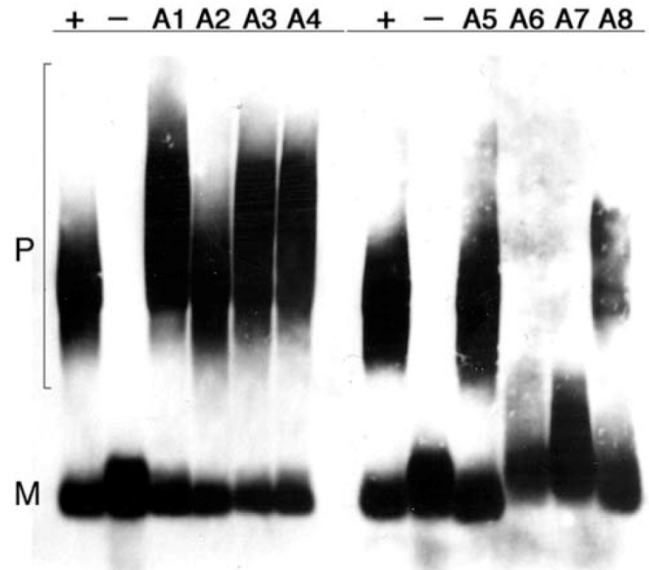


Figure 7. SDD-AGE analysis of Sup35p polymers in G402 [*PSI*⁺] cells. After inducing expression of Sup35-GFP fusion protein (NGMC) for 15 hours, log phase cells were harvested and cell lysates were incubated in SDS to dissolve all but the prion polymers. The lysates were then separated on agarose gels and immunoblotted using anti-GFP antibodies. Strain names are indicated at top. Horizontal line at top indicates origin. A1-A4 were processed separately from A5-A8. Wild type 779-6A [*PSI*⁺] and [*psi*⁻] controls processed independently for each blot show consistency between the blots. The high molecular weight smear represents polymers (P) of NGMC, faster migrating NGMC monomer (M) is at the lower part of the blot.
doi:10.1371/journal.pone.0006644.g007

size range. Although A3 and A4 had opposite effects on the [*PSI*⁺], both had polymers that were larger than normal (Figure 7). For A3, the data could be explained if replication remained efficient but Sup35-GFP was incorporated into prion polymers at a faster rate, which would confer a stronger phenotype. For strain A4, it could be that growth is normal but replication is inhibited, which would lead to larger but fewer polymers to deplete Sup35-GFP, and thus confer the weak prion phenotype. If so, then replication must not be reduced enough to prevent efficient transmission of prions during mitosis since [*PSI*⁺] is stable in strain A4. Overall these data suggest the different Hsp70s can affect both processes of growth and fragmentation and do so in distinct ways.

Ssa Hsp70s variously affect aggregation of Sup35p in [*PSI*⁺] cells

Although individual Sup35p polymers remain intact in the presence of SDS, any interaction between the polymers giving rise to higher-order aggregates is disrupted. To assess the overall aggregation status of Sup35p, and possibly determine the extent these polymers self associate into larger aggregates, we monitored fluorescence of Sup35-GFP in the same cells used for the SDD-AGE experiments. The fusion protein propagates like a typical [*PSI*⁺] prion and reports on the state of endogenous Sup35p [60]. Fluorescence in our wild type [*psi*⁻] cells was smoothly diffuse in the cytosol (Figure 8A). When [*PSI*⁺] was present in the same cells, fluorescence was somewhat granular in appearance, indicative of Sup35-GFP aggregation.

Aggregation of Sup35-GFP in cells expressing individual Ssa proteins was more intense and varied depending on the isoform being expressed (Figure 8A). While fluorescence in A1 and A2 cells resembled that of wild type cells, A3 and A4 cells had dimmer

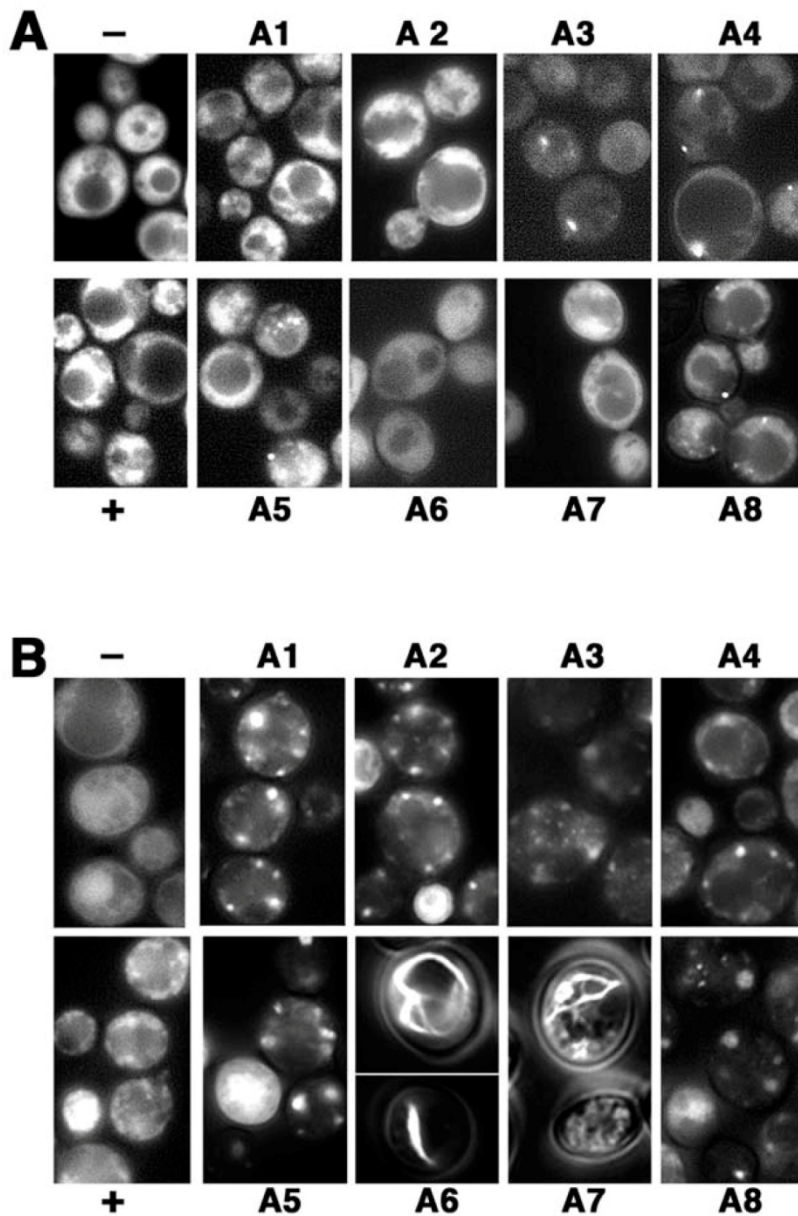


Figure 8. Aggregation of Sup35-GFP in vivo. (A) Fluorescent images of cells from cultures used for the SDD-AGE experiments were taken at the same time cells were processed for SDD-AGE. (B) Images of cells from the same cultures after incubation for an additional six days at 25° in the same medium. Strains A1-A8 are indicated, + and - are wild type 779-6A [*PSI*⁺] and [*psi*⁻] controls. doi:10.1371/journal.pone.0006644.g008

overall fluorescence and most cells contained one or two small bright foci. A5 cells had a combination of these patterns, with high overall brightness and granular appearance but also with bright foci. Fluorescence in A8 cells was similar to that of cells expressing Ssa3p or Ssa4p but more cells had multiple foci. A6 and A7 cells had mostly bright diffuse fluorescence with occasional (~5%) cells having a granular appearance.

We and others [61] reported that cells in aging cultures show changes in prion aggregation state. We therefore observed cells in these same Sup35-GFP cultures after allowing them to remain at 25 degrees for an additional 5–6 days to see if the Ssa proteins affected these changes differently (see figure 8B). There was no significant change in fluorescence of [*psi*⁻] cells but Sup35-GFP in [*PSI*⁺] was more extensively aggregated showing a large number of more defined foci. Aggregation of Sup35-GFP was similarly

increased in A1, A2 and A5 strains and these cells had many fewer but much larger aggregates. These and all the other cells expressing individual Ssa proteins had noticeably dimmer overall fluorescence. Sup35-GFP in A3 and A4 cells was not very different from log phase cells with the exception that more foci were visible. A8 cells also had larger, but dimmer foci. Thus, Sup35p in aged cells expressing any of these individual Ssa proteins aggregated similarly into a smaller number of larger foci than we saw in wild type cells.

Although we had difficulty distinguishing whether colonies of A6 and A7 cells were [*PSI*⁺] because of their very weak and unstable phenotype, aged cultures of these strains contained rare (0.5 - 2%) cells that were extremely bright and easy to identify among hundreds of dimmer cells. The fluorescence in these cells appeared as threads in web-like, branched and ring-shaped

patterns. We presume that cells with these aggregates represent *[PSI⁺]* cells in the originating culture, and that some effect of a particular Ssa6p and Ssa7p activity that leads to prion instability might eventually cause this type of aggregation as cells age. Additionally the brightness of these cells suggests that Sup35-GFP accumulates to a very high level, which likely contributes to the atypical aggregation.

These data show that different Hsp70s can affect aggregation of prion proteins differently. On the whole, however, the fluorescence patterns did not correlate with the prion phenotypes, indicating that the particular aggregation state of prion protein does not reflect how the strength or stability of the prion is being affected.

Individual Hsp70s affect *[URE3]* differently than *[PSI⁺]*

Earlier we showed that Ssa1p-Ssa4p affected *[URE3]* differently than *[PSI⁺]* [38]. In contrast to *[PSI⁺]*, *[URE3]* is unstable in cells expressing only Ssa1p and normal in cells expressing only Ssa2p, and it is weak and very unstable in cells expressing only Ssa3p. In cells expressing only Ssa4p *[URE3]* is weak, like *[PSI⁺]*, but somewhat unstable. We confirm these results (Figure 9A,B) and find that *[URE3]* phenotypes of 1161 strains A5-A8 were different than those in cells expressing only Ssa1p-Ssa4p (see Figure 9). Similar to that of A2 cells, *[URE3]* was both strong and stable in A5, A7 or A8 cells. We were unable to isolate A6 cells capable of propagating *[URE3]*. Since Ssa6p supports growth weakly and *[URE3]* reduces growth of our strains, the combination of effects might make cells inviable.

As we saw with *[PSI⁺]*, *[URE3]* was unstable in the strains expressing individual Ssa proteins that were retrieved from frozen storage. Roughly 1% of A2 cells from frozen stocks were *[ure-o]*, while about 5–10% of cells from A5, A7 and A8 stocks had no prion. Again, we have no explanation for this instability, which we

do not observe for wild type cells with an intact *SSA* genotype (strain 1075). Thus, although prions propagated efficiently in cells expressing individual Ssa proteins, freezing and thawing revealed a requirement for specific Hsp70 function to maintain prion stability under certain conditions.

Efficiency of *[URE3]* curing by Ydj1p depended on individual Ssa proteins

The very high identity of yeast Ssa proteins led us to suggest that previously observed differences in their effects on prions might be caused by differences in the way they are regulated by co-chaperones rather than by differences in their intrinsic Hsp70 activities [38]. Overexpressing Ydj1p or Ssa1p “cures” cells of *[URE3]* but increasing Ssa2p does not [36,62]. Our earlier work showed this curing depends on interaction of Ydj1p with Hsp70 but does not require Ydj1p substrate binding activity [63], suggesting Ydj1p eliminates *[URE3]* indirectly by affecting Hsp70 function.

To test if different Hsp70s affected Ydj1p curing of *[URE3]* differently, we monitored loss of *[URE3]* after inducing expression of Ydj1p from a galactose-inducible promoter in 1161 strains A1–A8 (see Figure 10). Since the strains grew at different rates, *[URE3]* loss was scored when cells divided 6–7 times. Like wild type cells, those expressing only Ssa2p were cured of *[URE3]* very efficiently so less than 10% of cells retained *[URE3]*. Because *[URE3]* is unstable in A1 cells, only 75–80% of A1 cells had *[URE3]* even after growth in non-inducing medium. Induction of Ydj1p also cured *[URE3]* very efficiently in these cells, with less than 5% of these cells retaining *[URE3]*. In contrast, although *[URE3]* was both weak and unstable in the strain expressing only Ssa3p, overproducing Ydj1p cured *[URE3]* much less efficiently in A3 cells. Untreated A3 cultures had a similar proportion of *[URE3]* cells as A1 cultures, but 65% of these still had the prion after

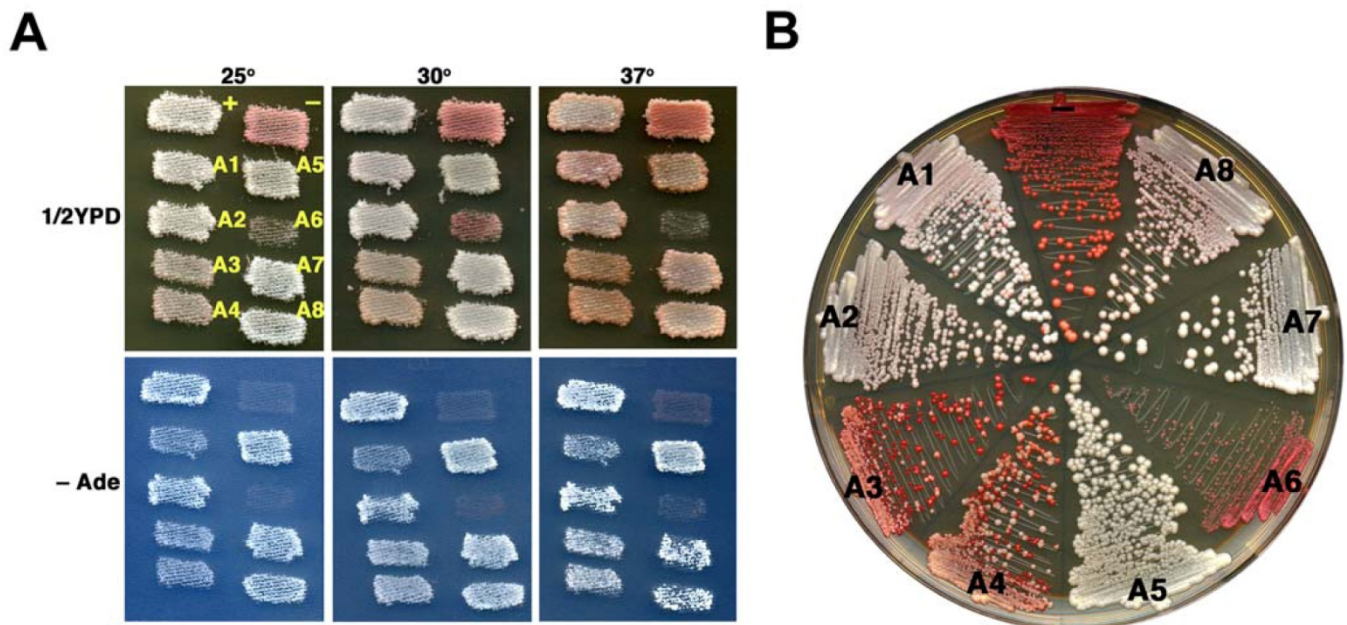


Figure 9. *[URE3]* prion phenotype of cells expressing different Ssa proteins. (A) Patches of 1161 strains A1-A8, as indicated (see Figure 5A), were grown on 1/2YPD and replica-plated onto 1/2YPD and –Ade plates as in Figure 5A. Plates were incubated at the indicated temperature for two days. Cells expressing Ssa6p are unable to propagate *[URE3]* and are uniformly *[ure-o]*. **(B)** Streaks of cells from the same cultures used in **(A)** were incubated two days at 30° followed by three days at 25°. Red colonies in streaks arise from cells that lost *[URE3]* before forming the colony. Red sectors in pink or white colonies are progeny of cells that lost *[URE3]* during growth of the colony. *[URE3]* (+) and *[ure-o]* (–) variants of wild type strain 1075 are included for comparison. doi:10.1371/journal.pone.0006644.g009

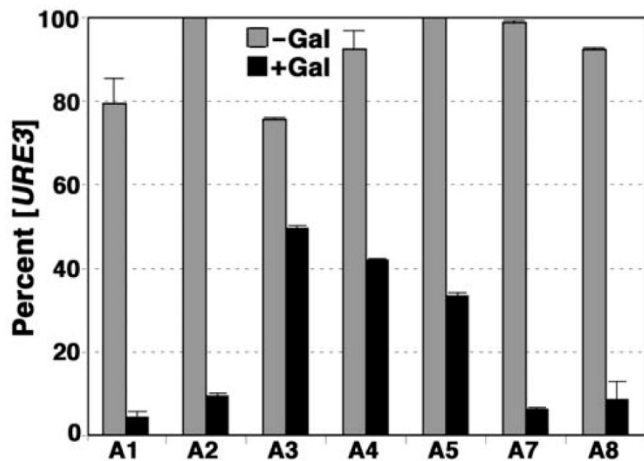


Figure 10. Efficiency of [URE3] curing by elevated Ydj1p depends on Hsp70 isoform. Cultures of 1161 [URE3] strains A1–A8 (except A6, which cannot propagate [URE3]), as indicated, were split into non-inducing (-Gal) and Ydj1p inducing (+Gal) conditions and grown to $OD_{600nm} = 1-2$. Aliquots were diluted to obtain 300–500 cells per plate onto 1/2YPD and the percentage of [URE3] cells remaining, scored as white colonies on the 1/2YPD plates, is shown. Data are averages of at least two experiments, error bars indicate standard deviation. For strains A2 and A5 we saw no loss of the prion among a total of about 1500 colonies. Overexpressing Ydj1p had no noticeable effects on fitness of any of the strains. doi:10.1371/journal.pone.0006644.g010

Ydj1p overexpression. [URE3] also was much less sensitive to elevated Ydj1p in cells expressing only Ssa4p or Ssa5p, but was eliminated efficiently from cells expressing Ssa7p or Ssa8p (figure 10). Thus, efficiency of curing did not depend on the strength or stability of [URE3] before elevating Ydj1p, but depended critically on the particular Hsp70 being expressed. These data confirm that Ydj1p eliminates [URE3] by a mechanism that requires Hsp70 and suggest that Ydj1p interacts differently with Ssa3p, Ssa4p, and Ssa5p than with the others. The data further suggest that the weak prion phenotype in cells expressing only Ssa3p or Ssa4p is not due to a Ydj1p effect.

CFTR degradation occurs at comparable rates in strains expressing individual Ssaps

The viability of the strains expressing individual Ssa proteins indicates that essential Hsp70-dependent functions such as protein folding, clathrin uncoating or protein translocation across the endoplasmic reticulum (ER) membrane are preserved, although with variable efficiencies as suggested by the differences in growth rates (Table 2). We then asked whether individual Ssaps would affect a specific Hsp70-dependent quality control mechanism other than prion propagation, and compared the degradation rates of the human cystic fibrosis transmembrane conductance regulator (CFTR) in our A1–A8 strains. CFTR is a polytopic plasma membrane chloride channel in which mutations cause cystic fibrosis [64]. CFTR folding at the endoplasmic reticulum (ER) membrane is a very inefficient process and most (~70–80%) of the wild-type protein is degraded by the proteasome through ER-associated degradation (ERAD), while a minor (20–30%) fraction of the protein pursues its route to the plasma membrane [65,66]. When expressed in yeast, wild-type CFTR is entirely retained at the ER membrane and degraded through ERAD in a process that requires Hsp70 function [67].

We transformed [psi⁻] and [PSI⁺] derivatives of the A1–A8 strains with pSM1152, a plasmid that allows the expression of HA-

tagged CFTR under the control of the constitutive *PGK1* promoter [68]. The degradation of CFTR through ERAD was then monitored by cycloheximide-chase as described in Material and Methods [67,68] (Figure 11 and Supplemental Figure S2). CFTR was degraded at similar rates in the A2, A3, A5, A6, and A7 [psi⁻] strains and in a wild-type control (Figure 11 and Supplemental Figure S2). The only difference was observed for the A8 and to a lesser extent A1 and A4 strains where CFTR levels decreased faster upon cycloheximide treatment compared to the other strains (Figure 11). These results indicate that all the Ssa proteins are able to maintain CFTR in a soluble form that is competent for proteasomal degradation. However, subtle differences in the way these Ssa proteins interact with CFTR or with other ERAD components may account for the observed differences in CFTR degradation rates.

We then asked whether the degradation of CFTR could be affected by the presence of the [PSI⁺] prion and therefore repeated these experiments in the [PSI⁺] derivatives of the wild-type and A1–A8 strains expressing CFTR (except for A6 and A7 where [PSI⁺] propagation is too unstable). The expression of CFTR did not affect the prion phenotypes of our strains (data not shown). As shown in Figure 11 and Supplemental Figure S2, CFTR degradation in the wild-type strain and in the A2, A3, and A8 strains occurred at the same rate regardless of the psi-state. In the A5 and to a lesser extent A1 and A4 strains, the degradation of CFTR was slower in the [PSI⁺] derivatives. These data suggest that in those strains, the activity of Hsp70 is limiting to cope with both Sup35 aggregates and CFTR misfolding, or that the ubiquitin proteasome system may be challenged by the presence of cellular aggregates [69].

Phenotypic differences among strains expressing individual Ssa proteins are not due to differences in Hsp104, Ydj1p or Sis1p abundance

We monitored the levels of Hsp70, Ydj1p, Sis1p and Hsp104 in [psi⁻] and [PSI⁺] derivatives of the strains expressing CFTR (Supplemental Figure S3), because differences in levels or activity of these chaperones affects different yeast prions and could account for our observed phenotypic differences. The levels of Ydj1p and Sis1p, two major regulators of Hsp70 in yeast, were similar in all the strains regardless of the presence of the prion (Supplemental Figure S3). The levels of Hsp104, while slightly higher in the A1–A8 strains compared to wild-type, were otherwise comparable and not affected by the prion (Supplemental Figure S3). The Ssa proteins were expressed from the same *SSA2* promoter and therefore Hsp70 levels should be similar in all the strains, although we can not rule out differences in protein turnover. The commercial anti-Hsp70 antibody we used (see Material and Methods) did not equally recognize each Ssa protein (Supplemental Figure S3) and it was therefore not possible to properly quantify Hsp70 levels in each strain. However, this antibody allowed us to show that Hsp70 levels were not affected by the presence of the prion (Supplemental Figure S3). Differences in Hsp70 levels among strains are unlikely to reflect the observed differences in growth rates and prion phenotypes because Ssa4p, Ssa5p and Ssa7p were detected at the same levels by our antibody, yet they conferred very different phenotypes. Similar protein levels were obtained in the same strains that do not contain the CFTR-expression plasmid (data not shown).

Discussion

The question of a functional specialization among the cytosolic Hsp70 isoforms of the Ssa-subfamily has never been systematically

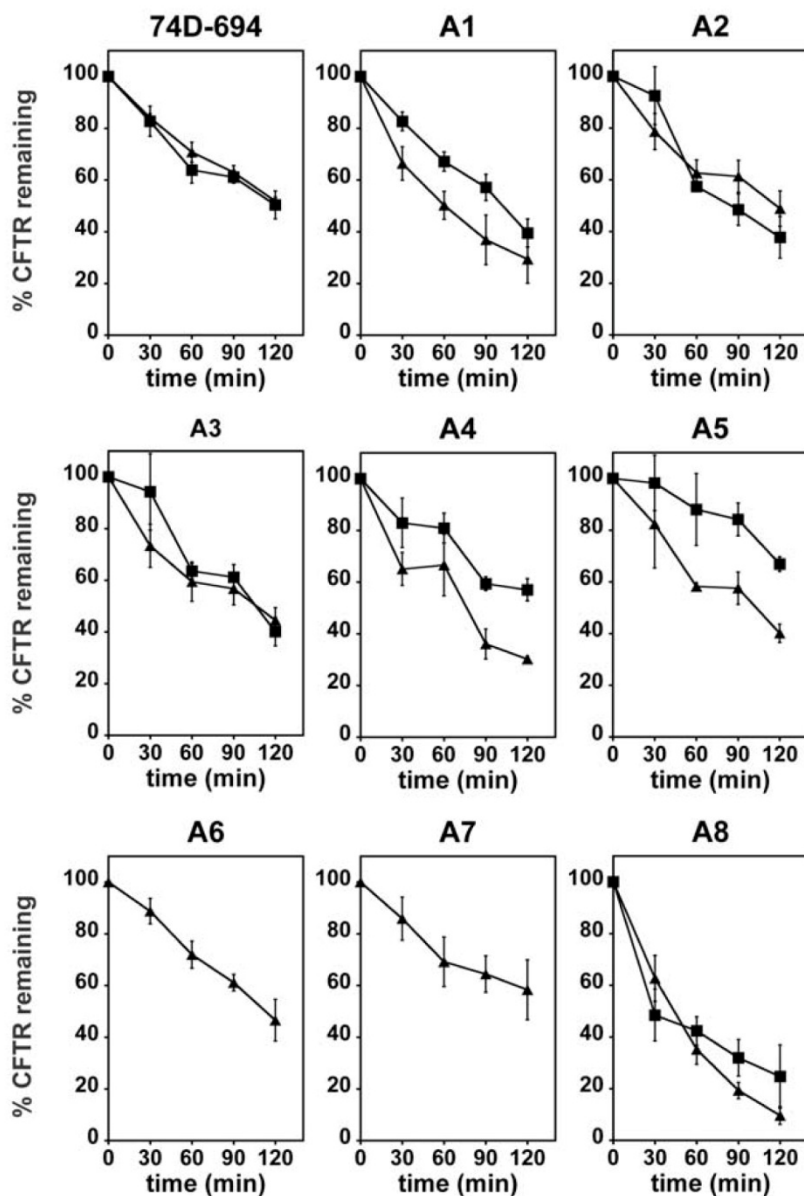


Figure 11. CFTR degradation monitored by cycloheximide-chase. The indicated strains, either *[psi⁻]* (triangles) or *[PSI⁺]* (squares), were grown to early log phase at 28°C, then cycloheximide was added at a final concentration of 200 µg/mL. Aliquots were taken periodically and total protein extracts were prepared and subjected to SDS-PAGE and immunoblot analysis as described in Materials and Methods. Immunoblots were quantified by PhosphorImager analysis and the amount of CFTR at time zero was set to 100% (representative blots for each strain are shown on Supplemental Figure S2). Error bars represent the standard error of 3 to 6 independent experiments. doi:10.1371/journal.pone.0006644.g011

tested, although several reports indicate such a possibility [11]. Here, we show that highly homologous intra- and inter-species Hsp70s have redundant yet clearly distinct functions. Not surprisingly, the four *S. cerevisiae* Ssa Hsp70s supported growth reasonably well, although the somewhat more divergent inducible Ssa3p and Ssa4p did not function as well as Ssa1p and Ssa2p. As anticipated, the interspecies orthologs Ssa5p–Ssa8p, whose divergence from Ssa1/2p is similar to that of Ssa3/4p, did not support growth of *S. cerevisiae* as well as Ssa1/2p. Somewhat unexpectedly, however, although Ssa5p–Ssa8p are significantly more homologous to each other, growth rates of strains A5–A8 ranged more widely. Here again the inducible isoforms (Ssa6 and Ssa7p) were less competent at supporting growth. The capacity of Ssa5p–Ssa8p to support growth of *Y. lipolytica* is unknown but our results suggest

that they also differ with regard to essential cellular functions in their native context, and that Ssa6p has evolved more specialized function, perhaps related to differences in this organism’s environment or metabolic activities.

Propagation of *[PSI⁺]* and *[URE3]* prions was affected differently by the different Hsp70s, which uncovered additional distinctions among the Hsp70s. For example, Ssa7p supported *[URE3]* well but *[PSI⁺]* very weakly. These differences could reflect differences in specific intrinsic Hsp70 activities, or in ways the Hsp70s interact with the prion proteins as substrates or with other components of the chaperone machinery. For example, the reduced curing of *[URE3]* by Ydj1p in strains A3 and A4 compared with A7 and A8 could be explained either by differences in affinity of these different Ssa proteins for Ure2p, or by

differences in efficiency that Ydj1p interacts with the interspecies Ssa7p and Ssa8p than with Ssa3p and Ssa4p. These results also confirm our earlier conclusion that curing of [URE3] by Ydj1p requires a specific interaction with Hsp70 [63].

Changes in prion strength and mitotic stability reflect effects primarily on processes of growth and replication, respectively. The disparity between the wide variation in [PSI⁺] phenotype and the similarity in length of polymers, in particular with respect to strains A3 and A4, imply that the commonly used SDD-AGE assay cannot on its own distinguish effects on growth or fragmentation of prion polymers, and may therefore not be used as a predictive tool for prion phenotype. Similarly, we saw no correlation between [PSI⁺] phenotype and fluorescence pattern of our Sup35-GFP fusion protein, suggesting effects on prion phenotype were not related to specific changes in gross aggregation state of Sup35p. In fact, here again effects of Ssa3p and Ssa4p were indistinguishable despite their opposite effects on [PSI⁺]. Thus, although aggregation of Sup35-GFP into visible fluorescent aggregates is diagnostic of the presence of [PSI⁺], it was not useful for predicting prion phenotype, which raises caveats in the field regarding interpretation of such data with respect to how various factors influence propagation of prions. Together our data suggest that the visible aggregates are byproducts that may or may not be directly involved in prion propagation, a suggestion proposed previously [59,60]. It is also possible that the different Hsp70s influence the ability of different types of Sup35p aggregates to participate in prion propagation.

Aside from the degree to which sequence specificity might determine differences in how chaperones interact with different prion proteins, the structural conformation of prion aggregates formed by them could contribute to determining which chaperone functions are more important for prion propagation of a particular prion. For example, if [URE3] prions are organized in a way that makes them less accessible to certain chaperones, then they might be more sensitive to changes in abundance or activity of these chaperones for certain aspects of their propagation. Differences in phenotype would then reflect differences in efficiency with which the different Hsp70s functionally interact, either positively or negatively, with other components of the disaggregation machinery.

The additional levels of distinction in Hsp70 activity uncovered by the prions did not necessarily correlate with Hsp70 function in essential cellular processes. For example, [PSI⁺] and [URE3] both propagated normally in A5 cells, which grew considerably slower than A3 cells, while in A3 cells [PSI⁺] was stronger than normal and [URE3] was weaker. Both of these Hsp70s functioned well with Hsp104 in refolding thermally denatured luciferase. We and others have found differences in Hsp104 functions with regard to prion propagation and thermotolerance [70–72], suggesting the disaggregation machinery interacts differently with amorphous aggregates than with highly ordered prion aggregates, and such differences might therefore be determined by specific Hsp70 components of the machinery. The prions therefore diagnose specific and non-essential Hsp70 activities that would be difficult to identify by growth phenotypes, and thus provide useful tools that will continue to help characterize the diverse interactions of the chaperone machinery in different cellular contexts.

Regardless of the mechanisms involved, the ability of the Hsp70s to support growth and prion propagation shows that the *Y. lipolytica* Ssa proteins functionally interact with other *S. cerevisiae* chaperones and co-chaperones to protect against protein aggregation, help proteins fold, facilitate protein transport, assist protein degradation and cooperate in protein disaggregation. It is not known if prions exist in *Y. lipolytica*, but our results imply that

Ssa5p-Ssa8p are capable of functioning in a way that is compatible with supporting prion propagation in their native context. We anticipate our system also will be useful to assess various functions of combinations of interspecies chaperones and co-chaperones.

The degradation of CFTR through ERAD in yeast is compromised in strains that are defective for Hsp70 function [67], yet CFTR was degraded in all our strains, although with different efficiencies (Figure 11). This indicates that the intra- and inter-species Ssa proteins all interacted with CFTR and help maintain it in a soluble degradation-competent form [67], regardless of the growth rates of the corresponding strains. Importantly, our cycloheximide-chase experiments only allow us to monitor the degradation of a soluble pool of CFTR present at the ER-membrane. In yeast, CFTR was shown to be concentrated at defined sites in the ER membrane that were proposed to be Russell bodies, an ER sub-compartment in which misfolded proteins are stored and can be targeted for degradation [73–75]. Hence, we can not exclude that the different Hsp70 orthologs while having minor effects on the degradation of the soluble pool of CFTR, may differentially cause the aggregation of a fraction of CFTR and its targeting instead to aggresome-like bodies. While completely speculative, such an effect on CFTR aggregation could explain the slowed CFTR degradation rates observed in some (but not all) [PSI⁺] cells compared to the corresponding [psi⁻] cells. Indeed, in such a situation the ubiquitin proteasome system and the cellular quality control machineries may be overwhelmed or inhibited by both Sup35p and CFTR aggregates [69].

Our work clearly demonstrates that cytosolic Hsp70 orthologs have distinct functional properties despite a high degree of sequence homology. This functional specialization is probably needed to cope with the wide panel of substrates and functions that Hsp70 encounter in the cytosol and suggests an evolutionary pressure to maintain multiple Hsp70s in the eukaryotic cytosol, while most organelles (ER; mitochondria; chloroplast) generally have only one Hsp70 [11]. Deciphering the molecular basis of the functional specificities among Hsp70 orthologs has major implications in humans where at least six Hsp70 of the HSPA subfamily coexist [8,11] that are implicated in conformational and cancer diseases. The distinctions we find, in particular with regard to differences in effects on growth and prion propagation, point to possible strategies for altering chaperone function to combat these diseases.

Materials and Methods

Strains media and plasmids

S. cerevisiae strains were G402 (MAT α , kar1–1, SUQ5, ade2–1, his3 Δ 202, leu2 Δ 1, lys2, trp1 Δ 63, ura3–52 ssa1::KanMX, ssa2::HIS3, ssa3::TRP1, ssa4::ura3–1f/pRDW10 [44]), and related strain 1161 (MAT α , kar1–1, SUQ5, P_{DAL5}::ADE2, his3 Δ 202, leu2 Δ 1, trp1 Δ 63, ura3–52 ssa1::KanMX, ssa2::HIS3, ssa3::TRP1, ssa4::ura3–2f/pJ401 [38]). The ade2–1 strain 1135, which is isogenic to strain 1161, was used previously to characterize the *S. cerevisiae* Ssa proteins for ability to support growth and [PSI⁺] propagation [38]. The current study was initiated before strain 1135 was constructed and we used it only to confirm that any phenotypic differences of the Ssa proteins were not due to strain background (unpublished observations). Our wild type ade2–1 and P_{DAL5}::ADE2 isogenic strains with intact SSA genes are 779–6A and 1075, respectively [38,76]. The *Y. lipolytica* strain 136463 (MatB, scr1::ADE1, his-1, leu2, ura3 [77]) was used as the source of the SSA5–8 genes and to determine their expression patterns.

Plasmids pRDW10 (strain G402) [48] and pJ401 (strain 1161) [38] are URA3-based, single-copy plasmids with SSA1 or SSA2,

respectively, under control of their own promoters. The *LEU2*-based plasmids pC210 (= pA1), pDCM62 (= pA2), pA3 and pA4 are described [36,38]. Plasmids pA5, pA6, pA7 and pA8 are pC210 with the coding region of *SSA5* (locus: YAL10F25289g; UniProt ID: Q6C0E9), *SSA6* (locus: YAL10E35046g; UniProt ID: Q6C3G5), *SSA7* (locus: YAL10D08184g; UniProt ID: Q6C9V0) and *SSA8* (locus: YAL10D22352g; UniProt ID: Q6C864) [11] on *NdeI-SphI* fragments, in place of *SSA1* (oligonucleotides used for cloning are described in Supplemental Table S1). Plasmid p316GalYDJ, used for galactose-inducible overexpression of Ydj1, is a *URA3*-based single copy vector with *YDJ1* controlled by the *Gall1-10* promoter. Plasmid p316CupNGMC is a *URA3*-based single-copy vector with the previously described *SUP35-GFP* fusion gene *NGMC* [60] under control of the *CUPI* promoter. Plasmid pDCM90 is a *URA3*-based single-copy plasmid containing a gene for expression of a thermolabile bacterial luciferase (LuxAB, [45]) on a *ClaI-SmaI* fragment.

Cultures were grown at 30°C unless indicated otherwise. Media were as described [38,77]. Liquid YPAD contains excess adenine. Solid 1/2YPD medium has limiting adenine. Defined media contain 9 mg/l (limiting) or 400 mg/l (excess) adenine.

Thermoresistance

Thermoresistance assays were done essentially as described in [40]. Briefly, 1 mL of yeast cells grown at 28°C to an $OD_{600\text{ nm}} \sim 0.15\text{--}0.3$ were placed in a 52°C circulating water-bath. At the indicated time points, 100 μL aliquots were removed and transferred to a microfuge tube on ice. Serial dilutions for each sample were spread on YPAD plates and incubated at 28°C for 3–5 days. The viability at time zero was set to 100%.

RT-PCR analysis

Total RNA was prepared from 50 mL cultures of a wild-type *Y. lipolytica* strain grown in the conditions indicated in the Results section and using the QIAGEN RNeasy kit. RNA preparations were devoid of genomic DNA contamination as standard PCR analysis failed to detect amplicons using the primers and conditions of the RT-PCR reactions described below (data not shown). Reverse transcription (RT) reactions were made using 1 to 100 ng of RNA, Ready-To-Go RT-PCR beads and were primed with an oligo-dT oligonucleotide according to the manufacturer's instructions (GE Healthcare). The RT reaction was then split in two equal parts. Primers allowing the selective amplification of individual Hsp70 isoforms were added to one half of the reaction, and those allowing the amplification of the actin-encoding gene *YACT1* (accession no. Q9UVF3) were added to the second half of the reaction. PCR amplification was then performed for 35 cycles, using an annealing temperature of 52°C and according to the manufacturer's instructions (GE healthcare). The primer pairs used for the RT-PCR analysis are described in Supplemental Table S1.

Luciferase refolding

Luciferase refolding was assayed as described [45]. Log phase cells containing pDCM90 grown at 30°C in dextrose medium lacking uracil were first diluted to $OD_{600\text{ nm}} = 0.2$ into the same medium. Diluted cultures were incubated with shaking at 37°C for 30 minutes to induce expression of heat shock proteins, and then transferred to a 42°C water bath and incubated with shaking for one hour. Cycloheximide was added 50 minutes after shifting to 42°C to prevent synthesis of luciferase during the recovery period. Cultures were shifted to 25°C for 30 minutes and luciferase activity of 200 μL aliquots was measured in a Zylux luminometer

(Model FB15) immediately after addition of 10 μL decanal (Sigma cat # D7384).

Monitoring prions

We monitored [*PSI*⁺] in strain G402, which has the *ade2-1* nonsense mutation and thus requires exogenous adenine for growth. G402 cells are red when adenine is limiting due to accumulation of a metabolite of the adenine biosynthetic pathway. Depletion of the translation termination factor Sup35p into [*PSI*⁺] prion aggregates reduces efficiency of termination, which, in combination with the *SUQ5* tRNA, suppresses *ade2-1*. Thus, while G402 [*psi*⁻] cells that lack prions require adenine and are red, G402 [*PSI*⁺] cells grow without adenine and are white. We monitored [*URE3*] in strain 1161 using the *P_{DAL5}::ADE2* reporter, in which the wild type *ADE2* gene is regulated by the *DAL5* promoter [78,79]. The nitrogen catabolite regulator Ure2p represses the *DAL5* promoter when a good source of nitrogen is available, so on standard growth media containing ammonium *ADE2* expression is repressed and, like *ade2-1* mutants, cells require exogenous adenine to grow and are red when adenine is limiting. When [*URE3*] is present, depletion of Ure2p into prion aggregates relieves repression of the *DAL5* promoter so *ADE2* is expressed and cells grow without adenine and are white.

Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE)

Cells with p316CupNGMC were grown in dextrose medium lacking uracil and diluted to $OD_{600\text{ nm}} = 0.05$ into the same medium and into identical medium with 25 μM CuSO_4 . Cells grown for 15 hours were harvested for analysis. SDD-AGE was performed as described [60,80]. Briefly, cell lysates were incubated with 2% SDS at 45°C for 10 min and then proteins were electrophoretically separated in 1.8% agarose gels containing 0.1% SDS. Proteins were then electrophoretically transferred to nylon membranes and probed with anti-GFP antibody.

Fluorescence microscopy

Cells from the agarose gel experiments at the time of harvesting were used. Fluorescent images were captured using an Olympus BX61 microscope with IPlab software and were processed using Adobe Photoshop software.

Curing of [*URE3*] by Ydj1p overexpression

Cells containing p316CupNGMC were grown in dextrose medium lacking uracil and diluted to $OD_{600\text{ nm}} = 0.02$ into the same medium and into similar medium containing 1% raffinose and 2% galactose in place of dextrose. Cultures were grown to $OD_{600\text{ nm}} = 1.5\text{--}2$ and then diluted and spread onto 1/2YPD to obtain 200–500 colonies per plate. Red colonies were scored as having lost [*URE3*]. After scoring, the plates were replica-plated onto –Ade and 1/2YPD plates containing 3 mM guanidine to verify that white colonies had the prion. Cultures of cells containing empty vector were treated identically as controls to monitor basal prion stability.

Induction of [*PSI*⁺] by transformation of yeast spheroplasts using cell extracts

Cell extracts were made as described in [81]. Briefly, 50 mL of yeast cultures grown overnight at 30°C were centrifuged and the cell pellet suspended in 500 μL of STC buffer (1,2 M sorbitol, 10 mM CaCl_2 , 10 mM Tris. Cl, pH 7.5) containing a protease inhibitor cocktail (Roche Diagnostics). Cells were broken by repeated glass-beads beating on ice and the cellular debris

removed by two centrifugation steps (5 min at 1100 g and at 4°C). Cell extracts were kept on ice and briefly sonicated (~10 sec with a Branson Sonifier 150 and a microtip at 20% intensity) just before use. Transformation of yeast spheroplasts was performed as described in [58] with minor modifications. The indicated strains were grown to mid-log phase ($OD_{600\text{ nm}} \sim 0.6$) in 50 mL of YPAD and spheroplasts were obtained as described [58] in 1 mL of STC buffer (1.2 M Sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$) and kept at 4°C until use (on the same day). Transformation mixtures contained 100 μ L of spheroplasts, 100 μ g/mL salmon sperm DNA, 20 μ g/mL of the *URA3*-based pRS316 plasmid, and 300 μ g/mL of the indicated yeast crude extract. Transformation mixtures without pRS316, without cell extract or containing extracts derived from a [*psi*⁻] strain were used as controls. After 30 min at room temperature, 9 volumes of PEG8000 buffer (10 mM Tris-HCl, pH 7.5, 20% PEG 8000, 10 mM $CaCl_2$) were added and the mixture incubated for 30 min at room temperature. Spheroplasts were then collected by centrifugation (5 min at 400 g), suspended in 150 μ L of SOS buffer (1M sorbitol, 7 mM $CaCl_2$, 0.25% yeast extract, 0.5% bactopectone, 0.5% glucose) and incubated for 30 min at 30°C. The cells were then gently mixed with 7 mL of top agar (synthetic complete medium containing 2.5% agar, 1M sorbitol and required aminoacids except adenine and uracil) kept at 45°C. The top-agar was poured onto defined medium plates lacking uracil and containing 6 mg/mL adenine and 1M sorbitol. The plates were then incubated at 30°C for up to ten days. [*PSI*⁺] candidate colonies were isolated from the transformation plates and further checked for prion propagation on defined medium lacking uracil and containing trace amounts of adenine or 1/2 YPD plates, and for prion curability on YPD plates containing 3 mM guanidium chloride.

CFTR degradation assay and Western blot analysis

The degradation of HA-tagged CFTR was performed as described [67,68] with minor modifications. Briefly, yeast cells carrying the pSM1152 plasmid were grown to exponential phase ($OD_{600\text{ nm}} \sim 0.4-0.6$) at 28°C then cycloheximide was added at a final concentration of 200 μ g/mL. At each indicated time point, an aliquot of 3 $OD_{600\text{ nm}}$ of cells was taken and total protein extracts were prepared with the sodium hydroxide/trichloroacetic acid (NaOH/TCA) method as described [68]. Proteins were resuspended in 60 μ L of TCA sample buffer (80 mM Tris-Cl, pH 8.0, 8 mM EDTA, 120 mM dithiothreitol, 3.5% SDS, 0.29% glycerol, 0.08% Tris base, 0.01% bromphenol blue), incubated at 37°C for 30 min and resolved by SDS-PAGE followed by blotting on a nitrocellulose membrane. Each membrane was then cut in half (approximately at the 90 kDa limit); the upper half was immunoblotted with anti-HA-peroxidase mouse monoclonal antibodies (Roche Applied Science) to detect CFTR, and the lower half with anti-BiP rabbit polyclonal antibodies as a loading

control (a generous gift from Jeffrey L. Brodsky, University of Pittsburgh). Immunoblots were analyzed by PhosphorImager and quantified with the ImageQuant software (Molecular Dynamics).

To assess the levels of Hsp70, Ydj1p, Sis1p, Hsp104 and RPP0, extracts were similarly prepared, without adding cycloheximide to log phase cultures, and analyzed by western blot. The polyclonal rabbit anti-Ydj1p, anti-Sis1p, anti-Hsp104p and anti-RPP0 antibodies were a generous gift from Ronald Melki (CNRS, Gif-sur-Yvette). The monoclonal mouse anti-Hsp70/Hsc70 antibody was purchased from Stressgen Biotechnologies, Victoria, Canada (catalog number SPA-822).

Supporting Information

Figure S1 Multiple Alignments of the *Saccharomyces cerevisiae* and *Yarrowia lipolytica* Ssa proteins. The alignments were generated using ClustalX 2.0.9 and GeneDoc V2.6.

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Figure S2 CFTR degradation monitored by cycloheximide-chase. Representative gels for the quantifications shown in Figure 11 (see Material and Methods for details). Immunoblots against the endoplasmic reticulum protein BiP were used as loading controls.

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Figure S3 Steady-state protein levels in strains expressing individual Ssaps. Total protein extracts were prepared from the indicated strains as described in figure 11 legend (without the addition of cycloheximide). Equal amounts of proteins were analyzed by SDS-PAGE and immunoblotting with antibodies against the indicated proteins.

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Table S1 Oligonucleotides used in this study.

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Author Contributions

Conceived and designed the experiments: DS CNM DCM MK. Performed the experiments: DS CNM MTLD MR DCM MK. Analyzed the data: DS CNM DCM MK. Contributed reagents/materials/analysis tools: DS CNM DCM MK. Wrote the paper: DS DCM MK.

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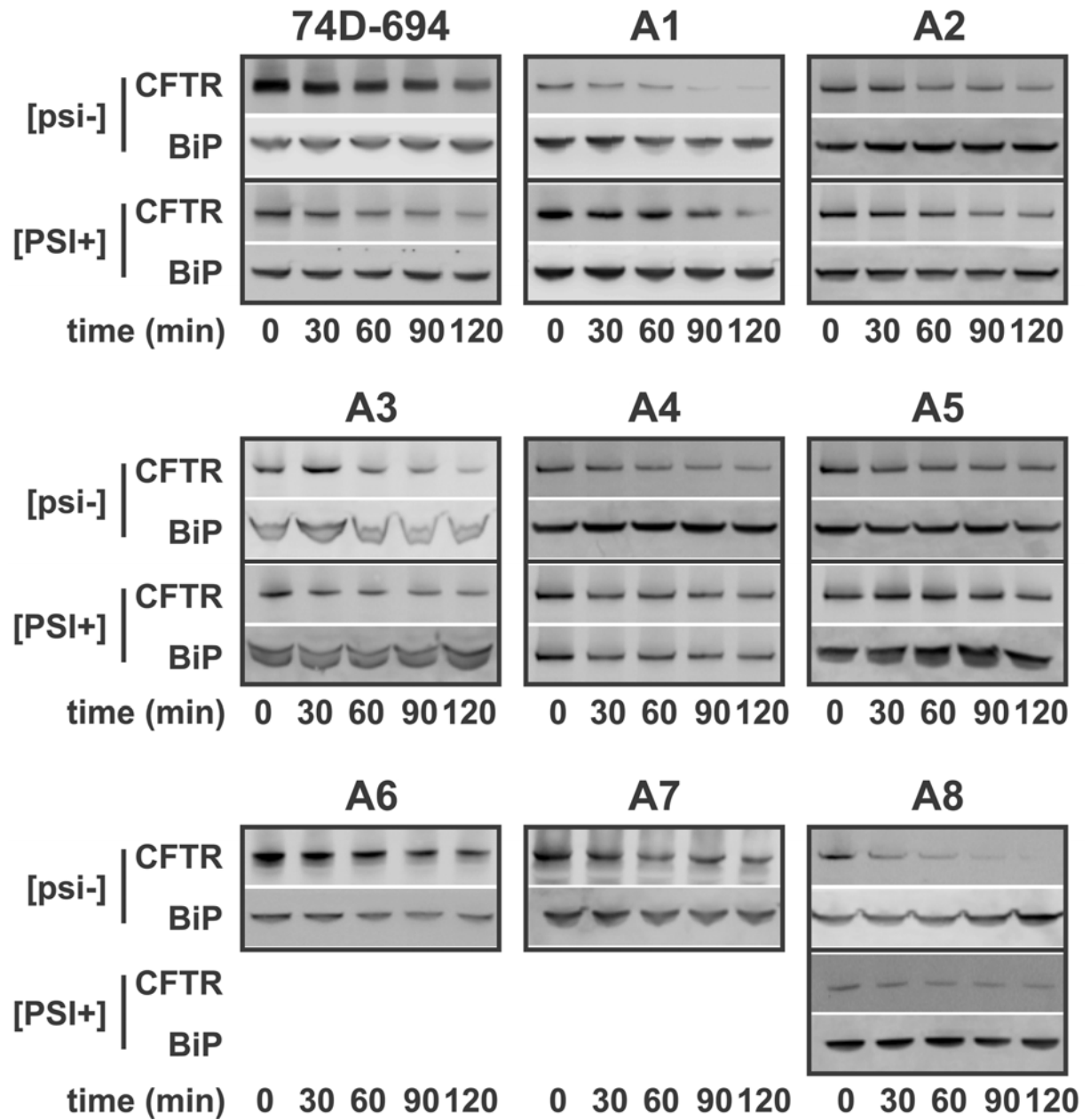


Figure S2: CFTR degradation monitored by cycloheximide-chase. Representative gels for the quantifications shown in Figure 11 (see Material and Methods for details). Immunoblots against the endoplasmic reticulum protein BiP were used as loading controls.

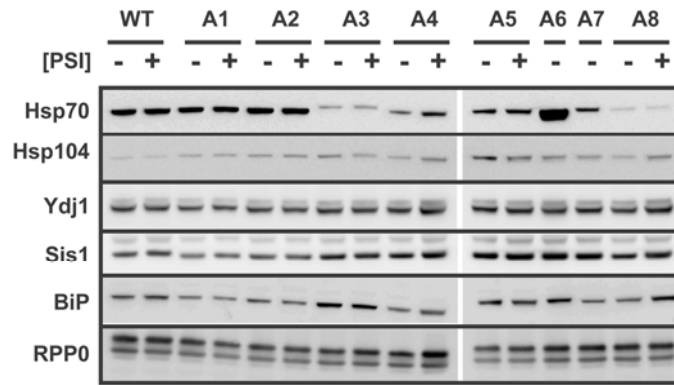


Figure S3: Steady-state protein levels in strains expressing individual Ssaps. Total protein extracts were prepared from the indicated strains as described in figure 11 legend (without the addition of cycloheximide). Equal amounts of proteins were analyzed by SDS-PAGE and immunoblotting with antibodies against the indicated proteins.

name	oligonucleotide sequence	purpose
SSA5-A ^a	GGGAATTCC <u>CATATGC</u> CTAACACATCTGTAGGAATTGACCTG	cloning in pC210
SSA5-B ^a	GAG GCATGC TTAGTCGACCTCCTCGACAGTGGGG	cloning in pC210
SSA6-A ^a	GGGAATTCC <u>CATATGT</u> CTAAAGCAGTAGGAATCGATCTTGG	cloning in pC210
SSA6-B ^a	GAG GCATGC TTAGTCAACCTCCTCAACGGTGGG	cloning in pC210
SSA7-A ^a	GGGAATTCC <u>CATATGT</u> CCAAAGCAGTAGGAATTGATCTTGG	cloning in pC210
SSA7-B ^a	GAG GCATGC TTAGTCAACCTCCTCAACGGTGGG	cloning in pC210
SSA8-A ^a	GGGAATTCC <u>CATATGT</u> CTAAAGCCGTCGGAATCGATCTTGG	cloning in pC210
SSA8-B ^a	GAG GCATGC TTAGTCAACCTCCTCAACGGTGGG	cloning in pC210
ACT1-5	TCGTTATCGATAACGGATCC	RT-PCR
ACT1-R	TAGTAACACTTTTCGGTGGAC	RT-PCR
SSA5-5	TCGATCCTTACAACACATTTCAACAACAAAATGCC	RT-PCR
SSA5-R	ACTAAAATACTTAGTCGACC	RT-PCR
SSA6-5	CACAACACGAAAGAAACACATTACAACAACATGTC	RT-PCR
SSA6-R	ATACAAGGAATCCCTTAGTC	RT-PCR
SSA7-5	AAGATTCTCTCCACAAAACTAACTGAAACACAATGTCC	RT-PCR
SSA7-R	CATGCACAAGAAGATTAGTC	RT-PCR
SSA8-5	GCTGGTCCTGGACTTTCTTCTTCTCCATACAACCACC	RT-PCR
SSA8-R	TACCTAGAAGTGATTTAGTC	RT-PCR
YI.SSB1-5	TAACAAAATGAGTGAAGGAACCTTTTGCTGGAGC	RT-PCR
YI.SSB1-R	ACTTAACTTATCGGGTAGCC	RT-PCR

^a these primers contain an *Nde*I (underlined) or a *Sph*I (bold) site used for cloning

Table S1: Oligonucleotides used in this study.

II. Le système Hsp70 est requis pour la formation de biofilms par *Saccharomyces cerevisiae* à travers des voies distinctes

II.A. Introduction aux Articles 2 et 3

La levure *Saccharomyces cerevisiae* est capable de former des biofilms, appelés *mats*, sur gélose molle (Reynolds and Fink, 2001). Leur structure à symétrie radiale, élaborée et hautement reproductible résulte d'un programme développemental complexe et finement régulé, ainsi que de forces physiques qui s'exercent entre les cellules d'une part et entre les cellules et la gélose d'autre part (Reynolds and Fink, 2001). La formation des *mats* est contrôlée par les conditions environnementales et implique un programme transcriptionnel spécifique (Reynolds, 2006). La formation de ces biofilms est strictement dépendante de l'expression d'une glycoprotéine de surface, l'adhésine Flo11 (Reynolds and Fink, 2001). La régulation de l'expression du gène *FLO11* est complexe et intègre plusieurs voies de signalisation, dont les voies MAPK (*Mitogen-Activated Protein Kinase* ; protéine kinase activée par les mitogènes) et AMP cyclique-PKA (*Protein Kinase A* ; protéine kinase A) (Rupp et al., 1999 ; Sengupta et al., 2007 ; Vinod et al., 2008). Seules les souches dérivées de $\Sigma 1278b$ expriment *FLO11*, contrairement aux souches de laboratoire dérivées de S288C où la transcription de ce gène est bloquée par une mutation dans le facteur de transcription Flo8 (Liu et al., 1996).

Flo11 est une protéine de 1367 acides aminés qui partage une structure commune aux autres adhésines de la famille FLO : un domaine lectine N-terminal ; un grand domaine central constitué de motifs répétés d'environ 10-50 acides aminés riches en sérines/thréonines et qui est fortement glycosylé (N-glycosylation au niveau du réticulum endoplasmique; O-mannosylation dans le Golgi) ; et un domaine C-terminal sur lequel est ajoutée une ancre GlycosylPhosphatidylInositol (GPI) permettant l'adressage à la paroi cellulaire (Verstrepen and Fink, 2009; Verstrepen and Klis, 2006).

Flo11 est strictement requise pour la formation de biofilms (Reynolds and Fink, 2001), mais aussi pour la croissance invasive pour les haploïdes et la formation de pseudo-hyphes en conditions de carence en azote pour les diploïdes (Lo and Dranginis, 1998).

Jusqu'à maintenant, toutes les mutations connues pour affecter la formation des *mats* résultaient dans une répression de *FLO11*, et affectaient également l'adhésion aux surfaces et la croissance invasive (Lo and Dranginis, 1998 ; Reynolds, 2006 ; Reynolds and Fink, 2001).

Le fonds génétique des souches peut jouer un rôle « tampon » en masquant ou en exacerbant les phénotypes induits par une ou plusieurs mutations. Ainsi, la délétion du gène *FESI* dans la

souche W303 s'accompagne d'une croissance thermosensible qui n'est pas observée lorsque cette mutation est introduite dans la souche $\Sigma 1278b$ (Kabani et al., 2002a ; Martineau et al., 2007). Mehdi Kabani a montré que la souche $\Sigma 1278b \Delta fes1$ est incapable de former des biofilms (Martineau et al., 2007). Ce résultat surprenant a servi de point de départ à une partie de mon travail de thèse visant à explorer le rôle du système Hsp70 dans la formation des *mats* chez *S. cerevisiae*.

II.B. Principaux résultats de l'article 2

Afin d'étudier le rôle de la machinerie Hsp70 cytoplasmique dans la formation de biofilms, nous avons construit une collection de mutants dans lesquels un ou deux gènes codant *Ssa1-4*, *Fes1*, *Ydj1* et *Sse1* ont été invalidés. Ces mutants ont été testés pour leur capacité à former des biofilms et à adopter une croissance invasive.

Dans un premier temps, nous avons montré que les souches $\Delta fes1$, $\Delta ydj1$ et $\Delta sse1$ sont totalement incapables de former des biofilms. Des expériences de complémentation fonctionnelle ont montré que la surexpression de *FES1* restaure la formation de biofilms dans une souche $\Delta sse1$, alors que la surexpression de *SSE1* dans le mutant $\Delta fes1$ ne le permet pas. Ce résultat confirme que les facteurs d'échange *Fes1* et *Sse1* ont des fonctions à la fois redondantes et spécifiques. L'invalidation de *SSA1*, et de *SSA2* dans une moindre mesure, affecte la formation de *mats* ; ces derniers sont plus petits et moins structurés. L'invalidation de *SSA3* ou de *SSA4* n'a pas d'effet notable sur la formation de biofilms, mais aggrave le phénotype des mutants $\Delta ssa1$ et $\Delta ssa2$. Il est important de noter que la souche $\Delta ssa1$ présente un défaut de formation de *mats* et ce malgré l'expression forte et constitutive de *SSA2*. Ce résultat suggère un rôle spécifique joué par *Ssa1* dans la formation de biofilms.

La capacité de nos mutants à adopter une croissance invasive a ensuite été testée et a montré que les souches $\Delta ydj1$ et $\Delta sse1$ sont hyper-invasives, alors que les souches $\Delta fes1$ ou Δssa ont un phénotype sauvage. L'hyper-invasivité du mutant $\Delta ydj1$ était connue et liée à une dérégulation de l'expression de *FLO11* (Palecek et al., 2000). Afin de pouvoir détecter la protéine Flo11 dans des extraits cellulaires, nous avons invalidé nos gènes d'intérêt dans une souche $\Sigma 1278b$ qui exprime une forme étiquetée de Flo11 (Flo11-HA). La séquence codant l'étiquette HémAgglutinine (HA) a été introduite au locus chromosomique *FLO11*, ce qui permet de conserver intacte la régulation de l'expression de ce gène. L'étiquette HA est située juste après la séquence signal d'adressage au réticulum endoplasmique en N-terminal de la

protéine (Park et al., 2006). Nous avons ainsi pu comparer les quantités de protéine Flo11-HA dans chaque souche par western blot et avons montré qu'elles étaient augmentées dans les souches $\Delta sse1$ et $\Delta ydj1$ en comparaison avec la souche sauvage. La quantité de Flo11-HA dans les souches $\Delta fes1$, $\Delta ssa1$ ou $\Delta ssa2$ était cependant similaire à celle observée chez la souche sauvage. De plus, des expériences d'immunofluorescence ont montré que Flo11-HA est retrouvée à la surface des cellules dans toutes les souches testées.

Ainsi, la machinerie Hsp70 intervient dans deux voies requises pour la formation de biofilms. Une première voie impliquant Ydj1 et Sse1 participe à la régulation de l'expression de Flo11, probablement *via* la voie AMP cyclique-PKA (Trott et al., 2005). L'augmentation de la quantité de Flo11 exprimée à la surface de la cellule suite à une dérégulation de cette voie engendre une croissance hyper-invasive et empêche le glissement des cellules le long de la surface d'agar, bloquant ainsi la formation de *mats*. La seconde voie qui implique principalement Ssa1 et Fes1 contrôle la formation de biofilms indépendamment de Flo11. Il reste à identifier lequel(s) parmi les nombreux processus cellulaires contrôlés par la machinerie Hsp70 est(sont) impliqué(s) dans la formation de biofilms chez la levure.

Ce travail a fait l'objet d'une publication dans *Genetics* (voir II.B. Article 2) (Martineau et al., 2007).

II.C. Principaux résultats de l'Article 3

Notre étude précédente a montré que les niveaux de Flo11 sont dérégulés dans un mutant $\Delta ydj1$ ce qui entraîne un phénotype hyper-invasif incompatible avec la formation de biofilms (Martineau et al., 2007). Dans ce second article consacré au rôle du système Hsp70 dans la formation de biofilms, nous avons voulu identifier les protéines à domaine J cytoplasmiques qui participent à ce processus, et notamment à la voie indépendante de Flo11.

La levure *S. cerevisiae* possède treize protéines à domaine J cytoplasmiques dont deux seulement sont essentielles, Cwc23p et Sis1p (Sahi and Craig, 2007). Nous avons donc invalidé les gènes codant les onze autres protéines dans la souche $\Sigma 1278b$ et directement testé leur capacité à former des *mats* sur gélose molle. Seuls deux mutants se sont avérés incapables de développer un biofilm : $\Delta ydj1$ et $\Delta swa2$. Le gène *SWA2* code l'orthologue de l'auxiline des mammifères, protéine impliquée dans la dissociation des manteaux de clathrine en coopération avec Hsc70 (Gall et al., 2000 ; Pishvae et al., 2000). Cette protéine est composée de trois domaines de liaison à la clathrine (domaines CB1 à CB3), d'un domaine

d'association à l'ubiquitine (UBA : *UBiquitin Association*), d'un domaine TPR et d'un domaine J à l'extrémité C-terminale. Seuls ces deux derniers domaines sont strictement requis pour la fonction de Swa2 *in vivo* (Xiao et al., 2006).

Le mutant $\Delta swa2$ présente les caractéristiques d'un mutant de la voie de formation de biofilms indépendante de Flo11, c'est-à-dire incapacité à former des *mats* mais croissance invasive parfaitement normale. Nous nous attendions donc à ce que Flo11 soit exprimée chez ce mutant à des niveaux comparables à ceux de la souche sauvage.

Afin de vérifier cette hypothèse, nous avons invalidé SWA2 dans la souche exprimant Flo11-HA afin d'évaluer l'impact de cette mutation sur l'expression et la localisation de Flo11. Lors de cette seconde étude, nous avons amélioré nos conditions d'extraction et d'électrophorèse, ce qui nous a permis d'obtenir des western blots de bien meilleure qualité qu'auparavant. Dans ces nouvelles conditions, Flo11-HA est détectée dans une souche sauvage sous deux formes de hauts poids moléculaires, une bande A d'environ 200 kDa et une bande B formant un *smear* (une traînée) au dessus de ~ 220 kDa. Le profil de Flo11-HA dans la souche $\Delta fes1$ est similaire à celui de la souche sauvage alors que les quantités de protéines dans les bandes A et B sont augmentées significativement dans les mutants $\Delta ydj1$ et $\Delta sse1$, conformément à nos observations précédentes. De manière surprenante, la quantité de protéine Flo11-HA dans $\Delta swa2$ est également plus élevée que dans la souche sauvage, ce qui contraste avec son phénotype invasif normal. Cependant, seule la quantité de protéines dans la bande B est augmentée dans $\Delta swa2$ alors que la bande A reste inchangée. Ainsi, Swa2 semble remplir une fonction importante pour la maturation de Flo11. Etant donné le rôle de l'auxiline dans le trafic intracellulaire des protéines, nous avons effectué des expériences de fractionnement cellulaire pour tenter d'identifier des différences de localisation subcellulaire entre la bande A et la bande B dans les différentes souches. Cependant, ces expériences n'ont pas permis d'attribuer des localisations subcellulaires différentes pour les bandes A et B, retrouvées majoritairement associées à la paroi cellulaire, ni d'observer de différences significatives entre les souches. Flo11 est facilement extraite de la paroi par un traitement SDS/ β -mercaptoéthanol à 90°C, indiquant que cette protéine n'est pas associée à la paroi de manière covalente, ce qui n'avait pas été démontré jusqu'à présent.

Nous avons montré que les domaines de Swa2 requis pour sa fonction *in vivo*, c'est-à-dire les domaines TPR et J, sont également requis pour assurer une maturation normale de Flo11. Une dynamique normale d'association-dissociation de la clathrine est donc nécessaire pour la maturation et l'activité de Flo11 dans la formation de biofilms. Afin de renforcer cette

hypothèse, nous avons construit une souche dans laquelle le gène codant la chaîne légère de la clathrine ($\Delta clc1$) est invalidé. La formation de *mats* dans cette souche est légèrement affectée, bien que les niveaux de Flo11 soient normaux. La chaîne lourde de la clathrine, Chc1, s'accumule sous sa forme assemblée dans un mutant $\Delta swa2$, et une quantité plus importante de Chc1 est ainsi retrouvée dans le culot après ultracentrifugation d'extraits cellulaires à 150000 g (Gall et al., 2000 ; Pishvae et al., 2000). La distribution de Chc1 dans le mutant $\Delta clc1$ est normale, et il est donc probable que Swa2 et Hsp70 compensent l'absence de la chaîne légère en contrôlant efficacement la dissociation des manteaux de clathrine formés par la chaîne lourde.

Par ailleurs, le gène *SWA2* a été identifié dans un crible de létalité synthétique avec un mutant du gène *ARF1*, codant une petite protéine G de la famille Ras impliquée dans le transport vésiculaire entre le réticulum endoplasmique et le Golgi. Un mutant $\Delta arf1$ est parfaitement capable de former des biofilms et présente une croissance invasive normale, mais accumule Flo11 de manière importante. Cependant, le profil obtenu en western blot diffère de celui obtenu pour $\Delta swa2$: la bande A est similaire en taille et en intensité, alors que le *smear* formé par la bande B migre à une taille inférieure et avec une intensité très supérieure à celle observée chez la souche sauvage. Des expériences de fractionnement cellulaires n'ont pas permis d'associer cette accumulation de Flo11 à une fraction sub-cellulaire précise. Ainsi, il semble qu'Arf1 joue un rôle dans le transport ou la maturation de Flo11, mais que cette fonction n'est pas cruciale au bon fonctionnement de cette adhésine.

Il a récemment été montré *via* l'utilisation d'une fusion Flo11-GFP que la localisation pariétale de Flo11 est dynamique au cours du développement d'une colonie (Vopalenska et al., 2009). Ainsi, Flo11 serait initialement focalisée au niveau des jonctions cellule mère-bourgeon, puis se retrouverait progressivement tout autour de la cellule. Lorsque le développement de la colonie est achevé, le signal de fluorescence de Flo11-GFP au niveau de la paroi est fortement réduit dans un grand nombre de cellules, notamment les plus âgées. Ce signal de fluorescence se retrouve au niveau des vacuoles, suggérant que Flo11 a été internalisée et transportée à la vacuole pour y être ultérieurement dégradée (Vopalenska et al., 2009). D'après ces données de la littérature et nos propres observations, nous suggérons que Swa2 intervient dans ce processus d'internalisation de Flo11 par endocytose clathrine-dépendante. De plus, nos travaux suggèrent que ce recyclage est crucial pour le développement des biofilms, mais pas pour la croissance invasive. Le mécanisme et la régulation de cette endocytose restent à élucider. Il serait intéressant de déterminer si des

mécanismes similaires existent pour d'autres adhésines, notamment chez les pathogènes fongiques comme *Candida albicans*, ce qui permettrait l'identification de nouvelles cibles thérapeutiques pour bloquer la capacité de ces organismes à former des biofilms.

Ce travail est décrit dans l'article 3 ci-après, soumis pour publication dans *Eukaryotic Cell*.

II.D. Article 2

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**FLO11P-INDEPENDENT CONTROL OF “MAT” FORMATION BY HSP70
MOLECULAR CHAPERONES AND NUCLEOTIDE EXCHANGE FACTORS IN YEAST**

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Flo11p-Independent Control of “Mat” Formation by Hsp70 Molecular Chaperones and Nucleotide Exchange Factors in Yeast

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ABSTRACT

The yeast *Saccharomyces cerevisiae* has been used as a model for fungal biofilm formation due to its ability to adhere to plastic surfaces and to form mats on low-density agar petri plates. Mats are complex multicellular structures composed of a network of cables that form a central hub from which emanate multiple radial spokes. This reproducible and elaborate pattern is indicative of a highly regulated developmental program that depends on specific transcriptional programming, environmental cues, and possibly cell–cell communication systems. While biofilm formation and sliding motility were shown to be strictly dependent on the cell-surface adhesin Flo11p, little is known about the cellular machinery that controls mat formation. Here we show that Hsp70 molecular chaperones play key roles in this process with the assistance of the nucleotide exchange factors Fes1p and Sse1p and the Hsp40 family member Ydj1p. The disruption of these cofactors completely abolished mat formation. Furthermore, complex interactions among SSA genes were observed: mat formation depended mostly on SSA1 while minor defects were observed upon loss of SSA2; additional mutations in SSA3 or SSA4 further enhanced these phenotypes. Importantly, these mutations did not compromise invasive growth or Flo11p expression, suggesting that Flo11p-independent pathways are necessary to form mats.

SEVENTY-kilodalton heat-shock proteins (Hsp70's) are a ubiquitous family of molecular chaperones that play essential housekeeping functions in protein folding, synthesis, transport across biological membranes, and degradation. They are also involved in quality control processes, such as protein refolding after a stress injury, and control the activity of regulatory proteins in signal transduction pathways (MAYER and BUKAU 2005). This functional pleiotropy is achieved through the evolutionary amplification and diversification of HSP70 genes, cofactors that recruit and regulate Hsp70's for specific cellular functions, and cooperation of Hsp70's with other chaperone systems such as TRiC/CCT or Hsp90 (MAYER and BUKAU 2005). All of these cellular activities depend on the ability of Hsp70's to interact with hydrophobic peptide stretches of proteins in an ATP-dependent manner. Hsp70's are composed of a highly conserved N-terminal 44-kDa ATPase domain, an 18-kDa peptide-binding domain, and a C-terminal 10-kDa variable “lid” domain. In the ATP-bound state, Hsp70's display fast on-and-off rates of peptide binding, whereas in the ADP-bound state these constants are slowed (SCHMID *et al.* 1994; McCARTY *et al.* 1995). The modulation of the affinity for polypeptide substrates is

triggered by a conformational change in the lid that is induced by ATP hydrolysis (ZHU *et al.* 1996; JIANG *et al.* 2005). The weak intrinsic ATPase activity of Hsp70's is stimulated by the Hsp40/DnaJ family of co-chaperones such as Ydj1p in yeast, whereas ADP–ATP exchange is catalyzed by several evolutionarily unrelated classes of nucleotide exchange factors (KABANI *et al.* 2003; MAYER and BUKAU 2005). These include GrpE homologs in prokaryotes and the Bag1 (Bcl2-associated athanogen 1) and HspBP1/Fes1 and Hsp110 families in eukaryotes. The reasons and biological implications for such a diversity of eukaryotic nucleotide exchange factors are still not understood (KABANI *et al.* 2003; MAYER and BUKAU 2005).

The cytosol of the yeast *Saccharomyces cerevisiae* contains four classes of Hsp70's, representing a total of 10 proteins (FRYDMAN 2001). The Ssa proteins are encoded by two constitutively expressed genes (SSA1, SSA2) and two stress-inducible genes (SSA3, SSA4) (WERNER-WASHBURN *et al.* 1987). These essential canonical Hsp70's are involved in protein folding, protein translocation across the endoplasmic reticulum and mitochondrial membranes, and in quality control processes such as endoplasmic-reticulum-associated degradation (YOUNG *et al.* 2003; NISHIKAWA *et al.* 2005). As only one Ssa protein is sufficient to support yeast viability if expressed at sufficiently high levels, it is generally assumed that Ssa proteins are functionally redundant (WERNER-WASHBURN

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et al. 1987). However, it is possible that individual Hsp70 isoforms evolved to perform specialized activities or to handle different types of substrates. In support of this hypothesis, the overexpression of Ssa1p but not of Ssa2p was shown to cure yeast cells from the [URE3] prion (SCHWIMMER and MASISON 2002). In human cells, where up to six Hsp70's can coexist simultaneously in the cytosol, the highly homologous inducible Hsp70 and constitutive Hsc70 were shown to play nonoverlapping essential functions in cancer cell growth and survival (ROHDE *et al.* 2005). The Ssb1/2 and Ssz1 proteins are ribosome-associated Hsp70's that cooperate in the folding of emerging nascent polypeptide chains (YOUNG *et al.* 2004). The Hsp110 family members Sse1/2 are divergent Hsp70's that cooperate in folding processes with Hsp70 and Hsp90 (OH *et al.* 1997, 1999; GOECKELER *et al.* 2002; SHANER *et al.* 2005; YAM *et al.* 2005) and function as nucleotide exchange factors for Ssa and Ssb proteins (DRAGOVIC *et al.* 2006; RAVIOL *et al.* 2006). The respective contribution of Sse1/2p and Fes1p to the cellular physiology is not clearly established. The overexpression of Fes1p only partially alleviated the lethality of a $\Delta sse1\Delta sse2$ mutant, suggesting that these proteins play both distinct and overlapping functions (RAVIOL *et al.* 2006; SHANER *et al.* 2006). The yeast *S. cerevisiae* also contains another Hsp70 nucleotide exchange factor, the Bag1-domain-containing protein Snl1p. Snl1p is tethered to the ER and nuclear envelope membranes through a transmembrane domain and its physiological role seems restricted to the nuclear pore complex assembly (SONDERMANN *et al.* 2002).

In this article, we provide evidence for the implication of Hsp70's and co-chaperones in mat formation, a poorly understood biological phenomenon that is observed when $\Sigma 1278b$ yeast cells are cultivated on the surface of low-agar (0.3%) petri plates where they form a complex multicellular structure. The mature mat is composed of a central hub made of a network of cables from which emanate multiple radial spokes and a leading edge (or rim) that is smooth in appearance (Figure 1) (REYNOLDS and FINK 2001). The radial symmetry of the mats and the reproducibility of the number of spokes and diameter of these structures are the hallmarks of a regulated developmental program that seems to be controlled by environmental cues (REYNOLDS and FINK 2001).

A specific transcriptional program is induced upon yeast growth on low-agar plates and controls mat formation (REYNOLDS 2006). The transcriptional profiles of mat cells resemble those of post-log-phase cells with the notable difference that protein synthesis genes continue to be highly expressed, while these are normally downregulated upon entry into the diauxic shift phase (REYNOLDS 2006).

The formation of mats occurs by sliding motility and depends on the viscosity of the medium and the availability of rich sources of nutrients and was shown to be strictly dependent on the expression of Flo11p, a cell

surface adhesion glycoprotein (Figure 1) (REYNOLDS and FINK 2001). Flo11p is also required for adhesion to plastic, haploid invasive growth, and diploid filamentous growth upon nitrogen starvation (LO and DRANGINIS 1998; REYNOLDS and FINK 2001; VERSTREPEN *et al.* 2004). The expression of *FLO11* is complex and depends on cross talk between the cAMP-protein kinase A (PKA) and mitogen-activated protein kinase signal transduction pathways that converge on the unusually large promoter of this gene (RUPP *et al.* 1999; SENGUPTA *et al.* 2007). Moreover, epigenetic control of *FLO11* expression by the histone deacetylase Hda1p results in a variegated expression of Flo11p at the cell surface of a clonal population derived from a single haploid cell (HALME *et al.* 2004).

Until now, all mutations known to affect mat formation resulted from the downregulation of *FLO11* and therefore also affected invasive and filamentous growth (REYNOLDS and FINK 2001; REYNOLDS 2006). In this report, we show that mutations in the Hsp70 system specifically compromised mat formation, but not invasive growth, through a Flo11p-independent way. Moreover, we show that specific Hsp70 isoforms are required for this function and that the Fes1p and Sse1/2p nucleotide exchange factors play essential nonredundant roles in this process.

MATERIALS AND METHODS

Yeast strains, plasmids, media, and growth conditions: All *S. cerevisiae* strains used in this study are described in Table 1 and were constructed in the $\Sigma 1278b$ derivatives TBR1 (*MAT α*), TBR2 (*MAT α*), or JK371 (Flo11p-HA) strains (generous gifts from Todd B. Reynolds and Jinmi Kim). Gene deletions were made using standard techniques (LONGTINE *et al.* 1998) and completely removed the indicated open reading frames from the start to the stop codons (construction details are available upon request). Yeast strains were propagated on YPD medium (1% yeast extract, 2% bacto peptone, 2% glucose, 2% bacto agar) or on synthetic complete (SC) medium [1.7 g/liter yeast nitrogen base without amino acids and ammonium sulfate (Difco), 5 g/liter ammonium sulfate, 2% glucose, 2% bacto agar] supplemented with uracil, leucine, and histidine when appropriate. The pGPD416-FES1, pGPD416-SSE1, and pGPD416-FLAG-SSE2 plasmids, a generous gift from Kevin A. Morano, are described in SHANER *et al.* (2006).

For mat assays, low-agar YPD plates containing only 0.3% bacto agar were poured and left for 2–3 days at room temperature. The indicated yeast cells were then inoculated with a toothpick on the center of the plates that were then wrapped in parafilm and incubated at 23° for 14 days (REYNOLDS and FINK 2001). For each strain, at least eight mats were grown at 23° and the number of spokes and diameter were measured after 7 days. We considered only those spokes that fully extended from the central hub to the external rim with the characteristic white color that contrasts well with the rest of the mat.

For invasive growth assays, the indicated strains were heavily streaked on YPD plates and allowed to grow for 5 days at 23°. The plates were photographed and then washed under a gentle stream of water before being photographed again. The plates were further washed and rubbed with a gloved finger to remove the remaining attached cells and photographed again.

TABLE 1
Yeast strains used in this study

Strain	Genotype	Reference
TBR1	<i>MATα ura3-52 leu2::hisG his3::hisG</i>	REYNOLDS and FINK (2001)
TBR2	<i>MATα ura3-52 leu2::hisG his3::hisG</i>	REYNOLDS and FINK (2001)
TBR5	<i>MATα ura3-52 leu2::hisG his3::hisG flo11::kanMX6</i>	REYNOLDS and FINK (2001)
JK371	<i>MATα ura3-52 leu2::hisG his3::hisG FLO11::HA</i>	PARK <i>et al.</i> (2006)
MKSC1	<i>MATα ura3-52 leu2::hisG his3::hisG ssa1::HIS3MX6</i>	This study
MKSC2	<i>MATα ura3-52 leu2::hisG his3::hisG ssa1::HIS3MX6</i>	This study
MKSC3	<i>MATα ura3-52 leu2::hisG his3::hisG ssa2::HIS3MX6</i>	This study
MKSC4	<i>MATα ura3-52 leu2::hisG his3::hisG ssa2::HIS3MX6</i>	This study
MKSC5	<i>MATα ura3-52 leu2::hisG his3::hisG ssa3::kanMX6</i>	This study
MKSC6	<i>MATα ura3-52 leu2::hisG his3::hisG ssa3::kanMX6</i>	This study
MKSC7	<i>MATα ura3-52 leu2::hisG his3::hisG ssa4::kanMX6</i>	This study
MKSC8	<i>MATα ura3-52 leu2::hisG his3::hisG ssa4::kanMX6</i>	This study
MKSC9	<i>MATα ura3-52 leu2::hisG his3::hisG ssa1::HIS3MX6 ssa3::kanMX6</i>	This study
MKSC10	<i>MATα ura3-52 leu2::hisG his3::hisG ssa1::HIS3MX6 ssa3::kanMX6</i>	This study
MKSC11	<i>MATα ura3-52 leu2::hisG his3::hisG ssa1::HIS3MX6 ssa4::kanMX6</i>	This study
MKSC12	<i>MATα ura3-52 leu2::hisG his3::hisG ssa1::HIS3MX6 ssa4::kanMX6</i>	This study
MKSC13	<i>MATα ura3-52 leu2::hisG his3::hisG ssa2::HIS3MX6 ssa3::kanMX6</i>	This study
MKSC14	<i>MATα ura3-52 leu2::hisG his3::hisG ssa2::HIS3MX6 ssa3::kanMX6</i>	This study
MKSC15	<i>MATα ura3-52 leu2::hisG his3::hisG ssa2::HIS3MX6 ssa4::kanMX6</i>	This study
MKSC16	<i>MATα ura3-52 leu2::hisG his3::hisG ssa2::HIS3MX6 ssa4::kanMX6</i>	This study
MKSC17	<i>MATα ura3-52 leu2::hisG his3::hisG fes1::HIS3MX6</i>	This study
MKSC18	<i>MATα ura3-52 leu2::hisG his3::hisG fes1::HIS3MX6</i>	This study
MKSC19	<i>MATα ura3-52 leu2::hisG his3::hisG sse1::HIS3MX6</i>	This study
MKSC20	<i>MATα ura3-52 leu2::hisG his3::hisG sse1::kanMX6</i>	This study
MKSC21	<i>MATα ura3-52 leu2::hisG his3::hisG sse2::HIS3MX6</i>	This study
MKSC22	<i>MATα ura3-52 leu2::hisG his3::hisG ydj1::HIS3MX6</i>	This study
MKSC23	<i>MATα ura3-52 leu2::hisG his3::hisG ydj1::kanMX6</i>	This study
MKSC24	<i>MATα ura3-52 leu2::hisG his3::hisG FLO11::HA fes1::HIS3MX6</i>	This study
MKSC25	<i>MATα ura3-52 leu2::hisG his3::hisG FLO11::HA sse1::HIS3MX6</i>	This study
MKSC26	<i>MATα ura3-52 leu2::hisG his3::hisG FLO11::HA ydj1::HIS3MX6</i>	This study
MKSC27	<i>MATα ura3-52 leu2::hisG his3::hisG FLO11::HA ssa1::HIS3MX6</i>	This study
MKSC28	<i>MATα ura3-52 leu2::hisG his3::hisG FLO11::HA ssa2::HIS3MX6</i>	This study

Preparation of protein extracts and Western blot analysis:

Protein extracts were prepared from mats grown for 7 days as indicated above. Mat cells were harvested from two to four low-agar plates with a spoon (REYNOLDS 2006), placed into 50-ml conical tubes containing 20 ml of cold water, vortexed to homogenize cells and agar, and then washed twice with cold water. Aliquots of 50 OD_{600nm} units of cells were resuspended in 600 μ l of cold lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF). Glass beads were added and cells were broken by repeated vortexing for 30 sec with 1 min on ice between each vortexing. Cellular debris and glass beads were removed by centrifugation at 500 \times g and the crude extracts were then centrifuged at 13,000 \times g and at 4° for 15 min. The crude membrane pellet (P13) was then resuspended in 20 μ l of lysis buffer. Proteins in the supernatant (S13) were precipitated with trichloroacetic acid (TCA) at a final concentration of 5% on ice for 30 min. The protein pellets were recovered by centrifugation at 13,000 \times g and at 4°, washed twice with cold acetone, and then resuspended in 20 μ l of TCA sample buffer (80 mM Tris-Cl, pH 8.0, 8 mM EDTA, 120 mM dithiothreitol, 3.5% SDS, 0.29% glycerol, 0.08% Tris base, 0.01% bromophenol blue).

Equal amounts of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting using 12CA5 mouse monoclonal anti-hemagglutinin (HA) antibodies (Roche Applied Science)

and rabbit polyclonal anti-BiP antibodies (a generous gift from Jeffrey L. Brodsky, University of Pittsburgh). Immunoblots were developed using enhanced chemiluminescence reagents (Pierce, Rockford, IL) and a LAS-3000 imager (Fuji).

Indirect immunofluorescence: Indirect immunofluorescent staining of HA-tagged Flo11p was performed essentially as described elsewhere (GUO *et al.* 2000; HALME *et al.* 2004). Briefly, cells were isolated from colonies on plates and fixed in phosphate-buffered saline (PBS) containing formaldehyde (3.7%) for 1 hr. The cells were then washed with PBS and incubated for 1 hr in PBS containing 2% bovine serum albumin (BSA). The cells were pelleted and resuspended in PBS containing 2% BSA and 12CA5 mouse monoclonal anti-HA antibody (Roche Applied Science; 1:1000 dilution) for 1 hr. The cells were then washed three times with PBS containing 2% BSA and resuspended in PBS containing 2% BSA and goat anti-mouse Alexa Fluor 595-conjugated IgG (Jackson ImmunoResearch; 1:1000 dilution). After 20 min of incubation, the cells were washed three times with PBS containing 2% BSA.

RESULTS AND DISCUSSION

The nucleotide exchange factors Fes1 and Sse1 play critical roles in mat formation: To identify physiological functions for the Hsp70 nucleotide exchange factors

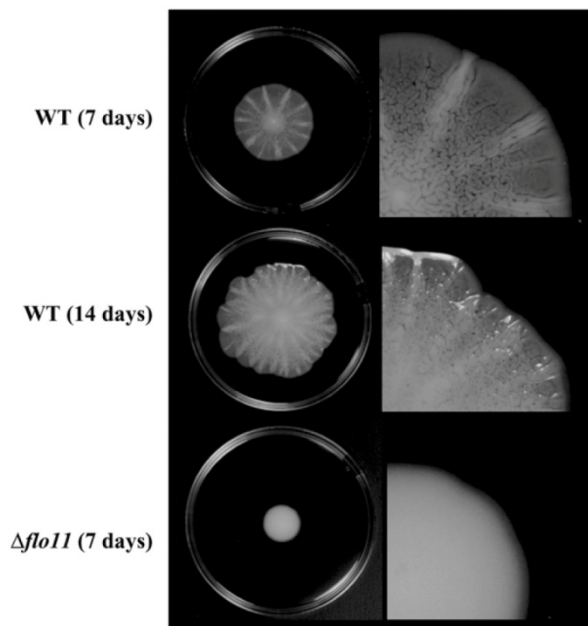


FIGURE 1.—The *S. cerevisiae* $\Sigma 1278b$ strain forms mats on low-agar plates. A wild-type strain or a $\Delta flo11$ mutant (both *MAT α*) was inoculated at the center of YPD plates containing 0.3% agar and grown at 23° for 7 or 14 days. Photographs of the whole plates and of a magnified portion of the plates are shown (note that in Figures 1–4 and 6 the photographs at 7 and 14 days do not necessarily show the same plate).

Fes1p and Sse1/2p in yeast, we constructed null alleles of the respective genes in the $\Sigma 1278b$ strain background. This strain has the ability to undergo a dimorphic transition from yeast to pseudohyphal growth as

well as to invade agar, to adhere to plastic surfaces, or to form mats on low-agar petri plates (Figure 1). We found that both $\Delta fes1$ and $\Delta sse1$ were severely impaired in mat formation (Figure 2 and Figure 3 for quantitative measures), yet exhibited different morphologies. The mats formed by the $\Delta fes1$ mutant were small and did not present the characteristic spokes and substructures, even after 14 days at 23° (Figure 2 and supplemental Figure S2 at <http://www.genetics.org/supplemental/>). They were, however, easily distinguishable from $\Delta flo11$ mats that had a smooth and brilliant aspect (Figure 1). In contrast, the deletion of *SIL1* (also known as *SLS1*), the gene encoding the homolog of Fes1p in the endoplasmic reticulum (KABANI *et al.* 2000; TYSON and STIRLING 2000), did not affect mat formation (data not shown), suggesting a specific involvement of cytosolic chaperones in this process.

It is noteworthy that the growth rates at 23° of $\Delta fes1$ and most of the other mutants described in this study were not significantly altered compared to the wild-type strain (supplemental Figure S1 at <http://www.genetics.org/supplemental/>), which indicates that the observed defects in mat formation are not due to a reduced fitness or sickness of these strains. Indeed, even after prolonged periods of time, these mutants were not able to form mats (photographs at 14 days in Figure 2). We previously showed that a $\Delta fes1$ mutant is thermosensitive in two genetic backgrounds (W303-1b and RSY801) (KABANI *et al.* 2002), but this is not the case in the $\Sigma 1278b$ background where this mutation does not impede growth at 37° (supplemental Figure S1). In contrast,

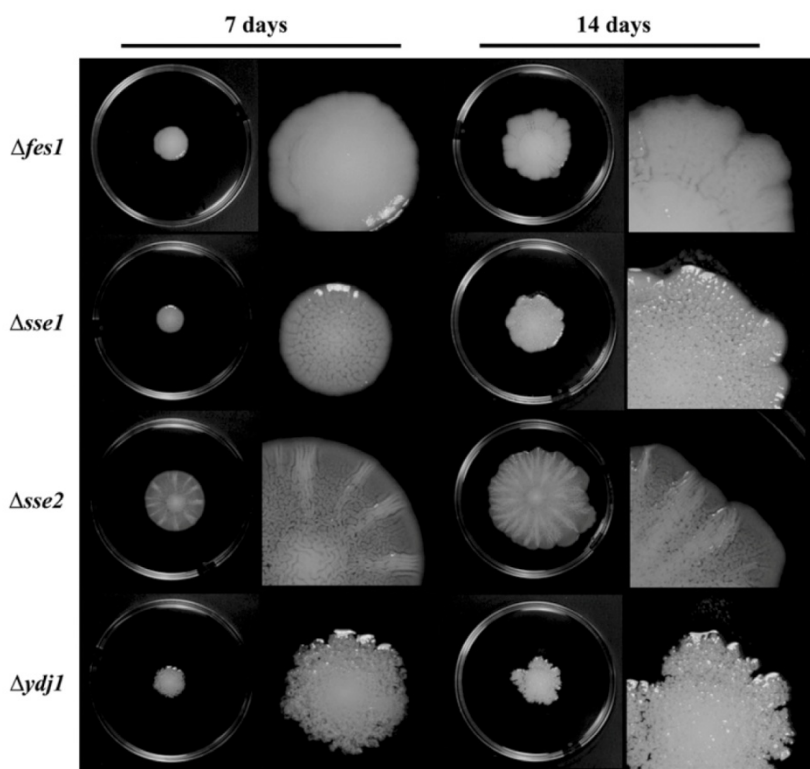


FIGURE 2.—The $\Delta fes1$, $\Delta sse1$, and $\Delta ydj1$ mutants are affected in mat formation. Mats were grown as indicated in Figure 1 (see supplemental Figure S2 at <http://www.genetics.org/supplemental/> for *MAT α* versions of these mutants and Figure 3 for quantitative measures).

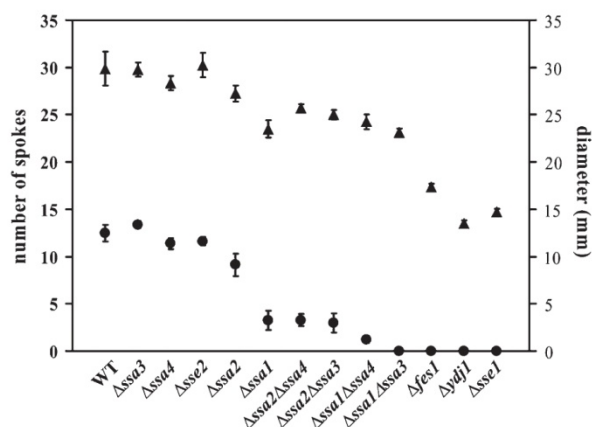


FIGURE 3.—Quantification of the diameter and number of spokes for each mat. For each indicated strain, eight mats were grown in parallel at 23° and the diameter (solid triangles) and number of spokes (solid circles) were measured after 7 days (the indicated numbers correspond to the average \pm SE).

the $\Delta sse1$ mutant in the $\Sigma 1278b$ background displayed the previously reported slow-growth phenotype at all temperatures tested (supplemental Figure S1) (MUKAI *et al.* 1993). This suggests a different utilization of nucleotide exchange factors among strains or species. For example, the deletion of *SLS1/SIL1* in the yeast *Yarrowia lipolytica* resulted in a thermosensitive phenotype whereas no obvious phenotype could be detected in several *S. cerevisiae* strains (BOISRAME *et al.* 1996; KABANI *et al.* 2000), and we showed that the *Y. lipolytica FES1* gene is essential (our unpublished data). Regardless, the strong defect in mat formation observed for $\Delta fes1$ contrasted with the overall healthiness of this strain and indicates a critical role for this factor in a specific cellular response induced by growth on low-agar plates.

The mats produced by the $\Delta sse1$ mutant also had a small diameter compared to wild type and contained no spokes but showed some level of substructures (Figure 2 and supplemental Figure S2 at <http://www.genetics.org/supplemental/>). In contrast, mats formed by the $\Delta sse2$ mutant were normal, which is expected because *SSE2* is expressed at low levels in wild-type cells and *Sse1p* alone is usually sufficient to fulfill Hsp110 activity (MUKAI *et al.* 1993; YAM *et al.* 2005; RAVIOL *et al.* 2006; SHANER *et al.* 2006). Importantly, the strong defect in mat formation observed for the $\Delta sse1$ mutant suggested either that Hsp110 levels are limiting for mat formation in this strain or that *Sse2p* cannot replace *Sse1p* in this particular cellular pathway. A functional distinction between these two Hsp110 paralogs has been suggested by previous studies that showed that *Sse1* is able to form stable complexes with both *Ssa*- and *Ssb*-type Hsp70's, while *Sse2* was able to bind only to the *Ssa* type (YAM *et al.* 2005; SHANER *et al.* 2006). Regardless, the strong and distinct morphological defects in mat formation observed upon loss of *FES1* or *SSE1* suggest that these two classes of nucleotide exchange factors play important

roles in this process where they cannot totally replace each other, at least at their normal physiological levels. This is in accordance with previous observations from Bukau and collaborators who showed that the lethal combination of $\Delta sse1$ and $\Delta sse2$ mutations can be only partially rescued by the overexpression of *FES1*; the resulting strain grew much slower at 30° than a similar strain expressing wild-type levels of *Sse1p*, and its viability was significantly reduced under stress conditions (RAVIOL *et al.* 2006). A recent study reported that while both *SSE1* and *FES1* are required for the propagation of [URE3], only the overexpression of the former cured yeast cells from this prion, again arguing against a complete functional redundancy of these two nucleotide exchange factors (KRYNDUSHKIN and WICKNER 2007).

To further investigate the contributions of nucleotide exchange factors in mat formation, we asked whether the overexpression of *Fes1p* would compensate for the loss of *Sse1p* and vice versa. The $\Delta fes1$ and $\Delta sse1$ strains were transformed with the pGPD416-FES1, pGPD416-SSE1, and pGPD416-FLAG-SSE2 plasmids that allow the constitutive overexpression of *Fes1p*, *Sse1p*, and *Sse2p*, respectively (SHANER *et al.* 2006). These plasmids are centromeric and we reasoned that they would not be significantly lost as cells divide to produce a mat on YPD. As shown in Figure 4, the $\Delta sse1$ mutant formed mats when transformed with pGPD416-SSE1 or pGPD416-FLAG-SSE2, but not with the empty vector. This result shows that *Sse2* can replace *Sse1* in its function in mat formation and suggests that the strong defect observed for the $\Delta sse1$ mutant results from the decrease in overall Hsp110 levels. Interestingly, the overexpression of *FES1* efficiently complemented the $\Delta sse1$ mutant and allowed mat formation (Figure 4), suggesting as previously reported (RAVIOL *et al.* 2006) that the functions of *Fes1p* and *Sse1/2p* partly overlap. Thus, *Fes1p* can replace *Sse1/2p* to allow mat formation but, strikingly, the opposite is not true. Indeed, while pGPD416-FES1 complemented the $\Delta fes1$ mutant as expected, pGPD416-SSE1 and pGPD416-FLAG-SSE2 did not (Figure 4). These results further demonstrate that separate cellular functions for *Fes1p* and *Sse1/2p* exist where these two classes of nucleotide exchange factors cannot replace each other. As a control, we overexpressed *FES1*, *SSE1*, or *SSE2* in a wild-type background and did not observe any notable effect on growth or on the ability to form mats (supplemental Figure S3 at <http://www.genetics.org/supplemental/>).

$\Delta yjd1$ is affected in mat formation: Hsp70's are also regulated by DnaJ-like proteins that contain a 70-amino-acid J-domain that is required for binding to Hsp70's and for activating their ATPase activity. We therefore investigated the contribution of the main Hsp40-family co-chaperone *Ydj1p* in mat formation. The $\Delta yjd1$ mutant totally failed to form mats and spread irregularly on low-agar petri plates, even after 14 days of incubation (Figure 2 and supplemental Figure S2 at <http://www.genetics.org/supplemental/>). The brilliant and spongy

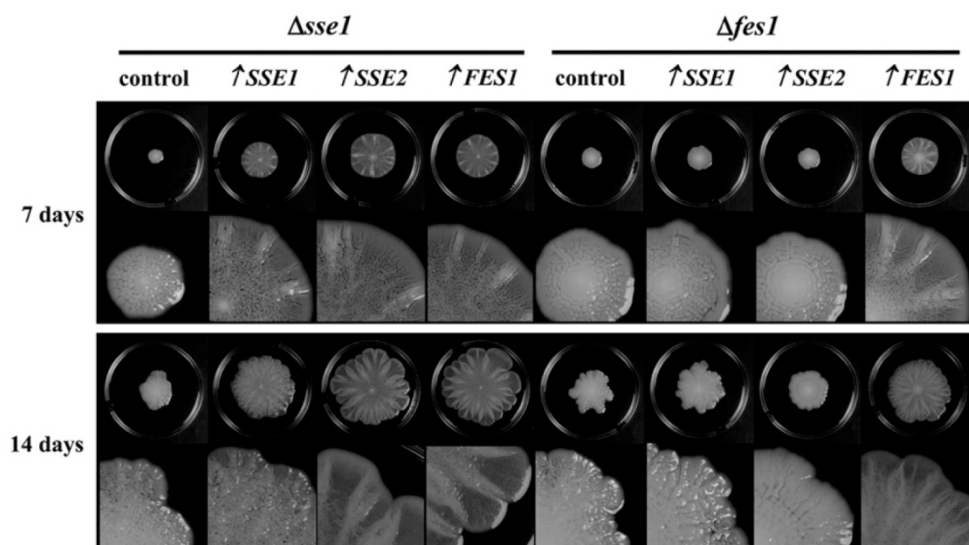


FIGURE 4.—The overexpression of *SSE1* or *FES1* does not equally complement the mat defect of the $\Delta fes1$ and $\Delta sse1$ mutants. The $\Delta fes1$ and $\Delta sse1$ strains were transformed with pGPD416 (control), pGPD416-*SSE1*, pGPD416-FLAG-*SSE2*, or pGPD416-*FES1* (see MATERIALS AND METHODS) and mats were then grown as indicated in Figure 1 (see supplemental Figure S3 at <http://www.genetics.org/supplemental/> for the wild-type control).

aspect of $\Delta ydj1$ mats was different from those observed for $\Delta fes1$ and $\Delta sse1$ mats. *YDJ1* was previously isolated in a transposon-mutagenesis screen for *dia* (*dig into agar*) mutants that enhance invasive growth; an *ydj1-100* mutant had a hyperinvasive phenotype due to elevated levels of the Flo11p adhesin (PALECEK *et al.* 2000). We made a similar observation with our $\Delta ydj1$ mutant that showed increased adherence to agar (see below). While Flo11p is absolutely required for mat formation, it may impede sliding motility if expressed at high levels by preventing cells from moving along the agar surface. Fink and collaborators showed that epigenetic control of *FLO11* expression results in a variegated expression of Flo11p in a cell population (HALME *et al.* 2004). Whether the expression of *FLO11* is differentially and strictly controlled in the different regions of the mats is an attractive hypothesis that needs future investigations. Ydj1p has also been recently implicated in the cell-wall integrity pathway as a $\Delta ydj1$ mutant was shown to be sensitive to cell-wall disrupting agents and its slow growth compensated by osmo-stabilizing reagents such as sodium chloride or sorbitol (WRIGHT *et al.* 2007). Unfortunately, mat formation does not occur on NaCl- or sorbitol-containing medium (our personal observations), preventing us from testing whether these compounds could rescue mat formation by the $\Delta ydj1$ mutant. Moreover, the overexpression of *FES1*, *SSE1*, or *SSE2* in the $\Delta ydj1$ mutant had no effect on mat formation (data not shown).

From these data, we cannot formally conclude that Ydj1p is specifically involved in mat formation. The yeast *S. cerevisiae* contains at least 22 J-domain-containing proteins, the functions of many of which remain unclear (WALSH *et al.* 2004; SAHI and CRAIG 2007). Future studies will be required to investigate the respective contributions of these co-chaperones to mat formation.

Complex interactions among Hsp70 isoforms during mat formation: Because mutations in known Hsp70 co-factors affected mat formation (Figure 2), we next asked

whether mutations in the constitutively expressed *SSA1* and *SSA2* genes or in the conditionally expressed *SSA3* and *SSA4* genes also result in similar phenotypes. As shown in Figure 5 (and supplemental Figure S4 at <http://www.genetics.org/supplemental/>), a $\Delta ssa1$ strain was significantly affected in mat formation but to a lesser extent than a $\Delta fes1$ or $\Delta sse1$ mutant. A $\Delta ssa1$ mutant formed significantly smaller mats than the wild-type strain and with much fewer spokes, even after 14 days of incubation (Figures 5 and 3). Mats formed by a $\Delta ssa2$ mutant had an almost normal diameter but showed a slight decrease in the number of spokes, and the difference with the wild type was less obvious after 14 days of incubation (Figures 5 and 3). Importantly, the level of expression of *SSA2* was shown to be several times that of *SSA1* under standard growth conditions (ELLWOOD and CRAIG 1984; WERNER-WASHBURNE *et al.* 1987), yet only the absence of the latter resulted in a marked defect in mat formation. This further emphasizes the possibility that the highly homologous Ssa1p and Ssa2p proteins may play distinct physiological roles. A functional distinction among these two Hsp70 isoforms has been suggested by earlier studies that showed that the overexpression of Ssa1p, but not of Ssa2p, was able to cure cells from the [URE3] prion (SCHWIMMER and MASISON 2002). To our knowledge, this is the first description of a phenotype associated with the deletion of *SSA1* in an otherwise wild-type background. Indeed, most mutations in *SSA1*, such as the thermosensitive *ssa1-45* allele (BECKER *et al.* 1996), result in detectable phenotypes only when at least *SSA2* and *SSA4* have also been deleted. Thus, mat formation appears as a sensitive mean for exploring chaperone function in the cell.

In contrast, the deletion of *SSA3* or *SSA4* did not affect mat formation (Figures 5 and 3, and Figure S4 at <http://www.genetics.org/supplemental/>); which is not surprising, given that these two Hsp70's are expressed only

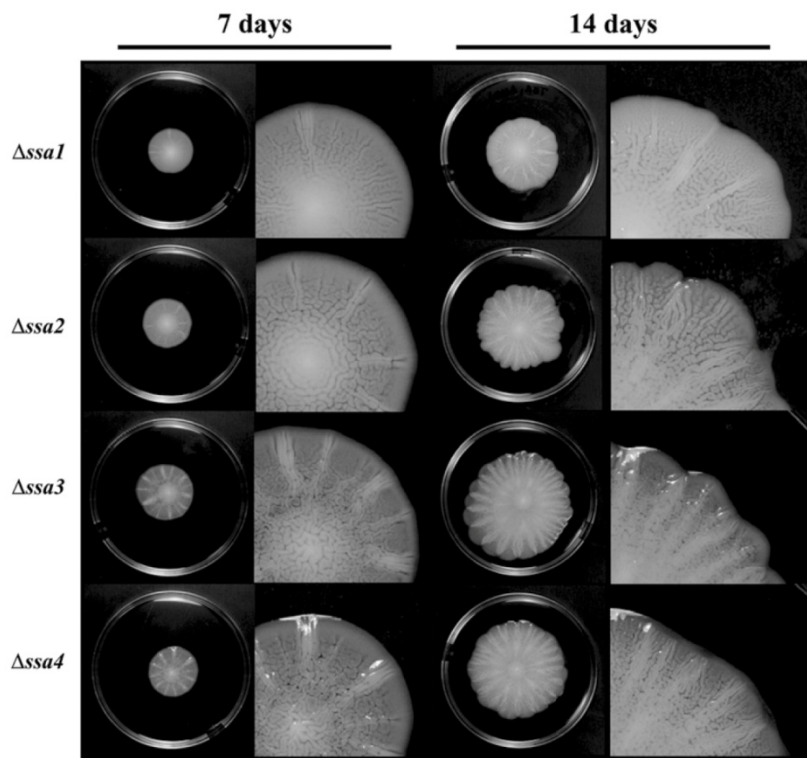


FIGURE 5.—Mutations in *SSA1-4* genes affect mat formation differently. Mats were grown as indicated in Figure 1 (see supplemental Figure S4 at <http://www.genetics.org/supplemental/> for *MATa* versions of these mutants and Figure 3 for quantitative measures).

under stress conditions or in stationary phase (WERNER-WASHBURNE *et al.* 1987; BOORSTEIN and CRAIG 1990). However, it should be noted that mats present a transcriptional profile that is very similar to stationary-phase cells, with the notable exception that genes required for protein synthesis continue to be actively expressed (REYNOLDS 2006). Therefore, *SSA3* is one gene that is overexpressed in mats compared to planktonic log-phase cells (REYNOLDS 2006), yet it seems dispensable for mat formation.

We then asked whether deletions in *SSA3* or *SSA4* genes would result in synthetic effects with mutations in *SSA1* or *SSA2*, and this was indeed the case. As shown in Figures 3 and 6, the additional disruption of *SSA3* or *SSA4* in a Δ *ssa1* or Δ *ssa2* context resulted in a further reduction in the number of spokes and diameter of the mats. The loss of *SSA3* seemed to induce more pronounced effects than *SSA4* in these double mutants, consistent with its greater expression in mats (REYNOLDS 2006). Surprisingly, defects in mat formation were more pronounced when the double mutants were constructed in an isogenic *MATa* strain, and this was most striking for the Δ *ssa2* Δ *ssa4* mutant that was more severely impaired in mat formation in a *MATa* compared to a *MATa* background (compare Figure 6 and supplemental Figure S5 at <http://www.genetics.org/supplemental/>). Because we analyzed at least two independent clones for each gene deletion and for each background; these differences are unlikely to be due to secondary mutations that arose after the transformation procedure. It is known that the mating type and ploidy affect the morphology of mats (REYNOLDS and FINK 2001), and biological differences

were observed between *MATa* and *MATa* cells in *Cryptococcus neoformans* (LEE *et al.* 2005).

Regardless, our observations suggest that the Hsp70 machinery effectively controls mat formation and highlight the intricate interactions among members of the SSA subfamily that may cooperate in this complex biological process.

Mutations in the Hsp70 machinery do not affect invasive growth or the expression and cellular localization of Flo11p: Few studies have addressed the molecular mechanisms that control mat formation and until now all known mutations in this process decreased or abolished *FLO11* expression, which in turn affected invasive growth and adherence to synthetic surfaces (REYNOLDS and FINK 2001; REYNOLDS 2006). We therefore tested our mutants in a standard agar invasion assay (see MATERIALS AND METHODS) where a Δ *flo11* mutant is readily washed away from a YPD plate with a gentle stream of water, whereas the wild-type strain remains attached to the agar surface (LO and DRANGINIS 1998) (Figure 7). Interestingly, none of our mutants in the Hsp70 system was affected in invasive growth (Figure 7) or in adherence to polypropylene or polystyrene plastic surfaces (data not shown), suggesting that these mutations do not result in decreased Flo11p expression at the cell surface. Moreover, we observed an increased invasiveness for the Δ *ydj1* mutant that was most apparent when the plates were washed with water and further rubbed with a gloved finger to remove more effectively noninvasive cells (Figure 7). This behavior is in agreement with increased Flo11p expression in Δ *ydj1* cells (PALECEK *et al.* 2000) (see below).

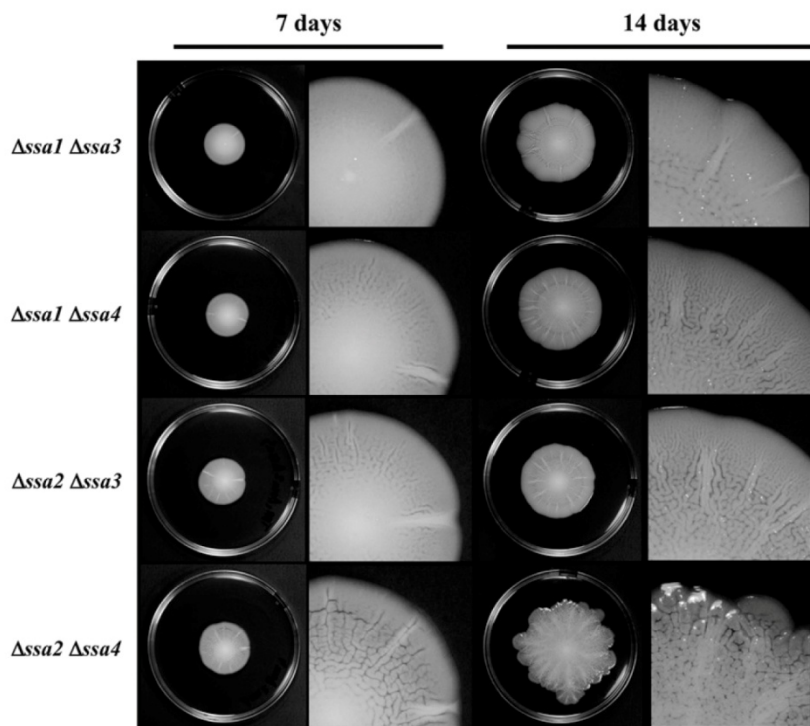


FIGURE 6.—Mutations in *SSA3* or *SSA4* affect mat formation in a Δ *ssa1* or Δ *ssa2* context. Mats were formed as indicated in Figure 1 (see supplemental Figure S5 at <http://www.genetics.org/supplemental/> for *MATa* versions of these mutants and Figure 3 for quantitative measures).

The fact that mutations in the Hsp70 system compromise mat formation without affecting invasive growth suggests that the expression and the cell-surface localization of Flo11p occur normally in those mutants. To verify this hypothesis, we deleted the *SSA1*, *SSA2*, *FES1*, *SSE1*, and *YDJ1* genes in a Σ 1278b-derivative strain expressing an HA-tagged Flo11 protein (PARK *et al.* 2006). These mutants behaved as those made in the wild-type background with respect to mat formation and invasive growth (data not shown).

Total lysates were then prepared from these strains and separated into crude membrane (P13) and soluble (S13) fractions as described in MATERIALS AND METHODS. Equal amounts of proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-HA antibodies to detect Flo11p-HA or anti-BiP (the endoplasmic reticulum Hsp70 family member) antibodies as a control. As shown in Figure 8A, the Flo11p-HA protein was expressed in all strains and was essentially detected in the P13 fractions as expected for a plasma

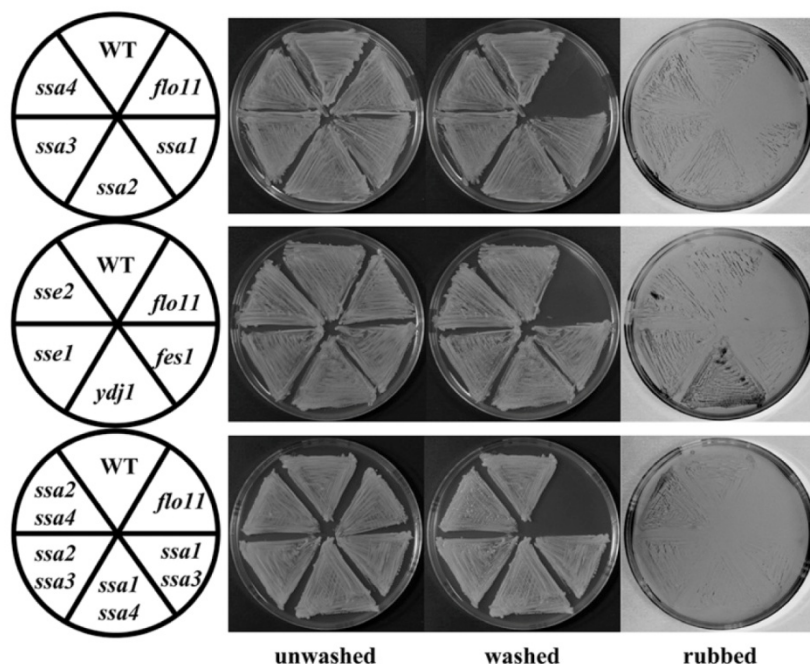


FIGURE 7.—Mutations in the Hsp70 system do not affect invasive growth. The indicated strains were heavily streaked on YPD plates containing 2% agar and grown for 5 days at 23°. The plates were photographed, washed with water to remove unattached cells, and photographed again. To remove the remaining cells more effectively, the plates were rubbed with a gloved finger under a gentle stream of water and photographed again.

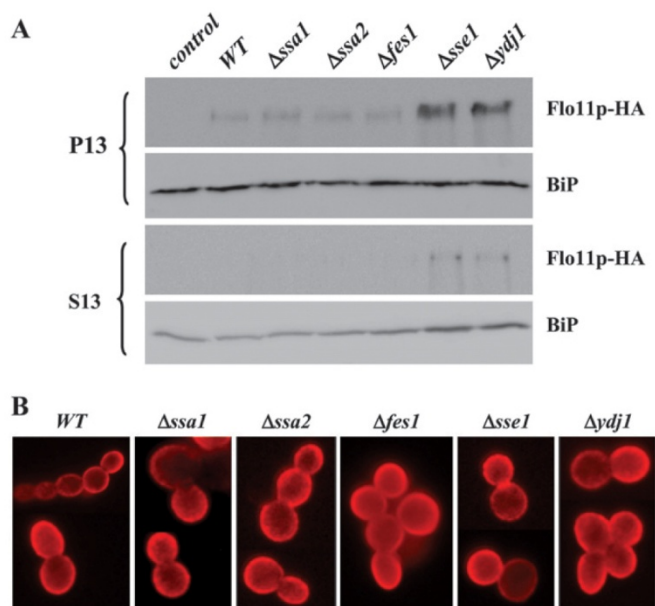


FIGURE 8.—Mutations in the Hsp70 do not affect Flo11p expression or cellular localization. (A) Cell lysates were prepared from the indicated strains (grown as mats on low-agar YPD plates at 23°) expressing HA-tagged Flo11p or from the untagged wild-type strain (control) and subjected to differential centrifugation at 13,000 × *g* to generate a crude membrane pellet (P13) and a supernatant (S13) containing cytosol and light vesicles. Equal amounts of proteins from each fraction were resolved by SDS-PAGE and analyzed by Western blotting with mouse anti-HA and rabbit anti-BiP antibodies. (B) The localization of Flo11p-HA was visualized by indirect immunofluorescence. The indicated strains were harvested from YPD plates, fixed, treated with mouse anti-HA antibody, and stained with Alexa Fluor 595-conjugated goat anti-mouse IgG antibody.

membrane/cell-wall-associated GPI-anchored protein. As expected from its hyperinvasive phenotype and from an earlier report (PALECEK *et al.* 2000), the $\Delta ydj1$ strain contained significantly higher levels of Flo11p-HA protein (Figure 8A). The $\Delta sse1$ strain also contained high levels of Flo11p-HA, probably due to the elevated PKA signaling that was reported for this strain (TROTT *et al.* 2005). Surprisingly, in spite of its elevated Flo11p levels, the $\Delta sse1$ mutant was only slightly hyperinvasive compared to the $\Delta ydj1$ strain (Figure 7). This could be due to the very slow growth of this mutant that might impede foraging into the agar. Alternatively, $\Delta sse1$ may be deficient in other cell-surface determinants that are required for agar invasion. In support of this hypothesis, we observed that $\Delta sse1$ cells are hypersensitive to cell-wall-disrupting agents (see below).

In light of these data, the observed defects in mat formation upon deletion of *SSA1*, *FES1*, *SSE1*, or *YDJ1* cannot be attributed to lowered Flo11p levels. It remained formally possible that, while Flo11p was normally expressed, its maturation or cellular localization was affected by mutations in the Hsp70 system. We therefore analyzed the cellular distribution of the Flo11p-HA

protein in cells harvested from colonies grown in regular YPD plates by indirect immunofluorescence (GUO *et al.* 2000; HALME *et al.* 2004). As shown in Figure 8B, the distribution of Flo11p-HA at the cell surface was comparable in wild-type and mutant strains. We obtained identical results using the same cells that were grown as mats on low-agar YPD plates, and we failed to observe any differences in the expression of Flo11p in the hub, spokes, or rim regions of the mats (data not shown).

Thus, mutations in the Hsp70 machinery do not affect the expression, maturation, and cellular localization of Flo11p. However, we cannot exclude that subtle differences in Flo11p folding or post-translational modifications such as *O*-glycosylation exist in these strains and could account for the observed phenotypes.

Because mat formation is tightly linked to cell-surface events and because a $\Delta ydj1$ mutant was shown to display defects in cell-wall integrity, we checked whether mutations in other components of the Hsp70 machinery would result in cell-wall-associated defects. We found that only $\Delta sse1$ and $\Delta ydj1$ were hypersensitive to SDS and Congo red (supplemental Figure S6 at <http://www.genetics.org/supplemental/> and data not shown), indicating that mat defects in the other mutants are not linked to major perturbations in cell-wall integrity.

The spreading of mats is correlated to the number of spokes: The phenotypic characterization of the mutants described in this study implied a quantification of the average number of spokes and diameter reached by each mat after 7 days of incubation at 23° (see MATERIALS AND METHODS). The number of spokes formed by a wild-type strain is rather constant and is known to depend on both carbon sources (PALECEK *et al.* 2002) and mating type, as *MAT α* mats produce more spokes than *MAT α* mats (REYNOLDS and FINK 2001).

We ranked each strain according to the number of spokes and the diameter of mats, from the highest to the lowest, and plotted these values on a graph (Figure 3). Strikingly, we observed that the average diameter reached by individual mats mirrored the average number of spokes. A linear regression analysis of the data revealed a linear relationship between the average diameter and the number of spokes with an R^2 value of ~ 0.96 ($P < 0.0001$) if the strains that never form spokes but displayed variable diameters (*i.e.*, $\Delta fes1$, $\Delta ydj1$, $\Delta sse1$) were excluded from the analysis. If these strains were also taken into account, the R^2 value drops to ~ 0.8 ($P < 0.001$) but nevertheless remains highly significant.

It is tempting to speculate that spokes are the main structures that control the spreading of the mats and to further emphasize the highly organized nature of these multicellular structures. In support of this hypothesis, mats formed on other carbon sources such as galactose or glycerol do not produce spokes and are significantly smaller than mats formed on glucose or fructose (PALECEK *et al.* 2002). However, because mats are also

a physical phenomenon, spokes may be the result of a complex equation with many parameters such as growth rate, cell–cell interactions, adhesion forces, sliding motility, and cell-surface protein production. Further investigations will be required to distinguish between the physical and biological contributions to mat formation. Because molecular chaperone dysfunctions may affect growth rate, cell-wall assembly, and signaling pathways, they constitute a solid starting point for addressing these questions.

Concluding remarks: In this study, we have provided evidence for a role of the Ssa-subfamily of Hsp70 molecular chaperones in mat formation and have highlighted a number of important features. First, in agreement with other studies (RAVIOL *et al.* 2006; SHANER *et al.* 2006; KRYNDUSHKIN and WICKNER 2007), we showed that the biological functions of Fes1p and Sse1p are mainly distinct since the overexpression of Sse1p did not compensate for the loss of Fes1p in our mat assays. The defects in mat formation in the $\Delta sse1$ and $\Delta fes1$ strains are unlikely to be due to alterations in a common pathway. Because $\Delta sse1$ cells are much sicker than $\Delta fes1$ cells, mat defects may be a secondary effect of more general perturbations in cellular physiology. Another explanation is that the elevated Flo1p levels in the $\Delta sse1$ as well as in the $\Delta ydj1$ mutants, by increasing adherence to the agar and foraging, impede the cells from sliding along the surface. Because the overexpression of Fes1p allowed mat formation in a $\Delta sse1$ mutant and because the strong mat phenotype contrasted with the apparent healthiness of the $\Delta fes1$ strain, we propose that Fes1p plays a more specific role in mat formation. Future work will be required to ask whether the separate biological functions observed for Sse1p and Fes1p can be attributed to their respective engagement in specific chaperone-containing complexes or to their interaction with particular regulatory proteins in distinct cellular pathways.

Second, our data are in agreement with the possible existence of a functional specialization among Ssa proteins suggested by earlier studies and challenge the generally admitted idea that Hsp70 isoforms are functionally redundant and differ only by their expression pattern. The fact that any of Ssa1–4 (WERNER-WASHBURNE *et al.* 1987) or even mammalian Hsp70 orthologs (TUTAR *et al.* 2006) allow the viability of $\Delta ssa1\Delta ssa2\Delta ssa3\Delta ssa4$ yeast cells if expressed at sufficient levels suggests that their general housekeeping and chaperoning functions are mostly conserved. However, the existence of specialized functions of individual isoforms remains an attractive hypothesis that has major significance in human cells where up to six Hsp70 isoforms potentially coexist in the cytosol (TAVARIA *et al.* 1996; ROHDE *et al.* 2005). Furthermore, we showed that the defects in mat formation observed upon deletion of the *SSA1* and *SSA2* genes were significantly aggravated by a deletion of either one of the inducible *SSA3* and *SSA4* genes. These data agree

with a functional distinction between constitutive Hsc70's and inducible Hsp70's, as previously shown (TUTAR *et al.* 2006), but also attractively suggest a possible cooperation between these isoforms in handling different client proteins, their respective contributions being dictated by the cellular conditions and hence their respective cellular levels.

Third, we showed that while attachment to the surface of the low-agar plates is strictly required (REYNOLDS and FINK 2001), *FLO11*-independent pathways affect mat formation. Whether mats represent a purely physical phenomenon, a regulated developmental program resulting in specialized regions within the mat, or a combination of these two aspects is still an open issue, but we clearly show that this biofilm-like behavior is highly sensitive to changes in the genotype that do not otherwise result in dramatic changes in growth rates or cell-wall integrity. We believe that mats constitute a remarkable biological model for investigating the functions of molecular chaperones and their engagement in specific cellular pathways and protein complexes.

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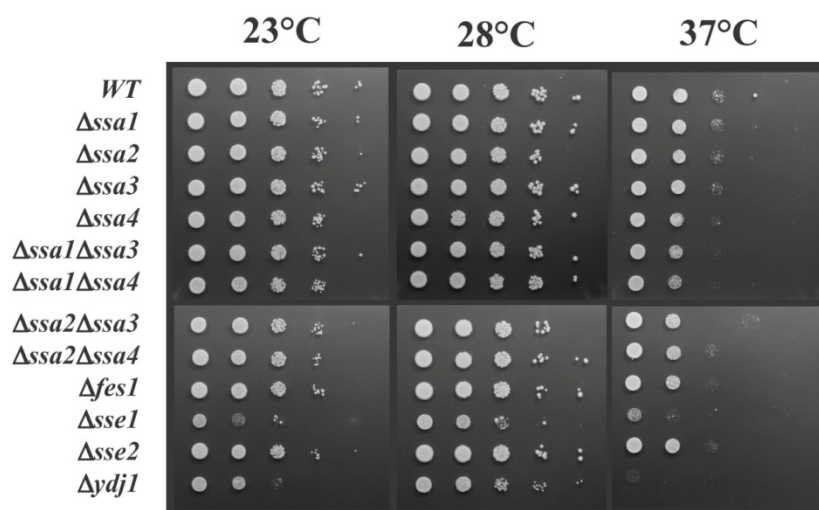
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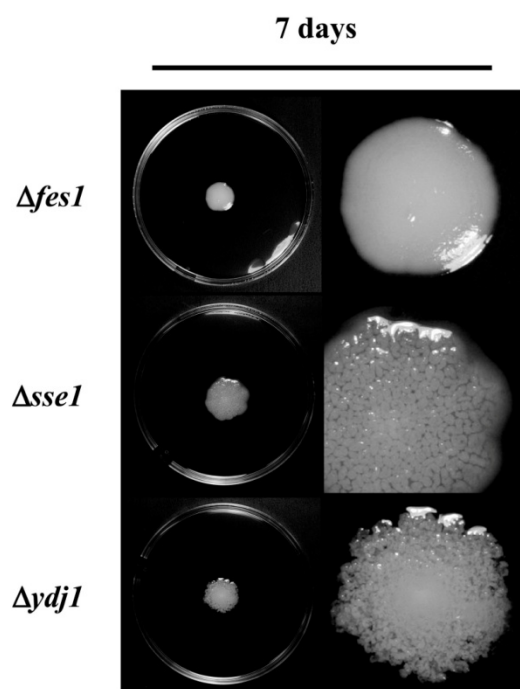
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Supplementary Figures



Supplementary Fig. S1: Growth properties of the strains used in this study. The indicated strains were grown to exponential-phase ($OD_{600nm} \sim 1.0$) and ten-fold serial dilutions were spotted on YPD plates and incubated for 2-3 days at 23°C, 28°C or 37°C.

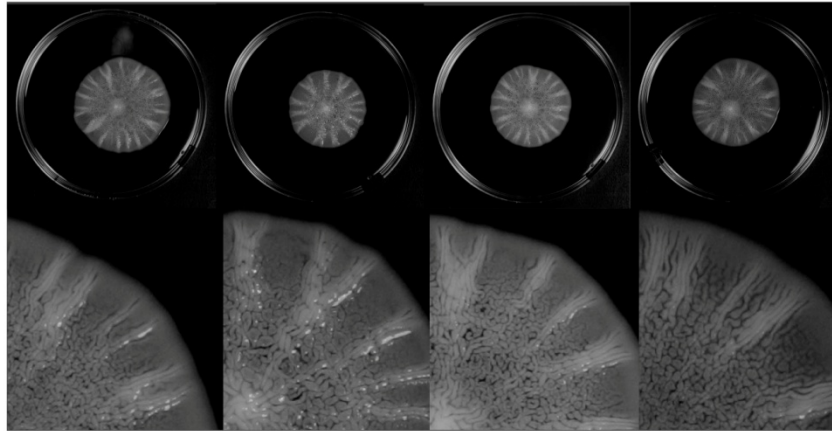


Supplementary Fig. S2: the same as Fig. 2 except that the mutations were made in a *MATa* strain.

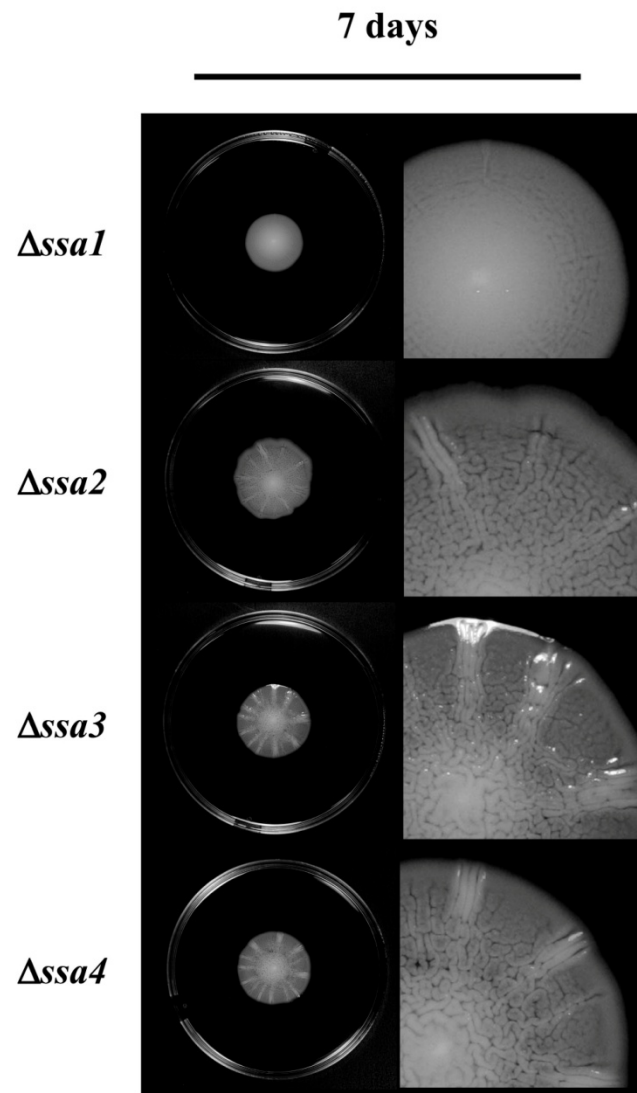
WT

control ↑*SSE1* ↑*SSE2* ↑*FES1*

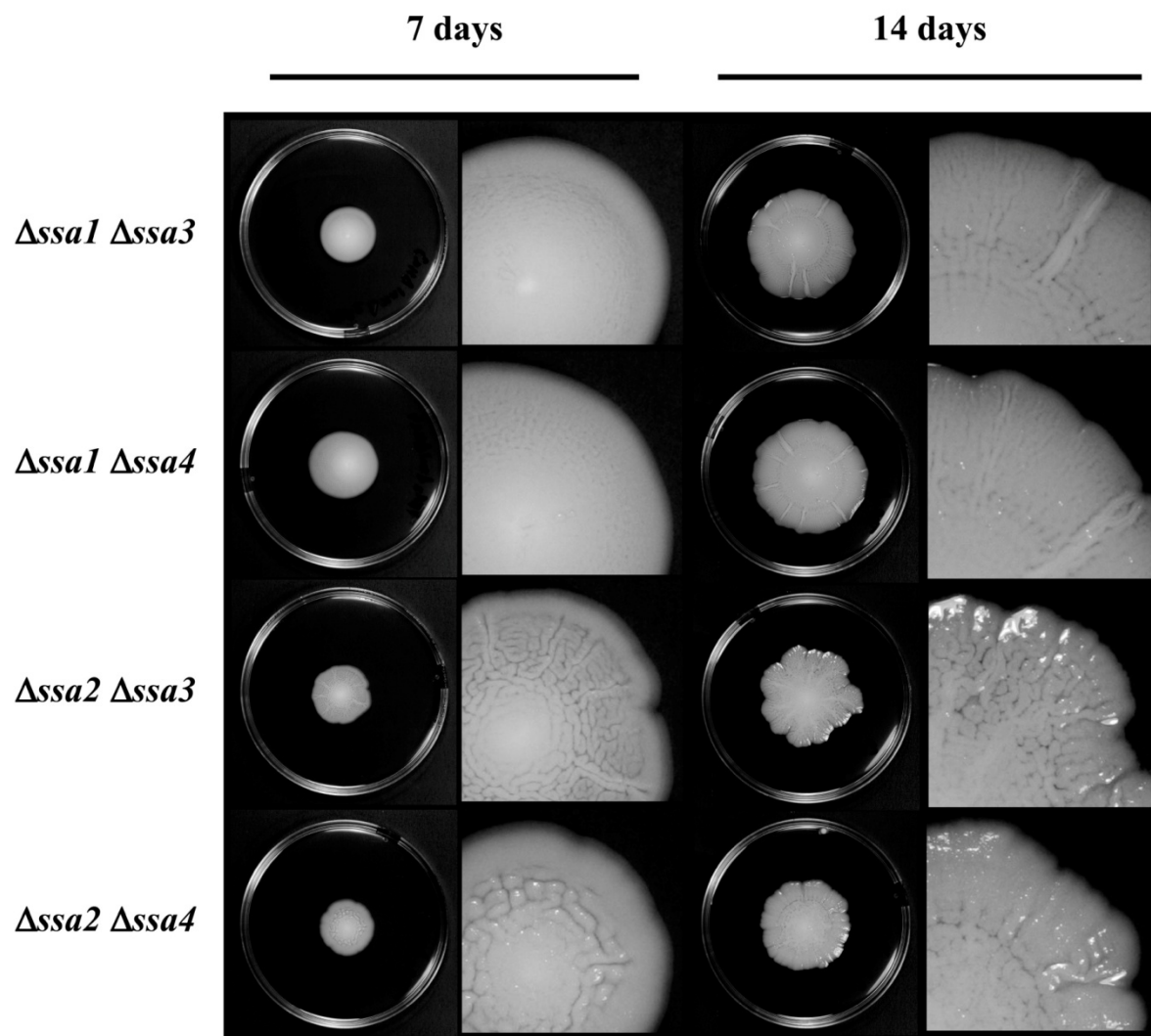
7 days



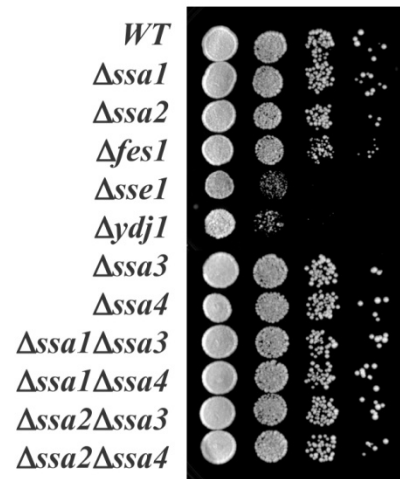
Supplementary Fig. S3: The wild-type strain was transformed with pGPD416 (control), pGPD416-SSE1, pGPD416-FLAG-SSE2 or pGPD416-FES1 and mats were grown at 23°C for 7 days.



Supplementary Fig. S4: the same as Fig. 5 except that the mutations were made in a *MATa* strain.



Supplementary Fig. S5: the same as Fig. 6 except that the mutations were made in a *MATa* strain.



Supplementary Fig. S6: SDS sensitivity assay. The indicated strains were grown to exponential-phase ($OD_{600nm} \sim 1.0$) and ten-fold serial dilutions were spotted on YPD plates containing 0.001% SDS and incubated at 28°C for 2 days.

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**SWA2P-DEPENDANT CLATHRIN DYNAMICS IS CRITICAL FOR FLO11P
PROCESSING AND MAT FORMATION IN THE YEAST *SACCHAROMYCES CEREVISIAE***

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Swa2p-dependent clathrin dynamics is critical for Flo11p processing and ‘Mat’ formation in the yeast *Saccharomyces cerevisiae*

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Running title: Swa2p is required for biofilm formation

Key words: Fes1, Sse1, Ydj1, Swa2, auxilin, fungal biofilm, clathrin, adhesin.

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ABSTRACT

The yeast *Saccharomyces cerevisiae* is able to form complex multicellular structures called mats via a process of sliding motility on low-density agar Petri plates. Mat formation strictly depends on Flo11p, a cell-surface mannoprotein that mediates the adhesion of yeast cells to the agar surface. We previously showed that the Hsp70 molecular chaperone machinery is required for mat formation through distinct pathways. Mutations in the genes encoding the Hsp40-family member Ydj1p or the nucleotide exchange factor (NEF) and Hsp110-family member Sse1p affected mat formation by increasing Flo11p expression. Mutations in the genes encoding cytosolic Ssa Hsp70s or the Fes1p NEF also affected mat formation, but without compromising Flo11p levels or cell-surface localization. To gain further insights into the function of Hsp70 co-chaperones in biofilm formation, we screened deletion mutants of all non-essential cytosolic J-proteins in the $\Sigma 1278b$ genetic background for their ability to form mats. We found that Swa2p, the yeast ortholog of auxilin, a protein required for clathrin-coated vesicles uncoating, is strictly required for biofilm formation. We show that the maturation and cellular levels of Flo11p are affected in $\Delta swa2$ cells, yet without compromising invasive growth. Both the TPR and J-domains of Swa2p are required for its function in biofilm formation, whereas its clathrin-binding and ubiquitin-association motifs are dispensable. Furthermore, $\Delta clc1$ cells lacking the clathrin light-chain showed slight defects in mat formation, in contrast to $\Delta arf1$ cells that formed normal biofilms despite a massive accumulation of Flo11p. Our results indicate that clathrin-dependent trafficking is required for proper function of Flo11p and for biofilm formation.

INTRODUCTION

In nature, in contrast to the way they have been studied for decades in most laboratories, microorganisms are seldom found as liquid cultures or clonal colonies on solid media. In the vast majority of

cases, microorganisms form biofilms which are complex communities of microbes that physically interact with each other and with their surrounding environment and that are often embedded in an extracellular polysaccharide matrix. Biofilm formation greatly increases the

resistance of microbes to environmental stress, including microbiocide treatments, which makes them a major cause of nosocomial infections. The attachment to biotic or abiotic surfaces as well as nutrients availability serve as stimuli that trigger specific changes in the transcriptome, proteome and metabolome of these organisms (d'Enfert, 2009; Stoodley et al., 2002; Verstrepen and Klis, 2006).

The yeast *Saccharomyces cerevisiae* adopts a variety of biofilm-like behaviours through its ability to flocculate and to form flor and mats (Verstrepen and Fink, 2009; Verstrepen and Klis, 2006). The latter are elaborated structures formed on semi-solid agar surfaces that depend on glucose concentration, pH, and the genetic background of the strain (Reynolds, 2006; Reynolds and Fink, 2001; Reynolds et al., 2008). The mature mat is composed of a central hub made of a network of wrinkles and water channels from which emanate multiple radial spokes and a leading edge (or rim) that is smooth in appearance (see Figure 2) (Reynolds and Fink, 2001). The ability to form mats is strictly dependent on the expression of Flo11p, a 1367 amino acids cell-surface adhesion glycoprotein (Reynolds and Fink, 2001). Like most fungal adhesins, Flo11p is composed of three domains: an N-terminal domain that protrudes from the cell surface; a large middle domain consisting in short (10-50 amino acids) serine- and threonine-rich repeats that are heavily glycosylated (N-glycosylation in the endoplasmic reticulum and O-mannosylation in the Golgi); and a C-terminal domain that links the protein to the cell-wall through a glycoposphatidylinositol (GPI)-anchor (Verstrepen and Fink, 2009; Verstrepen and Klis, 2006). Due to its ability to mediate homotypic cell-cell and cell-surface interactions, Flo11p is also required for adhesion to abiotic surfaces (e.g. plastic), for haploid invasive growth and diploid filamentous growth upon nitrogen starvation (Lambrechts et al.,

1996; Lo and Dranginis, 1996, 1998; Reynolds and Fink, 2001; Verstrepen et al., 2004).

The *FLO11* promoter is one of the largest in the yeast genome (~3.5 kb) and is regulated both positively and negatively by several trans-acting factors mainly through the cAMP-protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) signal transduction pathways (Rupp et al., 1999; Sengupta et al., 2007). Moreover, an elegant epigenetic control mechanism involving histone deacetylases and non-coding RNAs results in a variegated expression of *FLO11* in a clonal population (Bumgarner et al., 2009; Halme et al., 2004; Octavio et al., 2009). *FLO11* is not expressed in most laboratory strains (e.g. S288C) because of a mutation in the transcription activator Flo8p (Liu et al., 1996), and it is the only expressed adhesin of the *FLO* family in Σ 1278b derivatives (Guo et al., 2000).

Thus, it appears that mutations in any of the pathways required for the proper expression, synthesis, folding, post-translational modifications or the transport of Flo11p are likely to affect its functions in invasive growth and biofilm formation. We previously reported that the cytosolic Hsp70 machinery plays crucial roles in mat formation (Martineau et al., 2007). Hsp70s are ubiquitous molecular chaperones that play essential roles in the synthesis, folding, assembly, transport and degradation of proteins (Kabani and Martineau, 2008a; Mayer and Bukau, 2005). Hsp70s are regulated by an allosteric mechanism that couples ATP hydrolysis to conformational changes that increase the affinity for polypeptide substrates (Kabani and Martineau, 2008a; Mayer and Bukau, 2005). Members of the Hsp40 family (e.g. Ydj1p) stimulate ATP hydrolysis and substrate binding via their J-domain, while nucleotide exchange factors (e.g. Fes1p, Sse1p) trigger ADP release and substrate dissociation (Kabani and Martineau, 2008a; Mayer and Bukau, 2005).

Our previous work suggested that the Hsp70 machinery is implicated in mat formation through at least two distinct pathways that are dependent or independent of Flo11p (Martineau et al., 2007). Indeed, the deletion of the genes encoding Ydj1p or Sse1p dramatically affected mat formation and caused a hyperinvasive phenotype by increasing the expression of Flo11p at the cell surface (Martineau et al., 2007). Mutations in the cytosolic Hsp70s Ssa1p and Ssa2p had milder effects on mat formation (Martineau et al., 2007). Furthermore, the deletion of *FES1* completely blocked mat formation but did not compromise invasive growth nor Flo11p expression and cell-surface localization, suggesting that Flo11p-independent cellular processes are also critical for biofilm formation (Martineau et al., 2007).

Here, we present the results of a screen for cytosolic J-proteins that are required for mat formation. We show that the yeast auxilin ortholog Swa2p, which is required for clathrin disassembly, is required for mat formation but not for invasive growth. The deletion of *SWA2* results in an accumulation of Flo11p and defects in its processing. We also show that the same domains of Swa2p are required for its function in clathrin dynamics and Flo11p maturation. We thus propose that Flo11p recycling by endocytosis at the cell surface, which is permitted by the fact that Flo11p is not covalently linked to the cell-wall, is necessary for mat formation.

MATERIALS AND METHODS

Yeast strains, plasmids, media, and growth conditions.

All *S. cerevisiae* strains used in this study are described in Table 1 and were constructed in the Σ 1278b derivatives TBR1 or JK371 (expressing Flo11p-HA) strains (generous gifts from Todd B. Reynolds and Jinmi Kim, respectively) (Park et al., 2006; Reynolds and Fink,

2001). Gene deletions were made using standard techniques (Longtine et al., 1998) and completely removed the indicated open reading frames from the start to the stop codons (construction details are available upon request). For each gene deletion, the phenotypic characterization was carried out at least on two independent clones. Yeast strains were propagated on YPD medium (1% yeast extract, 2% bacto peptone, 2% glucose, 2% bacto agar) or Synthetic Defined (SD) medium (1.7 g/L Yeast Nitrogen Base without amino-acids and ammonium sulfate (Difco), 5 g/L ammonium sulfate, 2% glucose, 2% bacto agar) supplemented with uracil, leucine, and histidine when appropriate.

The pRS315 (*LEU2*)-based plasmids encoding deletion alleles of *SWA2* (Xiao et al., 2006) were a generous gift from Dr Todd R. Graham (Vanderbilt University, Nashville, USA). These plasmids were transformed into a $\Delta swa2$ mutant containing p*SWA2-URA3* (Xiao et al., 2006) which was subsequently counter-selected on 5-fluoroorotic acid (5-FOA) containing medium.

For mat assays, low-agar YPD plates containing 0.3% bacto agar were poured and left for 2-3 days at room temperature. The indicated yeast cells were then inoculated with a toothpick on the center of the plates that were then wrapped in parafilm and incubated at 23° or 28° for 7 days (Martineau et al., 2007; Reynolds and Fink, 2001) (Reynolds and Fink, 2001)(Reynolds and Fink, 2001)(REYNOLDS and FINK 2001).

For invasive growth assays, the indicated strains were heavily streaked on YPD plates and allowed to grow for 5 days at 23° or 28°. The plates were photographed, and then washed under a gentle stream of water before being photographed again. The plates were further washed and rubbed with a gloved finger to remove the remaining attached cells and photographed again (Martineau et al., 2007).

Preparation of protein extracts and western blot analysis

Total protein extracts were prepared by incubating 10 OD_{600nm} units of cells in 200 µl of Cracking Buffer (0.1 M NaOH, 0.05 M EDTA, 2% SDS, 2% β-mercaptoethanol) and heating at 90° for 10 min (von der Haar, 2007). The mixture was neutralized with 5 µL of 4 M acetic acid and further incubated at 90° for 10 min. Before adding 50 µL of Loading Buffer (0.25 M Tris-Cl, pH 6.8, 50% glycerol, 0.05% bromophenol blue) (von der Haar, 2007), we checked that protein concentrations were similar in all extracts by measuring the absorbance at 280nm.

For subcellular fractionation, 50-100 OD_{600nm} units of cells were recovered and resuspended in 1 volume of Lysis Buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl) supplemented with 1 mM PMSF and an anti-protease cocktail (Roche Applied Science). Glass beads were added to half the cell suspension volume and cells were broken by 8 cycles of vortexing for 30 sec with 1 min incubation on ice between each vortexing. The total lysate was centrifuged for 5 min at 4000g and at 4° to pellet cell-walls, debris and unbroken cells. The cleared lysate was centrifuged for 15 min at 13000g and at 4°, and the resulting supernatant was further centrifuged at 150000g for 90 min and at 4° to generate the P150 pellet (Golgi complex, endosomal membranes, transport vesicles) and the S150 fraction (soluble cytosolic proteins, soluble proteins from damaged organelles and released peripheral membrane proteins). The P150 pellet was resuspended in the same volume of buffer as the S150 fractions. The cell-walls were washed 4 times with 1 M NaCl, 1 mM PMSF to remove contaminating cytosolic proteins, and incubated for 10 min at 90° in Cell-Wall Extraction Buffer (50 mM Tris-Cl, pH 8.0, 100 mM EDTA, 2% SDS, 40 mM β-mercaptoethanol) to extract proteins that are non-covalently associated to the cell-wall (SDS/βME). The cell-walls were

pelleted by centrifugation for 5 min at 4000g, extensively washed with water and resuspended in Lysis Buffer containing 1 mM PMSF. Zymolyase 20T (~ 10 units) was added and the mixture was incubated for 3h at 30° with agitation. Cell-walls were removed by centrifugation and proteins covalently-associated with the cell-wall and released by the zymolyase treatment (Zymo) were hence recovered.

Equal amounts of proteins were electrophoretically resolved using 4-12% NuPAGE Novex bis-tris polyacrylamide gels (Invitrogen) and MOPS-SDS running buffer according to the manufacturer's instructions. Proteins were analyzed by Western blotting using 12CA5 mouse monoclonal anti-HA antibodies (Roche Applied Science), rabbit polyclonal anti-BiP antibodies (a generous gift from Jeffrey L. Brodsky, University of Pittsburgh, USA), rabbit polyclonal anti-Ydj1p antibodies, and mouse monoclonal anti-Chc1p antibodies (a generous gift from Sandra K. Lemmon, University of Miami, USA). Immunoblots were developed using enhanced chemiluminescence reagents (Pierce) and a LAS-3000 imager (Fuji), and quantified using the ImageJ software (NIH).

RESULTS

Deletion of non-essential cytosolic J-protein encoding genes reveals a role for Swa2p in biofilm formation

In order to identify which of the eleven non-essential cytosolic J-proteins are involved in mat formation, we constructed strains bearing deletions of each of the corresponding genes in the Σ1278b genetic background. As shown in Figure 1, these mutants were all viable in the Σ1278b background and their growth phenotypes at different temperatures were similar to those previously reported in other genetic backgrounds (Sahi and Craig, 2007). For example, the *Δydj1*, *Δzuo1*, *Δjjj1* and *Δswa2* strains grew slowly at low

temperatures while *Δydj1* was also temperature sensitive at 37° (Figure 1). We then assayed each of these strains for mat formation on low-density agar Petri plates at 23° (Figure 2). As expected, the wild-type strain formed mats with the characteristic spokes and substructures while the *Δydj1* strain was completely unable to form mats (Figure 2A). All the other strains were able to form mats except for *Δswa2* which was severely affected in this process. The aspect of the *Δswa2* strain on low-density agar plates was similar to that observed for *Δfes1* or *Δsse1* strains (Martineau et al., 2007), and was clearly different from the brilliant and spongy mats formed by the *Δydj1* strain (Figure 2A). Importantly, the inability of *Δswa2* to form biofilms is unlikely to be due to its slow growth phenotype because it could still not form mats when incubated at 28°, a temperature at which *Δswa2* is healthier (Figures 1 and 2B), and because other slow growing strains such as *Δzuo1* or *Δjji1* formed normal mats at 23° (Figure 2A). Finally, identical phenotypes were observed whether the mutants were constructed in *MATα* (Figure 2) or *MATa* strains (data not shown).

Invasive growth is not compromised in *Δswa2* cells

The Flo11p adhesin is required for biofilm formation, but also for haploid invasive growth (Lo and Dranginis, 1998), and mutations that increase or decrease the expression of *FLO11* result in hyperinvasive or non invasive phenotypes, respectively (Lo and Dranginis, 1998; Martineau et al., 2007; Palecek et al., 2000). Our J-protein mutants were then tested in a standard agar invasion assay where strains are grown for several days on YPD plates which are then washed with a gentle stream of water to remove non-adherent cells. In these conditions, the *Δflo11* strain was readily washed away, as previously described (Lo and Dranginis,

1998; Martineau et al., 2007), while all the other strains remained firmly attached to the agar surface (Figure 3). When the same plates were further washed with a gentle stream of water and rubbed with a gloved finger, no invasive cells could be detected for *Δflo11* as previously reported (Lo and Dranginis, 1998; Martineau et al., 2007) (Figure 3). Both *ZUO1* and *YDJ1* were isolated in a transposon-mutagenesis screen for *dia* (*dig* into agar) mutants that enhance haploid invasive growth (Palecek et al., 2000), yet only the lack of Ydj1p function resulted in an increase in the expression of *FLO11* (Martineau et al., 2007; Palecek et al., 2000). In our assay, we were not able to detect more invading cells in *Δzuo1* compared to the wild-type strain, while *Δydj1* clearly displayed the previously reported hyperinvasive phenotype (Figure 3) (Martineau et al., 2007; Palecek et al., 2000). Importantly, and like *Δfes1* cells (Martineau et al., 2007), *Δswa2* cells were not compromised in agar invasion, suggesting normal Flo11p function in this process (Figure 3).

Fewer *Δcaj1* cells invaded agar in this assay compared to the wild-type strain (Figure 3). Little is known about the function of Caj1p, a type-II J-protein (Mukai et al., 1994) which has been recently proposed to promote microtubule function in association with the Kap123p karyopherin (Ptak et al., 2009). Whether Caj1p plays a direct or indirect role in agar invasion has not been investigated further in the present study, as we have decided to focus on the role of Swa2p in biofilm formation.

Swa2p is required for proper maturation of Flo11p.

We previously hypothesized that at least two pathways control mat formation (Martineau et al., 2007). The Flo11p-dependent pathway regulates Flo11p levels and mutations in this pathway will result in a dramatic increase (*e.g.* *Δydj1*, *Δsse1* (Martineau et al., 2007)) or decrease of

Flo11p expression (e.g. $\Delta opi1$ (Reynolds, 2006)), as well as defects in invasive growth (Martineau et al., 2007; Reynolds, 2006). The Flo11p-independent pathway is also critical for mat formation, yet mutations in this category (e.g. $\Delta fes1$ (Martineau et al., 2007)) have no impact on Flo11p expression or invasive growth (Martineau et al., 2007). Because, $\Delta swa2$ cells are severely affected in biofilm formation while having normal invasive growth, we expected this mutant to belong to the Flo11p-independent pathway and to have wild-type Flo11p levels. To confirm this hypothesis, we deleted *SWA2* in JK371, a $\Sigma 1278b$ -derivative expressing hemagglutinin (HA)-tagged Flo11p (Flo11-HA) (Park et al., 2006), and the resulting $\Delta swa2$ mutant was unable to form mats as expected (Figure 4B). The HA-tag is located at the N-terminus of Flo11p, just after the signal sequence that is cleaved in the endoplasmic reticulum, and it is therefore exposed at the cell surface (Park et al., 2006). It should be noted that when made in JK371, $\Delta swa2$ cells displayed a normal invasive phenotype when the plates were simply washed with water, but fewer invasive cells were detected after gently rubbing the plates with a gloved finger (Figure 4C). The reason for this discrepancy is presently unknown. One likely explanation is that the HA-tag might have a subtle negative effect on Flo11p function, not detectable in wild-type or other mutant strains but exacerbated in a $\Delta swa2$ context.

Because $\Delta swa2$ cells poorly grow on semi-solid YPD plates, total cell extracts were prepared from standard liquid cultures grown at 23° and not from mats. However, our previous work showed that Flo11p levels and cell-surface localization are the same whether planktonic cells or mats are examined ((Martineau et al., 2007) and data not shown). Moreover, the transcription of *FLO11* was shown to be only modestly increased in mats compared to planktonic cells (Reynolds, 2006). We are therefore confident that our

observations with cells that are grown in liquid cultures are representative of the situation in mats.

Flo11p is a cell-wall associated GPI-anchored mannoprotein (Douglas et al., 2007; Guo et al., 2000; Martineau et al., 2007), but little is known about its intracellular trafficking and maturation steps. We previously detected Flo11-HA in the membrane fraction of cell extracts by western blot analysis. The Flo11-HA protein migrated as a high molecular weight fuzzy band, reflecting its glycosylated state (Martineau et al., 2007). By significantly improving our extraction and electrophoresis procedures (see Material and Methods), we now detect Flo11-HA as two major high-molecular weight bands (band A and band B; Figure 5A). Interestingly, Flo11-HA migrated similarly in the wild-type, $\Delta fes1$, $\Delta sse1$, and $\Delta ydj1$ strains, except that 5-6-fold more material in both bands A and B were detected in $\Delta sse1$ and $\Delta ydj1$. This result suggests that *Sse1p* and *Ydj1p* affect the expression, as previously shown (Martineau et al., 2007), but not the maturation of Flo11p (Figure 5A). In the $\Delta swa2$ strain however, Flo11-HA levels in band A decreased by 2-3-fold compared to the wild-type strain, whereas those in band B were increased by 5-6-fold (Figure 5A). To address the possibility that bands A and B represent two forms of Flo11-HA that may accumulate at different sites in the cell, total cell extracts were fractionated by differential centrifugation as indicated in Material and Methods. As expected for a GPI-anchored protein en route through the secretory pathway, Flo11-HA was found mainly associated to the membrane and cell-wall fractions (Figure 5B and data not shown). Importantly, Flo11-HA was readily extracted from the cell-wall with a SDS/ β -mercaptoethanol treatment, indicating that it is not covalently linked to the cell-wall glucans. Indeed, no additional amounts of Flo11-HA were released after treating the cell-walls with zymolyase (Figure 5B). For all the mutants we have

tested, both band A and B were found associated with the cell-wall (Figure 5B and data not shown). Additionally and although our differential centrifugation method may not be sufficiently resolutive, we were not able to obtain fractions enriched in band A or band B in any strain analyzed (data not shown). The defect in mat formation in $\Delta swa2$ is therefore not due to altered localization of Flo11p but rather defects in its maturation or activity. Impairment in Swa2p function results in a defect in clathrin disassembly and an increase in the amount of assembled clathrin heavy-chain, Chc1p, in the pellet fraction after centrifugation at 150000g (P150)(Gall et al., 2000; Pishvaei et al., 2000; Xiao et al., 2006). We indeed observed the accumulation of Chc1p in the P150 pellet (~45%) compared to the soluble S150 fraction (~55%) in our $\Delta swa2$ strain (Figure 5C). Most of Chc1p was found in the S150 fraction in the wild-type, $\Delta fes1$ and $\Delta ydj1$ strains (~80-85%) (Figure 5C), suggesting that Fes1p and Ydj1p have no role in clathrin (dis)assembly.

The TPR and J-domains of Swa2p are required for maintaining proper Flo11p levels.

Swa2p is a modular protein that contains at least three clathrin-binding (CB) motifs and an ubiquitin-association (UBA) domain in its N-terminal moiety, and a tetratricopeptide repeat (TPR) and a J-domain in its C-terminal moiety (Xiao et al., 2006) (Figure 6A). T.R. Graham and collaborators previously showed that deleting all CB motifs severely affects clathrin disassembly but does not abrogate Swa2p function *in vivo* (Xiao et al., 2006). The UBA domain also appeared dispensable for Swa2p function *in vivo* (Xiao et al., 2006). In contrast, the deletion of either the TPR or the J domains completely abolished Swa2p function, indicating critical and non-overlapping roles of these domains in Swa2p-mediated regulation of Hsc70 activity (Xiao et al.,

2006). Point mutations in the TPR (G388R) or in the J-domain (HPD→AAA) had much milder effects than the complete deletion of the corresponding domains (Xiao et al., 2006). However, combining these two mutations abolished Swa2p function *in vivo* similarly to the whole deletion of the *SWA2* gene (Xiao et al., 2006).

To address the role of these Swa2p domains in Flo11p processing and mat formation, we introduced these pRS315-borne *swa2* alleles into our $\Delta swa2$ strain expressing Flo11-HA (see Material and Methods). As expected from earlier studies (Xiao et al., 2006), strains bearing the empty vector or expressing the *swa2- Δ 2-362* (corresponding to the deletion of CB1, CB2, CB3 and UBA), *swa2- Δ TPR*, *swa2- Δ J* or *swa2-*tpr-j** (G388R and HPD→AAA) alleles grew much slower than those expressing wild-type *SWA2*, *swa2- Δ UBA*, *swa2- Δ 2-100* (corresponding to the deletion of CB1), *swa2- Δ 2-181* (corresponding to the deletion of CB1 and UBA), *swa2- Δ 2-287* (corresponding to the deletion of CB1, UBA, and half of CB2), *swa2- Δ 2-302* (corresponding to a deletion of CB1, UBA, and CB2), *swa2-*tpr** (G388R), or *swa2-*j** (HPD→AAA) alleles (Figure 6A and data not shown).

Total cell extracts were then prepared from each of these strains and Flo11-HA levels were analyzed by SDS-PAGE and western blot. As shown in Figure 6B, the $\Delta swa2$ strain bearing an empty pRS315 vector accumulated Flo11-HA, whereas the introduction of plasmid-borne wild-type *SWA2* restored Flo11-HA to normal levels. Flo11-HA levels in strains expressing *swa2* alleles with individual or combined deletions of the CB1, CB2 and UBA domains were similar to the strain expressing wild-type Swa2p (Figure 5B; *SWA2*, *swa2- Δ UBA*, *swa2- Δ 2-100*, *swa2- Δ 2-181*, *swa2- Δ 2-287*, and *swa2- Δ 2-302*). In contrast, the deletion of all the CB motifs impaired Swa2p function and Flo11-HA accumulated in the

corresponding strain (Figure 6B; *swa2-Δ2-362*). Similarly, the deletion of the TPR or the J-domain caused the accumulation of Flo11-HA (Figure 5B; *swa2-ΔTPR*, *swa2-ΔJ*), while point mutations in these domains caused an intermediate phenotype (Figure 6B; *swa2-tpr*, or *swa2-j* alleles). However, combining the two point mutations resulted in the inactivation of Swa2p and in the massive accumulation of Flo11-HA (Figure 6B; *swa2-tpr-j*). Hence, the behavior of these *swa2* alleles towards Flo11-HA accumulation perfectly mirrored their described effects on growth (Xiao et al., 2006), and suggests that the function of Swa2p in clathrin dynamics *in vivo* is required for proper Flo11-HA processing and hence for biofilm formation. We were unable to reliably test the ability of these *swa2* alleles to complement the defect in mat formation of $\Delta swa2$ because the plasmid borne alleles appeared unstable and readily lost on the rich low-agar medium. However, because the accumulation of Flo11-HA in *swa2-Δ2-362*, *swa2-ΔTPR*, *swa2-ΔJ*, and *swa2-tpr-j* is very similar to that observed in $\Delta swa2$ cells (Figure 6B), we can assume that mat formation in these mutants defective for Swa2p function is also strongly affected.

Clc1p and Arf1p are not required for biofilm formation and invasive growth, but Darf1 accumulate Flo11-HA.

We have shown so far that the function of Swa2p in regulating clathrin dynamics is essential for biofilm formation. We then asked whether other proteins known to be implicated in clathrin assembly or that functionally interact with Swa2p have a role in mat formation. First, we deleted *CLC1*, the gene encoding the clathrin light-chain that promotes the trimerization of clathrin heavy-chain (Chu et al., 1996; Huang et al., 1997; Silveira et al., 1990). The $\Delta clc1$ strain in the $\Sigma 1278b$ background grew well at all temperatures, in contrast to previous observations in other genetic backgrounds (Huang et al., 1997; Silveira et

al., 1990) (Figure 4A). The $\Delta clc1$ strain showed a defect in mat formation, although not as pronounced as that observed with the $\Delta swa2$ strain. The mats formed by the $\Delta clc1$ strain were smaller and smoother with fewer substructures (Figure 4B). SWA2 was originally isolated in a synthetic lethality screen with a $\Delta arf1$ mutant revealing a potential role for Arf proteins in clathrin function (Gall et al., 2000). The *ARF1* gene encodes a well characterized ADP-ribosylation factor and GTPase of the Ras superfamily required for vesicle budding at the Golgi membrane (Schekman and Orci, 1996). The $\Delta arf1$ strain grew slower than the wild-type at low temperature (Figure 6A), as previously reported (Stearns et al., 1990a). In contrast to the $\Delta swa2$ and $\Delta clc1$ strains, $\Delta arf1$ had no defects in mat formation (Figure 4B) and both $\Delta clc1$ and $\Delta arf1$ displayed a normal agar invasion phenotype (Figure 4C). We were not able to delete the clathrin heavy-chain encoding gene (*CHC1*) in our $\Sigma 1278b$ derived-strain, indicating that it might be essential in this genetic background as previously shown for some (but not all) yeast strains (Lemmon and Jones, 1987; Munn et al., 1991).

We then prepared total cell extracts from the wild-type, $\Delta clc1$ and $\Delta arf1$ strains and analyzed the amount of clathrin heavy-chain and Flo11-HA by western blot. Surprisingly, Flo11-HA levels in $\Delta clc1$ were similar to those obtained in the wild-type strain (Figure 5A), indicating either that mat formation is affected independently of Flo11p in this mutant or that the Swa2p/Hsc70-mediated assembly of clathrin heavy-chain alone can efficiently compensate for the lack of clathrin light-chain *in vivo*. In support with the latter hypothesis, we found that the intracellular levels of Chc1p were not affected in our $\Delta clc1$ mutant (Figure 7A), in contrast to what has been previously reported in another genetic background (Huang et al., 1997). Moreover, the amount of Chc1p found in the P150 pellet

after cell fractionation was the same in the wild-type and in the $\Delta clc1$ strains, arguing against a major defect in clathrin function in the latter (Figure 7B). On another hand, Flo11-HA significantly accumulated in $\Delta arf1$ cells, yet the band pattern was clearly different from that obtained with the $\Delta swa2$ strain (Figure 5A). The size and amount of Flo11-HA in band A was similar to the wild-type, but the band B was 5-6-fold more intense, as seen in $\Delta swa2$, but reproducibly migrated at a lower molecular weight (Figure 5A). As for the wild-type and $\Delta swa2$ strains, Flo11-HA was mainly detected in the membrane and cell-wall associated proteins fractions of $\Delta arf1$ and $\Delta clc1$ in cell fractionation experiments (data not shown). Moreover, the levels of the ER-resident Hsp70 molecular chaperone BiP were elevated in $\Delta arf1$ strain, correlating with an up-regulation of the unfolded protein response (UPR) following alterations in the ER-to-Golgi transport (Figure 7). $\Delta arf1$ cells showed no apparent defect in clathrin dynamics as the distribution of Chc1p after a 150000g centrifugation of cell extracts was the same as in the wild-type and $\Delta clc1$ strains (Figure 7B). Hence, while the $\Delta arf1$ mutation has dramatic effects on Flo11-HA processing, these defects appear compatible with its function in mat formation, and thus are different from those induced by the lack of Swa2p.

DISCUSSION

Our prior studies suggested that the cytosolic Hsp70 machinery plays crucial roles in biofilm formation in the yeast *S. cerevisiae* (Martineau et al., 2007). To extend this work, we report the results of a screen for non-essential cytosolic J-proteins required for mat formation. The deletion of two of the eleven corresponding genes, *YDJI* and *SWA2*, completely blocked biofilm formation (Figure 2). The implication of Ydj1p in biofilm formation has been previously reported (Martineau et al., 2007). Cells

defective for Ydj1p or Sse1p activity show elevated expression of *FLO11* resulting in a hyperinvasive growth phenotype that blocks the sliding motility of cells along the agar surface (Martineau et al., 2007; Palecek et al., 2000). The second gene identified in our screen is *SWA2* encoding the yeast ortholog of mammalian auxilin, a protein required for the uncoating of clathrin-coated vesicles (Gall et al., 2000; Pishvaei et al., 2000; Ungewickell et al., 1995). The deletion of the *SWA2* gene leads to pleiotropic phenotypes, including slow growth and defects in protein trafficking (Pishvaei et al., 2000). This raised the question of whether the inability of $\Delta swa2$ cells to form mats merely results from the sum of these defects or rather reflects a specific function played by Swa2p in this process. We faced the same dilemma when we identified *FES1* as a gene required for mat formation (Martineau et al., 2007). Indeed, Hsp70 is involved in a variety of cell functions that could potentially be affected by the loss of Hsp70's NEF Fes1p, ultimately and indirectly leading to defects in biofilm formation. However, the limited effect that deleting *FES1* had on growth contrasted with the strong inhibition of biofilm formation, and led us to conclude that Fes1p plays a specific, yet uncharacterized, role in mat formation (Martineau et al., 2007). Here again, mat formation was completely blocked in $\Delta swa2$ (Figure 2), resembling $\Delta flo11$ or $\Delta fes1$ strains, yet it cannot be accounted for by the differences in growth rates between $\Delta swa2$ and wild-type cells (Figure 1). $\Delta zuo1$ or $\Delta jji1$ cells are perfectly able to form mats, further indicating that a slow growth phenotype is not an obstacle to sliding motility (Figure 2). Thus, we believe that Swa2p fulfills a specific function required for mat formation, regardless of its broader impacts on cell physiology. We found that Flo11p accumulated in $\Delta swa2$ cells (Figure 5A) but this did not lead to a hyperinvasive phenotype as in $\Delta sse1$ or $\Delta ydj1$ cells (Figure 3). In the latter strains, their

hyperinvasive phenotype is likely to be due to an excess of Flo11p exposed at the cell-surface, causing increased cell-cell and cell-agar attachment (Martineau et al., 2007; Palecek et al., 2000). The situation in $\Delta swa2$ cells must therefore be different, and not all Flo11p molecules may be exposed at the cell-surface and/or functional. In support with this hypothesis, the band pattern of Flo11p in total cell extracts derived from the $\Delta swa2$ strain differed from that obtained from the wild-type, $\Delta fes1$ and $\Delta sse1$ strains (Figure 5A). Flo11p was detected as two major products, both of higher molecular weight than the predicted size of ~150 kDa, indicating they are both post-translationally modified forms of the proteins. Such modifications may include the addition of a GPI-anchor, N-glycosylation and O-mannosylation (Douglas et al., 2007). Dranginis and collaborators expressed a GPI-anchor-less variant of Flo11p that was secreted in the medium and migrated as a polypeptide with an apparent molecular weight of ~200 kDa similar to the band A in our western blots (Douglas et al., 2007). Upon purification and concentration, the protein was retained in the stacking gel and considered aggregated (Douglas et al., 2007). We do not believe that band B represents an aggregated form of the protein, because we examined Flo11p in its native context and because we were able to detect band B in the supernatant after high speed centrifugation of cell extracts (data not shown). Moreover, whereas band A remained unchanged in $\Delta arf1$ cells, band B reproducibly migrated faster (Figure 5A) suggesting fewer post-translational modifications.

Both bands A and B were found associated to the cell-wall (Figure 5B), but it is presently unclear whether they correspond to two distinct forms of Flo11p that have different post-translational modifications. Another possibility is that these bands reflect the recycling and degradation of Flo11p at the cell surface (see below).

The regulation of the expression of FLO11 is complex and integrates signals from different signaling pathways, including the MAPK and PKA pathways (Rupp et al., 1999; Sengupta et al., 2007). Moreover, as a result of an epigenetically controlled variegated expression, not all cells in a clonal population transcribe the *FLO11* gene (Bumgarner et al., 2009; Halme et al., 2004; Octavio et al., 2009). This mechanism may allow the cells to colonize or escape natural surfaces by turning on or off, respectively, the expression of *FLO11* (Octavio et al., 2009). It is not clear whether variegated expression of *FLO11* is required for proper mat formation. Additionally, it has been recently proposed that the localization of Flo11p is highly dynamic during colony formation (Vopalenska et al., 2009). A GFP-tagged variant of Flo11p was localized at the bud-neck regions of dividing cells at the early stages of colony formation, then spread throughout the cell surface at later stages (Vopalenska et al., 2009). Interestingly, when the colonies were fully developed, the GFP fluorescence weakened at the cell-surface but a strong signal appeared in the vacuoles, suggesting that GFP-Flo11p has been endocytosed and targeted for vacuolar degradation (Vopalenska et al., 2009). Hence, tight control of Flo11p expression, localization and degradation may be required to ensure proper mat formation. Thus, we propose that Flo11p is internalized via clathrin-dependent endocytosis and that the function of Swa2p in clathrin function is required for this process to occur normally. Indeed, the TPR and J-domains of Swa2p that are essential for its function *in vivo* and to maintain normal clathrin distribution (Xiao et al., 2006) are also required for proper Flo11p processing (Figure 6). Moreover, we show that Flo11p is not covalently linked to the cell-wall (Figure 5), which may facilitate its recycling by endocytosis. The fact that $\Delta clc1$ cells are slightly affected in mat formation (Figure 4B) supports this hypothesis despite normal Flo11p band

pattern and clathrin distribution (Figure 7) that may be due to the efficient activity of Swa2p in regulating the assembly of the clathrin heavy-chain alone. Because its levels are decreased in $\Delta swa2$ (Figure 5A), band A may represent a recycling/degradation-competent form. In the absence of clathrin-dependent endocytosis, band A may fail to form and band B then accumulates as seen in $\Delta swa2$, which causes defects in mat formation. It is unclear why the accumulation of band B at the cell surface of $\Delta swa2$ cells does not cause a hyperinvasive phenotype (Figure 3, 4C). We can only hypothesize at the moment that in this strain, Flo11p may not be fully active or properly exposed at the cell surface. Flo11p accumulation in $\Delta arf1$ cells did not lead to a defect in mat formation (Figure 4B). This suggests that the function of Arf1p in vesicular ER-to-Golgi transport, (Rothman and Wieland, 1996; Schekman and Orci, 1996; Stearns et al., 1990a; Stearns et al., 1990b) has distinct effects on the maturation of Flo11p, consistent with the observed difference in the electrophoretic mobility of band B (Figure 7).

Further efforts are needed to explore the dynamic processing of Flo11p and understand how perturbations in the pathways that regulate the expression, localization and possibly degradation of this protein affect the formation of biofilms. Our improved procedures to detect the different forms of Flo11p may be combined with the screen of a larger collection of mutants for defects in mat formation and invasive growth, and with the characterization of the post-translational modifications that are added to this adhesin at the different stages of its life cycle. Investigating whether similar mechanisms exist in pathogenic fungi to regulate their interaction with abiotic surfaces or their hosts will be of great interest and may provide alternate targets for drug design.

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FIGURES

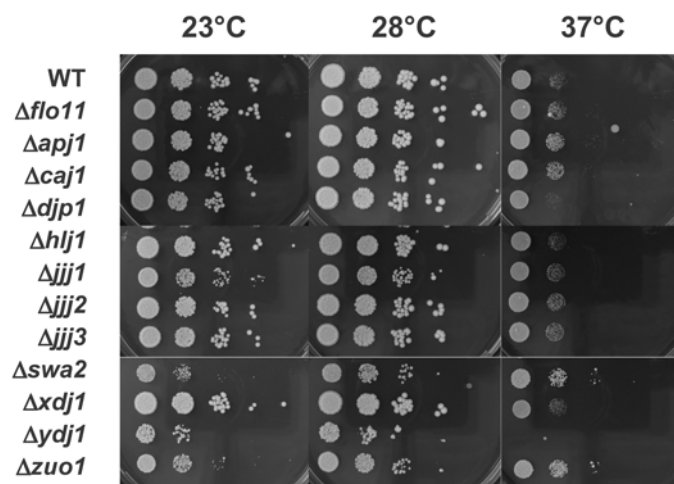


Figure 1: Growth properties of cytosolic J-proteins mutants in the $\Sigma 1278b$ genetic background. The indicated strains were grown to exponential phase ($OD_{600nm} \sim 1.0$) and ten-fold serial dilutions were spotted onto YPD plates and incubated for 2-3 days at the indicated temperatures.

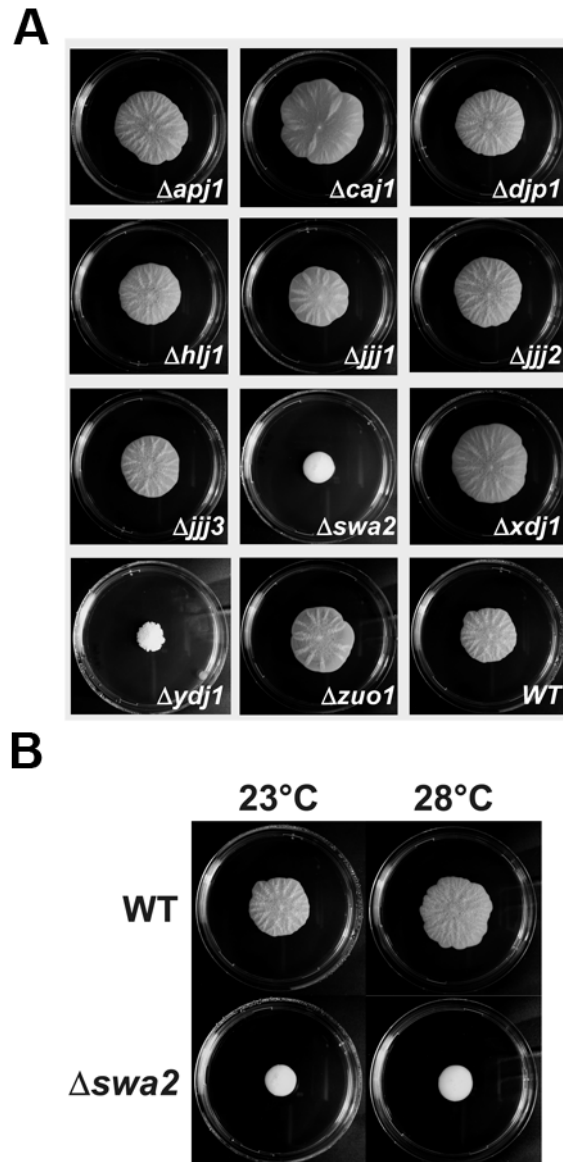


Figure 2: Ydj1p and Swa2p are required for biofilm formation. (A) The indicated strains were inoculated at the center of low-agar YPD plates and grown at 23° for 7 days before being photographed. (B) Mat formation by the wild-type and $\Delta swa2$ strains at 23° and 28°.

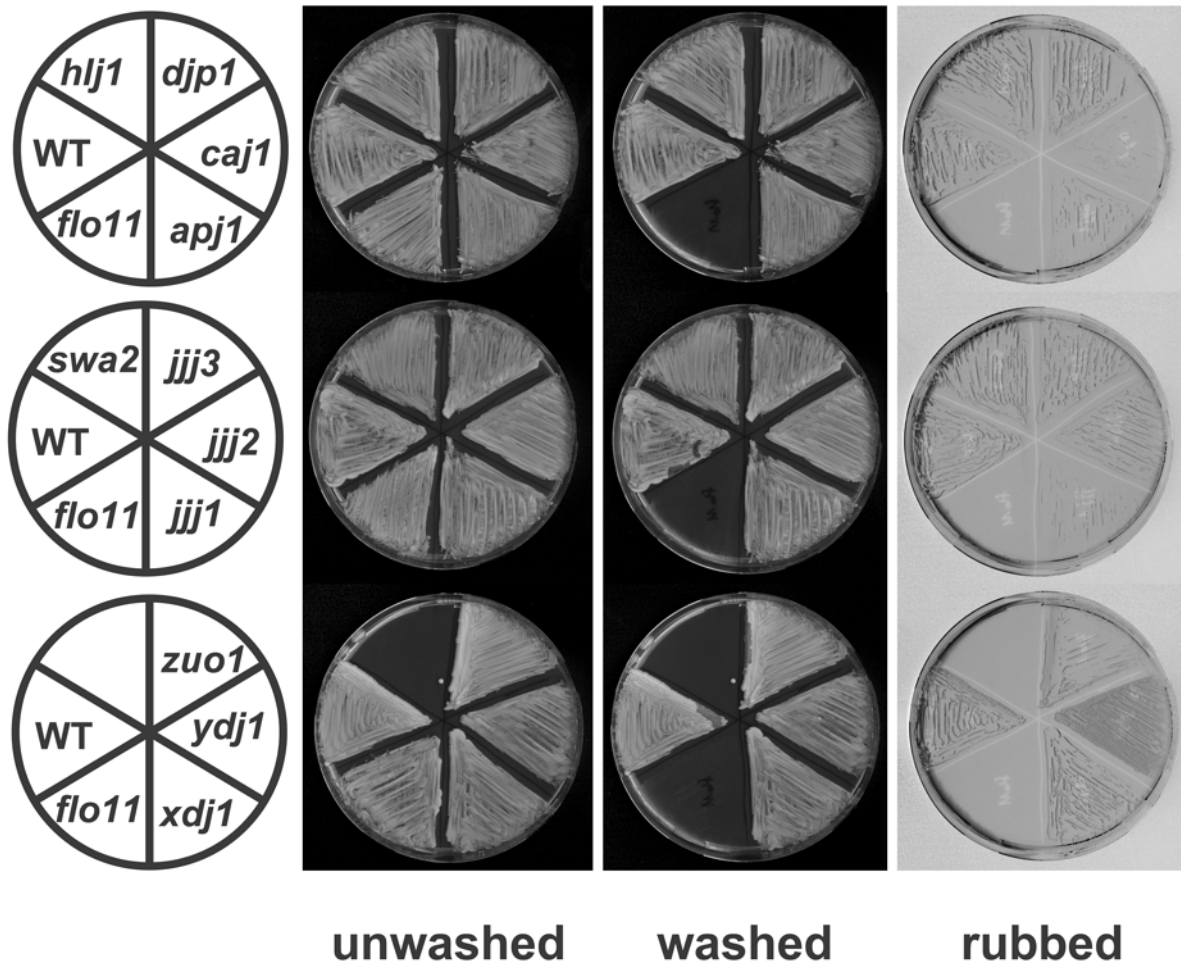


Figure 3: The $\Delta swa2$ strain exhibit normal invasive growth. The indicated strains were heavily streaked on YPD plates containing 2% agar and grown for 5 days at 28°. The plates were photographed, washed with water to remove unattached cells, and photographed again. To remove the remaining cells more effectively, the plates were rubbed with a gloved finger under a gentle stream of water and photographed again.

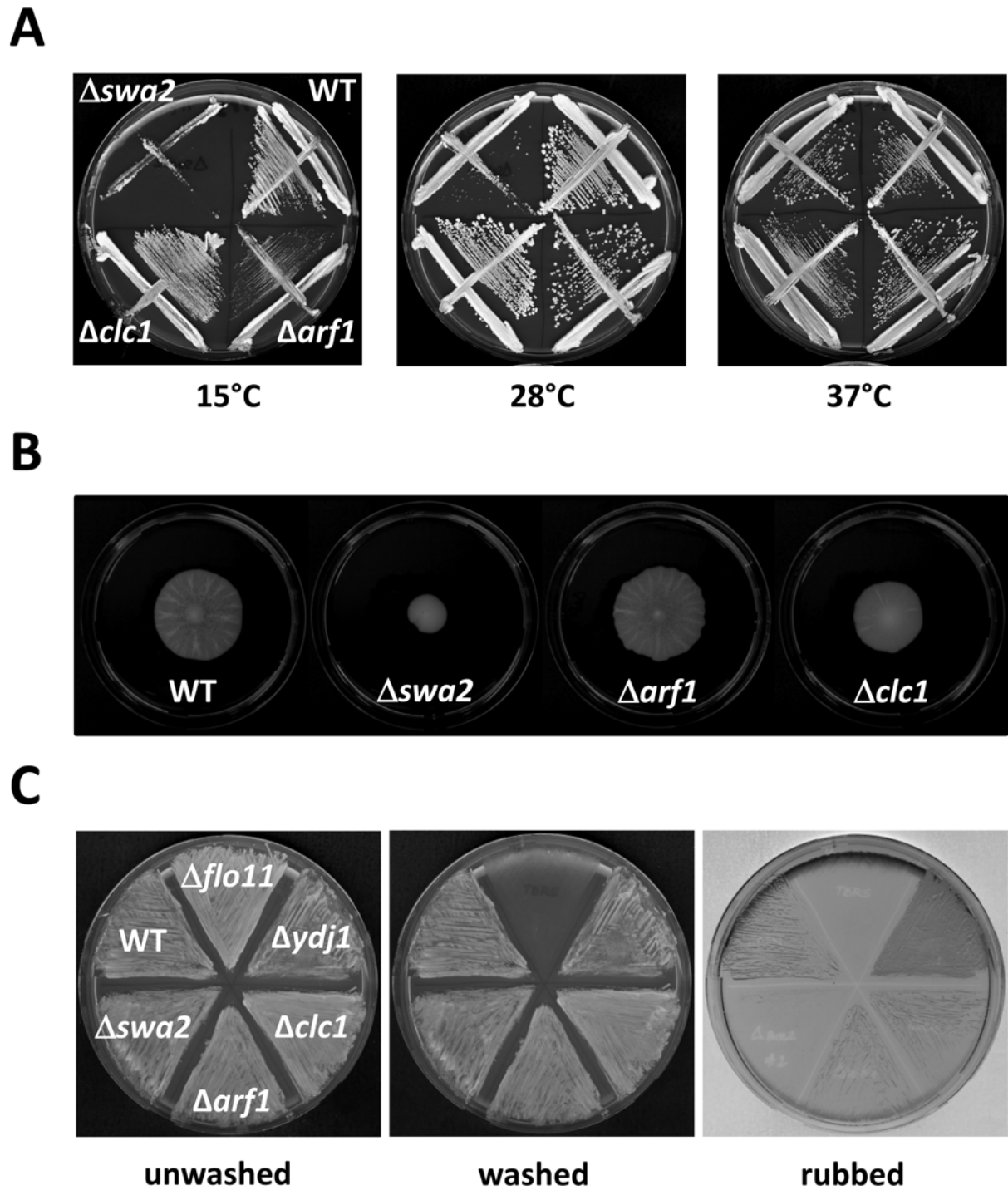


Figure 4: Mat formation and invasive growth in $\Delta arf1$ and $\Delta clc1$ mutants. (A) The indicated strains (in the JK371 background) were streaked onto YPD plates and incubated for 3-7 days at the indicated temperatures. (B) The indicated strains were tested for mat formation for 7 days at 23°. (C) The invasive growth of the indicated strains grown for 5 days at 23° was tested as described in figure 3.

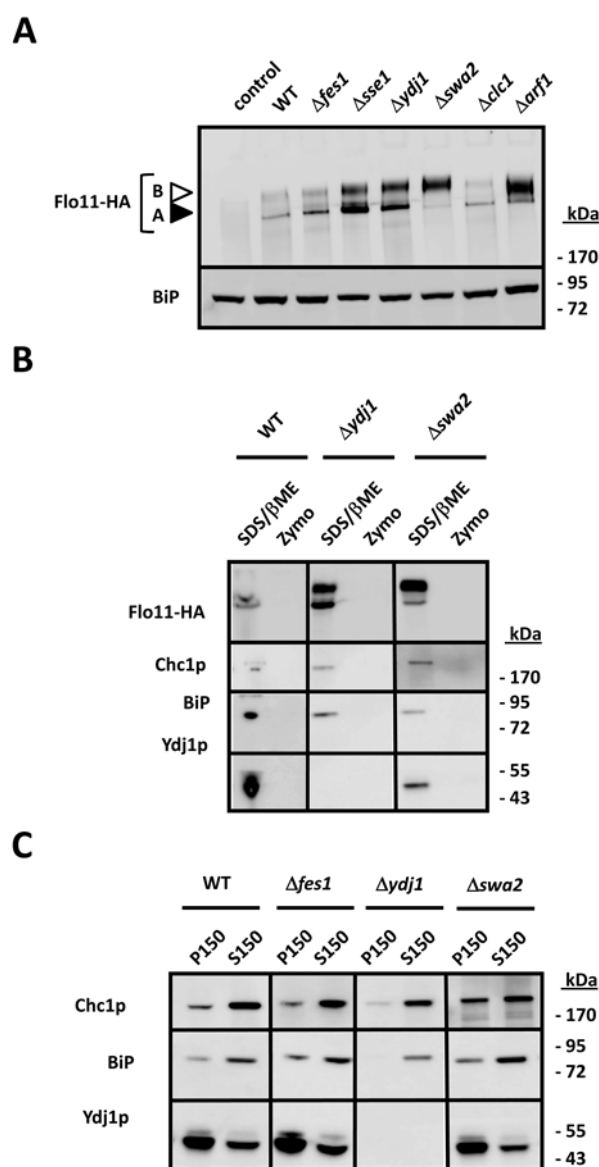


Figure 5: The maturation of Flo11p is compromised in $\Delta swa2$ and $\Delta arf1$ cells. (A) Total cell extracts were prepared as described in (von der Haar, 2007) from the untagged wild-type strain (control) or from the indicated strains expressing HA-tagged Flo11p that were grown at 23° to late exponential phase. Equal amounts of proteins were resolved by SDS-PAGE on 4-12% polyacrylamide gels and analyzed by Western blotting with anti-HA and anti-BiP antibodies. Flo11-HA is detected as two high molecular weight bands: band A (filled arrow) and band B (empty arrow). (B) Total cell extracts were prepared from the indicated strains grown at 23° to late exponential phase (see Materials and Methods). Cell-walls were recovered by centrifugation of the lysates at 4000g for 5 min followed by extensive washes with 1M NaCl, 1 mM PMSF. Proteins not covalently associated to the cell-walls were extracted with SDS/ β -mercaptoethanol (SDS/ β ME) for 10 min at 90°. The cell-walls were subsequently washed with water and then treated with Zymolyase (Zymo) for 3 h at 30° to extract covalently-linked proteins. Proteins were loaded onto 4-12% polyacrylamide gels and analyzed by Western blotting with the appropriate antibodies. (C) Cell extracts were prepared from the indicated strains grown at 23° to late exponential phase (see Materials and Methods). The lysates were centrifuged at 4000g for 5 min to pellet cell-walls, debris and unbroken cells; the cleared lysates were then centrifuged at 13000g for 15 min and the resulting

supernatants were subsequently centrifuged at 150000g for 90 min to generate the P150 pellet and S150 supernatant fractions. The P150 pellets were resuspended in the same volume of buffer as the S150 supernatants, and these fractions were analyzed by SDS-PAGE and western blot using antibodies against the indicated proteins.

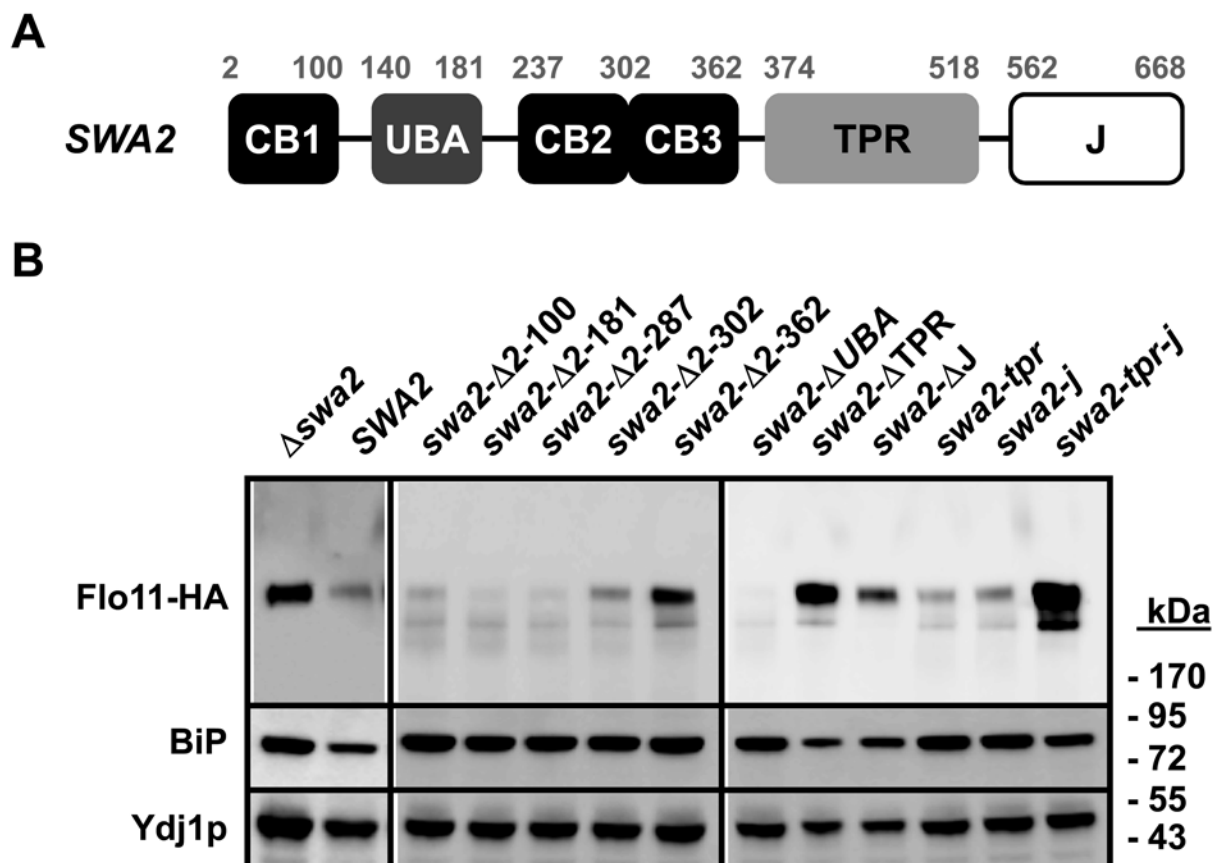


Figure 6: Requirement for Swa2p domains for its function in mat formation. (A) Scheme of the modular structure of Swa2p (CB1-3: clathrin-binding domains; UBA: ubiquitin-association domain; TPR: tetratricopeptide repeat domain; J: J-domain). Numbers point to residues delimiting each domain (Xiao et al., 2006). (B) Total cell extracts were prepared as described in Figure 5A from $\Delta swa2$ cells bearing plasmids expressing the indicated *swa2* alleles (Xiao et al., 2006) and grown in SD medium without leucine at 23° to late exponential phase. Equal amounts of proteins were resolved by SDS-PAGE on 4-12% polyacrylamide gels and analyzed by Western blotting with anti-HA, anti-BiP and anti-Ydj1p antibodies (see Material and Methods).

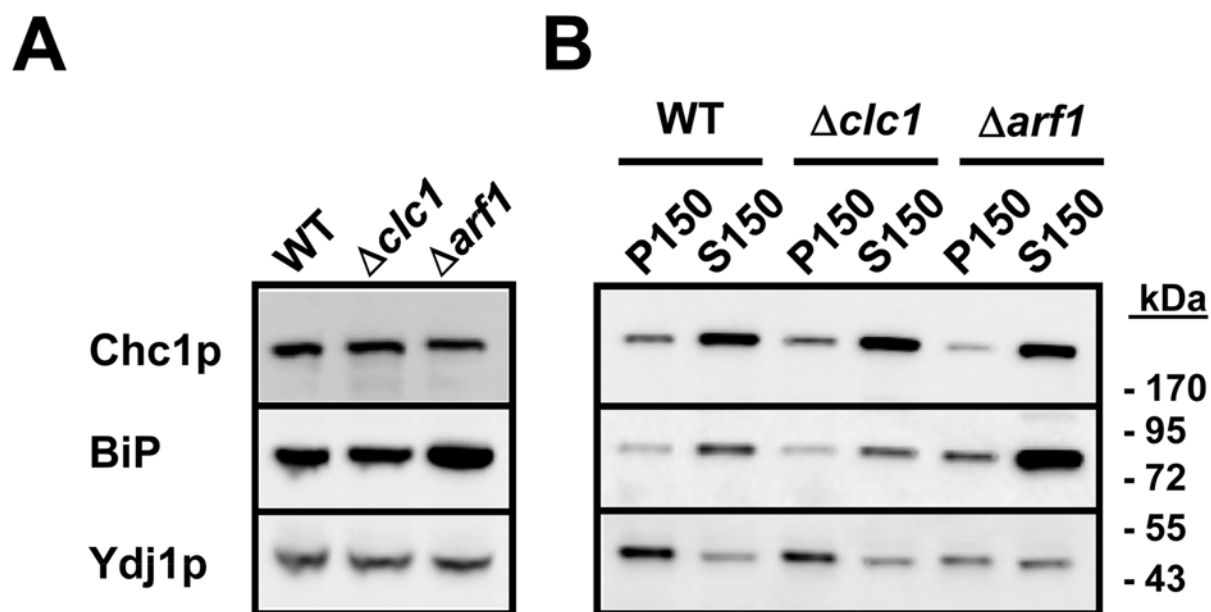


Figure 7: $\Delta arf1$, but not $\Delta clc1$, exhibit a defect in Flo11-HA processing which is not related to a defect in clathrin function. (A) Total cell extracts from the indicated strains were prepared as described in Figure 5A and analyzed by SDS-PAGE and western blot using antibodies against the indicated proteins. (B) P150 and S150 fractions were obtained from the indicated strains as described in Figure 5C and analyzed by SDS-PAGE and western blot using appropriate antibodies.

Table 1: Yeast strains used in this study

Strain	Genotype	Reference
TBR1	MAT α <i>ura3-52 leu2::hisG his3::hisG</i>	(Reynolds and Fink, 2001)
TBR5	MAT α <i>ura3-52 leu2::hisG his3::hisG flo11::kanMX6</i>	(Reynolds and Fink, 2001)
JK371	MAT α <i>ura3-52 leu2::hisG his3::hisG FLO11::HA</i>	(Park et al., 2006)
MKSC17	MAT α <i>ura3-52 leu2::hisG his3::hisG fes1::HIS3MX6</i>	(Martineau et al., 2007)
MKSC19	MAT α <i>ura3-52 leu2::hisG his3::hisG sse1::HIS3MX6</i>	(Martineau et al., 2007)
MKSC22	MAT α <i>ura3-52 leu2::hisG his3::hisG ydj1::HIS3MX6</i>	(Martineau et al., 2007)
MKSC24	MAT α <i>ura3-52 leu2::hisG his3::hisG FLO11::HA fes1::HIS3MX6</i>	(Martineau et al., 2007)
MKSC25	MAT α <i>ura3-52 leu2::hisG his3::hisG FLO11::HA sse1::HIS3MX6</i>	(Martineau et al., 2007)
MKSC26	MAT α <i>ura3-52 leu2::hisG his3::hisG FLO11::HA ydj1::HIS3MX6</i>	(Martineau et al., 2007)
MKSC29	MAT α <i>ura3-52 leu2::hisG his3::hisG caj1::HIS3MX6</i>	This study
MKSC30	MAT α <i>ura3-52 leu2::hisG his3::hisG apj1::HIS3MX6</i>	This study
MKSC31	MAT α <i>ura3-52 leu2::hisG his3::hisG djp1::HIS3MX6</i>	This study
MKSC32	MAT α <i>ura3-52 leu2::hisG his3::hisG hlj1::HIS3MX6</i>	This study
MKSC33	MAT α <i>ura3-52 leu2::hisG his3::hisG jjj1::HIS3MX6</i>	This study
MKSC34	MAT α <i>ura3-52 leu2::hisG his3::hisG jjj2::HIS3MX6</i>	This study
MKSC35	MAT α <i>ura3-52 leu2::hisG his3::hisG jjj3::HIS3MX6</i>	This study
MKSC36	MAT α <i>ura3-52 leu2::hisG his3::hisG swa2::HIS3MX6</i>	This study
MKSC37	MAT α <i>ura3-52 leu2::hisG his3::hisG xdj1::HIS3MX6</i>	This study
MKSC38	MAT α <i>ura3-52 leu2::hisG his3::hisG zuo1::HIS3MX6</i>	This study
MKSC39	MAT α <i>ura3-52 leu2::hisG his3::hisG FLO11::HA swa2::HIS3MX6</i>	This study
MKSC40	MAT α <i>ura3-52 leu2::hisG his3::hisG FLO11::HA clc1::HIS3MX6</i>	This study
MKSC41	MAT α <i>ura3-52 leu2::hisG his3::hisG FLO11::HA arf1::HIS3MX6</i>	This study

III. Caractérisation du système Hsp70 de *Yarrowia lipolytica*

III.A. Contexte

Yarrowia lipolytica (nommée successivement *Candida*, *Endomycopsis* ou *Saccharomyces lipolytica*) est une levure non conventionnelle, par opposition aux levures modèles *Saccharomyces cerevisiae* ou *Schizosaccharomyces pombe*. *Yarrowia lipolytica* est un ascomycète hétérothallique, non pathogène, aérobic stricte et se développant à une température optimale de 28°C (Barth and Gaillardin, 1997). C'est une levure dimorphique se développant par croissance végétative ou en formant des pseudo-hyphes ou des hyphes septées, en fonction des conditions environnementales. *Yarrowia lipolytica* a été isolée à partir de produits laitiers et carnés fermentés. Elle peut se développer sur glucose, *n*-alcanes, éthanol, 1-alcènes, acides gras ou acétate comme seule source de carbone (Barth and Gaillardin, 1997). *Yarrowia lipolytica* est également capable de sécréter des métabolites et des protéines en grande quantité afin de s'adapter à son milieu en croissance (Barth and Gaillardin, 1997).

Elle fut étudiée à l'origine pour des raisons industrielles, notamment pour sa capacité à se développer sur *n*-paraffines comme seule source de carbone. Sa capacité naturelle de sécrétion en a fait un candidat de choix pour l'étude la voie sécrétoire d'un point de vue fondamental mais également biotechnologique pour la production de protéines hétérologues. L'étude de la voie sécrétoire chez cette levure a également permis la découverte du facteur d'échange Sls1 (Boisrame et al., 1998 ; Kabani et al., 2000a).

Le génome nucléaire de *Yarrowia lipolytica* a une taille d'environ 20 Mpb, réparties en six chromosomes ; et il a été entièrement séquencé dans le cadre du projet Genolevures (Casaregola et al., 1997 ; Dujon et al., 2004). Dans l'arbre phylogénétique des Hémiascomycètes, elle est localisée sur une branche isolée, divergeant de celles des autres levures dès l'origine de l'arbre (Figure 16) (Barns et al., 1991 ; Fischer et al., 2006).

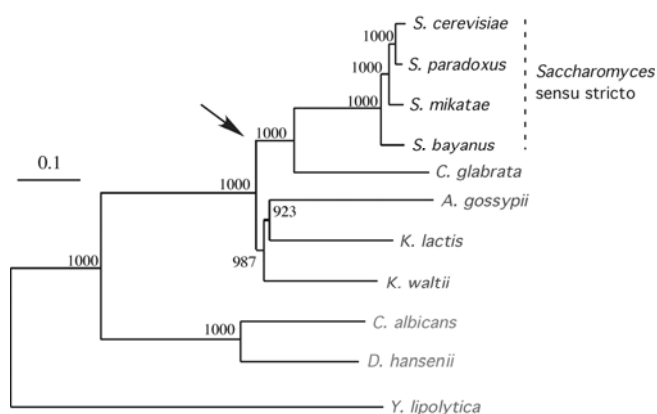


Figure 16 : Arbre phylogénétique des Hémiascomycètes

Cet arbre a été construit à partir des séquences concaténées de 25 protéines ayant des orthologues définis chez les 11 espèces. La flèche situe l'évènement de duplication totale du génome.

Figure adaptée d'après (Fischer et al., 2006).

Les analyses phylogénétiques indiquent que *Yarrowia lipolytica* a considérablement divergé des autres levures et serait phylogénétiquement plus proche des eucaryotes supérieurs : génome à haut pourcentage en GC (environ 50%), ARNsn et ARN7S proches de ceux des eucaryotes supérieurs, séquences des ADNr 18S et 26S divergentes de celles des autres levures par exemple (Casaregola et al., 1997 ; Casaregola et al., 2000 ; Fischer et al., 2006). *Yarrowia lipolytica* possède également des homologues de protéines eucaryotes comme Bag1 et CHIP, inexistantes chez *Saccharomyces cerevisiae* notamment.

La culture de cette levure est maîtrisée et de nombreux outils génétiques sont disponibles, comme par exemple les transformations répllicative et intégrative, des techniques d'inactivation, des vecteurs de clonage et d'expression, et différentes banques (génomique, de mutagenèse, double-hybride, etc) (Fickers et al., 2003 ; Gaillardin et al., 1985 ; Kabani et al., 2000b; Neuveglise et al., 1998).

III.B. Approche

Une carte d'interactions génétiques entre des composants de la machinerie Hsp70 et d'autres chaperons ou cofacteurs a été réalisée chez la levure *Yarrowia lipolytica* par la construction de simples et doubles mutants (présentés dans la Table 1, page suivante). Des spécificités fonctionnelles entre Hsp70 ont été recherchées par une analyse phénotypique des souches. Différents phénotypes ont été analysés : la croissance en conditions optimales ou sur différents substrats, la croissance en conditions de stress (thermique, drogue-dépendant) et la capacité de thermotolérance des mutants.

L'ensemble de ce travail a été réalisé en étroite collaboration avec Marie-Thérèse Le Dall dans le groupe du Dr Jean-Marie Beckerich, du Laboratoire de Microbiologie et Génétique Moléculaire (UMR CNRS-INRA-AgroParisTech, Thiverval-Grignon, France). Marie-Thérèse Le Dall a participé à toutes les étapes de ce projet, et sa contribution est équivalente à la mienne.

Souche sauvage	
WT136463 (<i>MatB, scr1::ADE1, his-1, leu2, ura3</i>)	
Simplees invalidations	Doubles invalidations
$\Delta ssa5$	$\Delta ssa5\Delta bag1$
$\Delta ssa6$	$\Delta ssa6\Delta bag1$
$\Delta ssa7$	$\Delta ssa7\Delta bag1$
$\Delta ssa8$	$\Delta ssa8\Delta bag1$
$\Delta bag1$	$\Delta ssa5\Delta chip$
$\Delta chip$	$\Delta ssa6\Delta chip$
$\Delta hsp104$	$\Delta ssa7\Delta chip$
	$\Delta ssa8\Delta chip$
	$\Delta ssa5\Delta hsp104$
	$\Delta ssa6\Delta hsp104$
	$\Delta ssa7\Delta hsp104$
	$\Delta ssa8\Delta hsp104$
Souches de <i>Pop-in</i>	
<i>Pop-in BAG1</i>	
<i>Pop-in FES1</i>	
<i>Pop-in SSE1</i>	

Table 1 : Souches utilisées lors de cette étude

III.C. Stratégie de construction des mutants

Les mutants ont été construits par invalidation de l'ORF (*Open Reading Frame* ; phase ouverte de lecture) des gènes correspondants en utilisant des cassettes d'invalidation (Fickers et al., 2003) ou la technique de *Pop-in/Pop-out*. Cette dernière a été utilisée pour l'invalidation de gènes dont nous pouvions supposer qu'ils étaient essentiels.

III.C.1. Exemple d'invalidation par intégration homologue d'une cassette PHT

Les cassettes d'invalidation sont constituées du promoteur (P) et du terminateur (T) du gène à invalider, encadrant un gène de résistance à l'Hygromycine (H) flanqué des régions d'excision *loxR/P*. Une cassette PHT a par exemple été utilisée pour l'invalidation du gène *HSP104* (Figure 17, page suivante).

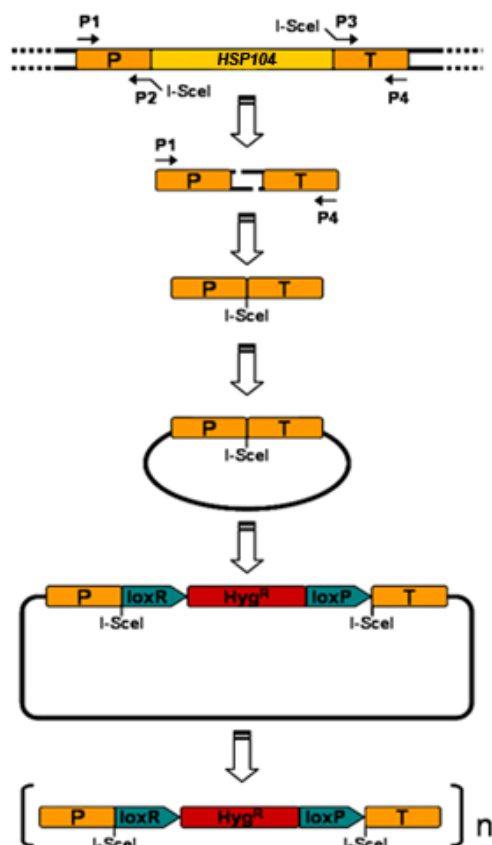


Figure 17 : Construction de la cassette PHT pour l'inactivation du gène *HSP104*

Le détail des étapes est décrit ci-dessous.

Le promoteur et le terminateur de *HSP104*, correspondant respectivement à des régions de 1 kpb en amont et en aval de l'ORF, ont été amplifiés à partir d'ADN génomique de la souche WT136463 avec les oligonucléotides P1 et P2 pour le promoteur et P3 et P4 pour le terminateur. Les oligonucléotides P2 et P3 possèdent une extension en 5' comprenant le site de restriction rare *I-SceI*. Les produits d'amplification par PCR P et T ont été réunis par une seconde étape d'amplification avec les oligonucléotides P1 et P4. La cassette PT ainsi obtenue a été clonée dans un vecteur pBluescript SK⁻ au site *EcoRV*. Le gène de résistance à l'hygromycine, flanqué des régions *loxR* et *loxP*, a été cloné au niveau du site *I-SceI* (Fickers et al., 2003). La cassette PHT a été excisée du vecteur et utilisée pour la transformation de la souche WT136463. Les transformants ont été sélectionnés sur milieu YPD (*Yeast-Extract-Peptone-Dextrose* ; 1% d'extrait de levure, 2% de bacto-peptone, 2% de glucose, 2% de bacto-agar) contenant 200 µg/mL d'hygromycine. Ils ont ensuite été analysés par extraction d'ADN génomique, PCR et Southern Blot (Figure 18, page suivante).

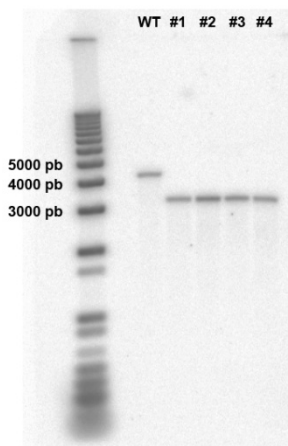


Figure 18 : Analyse par Southern blot des transformants de la souche WT136463 par la cassette PHT(*HSP104*)

Les ADN génomiques ont été digérés par *Nsi*I et le terminateur de *HSP104* a été utilisé comme sonde. La taille des bandes attendues est de 4183 pb pour la souche sauvage WT136463 (WT) et 3144 pb pour un mutant $\Delta hsp104$. #1 à #4 indique la numérotation des transformants.

Les mutants ont été transformés par un plasmide *LEU2* permettant l'expression de la recombinase Cre (pLEU2-Cre) et sélectionnés sur milieu YNB (*Yeast Nitrogen Base* ; 0,17% de YNB (Difco®) sans acide- aminé ni sulfate d'ammonium, 0,5% de sulfate d'ammonium, 2% de glucose, 2% de bacto-agar) sans leucine (Fickers et al., 2003). Cette étape a permis de recycler le marqueur de résistance à l'hygromycine par recombinaison spécifique entre les régions *loxR* et *loxP*. Le plasmide pLEU2-Cre a été éliminé par culture en milieu riche. L'élimination du marqueur et du plasmide a été vérifiée par répliques sur milieu YNB sans leucine et sur milieu additionné d'hygromycine.

III.C.2. Exemple d'invalidation par *Pop-in/Pop-out*

La technique de PopIn-PopOut utilise le vecteur pBluescript SK⁻ contenant la cassette PT correspondant au gène à invalider (présenté ci-dessus). La cassette PT est excisée du vecteur pBluescript SK⁻ au niveau des sites *Cla*I et *Bam*HI et clonée aux mêmes sites dans un vecteur pINA300' (*URA3*).

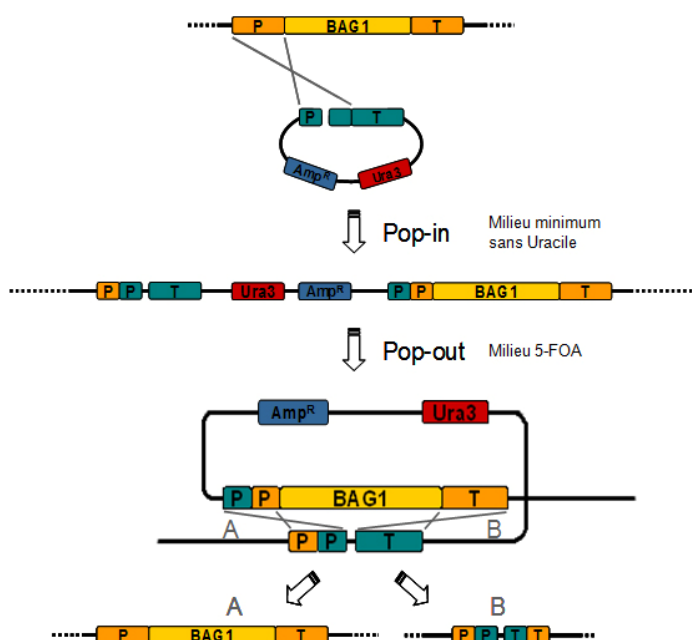


Figure 19 : Construction de la souche $\Delta BAG1$ par technique de *Pop-in/Pop-out*

Les différentes étapes sont décrites ci-après.

Le *Pop-in/Pop-out* a par exemple été utilisé pour la construction du mutant $\Delta bag1$ (Figure 19, page précédente). La cassette PT correspondante a été clonée dans le vecteur pINA300'-*URA3*. Le vecteur a été linéarisé au niveau du site unique *NotI* présent dans le promoteur de *BAG1*. La souche WT136463 a été transformée par le vecteur linéarisé et les transformants sélectionnés sur milieu YNB dépourvu d'uracile. Les clones ont été analysés par Southern blot pour identifier les clones *Pop-in* (intégration du vecteur en une seule copie au locus *BAG1*) (Figure 20.a). Le PopOut correspond à l'excision du vecteur pINA300' par recombinaison homologue entre les régions répétées, promoteur ou terminateur. Dans le cas de *BAG1*, si le *Pop-out* a lieu au niveau du promoteur (P), le gène sauvage est conservé (cas A). S'il a lieu au niveau du terminateur (T), alors le gène est invalidé (cas B). Il a été réalisé à partir de deux clones *Pop-in* indépendants (#1 et #3). Les clones ont été cultivés sur milieu YPD puis sur milieu YNB dépourvu d'uracile. La recombinaison a été provoquée par une culture sur milieu minimum additionné de 1,25 g/L de 5-FOA, un analogue toxique de l'uracile permettant la contre-sélection des cellules [*ura3+*]. Les clones se développant sur ce milieu ont été analysés par Southern blot (Figure 20.b).

Les doubles mutants ont été réalisés par invalidation des gènes codant *Ssa5-8* dans les souches $\Delta bag1$, $\Delta chip$ et $\Delta hsp104$.

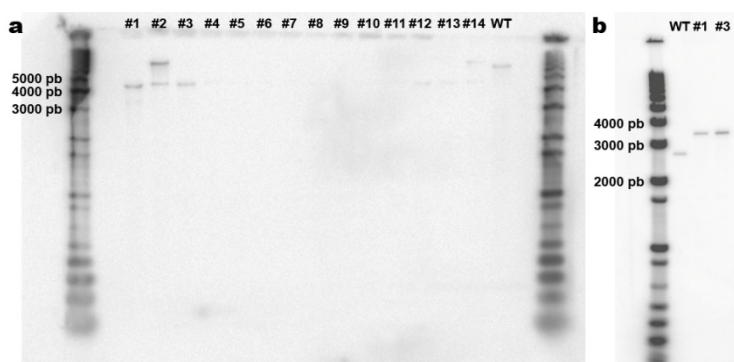


Figure 20 : Southern blots des souches *Pop-in* et *Pop-out* de *BAG1*

a. Southern blot des souches de *Pop-in*. Les ADN génomiques ont été digérés par *PstI* et l'ORF *BAG1* a été utilisée comme sonde. La taille des bandes attendues est de 6767 pb pour la souche sauvage WT136463 (WT) et de 4517 pb pour un mutant *Pop-in* *BAG1*.

b. Southern blot des souches de *Pop-out*. Les ADN génomiques ont été digérés par *SphI* et le terminateur de *BAG1* a été utilisé comme sonde. La taille des bandes attendues est de 2591 pb pour la souche sauvage WT136463 (WT) et 3257 pb pour un mutant $\Delta bag1$.

III.D. Recherche de spécificités fonctionnelles des *Hsp70* par analyse phénotypique des mutants

III.D.1. Croissance à différentes températures

Dans des conditions optimales de croissance, c'est-à-dire une culture sur milieu YPD à 28°C, la redondance fonctionnelle des *Hsp70* ne permet pas d'observer de différence de

croissance entre les simples mutants $\Delta ssa5$ à $\Delta ssa8$ (Figure 21). De plus, la croissance des mutants est similaire à celle de la souche WT136463, cette redondance fonctionnelle compense donc en apparence l'invalidation des gènes *SSA5* à *SSA8*. Les mêmes observations sont faites pour une croissance à 23°C ou en conditions de stress thermique (34 et 35°C), 35°C étant la température maximale de croissance de *Yarrowia lipolytica*.

Les simples mutants $\Delta bag1$, $\Delta chip$ et $\Delta hsp104$ et les doubles mutants $\Delta bag1\Delta ssa$, $\Delta chip\Delta ssa$ et $\Delta hsp104\Delta ssa$ se comportent de la même manière que la souche WT136463 quelle que soit la température de croissance. Seul le mutant $\Delta chip\Delta ssa6$ a une croissance légèrement ralentie (Figure 21).

Ainsi, la machinerie Hsp70 semble robuste et tolère la perte de certains composants qui est compensée par une redondance fonctionnelle.

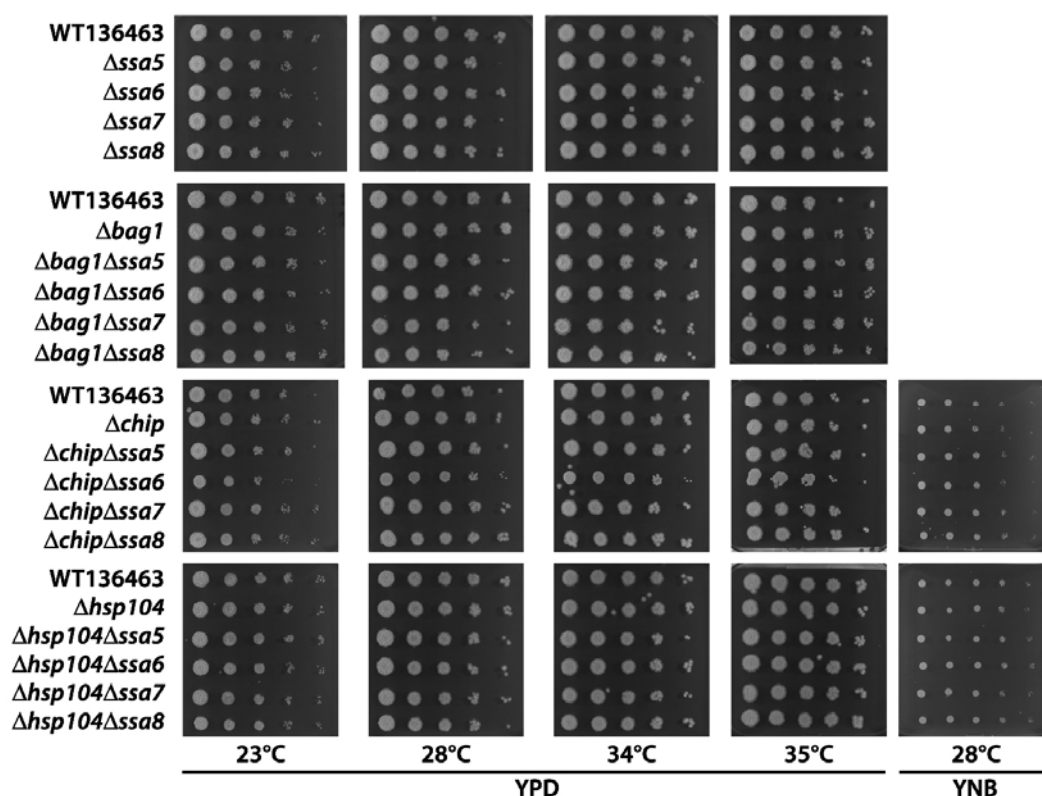


Figure 21 : Croissance des mutants à différentes températures

YPD. Pour chaque souche, une préculture de nuit en YPD à 28°C sous agitation a été diluée à une DO_{600nm} (Densité Optique) de 0,2 en YPD et incubée à 28°C sous agitation jusqu'à une DO_{600nm} de 0,6 (phase exponentielle de croissance). Les cultures ont été normalisées à une DO_{600nm} de 0,5 puis diluées en série au $1/10^{ème}$ jusqu'à la dilution 10^{-4} . 5 μL des dilutions 1 à 1.10^{-4} ont été déposés sur milieu YPD.

YNB. Le même protocole que ci-dessus a été suivi avec deux modifications. Les précultures ont été diluées en milieu minimum. 5 μL des dilutions 1 à 1.10^{-4} ont été déposés sur ce même milieu.

Les boîtes de Petri ont été incubées 48 heures aux températures indiquées. Ces manipulations ont été réalisées une fois avec dépôt des gouttes en duplicate.

III.D.2. Croissance sous diverses conditions de stress et sur différents substrats

Des tests en gouttes en présence de divers agents toxiques (cadmium, DiThioThreitol (DTT), hygromycine, L-canavanine) ont été réalisés pour analyser la réponse des mutants à différents types de stress. Quel que soit le type de stress (lié aux métaux lourds, aux agents réducteurs, ...) aucune différence de croissance réellement significative n'a pu être observée entre les mutants (Figure 22). De plus, la croissance des mutants est comparable à celle de la souche WT136463 dans une grande majorité des cas.

L'effet des invalidations sur le métabolisme particulier de *Yarrowia lipolytica* a également été analysé par une croissance sur acide gras, substrat qu'elle est capable d'assimiler comme seule source de carbone, et par une croissance sur gélose au lait qui révèle la sécrétion de protéases dans le milieu extérieur (1 % de lait écrémé) (Figure 22). L'activité protéolytique, détectée par la formation d'un halo autour des levures n'est pas significativement affectée dans les mutants, tout comme la croissance sur acide gras. Certaines différences de croissance peuvent tout de même être observées. Par exemple, les mutants $\Delta bag1\Delta ssa7$, $\Delta bag1\Delta ssa8$ et $\Delta hsp104\Delta ssa7$ semblent mieux se développer sur milieu oléate que les autres souches.

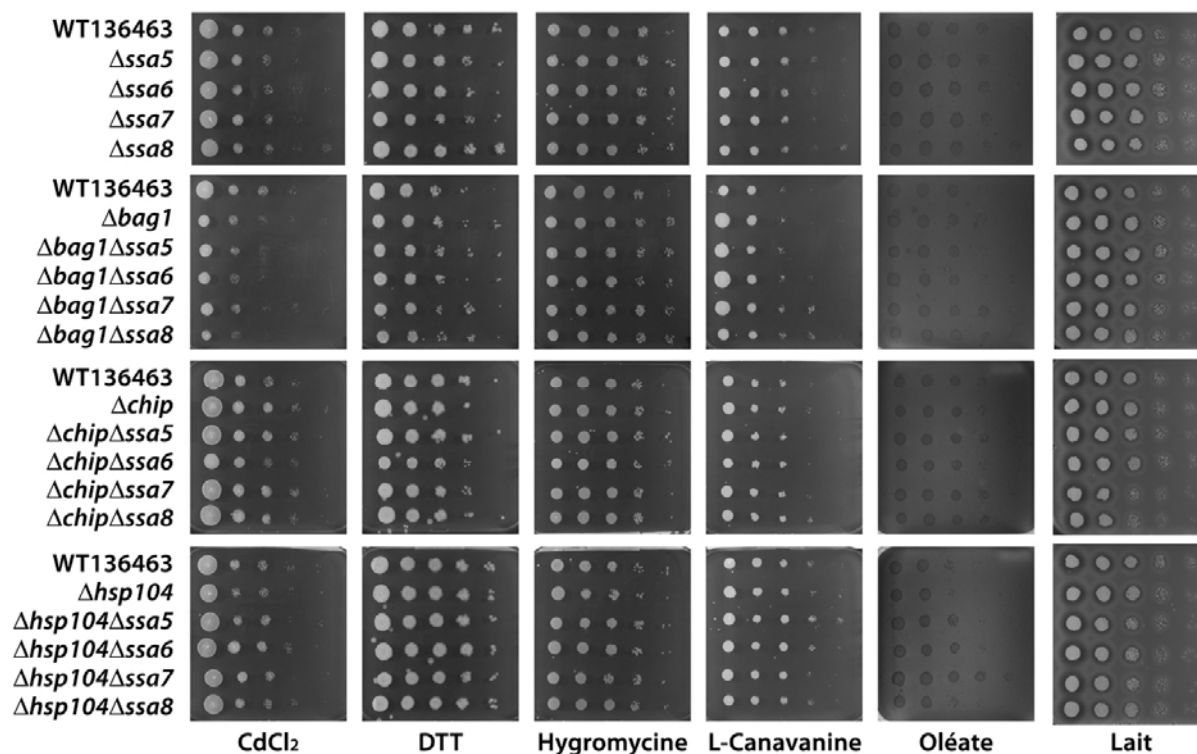


Figure 22 : Croissance des mutants en présence d'agents toxiques ou sur différents substrats

Le protocole suivi est le même qu'en Figure 21. Les précultures ont été diluées en milieu YPD ou milieu minimum en fonction du milieu de dépôt des gouttes. 5 µL des dilutions 1 à 1.10⁻⁴ ont été déposés sur les milieux suivants et les boîtes de Petri ont été incubées à 28°C.

CdCl₂ : YPD contenant 15 µM de CdCl₂; **DTT** : YPD contenant 5 mM de DTT ; **Hygromycine** : YPD contenant 10 µg/mL d'hygromycine ; **L-Canavanine** : YNB (+ Pro, His, Leu, Ura) contenant 0,05 µg/mL de L-canavanine ; **Oléate** : 0,17 % YNB, 0,5 % (NH₄)₂SO₄, 50 mM KH₂PO₄/KHPO₄ pH 6.8, 1 % oléate, 0,125 % Tween 40, 2 % bacto agar, proline, histidine, leucine, uracile (photos en contraste inversé); **Lait** : 0,17 % YNB, 100 mM KH₂PO₄/Na₂HPO₄ pH 6.8, 1 % lait écrémé Difco[®], 1,7 % bacto-agar, proline, histidine, leucine, uracile.

Ces manipulations ont été réalisées une fois avec dépôt des gouttes en duplicate.

Chez *Saccharomyces cerevisiae*, une mort cellulaire proche de l'apoptose peut-être déclenchée naturellement par le vieillissement de la cellule, et peut être induite par des stress environnementaux dont l'exposition à des métabolites toxiques (Sharon et al., 2009). Il a été montré que la production d'espèces réactives de l'oxygène provoque ce type de mort cellulaire (Madeo et al., 1999). De plus, l'acide acétique peut engendrer un tel stress oxydatif conduisant à l'apoptose (Ludovico et al., 2001).

Des tests de croissance après exposition à différentes concentrations d'acide acétique ont donc été réalisés. La croissance de la souche sauvage WT136463 est affectée après augmentation progressive de la concentration en acide acétique (Figure 23). Ici encore, les différences de développement entre les souches sont faibles. Les mutants $\Delta bag1\Delta ssa7$, et $\Delta chip\Delta ssa6$ semblent plus sensibles à ce type de stress que les autres mutants. En revanche, l'inactivation de *HSP104* semble augmenter la résistance des souches au traitement à l'acide acétique. Ici encore, ces résultats demandent à être reproduits voire affinés avant d'être interprétés.

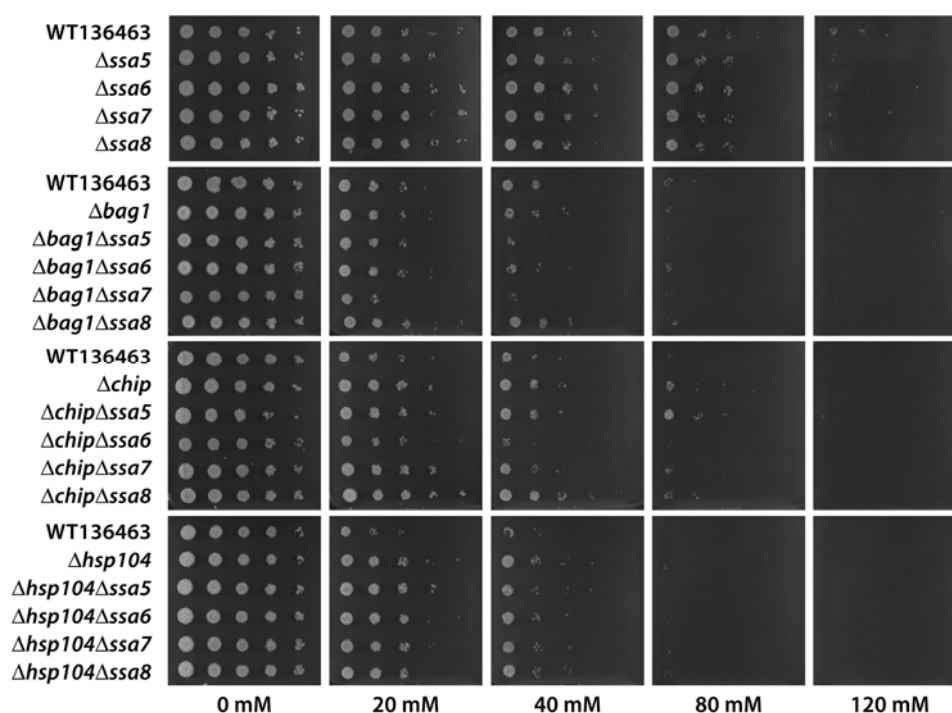


Figure 23 : Résistance des mutants à un agent pro-apoptotique

Pour chaque souche, une préculture de nuit en YPD à 28°C sous agitation a été diluée à une DO_{600nm} de 0,2 en YPD et incubée à 28°C sous agitation jusqu'à une DO_{600nm} de 0,6 (phase exponentielle de croissance). Les cultures ont été normalisées à une DO_{600nm} de 0,5 et réparties en 5 tubes de 1 mL. Les tubes ont été centrifugés 2 minutes à 800 g. Les culots ont été repris dans 1 mL de YPD-HCl pH 3.0 contenant de 0, 20, 40, 80 ou 120 mM d'acide acétique. Ces cultures ont été incubées sur roue tournante 200 minutes à 28°C puis diluées en série au $1/10^{ème}$ jusqu'à la dilution 10^{-4} . 5 μ L des dilutions 1 à 1.10^{-4} ont été déposés sur milieu YPD et les boîtes de Petri ont été incubées à 28°C.

III.D.3. Thermotolérance

Les capacités de thermotolérance basale et acquise de chaque mutant ont été comparées.

La thermotolérance basale correspond à la réponse des cellules à l'exposition à une température létale, sans phase d'adaptation et pendant un temps déterminé.

La viabilité du mutant $\Delta hsp104$ est légèrement réduite en comparaison de celle de la souche WT136463 (Figure 24). De plus, la viabilité des doubles mutants $\Delta hsp104\Delta ssa$ est nulle dès 10 minutes de choc thermique. Ces données montrent donc que chez *Yarrowia lipolytica*, Hsp104 joue un rôle primordial qui n'est que partiellement compensé par la machinerie Hsp70 (et autres chaperons moléculaires). Une approche quantitative devra être réalisée pour confirmer ces résultats.

La thermotolérance acquise correspond à la réponse à l'exposition à une température létale après une phase d'adaptation à une température intermédiaire permettant la synthèse de protéines de stress thermique. Comme attendu, la viabilité des mutants $\Delta hsp104$ et $\Delta hsp104\Delta ssa$ est supérieure à celle observée sans phase d'adaptation (en raison de la surexpression des chaperons moléculaires inductibles) tout en restant inférieure à celle de la souche sauvage (Figure 25, page 160). Contrairement aux résultats obtenus pour la thermotolérance basale, la viabilité de $\Delta hsp104$ et celles des doubles mutants $\Delta hsp104\Delta ssa$ sont comparables, ce qui suggère l'induction de chaperons compensant l'activité de Hsp104 pendant la phase d'adaptation.

Qu'il s'agisse de thermotolérance acquise ou basale, nous n'avons pas montré de différence fonctionnelle significative entre les Ssa.

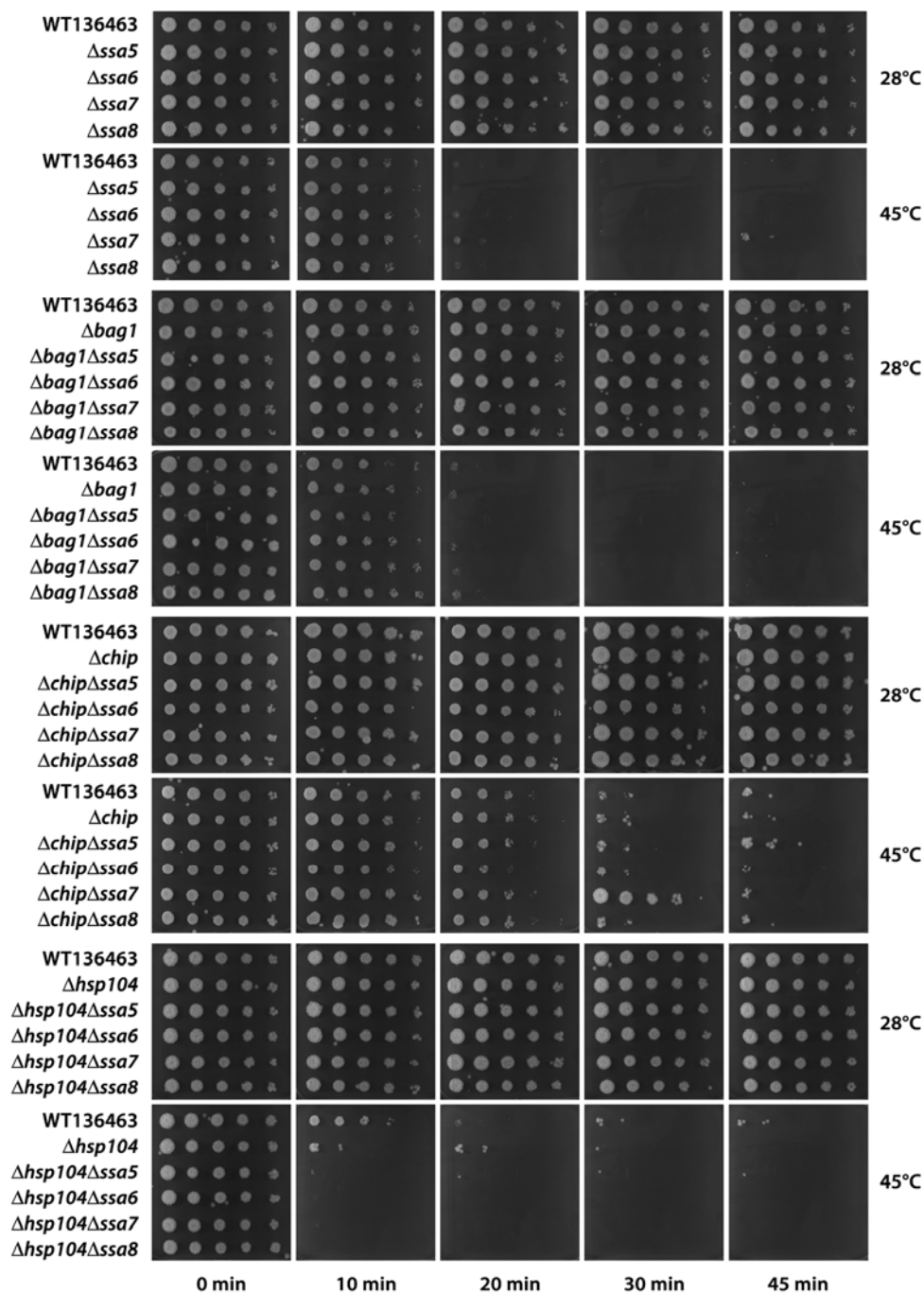


Figure 24 : Comparaison de la thermotolérance basale des mutants

Pour chaque souche, une préculture de nuit en YPD à 28°C sous agitation a été diluée à une DO_{600nm} de 0,2 en YPD et incubée à 28°C sous agitation jusqu'à une DO_{600nm} de 0,6 (phase exponentielle de croissance). Les cultures ont été normalisées à une DO_{600nm} de 0,5 et réparties en 10 tubes de 1 mL (un tube par température et par temps). Les deux séries de 5 tubes ont été incubées en parallèle à 28°C et 45°C pendant 0, 10, 20, 30 ou 45 minutes. A la fin du temps d'incubation correspondant, les tubes ont été conservés sur glace. Ces cultures ont été diluées en série au $1/10^{ème}$ jusqu'à la dilution 10^{-4} . 5 μ L des dilutions 1 à 1.10^{-4} ont été déposés sur milieu YPD et les boîtes de Petri ont été incubées à 28°C. Les souches présentes sur une même boîte ont été traitées simultanément.

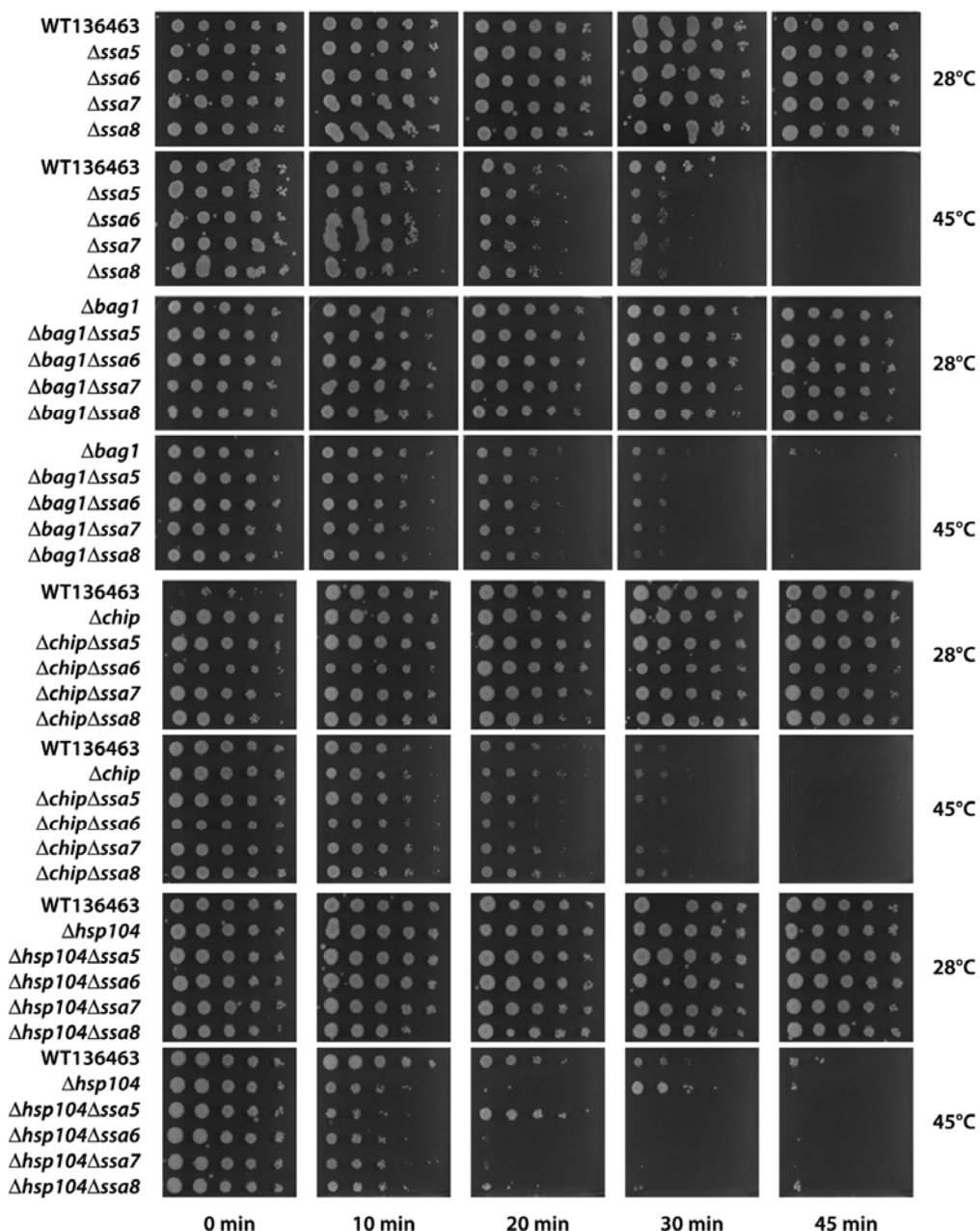


Figure 25 : Comparaison de la thermotolérance acquise des mutants

Le protocole suivi est le même que celui de la Figure 24 avec une modification. Les deux séries de 5 tubes ont été incubées 1 heure à 37°C avant d’être incubées à 28°C et 45°C.

III.E. Facteurs d’échange

L’invalidation du facteur d’échange Fes1 a été tentée par la technique de *Pop-in/Pop-out*. Aucun mutant n’a pu être obtenu suite au *Pop-out*, sur un nombre représentatif de 200 clones testés. Contrairement à chez *Saccharomyces cerevisiae*, *FES1* pourrait donc être essentiel chez *Yarrowia lipolytica* (données non publiées obtenues par Mehdi Kabani).

L’invalidation de *SSE1* a donc été abordée par la technique de *Pop-in/Pop-out*, et des tests de complémentation de mutants des facteurs d’échange ont été débutés à partir des souches

Pop-in de *BAG1*, *FES1*, et *SSE1*. Ces souches ont été transformées par les plasmides d'expression pMK1 (*LEU2*), pMK1-*BAG1*, pMK1-*FES1* et pMK1-*SSE1* de manière indépendante. Ce travail a été interrompu par manque de temps. Cependant, parmi la vingtaine de clones testés issus du *Pop-out* de la souche *Pop-in-SSE1* transformée par pMK1, aucun mutant Δ *sse1* n'a été obtenu.

III.F. Conclusion et perspectives

L'absence de différence phénotypique entre nos mutants lors de l'exposition à des stress variés confirme la robustesse du système Hsp70. Cependant, l'absence quasi-systématique de différences phénotypiques entre la souche sauvage et les mutants pourrait être expliquée par une induction permanente, même faible, des chaperons moléculaires de *Yarrowia lipolytica*. Ainsi, Anna Babour *et al.* ont montré que la voie UPR est faiblement activée chez une souche sauvage même en conditions de croissance *a priori* favorables (Babour *et al.*, 2008). Il se pourrait que dans nos conditions de culture, *Yarrowia lipolytica* soit en condition de stress permanent, ce qui lui conférerait une certaine protection basale.

Pour que les résultats obtenus puissent être interprétés de manière plus précise, chaque manipulation devrait être reproduite en comparant quantitativement la viabilité cellulaire des souches pour chaque stress. Ce type de manipulations pourrait être réalisé pour l'étude de mutants d'inactivation de plusieurs Ssa (doubles et triples mutants).

Le ralentissement de croissance de la souche Δ *chip* Δ *ssa6* corrobore des résultats obtenus précédemment. Ainsi, Mehdi Kabani a montré que l'ubiquitine ligase CHIP de *Yarrowia lipolytica* est impliquée dans la thermotolérance (données non publiées) et nous avons montré que Ssa6 est une forme inductible qui procure la plus forte thermotolérance à une souche de *Saccharomyces cerevisiae* lorsqu'elle est exprimée comme unique Hsp70 cytoplasmique (Sharma *et al.*, 2009). L'étude du rôle de CHIP dans la thermotolérance, en conjonction avec les Hsp70, Hsp104 et le système de dégradation par le protéasome pourrait être envisagée.

Yarrowia lipolytica codant *BAG1* et *CHIP*, le rôle antagoniste suggéré de Bag1 et Fes1 (induisant la dégradation ou le repliement) lorsqu'ils sont en complexe avec CHIP et une Hsp70, pourrait être étudié et rapporté à chaque paralogue de Hsp70 par une étude génétique chez cette levure (Alberti *et al.*, 2004; Ballinger *et al.*, 1999 ; Demand *et al.*, 2001 ; Meacham *et al.*, 2001). Cependant, il faudra au préalable confirmer la fonction facteur d'échange de *Yl.Bag1*. J'ai exprimé et purifié cette protéine dans ce but, mais n'ai pas poursuivi cette étude par manque de temps.

CONCLUSION ET PERSPECTIVES

Cette thèse a pour objet l'étude des spécificités fonctionnelles des Hsp70 cytoplasmiques chez la levure. Nous nous sommes fixés deux objectifs, décrire ces spécificités le plus exhaustivement possible, et comprendre leur origine.

Au point de départ ...

Les Hsp70 constituent une famille ubiquitaire de chaperons moléculaires située au cœur de la protéostase à travers les processus essentiels de repliement, d'assemblage, de contrôle qualité, de transport et de dégradation des protéines. Le cytoplasme de la plupart des cellules eucaryotes contient plusieurs paralogues de Hsp70 hautement conservés qui diffèrent essentiellement par leur expression spatio-temporelle. Cependant, ils possèdent également des spécificités fonctionnelles, qui étaient peu décrites au commencement de cette thèse.

Nous savions notamment que chez la levure, les formes constitutives Ssa1 et Ssa2 possèdent des activités différentes dans la dissociation des manteaux de clathrine (Gao et al., 1991) et que l'effet de ces formes constitutives sur la propagation des prions [*PSI+*] et [*URE3*] est complexe et prion-dépendant (Chernoff, 2007). Chez les eucaryotes supérieurs, les efforts ont été portés sur une distinction entre paralogues constitutifs et inductibles, mais n'ont généralement pas été menés de manière systématique, comme l'indiquent les revues suivantes (Daugaard et al., 2007 ; Kabani and Martineau, 2008b).

De plus, les différents paramètres régissant ces spécificités n'avaient été que peu étudiés. Le groupe de Daniel C. Masison proposait alors que les spécificités fonctionnelles des Hsp70 observées vis-à-vis de la propagation de [*PSI+*] résideraient essentiellement dans des coopérations différentes avec leurs co-chaperons, et ne dépendraient pas forcément de l'affinité des Hsp70 pour Sup35 (Tutar et al., 2006). Chez les eucaryotes supérieurs, la surexpression du facteur d'échange HspBP1 dans différentes lignées cancéreuses confortait l'idée d'une spécialisation liée aux facteurs d'échange (Raynes et al., 2003). De plus, les rôles *a priori* antagonistes de HspBP1 et Bag1 dans la dégradation de CFTR abondaient dans ce sens (Alberti et al., 2004; Demand et al., 2001). La spécialisation fonctionnelle apportée par les protéines à domaine J était connue, mais n'avait pas été clairement mise en relation avec les différents paralogues de Hsp70.

Cette thèse avait ainsi pour objectif de répondre à deux grandes questions. Pour quels processus cellulaires existe-t-il une spécialisation fonctionnelle des Hsp70 et quels paralogues y sont impliqués? En d'autres termes, les différences entre Hsp70 se résument-

elles à une « fonction de ménage » cellulaire pour les formes constitutives et une fonction de réponse au stress pour les formes inductibles ?

Deuxièmement, quels sont les paramètres régissant ces spécificités fonctionnelles ? Sont-elles liées aux différents types de substrats, à la coopération avec les co-chaperons, à la coopération avec d'autres chaperons ou protéines impliquées dans la protéostase ?

La réponse à ces questions devait permettre de mieux comprendre le rôle que peut jouer chaque paralogue dans la cellule, et sous quelles conditions. De plus, ces données seraient utiles pour mieux aborder le rôle joué par les Hsp70 dans les nombreuses pathologies dans lesquelles elles sont impliquées, comme les cancers et les maladies neurodégénératives.

Principaux résultats

Dans une première partie, réalisée en collaboration avec le groupe de Daniel C. Masison, nous avons comparé de manière systématique la capacité des Ssa de *Saccharomyces cerevisiae* et de *Yarrowia lipolytica* à assurer différents processus cellulaires *in vivo*. Cette étude a permis de montrer que les Hsp70 possèdent des propriétés distinctes permettant aux cellules de faire face à différents types de substrats et de conditions de stress.

Nous avons tout d'abord analysé l'expression des Hsp70 de *Yarrowia lipolytica* et supposé que Ssa6 et Ssa7 seraient fonctionnellement similaires aux formes inductibles Ssa3 et Ssa4 de *Saccharomyces cerevisiae*, et que Ssa5 et Ssa8 s'apparenteraient davantage aux formes constitutives Ssa1 et Ssa2. Nous avons montré que malgré une plus forte conservation de séquence, l'expression des Hsp70 de *Yarrowia lipolytica* dans un contexte hétérologue exacerbe les différences observées entre les souches correspondantes, probablement en raison d'interactions moins robustes avec les co-chaperons de *Saccharomyces cerevisiae*.

Ssa1 à Ssa8, exprimées comme seule Hsp70 cytoplasmique dans une souche de *Saccharomyces cerevisiae*, assurent la viabilité des cellules mais avec des taux de croissance très différents. Les formes inductibles provoquent une augmentation du temps de génération, suggérant que leur capacité à assurer des « fonctions de ménage » est plus faible que celle des formes constitutives. Les orthologues inductibles procurent aux souches une meilleure capacité de thermotolérance, indépendamment de leur taux de croissance. Ils pourraient donc posséder une affinité particulière pour les substrats dénaturés, ou être plus efficaces dans la coopération avec Hsp104. Cette donnée confirme une spécialisation fonctionnelle entre orthologues constitutifs et inductibles. Les Ssa varient également dans leur fonction de repliement d'un substrat dénaturé (luciférase), mais cette variation n'est pas strictement

corrélée aux résultats observés pour la thermotolérance. Cela suggère que les spécialisations fonctionnelles des Hsp70 dépendent à la fois du substrat, et de leur coopération avec le reste du système chaperon.

Concernant les prions, leur propagation et leur stabilité sont Ssa-dépendantes. Un point important est que les formes inductibles Ssa3 et Ssa4 induisent des phénotypes radicalement différents pour le prion [*PSI+*], et identiques pour le prion [*URE3*]. Une spécificité fonctionnelle existe donc entre paralogues inductibles. Cependant, l'étude du rôle des Hsp70 dans la propagation de [*PSI+*] a montré que les orthologues n'induisent pas la formation de variants différents, et que l'état d'oligomérisation de la protéine Sup35 ne semble pas corrélé à la force du phénotype prion. Enfin, les Ssa coopéreraient de manière différente avec Ydj1 lors de l'élimination du prion [*URE3*]. La propagation et la stabilité des prions impliquent des processus cellulaires complexes prion-dépendants, faisant intervenir des chaperons moléculaires différents. Il est donc difficile de conclure quant au rôle précis qu'y jouent les Hsp70.

Enfin, la dégradation de CFTR par le protéasome est comparable quelque soit l'orthologue exprimé.

Dans une seconde partie, nous avons montré que la machinerie Hsp70 est requise pour la formation de biofilms chez la levure. Elle contrôle, *via* des voies distinctes, l'expression, la maturation et le recyclage de l'adhésine de surface Flo11 requise pour ce processus.

Concernant la comparaison des Hsp70, la formation de biofilms est davantage affectée lorsque les paralogues constitutifs sont invalidés. Les facteurs d'échange sont quant à eux impliqués dans des voies distinctes. Fes1 est requise pour la formation des biofilms *via* une voie Flo11 indépendante, qui n'affecte pas la croissance invasive. Au contraire, Sse1 et la protéine à domaine J Ydj1 interviennent dans une voie contrôlant l'expression de Flo11. Le criblage des mutants des protéines à domaine J non essentielles montre que seules Ydj1 et Swa2 sont requises dans ce processus. Swa2, orthologue de l'auxiline, jouerait un rôle encore différent à travers une voie contrôlant à la fois la maturation et le recyclage de Flo11.

Ici encore, les résultats indiquent que les spécificités fonctionnelles des Hsp70 sont probablement liées à leur interaction avec différents co-chaperons.

Enfin, nous avons caractérisé la machinerie Hsp70 de la levure *Yarrowia lipolytica*. Cette partie n'a pas pu nous renseigner sur des spécificités fonctionnelles des paralogues de Hsp70

liées à leurs co-chaperons, ou à Hsp104 ou CHIP. Elle a néanmoins renforcé l'idée d'une grande robustesse du système Hsp70.

Retour sur les questions posées

L'objectif descriptif de cette thèse a été rempli par la première approche et une partie de la seconde approche. Des différences fonctionnelles ont pu être observées entre les paralogues de Hsp70 pour plusieurs, mais pas tous les processus cellulaires étudiés. De plus, elles peuvent être corrélées au caractère constitutif ou inductible des Hsp70, mais pas de manière systématique. Enfin, comme cela avait déjà été montré, certains paralogues influencent la propagation et la stabilité des prions de manière radicalement différente.

Ces données confirment que les spécificités fonctionnelles des Hsp70 ne se limitent pas à une simple différenciation entre « fonctions de ménage » et fonctions de réponse au stress, ou entre paralogues constitutifs et paralogues inductibles.

De manière générale, la complexité d'un système augmente sa dépendance à l'environnement. Ainsi, les Hsp70 possédant des fonctions essentielles à la protéostase et donc à la survie de l'organisme, une forte redondance fonctionnelle aurait été conservée au cours de l'évolution, parallèlement au développement de spécificités fonctionnelles. Si ces spécificités ne sont pas systématiques vis-à-vis de chaque substrat ou processus cellulaire, l'hypothèse d'une régulation fine de chaque paralogue n'est cependant pas à exclure.

Le second objectif était de comprendre l'origine des spécificités fonctionnelles des Hsp70. Si nous n'avons pas pu réellement le remplir, nous apportons cependant des éléments de réponse. En effet, les résultats, quand ils ne peuvent le confirmer, abondent dans le sens d'une coopération préférentielle des paralogues de Hsp70 avec certains co-chaperons ou chaperons moléculaires. Ceci est le cas pour Ydj1, et pourrait également être vrai pour Swa2 et les facteurs d'échange, au vu des résultats obtenus quant à la formation des biofilms. De plus, l'ensemble des résultats indique que les paralogues de Hsp70, en coopération avec les autres membres du système chaperon, pourraient agir sur des substrats préférentiels.

Perspectives

En raison de la pléiotropie des Hsp70, ainsi que de leur régulation fine, il apparaît primordial de coupler des données obtenues *in vivo* et *in vitro* pour caractériser leurs spécificités fonctionnelles.

Le suivi de la polymérisation *in vitro* des protéines Sup35 et Ure2 en fibres est une technique maîtrisée et éprouvée pour l'étude des chaperons (Krzewska and Melki, 2006 ; Savistchenko et al., 2008). Il pourrait être un bon modèle pour déterminer si l'origine de la spécialisation fonctionnelle des Hsp70 provient de leur affinité pour certains substrats et/ou d'une coopération avec le reste du système chaperon. L'effet de chaque Ssa sur la polymérisation de Sup35 et Ure2 en fibres pourrait nous fournir plusieurs informations. Premièrement, l'observation de différences, ou non, nous indiquera si la spécialisation fonctionnelle des Hsp70 peut être liée de manière significative à des affinités différentes pour les substrats. Ensuite, l'effet de chaque Ssa pourra être testé en présence de différents co-chaperons, voire de plusieurs combinaisons de co-chaperons. Enfin, l'effet des Ssa les unes sur les autres pourra également être investigué. Ainsi, les données obtenues pourront être reliées aux connaissances que nous avons sur le rôle des chaperons *in vivo*, afin de comprendre à la fois les fonctions des Hsp70, mais aussi le mécanisme de propagation des prions.

La poursuite de l'étude des biofilms de *Saccharomyces cerevisiae* pourrait également s'avérer prometteuse pour l'étude des interactions entre Hsp70 et co-chaperons.

Tout d'abord, la construction de doubles mutants $\Delta ssa\Delta fes1$ et $\Delta ssa\Delta sse1$ pourrait nous permettre de savoir si des paralogues différents sont impliqués dans les voies Flo11-dépendante et Flo11-indépendante, et par la même occasion d'identifier une nouvelle spécialisation fonctionnelle des Hsp70 liée aux facteurs d'échange. De la même manière, la construction de doubles mutants $\Delta ssa\Delta swa2$ nous renseignerait sur la spécificité fonctionnelle entre Ssa1 et Ssa2 pointée par Gao *et al.* (Gao et al., 1991). De plus, nos résultats indiquent que la formation de biofilms serait conditionnée par un recyclage de Flo11 clathrine dépendant. Ce point pourrait être confirmé par la localisation cellulaire de ces deux protéines observée en microscopie confocale. L'identification des modifications post-traductionnelles présentes sur l'adhésine Flo11 depuis sa sécrétion jusqu'à son recyclage devrait également nous renseigner sur la dynamique de cette protéine.

La réelle compréhension du rôle de la machinerie Hsp70 dans la formation de biofilms nécessitera une étude beaucoup plus approfondie. Un criblage de la collection de mutants d'inactivation dans la souche $\Sigma 1278b$ (banque réalisée par le groupe de Charlie Boone) vis-à-vis de la formation de biofilms et de la croissance invasive pourrait fournir des informations précieuses quant à l'orientation à prendre.

Chaque organisme, tissu, ou cellule, exprime un set différent de Hsp70 cytoplasmiques qui lui est propre, et qui réagit aux contraintes cellulaires et aux conditions environnementales. La description systématique des spécificités fonctionnelles des Hsp70 en tant que telle, si toutefois elle a un sens, restera donc ouverte. Cependant, la preuve de leur existence, leur caractérisation, et leur étude dans des cas précis doit nous permettre de mieux appréhender le rôle des Hsp70 dans les nombreuses pathologies dans lesquelles elles sont impliquées.

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Résumé - Abstract

Les Hsp70 constituent une famille de chaperons moléculaires ubiquitaires qui jouent des rôles essentiels dans le repliement, le transport ou la dégradation des protéines. Le cytoplasme des cellules eucaryotes contient plusieurs paralogues de Hsp70 fortement conservés qui diffèrent essentiellement par leur expression spatio-temporelle. Plusieurs travaux suggèrent que ces paralogues ont des spécificités fonctionnelles que nous avons cherché à mettre en lumière et caractériser par des approches génétiques.

Dans une première étude, nous avons comparé les activités des Hsp70 des levures *Saccharomyces cerevisiae* (Ssa1-4) et *Yarrowia lipolytica* (Ssa5-8) lorsqu'elles sont exprimées comme unique Hsp70 chez *S. cerevisiae*. Nous avons montré que ces Hsp70 : 1) assurent la viabilité des cellules mais avec des taux de croissance très différents ; 2) ont des effets variables sur la propagation et la stabilité des prions [*URE3*] et [*PSI+*] ; et 3) permettent la dégradation protéasomale de CFTR avec des cinétiques comparables. Dans une seconde étude, nous avons montré que la formation de biofilms chez la levure dépend de la machinerie Hsp70 qui contrôle, *via* des voies distinctes, l'expression, la maturation et le recyclage d'une adhésine de surface (Flo11) requise pour ce processus. Enfin, nous avons construit et caractérisé des mutants de *Y. lipolytica* dans lesquels un ou plusieurs gènes codant des chaperons moléculaires et autres acteurs de la protéostase (*e.g.* Hsp70, Hsp104, CHIP) ont été invalidés.

Malgré une forte homologie et une redondance fonctionnelle, les Hsp70 possèdent des propriétés distinctes permettant aux cellules de faire face à différents types de substrats et de conditions de stress.

Mots clés : Hsp70 ; Hsp40 ; levure ; prion ; biofilm

Functional specificities among yeast cytosolic Hsp70s

Hsp70s are a highly conserved family of ubiquitous molecular chaperones that play essential roles in protein folding, transport or degradation. The cytosol of most eukaryotic cells contains multiple highly conserved Hsp70 orthologs that differ mainly by their spatio-temporal expression patterns. While several reports suggest that specialized functions of Hsp70 orthologs were selected through evolution, few studies addressed systematically this issue.

First, we compared the ability of Ssa1-Ssa4 from *Saccharomyces cerevisiae* and Ssa5-Ssa8 from the evolutionary distant yeast *Yarrowia lipolytica* to perform Hsp70-dependent tasks when expressed as the sole Hsp70 for *S. cerevisiae* *in vivo*. We showed that Hsp70 isoforms: 1) supported yeast viability yet with markedly different growth rates; 2) influenced the propagation and stability of the [*PSI+*] and [*URE3*] prions; but 3) did not significantly affect the proteasomal degradation rate of CFTR. Second, we showed that biofilm formation in yeast depends on the Hsp70 machinery that controls, through distinct pathways, the expression, maturation and recycling of a cell-surface adhesin (Flo11) required for this process. Finally, we constructed and analyzed *Y. lipolytica* mutants bearing one or multiple deletion(s) in genes encoding molecular chaperones and others proteostasis modulators (*e.g.* Hsp70, Hsp104, CHIP).

Despite very high homology and overlapping functions, the different Hsp70 orthologs have evolved to possess distinct activities that are required to cope with different types of substrates or stress situations.

Keywords: Hsp70; Hsp40; yeast; prion; biofilm