The stress-induced nucleolar accumulation of hsc70 is mediated by specific signals, nucleolar chaperone networks and RNA

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September 2011

A thesis submitted to McGill University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

To Wanda and Mateusz, my mother and brother… the most precious in my life

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Abstract

Heat shock cognate 70 kDa protein (hsc70) is a well conserved molecular chaperone involved in many physiological functions such as the stress response, protein folding, and protein transport. Moreover, hsc70 plays roles in human disease, such as cancer, neurodegenerative disease, heart and brain ischemia, and it also takes part in the aging process. With the help of co-chaperones and ATP hydrolysis, hsc70 can restore proper protein folding by binding to normally inaccessible hydrophobic residues on its target.

Following heat shock, hsc70 traffics from the cytoplasm to the nucleoplasm. Hsc70 further accumulates in nucleoli during heat stress recovery. Using GFP-tagged portions of hsc70, I identified the nucleolar localization sequence (NoLS) of hsc70. Following quantification of the fluorescence signal, I showed that the nucleolar accumulation of different constructs varied. This allowed me to identify a heat inducible segment on hsc70, residues 225 to 297, which is sufficient for nucleolar accumulation during recovery from stress. The analysis of deletion mutants generated for segment 225-297 revealed that some constructs accumulated in the nucleolus constitutively, while others did not target GFP to nucleoli. Using pharmacological inhibitors, I also showed that PI3-kinase, MEK (MAP kinase kinase) and tyrosine dephosphorylation are involved in the hsc70 nucleolar accumulation process.

The nucleolus is a well defined compartment in the nucleus that is dynamic and characterized by multiple functions. Nucleoli are currently at the center of important discoveries as they play a role in diseases such as cancer. The nucleolus participates in the control of cell cycle progression and viral infections, but its fundamental function is the assembly of ribosomes. Several thousand proteins have been identified in the nucleolus; however, as the nucleolus is dynamic, this composition depends on physiological and environmental conditions. My work focused on reviewing our knowledge of the possible chaperone networks present in the nucleolus. A new set of proteins, the nucleolar multitasking proteins (NoMPs), has been identified as chaperones that are necessary for the specific function of the nucleolus. These multitasking proteins include B23 and nucleolin. This work provided the basis for us to predict interactions between chaperones in the nucleolus.

Many proteins of the nucleolus are dynamic: they can traffic to other cellular compartments when environmental conditions change. To define the mechanisms of hsc70 nucleolar accumulation during stress recovery, my work aimed at identifying the nucleolar binding partners of hsc70. I provided evidence that RNA plays a role for hsc70 nucleolar accumulation by treating heat shocked cells with detergent and RNase. Furthermore, inhibition of RNA polymerase I increased the retention time of endogenous hsc70 in the nucleolus. In further studies I examined the GFP-tagged hsc70 NoLS in transfected cells. This reporter displayed increased nucleolar accumulation and delayed release from the nucleolus during the recovery from heat shock when cells were treated with actinomycin D. On the

other hand, RNA polymerase I inhibition was not sufficient to accumulate hsc70 or the GFP-tagged NoLS in nucleoli under normal growth conditions. In addition to the links between nucleolar RNA and hsc70, my research demonstrated that hsc70 and its NoLS bind to polyA$^+$ RNA under normal growth conditions.

Taken together, my studies advance our current understanding of the nucleolar accumulation of hsc70 during heat stress recovery. I identified important sequence elements, pathways and possible nucleolar anchors that contribute to hsc70 nucleolar accumulation. My work congregates important concepts that will likely be beneficial to human health.

Résumé

La protéine apparentée à la protéine de choc thermique 70 kDa (hsc70: *heat shock cognate 70 kDa protein*) est une chaperonne moléculaire bien conservée impliquée dans plusieurs fonctions physiologiques telles que la réponse au stress, le repliement des protéines, ainsi que dans le transport de celles-ci. Hsc70 joue des rôles dans les maladies humaines, comme le cancer, les maladies neurodégénératives, l'ischémie du cerveau et du cœur, et elle prend part au processus de vieillissement. Avec l'aide des co-chaperonnes et de l'hydrolyse de l'ATP, hsc70 peut replier correctement les protéines en se liant aux résidus hydrophobes normalement inaccessibles.

Suivant un choc thermique, hsc70 se déplace du cytoplasme vers le nucléoplasme. Hsc70 s'accumule subséquemment dans les nucléoles, pendant la récupération du stress thermique. Utilisant des portions de hsc70 liées à GFP, j'ai identifié la séquence de ciblage au nucléole (NoLS: *nucleolar localization sequence*) de hsc70. Après avoir quantifié le signal fluorescent, j'ai démontré que l'accumulation nucléolaires des différentes constructions variait. Ceci m'a permis d'identifier un fragment inductible par la chaleur sur hsc70, aux résidus 225 à 297, qui est suffisant pour l'accumulation au nucléole pendant la récupération du stress. L'analyse de mutants contenant des délétions générées dans le segment 225-297 a révélé que certaines constructions s'accumulaient constitutivement au nucléole, alors que d'autres ne ciblaient jamais GFP au nucléole. Utilisant des inhibiteurs pharmacologiques, j'ai également démontré que PI3-kinase, MEK (MAP kinase

kinase) et la déphosphorylation des tyrosines, sont impliqués dans le processus de l'accumulation nucléolaire de hsc70.

Le nucléole est un compartiment bien défini du noyau, qui est dynamique et caractérisé par de multiples fonctions. Les nucléoles sont actuellement au centre de découvertes importantes étant donné qu'ils jouent un rôle dans les maladies telles les cancers. Le nucléole participe au contrôle de la progression du cycle cellulaire et aux infections virales, mais sa fonction fondamentale est l'assemblage des ribosomes. Plusieurs milliers de protéines ont été identifiées dans le nucléole, cependant, comme le nucléole est dynamique, cette composition dépend des conditions physiologiques et environnementales. Mon projet focalise aussi sur la revue des connaissances portant sur les possibles réseaux de chaperonnes présents dans le nucléole. Une nouvelle catégorie de protéines, les protéines multitâches (NoMP: *nucleolar multitasking protein*), a été identifiée comme étant des chaperonnes nécessaires pour la fonction spécifique du nucléole. Ces protéines multitâches incluent B23 et la nucléoline. Ce travail nous a procuré les bases permettant de prédire les interactions entre les chaperonnes dans le nucléole.

Plusieurs protéines du nucléole sont dynamiques: elles peuvent se déplacer vers d'autres compartiments cellulaires lorsque les conditions environnementales changent. Afin de définir les mécanismes régissant l'accumulation nucléolaire de hsc70 lors de la récupération du stress, mon projet a visé à identifier les partenaires nucléolaires qui se lient à hsc70. J'ai démontré que l'ARN joue un

rôle dans l'accumulation nucléolaire de hsc70 en traitant les cellules exposées à un stress à la chaleur avec un détergent et de l'ARNase. De plus, l'inhibition de l'ARN polymérase I augmente la duré de rétention nucléolaire de la hsc70 endogène. Lors d'études subséquentes sur des cellules transfectées, j'ai examiné le NoLS lié à GFP. Cette construction a démontré une accumulation nucléolaire plus élevée ainsi qu'un délai dans le relâchement du nucléole pendant la récupération du stress à la chaleur, lorsque les cellules étaient traitées avec l'actinomycine D. D'autre part, l'inhibition de l'ARN polymérase I n'était pas suffisante pour accumuler hsc70, ou le NoLS liée à GFP, dans le nucléole lors de conditions normales de croissance. En plus des liens entre l'ARN nucléolaire et hsc70, ma recherche a démontré que hsc70 et son NoLS se lient à l'ARN polyA$^+$ dans les conditions normales de croissance.

Ensemble, mes travaux contribuent à l'avancement de notre compréhension actuelle de l'accumulation de hsc70 dans le nucléole lors de la récupération du stress à la chaleur. J'ai identifié d'importants éléments de séquence, des voies de signalisation et des ancres nucléolaires potentielles qui contribuent à l'accumulation de hsc70 au nucléole. Mes travaux regroupent d'importants concepts qui vont possiblement être bénéfiques à la santé humaine.

Preface

Acknowledgements

Above all, I would like to thank McGill University for all the unique and unforgettable academic, scientific, social and professional experiences that will contribute to my future. I've crossed paths with many extraordinary people in the past years, and this would have been impossible without my supervisor, Dr. Ursula Stochaj, whom I must thank for the opportunity she has given me by letting me conduct my studies in her laboratory, the scientific experience, the environment that she offered, and the so much appreciated financial support. My work in her laboratory permitted me to contribute to 7 scientific publications. Many thanks to my funding agencies, Fonds de recherche Santé Québec and Heart and Stroke Foundation of Canada, for their trust and support of my scientific fulfillment. I also want to thank my present and past PhD committee members: Dr. John Orlowski, the chair of the Department of Physiology, Dr. Jason Young, Dr. John Presley, and Dr. Peter Swain; for both encouraging and critical discussions. Their expertise, brilliant advice and contributions to my success will undoubtedly have the most impact on a rewarding future scientific profession.

I would like to thank the Department of Physiology staff for all the help and support they offered. Above all, I want to thank Christine Pamplin for her flawless and constant feedback about my university records. Her professionalism and

friendly approach was always greatly appreciated. Many thanks to Maria Dimas and Rosetta Vasile for their support, encouragements, and human approach.

I also want thank all the students that I have been working with and that I had the pleasure to meet. I must thank my present and past laboratory colleagues: Dr. Mohammed Kodiha, Dr. Xinxin Quan, Rui Zhang, and Neola Matusiewicz. Also, I am grateful for the help and effort of the countless undergraduate students, volunteers and other students that contributed to my professional experience. Among them are: Hicham Mahboubi, Sanhita Shrivastava, Cynthia Kanagaratham, Laura Johns, Seung Pyo Hong, Tania Morielli, and Xiaoxiao Wang. I wish to thank all my friends (you know who you are), who were by my side when there was reason to celebrate, but also when times were hard. I will never forget your presence, your support, and the effort you put to understand.

This PhD would have been impossible without the support and encouragement of my mother, brother, and Isabelle Guart: I will never be able to thank them enough. My most profound thanks go to my beloved family in Poland, mainly Damazy Bański and Lucyna Bańska-Dobosz: may they rest in peace; and their daughters, who made it through such incredibly hard times and uncommon life experiences, and who are the foundation of my energy, perseverance and will to make it through all rough times of my own life. - They will never be forgotten. -

Contributions of Authors

As permitted by the Graduate and Postdoctoral Studies of McGill University (Montréal, Canada), the following work is composed of one published research article and one manuscript to be published, in both of which I am the first author. Chapter II is composed of a review of which I am first author with Kodiha, M. All work has been done in Dr. Stochaj's laboratory, under her guidance and supervision.

Chapter I:

The content of this chapter was published.

Nucleolar targeting of the chaperone hsc70 is regulated by stress, cell signaling, and a composite targeting signal which is controlled by autoinhibition.

Bański P, Mahboubi H, Kodiha M, Shrivastava S, Kanagaratham C, Stochaj U.

J Biol Chem. 2010 Jul 9;285(28):21858-67.

and doi: 10.1074/jbc.A110.117291 August 19, 2011 The Journal of Biological Chemistry, 286, 29441.

Most of the work was designed and conducted by me. This scientific article was published and written with the help of Mahboubi H. Kodiha M. did some signal quantification (Figures 9A and 9B; graphs) and contributed to the writing of the manuscript. Shrivastava S. and Kanagaratham C. assisted with technical work to generate and test the various GFP constructs. Dr. Stochaj did western

blots (Figures 8 and 10, Figures 11B and 11C, and Figure 13C) and contributed to the writing of the manuscript.

Chapter II:

The content of this chapter was published.

Chaperones and multitasking proteins in the nucleolus: networking together for survival? Bański P, Kodiha M, Stochaj U. Trends Biochem Sci. 2010 Jul;35(7):361-7.

Both I and Kodiha M. have contributed equally to this work. Dr. Stochaj contributed to the writing.

Chapter III:

The content of this chapter will be submitted for publication.

Most of the work was designed and conducted by me. Johns L. contributed to this work by helping with the *in vitro* treatments. Dr. Stochaj did the statistical analysis in figure 22A and contributed to the writing of the manuscript.

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ActD	actinomycin D
ADP	adenosine diphosphate
Akt	serine/threonine protein kinase
ANOVA	analysis of variance
ARE	A+U-rich element
ATP	adenosine triphosphate
AUF1	AU-rich element binding factor 1
B23	nucleophosmin
BAG	B-cell CLL/lymphoma 2-associated athanogene
BiP	binding immunoglobulin chaperone
C23	nucleolin
CAS	cellular apoptosis susceptibility gene
CCT	chaperonin-containing T-complex 1
CCT1	chaperonin-containing T-complex 1 subunit alpha
CCT2	chaperonin containing TCP1, subunit 2
Cct6p	chaperonin-containing T-complex zeta subunit
CCT8	chaperonin-containing T-complex 1 subunit theta
CDC37	cell division cycle 37
CDK4	cyclin-dependent kinase 4
CDK9	cyclin-dependent kinase 9
CHIP	carboxyl terminus of hsc70 interacting protein
CK2	casein kinase 2

CV1	African green monkey *Cercopithecus aethiops* kidney fibroblast cell line
Cy3	Cyanine dye 3
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DFC	dense fibrillar component
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAJ B1	DnaJ (Hsp40) homolog, subfamily B, member 1
DnaJ	heat shock 40 kDa protein family
DnaK	heat shock 70 kDa protein
DTT	Dithiothreitol
ECL	enzymatic chemiluminescence
EGTA	ethylene glycol tetraacetic acid
eIF2AK2	eukaryotic translation initiation factor 2 alpha kinase 2
ERK1/2	extracellular signal-regulated kinase 1 and 2
ERK5	extracellular signal-regulated kinase 5
Exp	Exportins
FC	fibrillar center
FITC	fluorescein isothiocyanate
GC	granular component

GDP	guanine diphosphate
GFP	green fluorescent protein
GTP	guanine triphosphate
HDM2	human double minute 2 protein
HeLa S3	Henrietta Lacks S3 cell line
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hip	heat shock 70 kDa protein interacting protein
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type 1
Hop	hsp70/hsp90 organizing protein
HS	heat shock
HSC70	heat shock cognate 70 kDa gene
hsc70	heat shock cognate 70 kDa protein
HSP10	heat shock 10 kDa protein
hsp1A	heat shock 70 kDa protein 1A
Hsp40	heat shock 40 kDa protein family
hsp60	heat shock 60 kDa protein
HSP60	heat shock 60 kDa protein
HSP70	heat shock 70 kDa gene
hsp70	heat shock 70 kDa protein
Hsp70	heat shock 70 kDa protein family
HSP70s	heat shock 70 kDa proteins
hsp72	heat shock 70 kDa protein 2
hsp73	heat shock 70 kDa protein

hsp90	heat shock 90 kDa protein
HSP90s	heat shock 90 kDa proteins
HSPA8	heat shock 70 kDa protein 8 gene
hspBP1	heat shock 70 kDa protein binding protein 1
HSPs	heat shock proteins
IL-10	Interleukin-10
Imp	Importins
LY	LY294002
MAPKs	mitogen-activated protein kinases
MEK	mitogen-activated protein kinase/extracellular signal-regulated kinase kinase
mRNA	messenger ribonucleic acid
NA	numerical aperture
NCBI	National Center for Biotechnology Information
NLS	nuclear localization signal
$NoDL^{H+}$	nucleolar detention signal regulated by hydrogen ions
NoLS	nucleolar localization sequence/signal
NoMP	nucleolar multitasking protein
NOPdb3.0	Nucleolar protein database 3.0
NP-40	Nonidet P-40
NPC	nuclear pore complex
NTF2	Nuclear Transport Factor 2
Nups	nucleoporins

$p14^{Arf}$	cyclin-dependent kinase inhibitor 2A, isoform 4, alternative reading frame
p53	protein 53
PAO	phenylarsine oxide
PARK2	parkin 2
PARP1	poly[ADP-ribose] polymerase I
PCR	polymerase chain reaction
PD	PD98059
Pi	inorganic phosphate
PI3K	phosphatidylinositol 3 kinase
PIH1	protein interacting with Hsp90
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
pre-rRNA	pre-ribosomal ribonucleic acid
RanBP1	Ras-related protein specific binding protein 1
RanGAP	Ras-related protein GTPase activating protein
RanGDP	Ras-related protein guanine diphosphate complex
RanGTP	Ras-related protein guanine triphosphate complex
RCC1	regulator of chromosome condensation 1
rDNA	ribosomal DNA
Rev	regulator of expression of viral proteins
RNA	ribonucleic acid
rpS6	ribosomal protein S6
rRNA	ribosomal ribonucleic acid

S phase	synthesis phase
S.D.	standard deviation
S.E.	standard error
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SGD	Saccharomyces Genome Database
siRNA	small interfering ribonucleic acid
SL1	selectivity factor 1
snoRNPs	small nucleolar ribonucleoproteins
Src	tyrosine-protein kinase
SRP	signal recognition particle
STAD	subnuclear targeting arginine domain
STHD	subnuclear targeting hydrophobic domain
TAH1	TPR (tetratricopeptide repeat)-containing protein associated with Hsp (heat-shock protein) 90
TBST	Tris-Buffered Saline and Tween 20
Tcp-1	T-complex 1
Tom20	translocase of the mitochondrial outer membrane 20 kDa subunit
Tom70	translocase of the mitochondrial outer membrane 70 kDa subunit
TRAP1	tumor necrosis factor type 1 receptor-associated protein

TRiC	T-complex 1 Ring Complex
UBA1	ubiquitin-activating enzyme E1
UBC4	ubiquitin-conjugating enzyme
UTR	Untranslated region
VAC	Vanadyladenosine Complex
WRN	Werner Syndrome gene
XRCC1	X-ray repair cross complementing group
β-gal	β-galactosidase

1. INTRODUCTION

1.1. Chaperones

The evolution of living organisms contributed to the development of intracellular mechanisms allowing their survival in diverse habitats. Not only are phenotypes adapted to specific environmental conditions, but intracellular modifications were at the basis of growth and survival. As such, DNA repair mechanisms, which rely on specific proteins, minimize genetic mutations. Although DNA is the fundamental material for protein synthesis, the complexity of functional protein structures makes them very sensitive to insults such as heat or cold stress, oxidative stress, disease, and aging [1]. Chaperones are proteins that contribute to the proper production of functional proteins. Thus, by limiting the amount of misfolded proteins, they prevent the formation of dangerous potentially cytotoxic protein aggregates and target aggregates to the 26S proteasome. Furthermore, chaperones can act as carriers to distribute molecules to specific compartments or organelles [2] where they are mostly needed, thereby constantly maintaining proper protein function, proper development [3, 4], and overall cell survival.

As proteins are being synthesized by ribosomes, they undergo rapid conformational changes that appear to be controlled in a way where hydrophobic residues are being forced into the protein core, away from the aqueous

intracellular milieu ([5] and references therein). The current understanding is that chaperones recognize unfolded and misfolded polypeptides *via* linear sequences where hydrophobic residues are particularly abundant [6-8]. Moreover, proteins that do not achieve their proper conformation can interact with each other through their hydrophobic segments ([9] and references therein) and generate aggregates. These protein aggregates can contribute to some human diseases such as Alzheimer's [10] and Huntington's disease [11]. However, just as it is with all vital proteins in cells, a lack of chaperone expression control can have negative effects on cell health [12].

Heat shock 70 kDa protein family (Hsp70) is one of the most conserved families of proteins in living organisms, both structurally and functionally. In humans, 8 homologous proteins compose this family ([13] and references therein). Hsp70 is a heat inducible chaperone [14-16], which means that its expression is increased when stressors are perceived. It was shown that its maximal rate of production occurs 3-5 hours post heat stress and diminishes after 8 hours [17]. The HSP70 gene, giving rise to hsp70 [also known as heat shock 70 kDa protein 1A (hspa1A)], is situated on chromosome 6, location 6p21.3. Hsp70 is intronless and is 641 residues in length [18]. This chaperone primarily functions as a monomer and it is an ATPase that has low affinity for substrate when bound to ATP. As the bound ATP is hydrolyzed, the substrate affinity of hsp70 is increased [19] (Figure 1). The majority of Hsp70 family members is composed of the N-terminal end (44 kDa), which carries the functional ATPase portion within the nucleotide binding domain, a substrate binding domain (17 kDa) and a small C-

terminal variable domain (10 kDa) [20]. Hsp70's function can be modulated through the interaction with other chaperones and co-chaperones. Hsp90s, an important class of molecular chaperones [21], DnaJs (members of the Hsp40 family) [9, 19, 22], BCL2-associated athanogene (BAG) family members [23-27], hsp70 binding protein 1 (HspBP1) [28, 29] are examples of such interacting partners which can stimulate hsp70 and other hsp70 family members, such as hsc70 [23, 26, 27, 30-33]. For example, DnaJ proteins stimulate, through their J domains, both the ATP to ADP and substrate binding reactions of hsc70 [34]. Other proteins can control and modulate chaperone function. Hip (hsc70 interacting protein [31]) interacts with the ATPase domain of hsc70 [35], and Hop (hsp70/hsp90 organizing protein) contributes to the communication between hsp70 and hsp90 and modulates their ATPase function [36, 37]. Another such interacting co-chaperone is the carboxyl terminus of hsc70 interacting protein (CHIP), which negatively regulates the substrate binding of both hsc70 and hsp70 by inhibiting the hsp40 mediated activation of their ATPase activity [38]. Among other functions, CHIP ubiquitinates chaperone clients which targets the defective proteins to the 26S proteasome [39]. Moreover, CHIP associates with both hsp70 and hsp90 and can ubiquitinate them [40, 41], which emphasizes the importance of CHIP in the overall protein quality control mechanisms. Hsp70 family members are an important family of proteins whose functions are constantly modulated by the environment and cellular events that may lead to protein damage. This underlies Hps70's immediate role in cell survival.

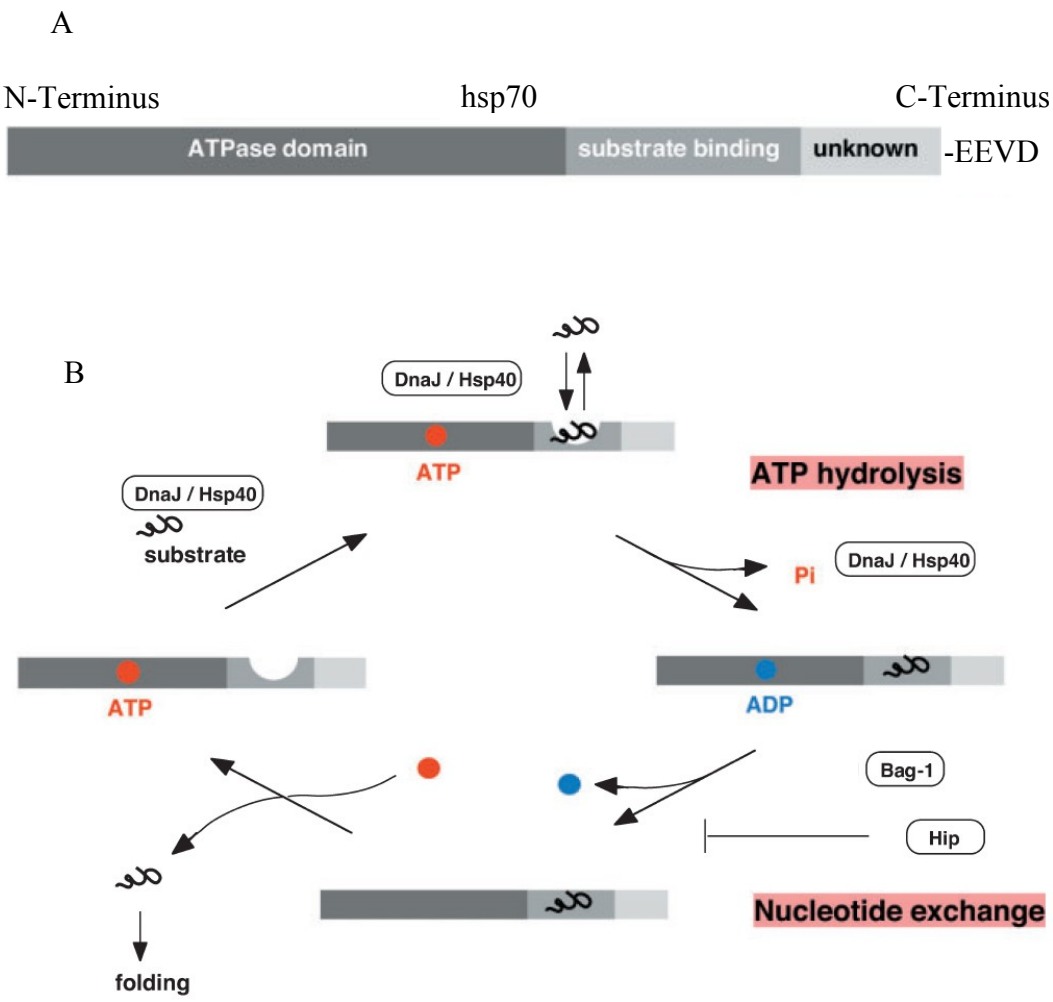

Figure 1: ATPase cycle of the hsp70 chaperone. **A**: The ATPase, substrate binding and variable (unknown) domains of hsp70 are shown. **B**: Hsp70 ATPase cycle where ATP/ADP conversion and binding steps are shown. Hsp70 binds peptides in the ATP-bound form. ATP to ADP conversion is enhanced by DnaJ and Hsp40, which causes release of the nucleotide and binding of a new ATP. This, with the help of Bag-1, releases the substrate. Hip stabilizes the ADP form. Adapted from Morishima 2005 [42].

Chaperonins are another category of conserved proteins that play important roles in the maintenance of protein function. Two groups are among this family of proteins. The first is GroEL in *Escherichia coli*, and hsp60 in eukaryotes which, when not properly functional, can contribute to the development of neurodegenerative diseases ([43] and references therein). The Tcp-1 Ring Complex (TRiC), also known under the chaperonin-containing TCP1 (CCT) name, is present as a ring-shaped complex that is composed of 8 homologous subunits [43] called CCT1 to CCT8 [44]. This second group of chaperonins is mainly involved in protein synthesis, rather than in stress response, and also plays important roles in neurodegenerative diseases ([43] and references therein). It is important to note that chaperones and their interacting partners are present throughout the cell, but can also be compartment specific, such as specific mitochondrial chaperones (e.g.: prohibitin, mortalin) [45], or endoplasmic reticulum chaperones, such as the binding immunoglobulin chaperone (BiP) [46] and calreticulin [47]. Tom20 and Tom70 (translocase of the mitochondrial outer membrane) are examples of mitochondrial proteins that interact with hsc70 and hsp90 chaperones. These mitochondrial proteins are needed for the translocation of various polypeptides, and confer shuttling through the inner and outer membranes of the organelle [34]. This shows the multitude of chaperone roles, some of which function as multitasking proteins [44]. As presented above, some chaperones can be compartment specific, whereas others can traffic from one organelle to another while carrying client proteins. Moreover, many of the chaperones/co-chaperones/chaperonins cooperate, and their function or distribution can be modulated upon stress.

1.2. Chaperones, stress and protein trafficking

Cells have to cope with various environmental stresses during their existence. Among the common stresses are heat and cold shocks. Although the majority of stressors come from the surrounding environment, intracellular processes that generate reactive oxygen species can lead to oxidative stress and trigger stress responses. Furthermore, stress can come from other molecules such as reactive nitrogen species [48]. The damage caused by such stressors can be extensive, both at the genomic and protein level. Genetic mutations can cause death via apoptosis at the cellular level, but can also contribute to tumor development in a higher organism [49]. As such, chaperones of the Hsp70 family, as well as other stress proteins, play roles in the control of apoptosis, aging ([50, 51] and references therein, and reviewed in [52, 53]), heart ischemia [54-56], diabetes [54, 56], and brain ischemia [57, 58].

As presented above, protein quality control is maintained by several key players. Chaperones, proteases and antioxidant molecules are all such partners that prevent, or at least limit overall cellular injury [48]. The most common event that generates reactive oxygen species is the leakage of superoxides from the mitochondrial respiratory chain [48]. Oxidative stress is believed to be involved in several human pathologies such as Alzheimer's disease [59, 60], amyotrophic lateral sclerosis, Friedreich's ataxia, cancer, atherosclerosis and chronic inflammation [61, 62]. Considering the variety of stressors and large numbers of various specialized proteins that interact upon cellular insults, the concept of

protein networks can be regarded as an important and general feature contributing to proper cell function.

The trafficking of proteins is an important process that needs constant regulation. For example, Hsp70 family members need to move from one compartment to another at all times in order to maintain protein quality. As such, controlled trafficking mechanisms are extremely important during stress, as chaperones need to reach their targets for proper refolding. Hsp70, for example, is expressed starting 3 hours after heat exposure, and another expression peak occurs at 12 hours. Furthermore, hsp70 moves into the nucleus in a time-sensitive fashion [63]. This is only one example of controlled trafficking of a chaperone during stress recovery, but protein trafficking can be controlled through several different mechanisms [51, 64-74].

An important element in proper protein trafficking and distribution is the targeting signal, which allows specific delivery of molecules to well defined cellular compartments. A good example of such a targeting sequence is the nuclear localization signal (NLS) [70, 75, 76]. The classic NLS can have 2 different structures: monopartite or bipartite [77]. The bipartite NLS is composed of 2 stretches of basic residues, separated by approximately 10 [78, 79] to 12 residues [80]. A common bipartite NLS sequence is KKxxxxxxxxxxKxxKK. The K's can be either arginines or lysines, while the x's can be replaced by any amino acid. In general, the NLS contains a high level of positively charged residues [75]. The non classical type of NLS lacks basic residues [81]. Targeting of molecules to

the nucleus does not only rely on the presence or absence of the NLS. This means that a non-nuclear protein containing a classic NLS does not necessarily traffic to the nucleus; the signal must be somehow made available, for example, to the nuclear import molecules. This sort of control could be true because of various conformational changes, or phosphorylation states of a protein, which can expose or hide the NLS. Furthermore, several NLSs can be present in one protein sequence. In this scenario, the nuclear accumulation of the protein can be increased. Another level of control can occur through the interaction with other molecules. For instance, an NLS-containing protein can be sequestered in the cytoplasm and its nuclear accumulation will therefore be modified [82]. This brief overview shows that not only is the NLS important to target a protein to the nucleus, but the context in which the signal is presented can modulate its function [64, 75].

The protein content of cellular compartments must be controlled to assure their proper function and cellular integrity. An obvious example of such a compartment is the nucleus: in order to protect the genome, access of molecules to the nucleus has to be limited. To achieve this control, the nucleus is protected by the nuclear envelope, which contains specific gates, called nuclear pores. Molecules can only move in and out of the nucleus through these pores, which are composed of nucleoporins that together form the nuclear pore complex (NPC) (Figure 2). The number of NPCs can vary tremendously depending on the cell type, trafficking demand, cell size and activity. The average number of NPCs is between 3000-5000 in a human proliferating cell. In higher organisms, the nuclei

of differentiated cells can have between 2000 to 4000 NPCs, which is equivalent to about 10 to 20 pores/μm^2. This value can go up to 60 pores/μm^2 in the case of mature *Xenopus* oocytes [79, 81, 83, 84]. NPCs are made of an 8 fold symmetrical structure, cytoplasmic and nucleoplasmic extensions, and a nuclear basket, with a total mass of about 125 MDa in higher eukaryotes. It is estimated that about 30 different proteins called nucleoporins (Nups) ([70, 81, 84, 85] and references therein) form the NPC. Since all of the Nups are present in multiple copies, at least 400 individual proteins are present in a single NPC [70, 86]. The functioning of the NPC as a gate can be affected by age and therefore impact on nuclear integrity [87], demonstrating the importance of the NPC for proper intracellular transport.

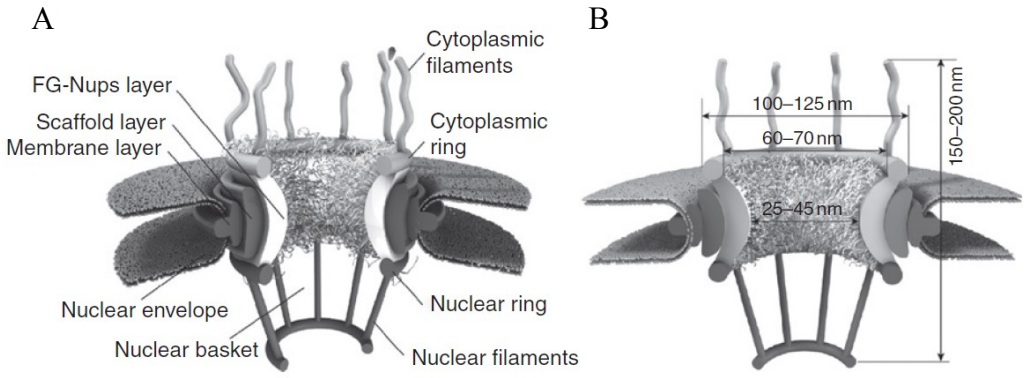

Figure 2: The nuclear pore complex. **A**: Possible representation of a NPC and its position within the nuclear envelope; substructures are shown. **B**: The NPC and its dimensions. Adapted from Jamali *et al.* 2011 ([81] and references therein).

In the many years since its discovery, our knowledge about the NPC changed considerably. Based on a 2011 review [81], some of its features will be mentioned

below. Molecules can either passively or actively traffic through the NPC, which is believed to be 25 nm to 45 nm at its narrowest diameter, limiting passive transport to molecules that are below 5 nm to 9 nm in diameter ([81, 84] and references therein). The widest molecules that can traffic through the NPC are established to be at about 40 nm [81]. Data indicates that this pore size can passively let through molecules in size lower than a 30 kDa to 60 kDa range ([79] and references therein, [88]); others state that it can allow the passive movement of molecules smaller than 40 kDa to 60 kDa [89]. The overall length of the NPC, from the tip of the fibrils at the cytoplasmic side, to the end of the nuclear basket on the nucleoplasmic side, is evaluated to range between 150 nm to 200 nm. Trafficking of molecules from the cytoplasm to the nucleoplasm through the classic nuclear import pathway can occur at a rate of between 100 to 1000 cargoes per minute per NPC ([71, 81], and references therein; [90]). In molecule size, this is about 80 MDa per NPC per second, rendering approximately 800 translocations per second of a 100 kDa molecule ([70] and references therein). These values emphasize the fact that nuclear trafficking is an utmost important process that needs extensive control. Furthermore, this suggests that the targeting of cytoplasmic proteins to the nucleolus is not a straight forward event. Despite the current knowledge of the NPC and our understanding of its function, it is still not completely known what forms the opening of the pore and controls the movement of molecules. The movement through the NPC is described by several interesting models such as the “Brownian affinity gate” based on the Brownian movement of molecules and their interaction with both cytoplasmic and nucleoplasmic filaments [91], the “selective phase” model suggesting the presence of a flexible

meshwork or nucleoporins that interact and control the passage of molecules [90], and finally, the oily-spaghetti model [92], which is also based on the presence of nucleoporins on the inner walls of the NPC, that function as binding sites for trafficking molecules. Furthermore, this model suggests the presence of a central 10 nm passage ([70] and references therein). The research area of nucleocytoplasmic trafficking of molecules and the understanding of the NPC is one where many discoveries may still be made. This renders the nucleolar accumulation of proteins, which is the main topic of this work, more complex than a simple and passive displacement of molecules. Not only must proteins have some sort of signaling mechanism to be sent to the nucleolus, they must also pass through the nuclear pore complex, suggesting the possible presence of two or more signaling sequences. Many gaps need yet to be filled as to the understanding of mechanisms through which the nucleolar accumulation of proteins occurs.

The classic nuclear import/export mechanism is complex and requires several key molecules such as import and export factors. Moreover, active transport in and out of the nucleus is an energy-dependent process. The RanGTPase is the molecule at the center of the classical nuclear import/export pathway: it generates the RanGTP/RanGDP gradient which is necessary for the active transport of molecules. Importin-α and importin-β, as well as exportins and other molecules, are necessary for the classical nuclear import/export pathway ([84], [70] and references therein). The overall progress of nuclear import occurs when a cargo binds to the nuclear import carrier. The assembled complex then binds to the fibrils, goes through the NPC and is released into the nucleoplasm where it

dissociates into its original interacting subunits. The import factors are then recycled to the cytoplasm allowing other import/export cycles to go on. Figure 3 and figure 4 show some of the fundamental processes occurring during nuclear import and export of macromolecules.

Cells rely on various inputs to control their metabolism. As such, under stress conditions, a cell can activate or inactivate specific signaling pathways to adjust to the changing environmental conditions. Alternatively, if the cell cannot recover from the insult and threaten the survival of the organism, apoptotic pathways can be activated to prevent the acquisition of mutations that could lead to tumor formation. Phosphatidylinositol 3-kinase (PI3K) is a crucial component of signaling pathways linked to cell cycle progression, protein synthesis, and metabolism [93]. Heat stress, for instance, activates the MEK and PI3K pathways [64], whereas oxidative stress activates PI3K/Akt and p38 [94]. It was further demonstrated that hypoxic conditions can activate PI3K/Akt [95], but do not activate ERK, p38 nor JNK [96], which emphasizes that stress can differentially trigger the activation of various pathways. Using well defined pharmacological tools, it is possible to inhibit specific pathways and observe their intracellular effects. For example, PD98059 specifically prevents the activation of the mitogen-activated protein kinase kinase (or MEK), which is upstream of ERK1/2. Moreover, there is crosstalk between certain cellular signaling pathways; an example of such crosstalk is the interplay between MEK and PI3K during oxidative stress [67]. Several reviews present important cellular signaling pathways [97-99].

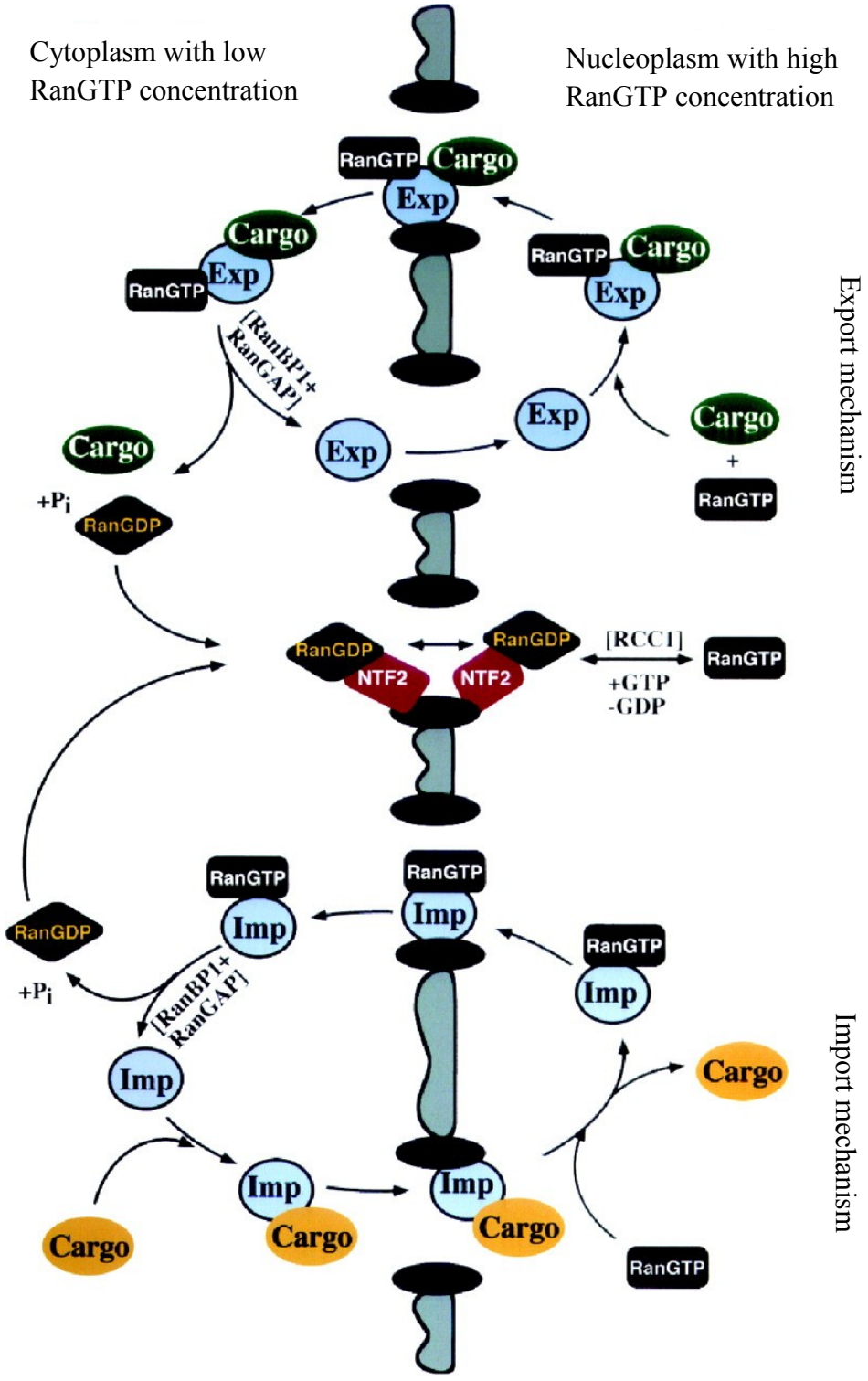

Figure 3: Importins (Imp) and exportins (Exp) and their transport. RanGTPase is an important element in the nucleocytoplasmic trafficking of molecules. NTF2: Nuclear transport factor 2. Adapted from Görlich & Kutay, 1999 [84].

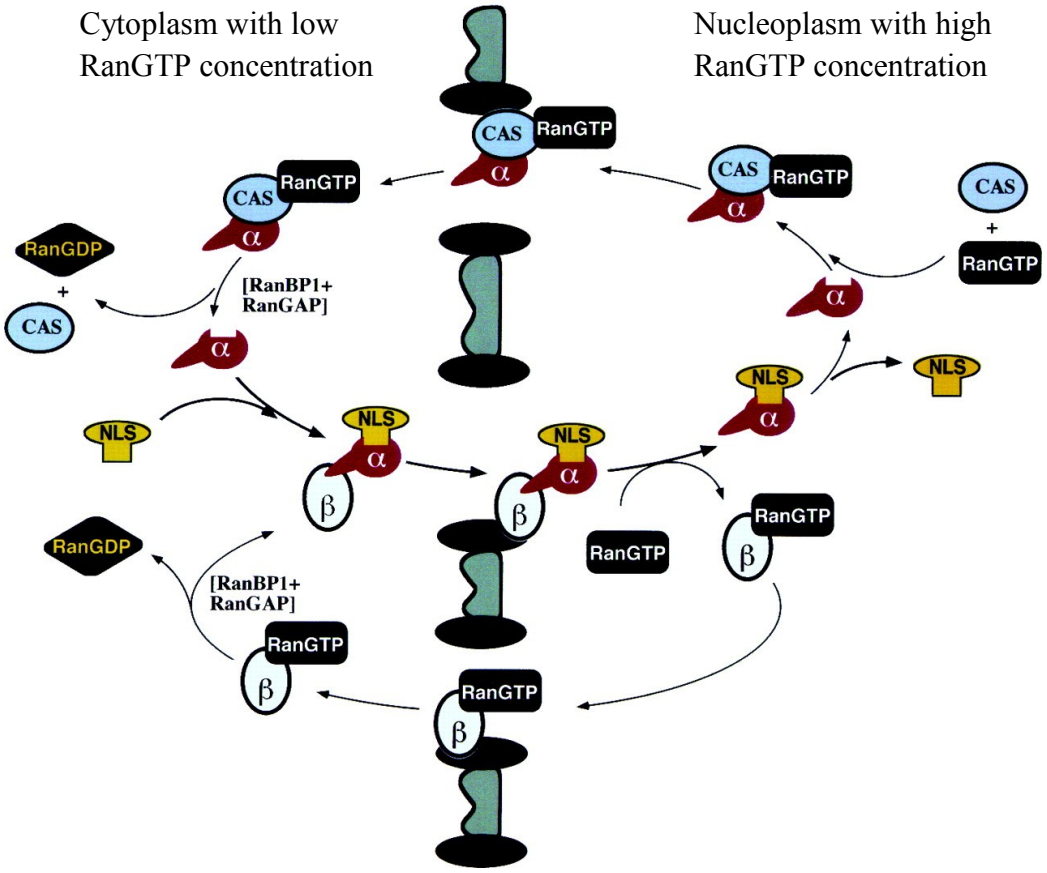

Figure 4: Classical nuclear accumulation pathway involving an NLS carrying molecule. RanGTP to RanGDP conversions are shown. Abbreviations: α and β, Importin α and β, respectively; NLS (nuclear localization signal) indicates the import substrate. Adapted from Görlich & Kutay, 1999 [84].

As signaling is an important mechanism for many cellular events, targeting of molecules to specific compartments is necessary to control proper growth and development. It is important to view the intracellular environment as a pool of highly specialized proteins, but also as an environment that needs to process extracellular inputs. Therefore, the idea of "protein networks" is a topic of several publications [44, 100-103]. As such, my work has focused on the targeting of

hsc70 to the nucleolus [64] and the possible roles and interactions it might be involved in, which will be discussed in the following sections.

1.3. Hsc70

Heat shock cognate 70 kDa protein (hsc70) is a molecular chaperone member of the Hsp70 family. Searching through the National Center for Biotechnology Information (NCBI) database shows that the first description of hsc70 was done in 1982 [104]. It was identified in *Drosophila melanogaster* and described as follows:

> "The experiments reported here establish the existence of a gene related to the hsp70 gene that is expressed at the normal growth temperature but not induced by heat shock. The protein product of this hsc gene may perform a function analogous to that of hsp70. This is the first indication that a function similar to that performed by hsp70 may be required for normal growth of the fly." [104].

In humans, hsc70 was first described as a "clathrin-uncoating" protein [105]. The clathrin cage uncoating steps by hsc70 require ATP and the J-domain protein auxilin [106]. Beside being a member of the Hsp70 chaperone family and having all respective chaperone functions, more specifically, experiments carried out in HeLa cells demonstrated that hsc70 is involved in the recycling of

nucleocytoplasmic transport factors such as importin α, importin β and transportin. When hsc70 was depleted with siRNA, these transport factors accumulated in the nucleus. The same group suggested that the nuclear migration of hsc70 is Ran-dependent, but is not dependent on importin-β or transportin [89]. Other interesting experiments focused on the nuclear accumulation of hsc70 itself. Following heat shock, hsc70 does not accumulate in nuclei of HeLa cells that are highly confluent. When the cell confluency is maintained at a 50% to 60% range, nuclear accumulation of hsc70 occurs very quickly and is complete after a 1 hour heat shock at both 45.5°C and 42°C. Furthermore, the heat shock induced nuclear accumulation of hsc70 was efficiently inhibited with okadaic acid and sodium orthovanadate, both functioning as phosphatase inhibitors. Importantly, although the nuclear accumulation of hsc70 is inhibited in confluent cells upon heat shock, the nuclear accumulation of other molecules still occurs [69].

Hsc70, also called hsp73, is encoded by the HSPA8 gene found on chromosome 11 at the 11q24.1 location [107]. Although the human hsp70 gene is intron-less, hsc70 is split into 9 fragments [108]. Hsc70 mRNA is 2276 base pairs long, which translates into a protein of 646 amino acids with a molecular weight of 70898 Da [109]. Similarly to hsp70, hsc70 is divided into 2 major domains: the 44 kDa N-terminal domain (residues 1 to 384), carrying the ATPase portion, and the 30 kDa C-terminal portion. The C-terminal end is composed of two subdomains of 18 kDa (residues 385 to 543) and 10 kDa (residues 544 to 646) [110-115]. The 18 kDa domain and 10 kDa domain are called binding domain and variable domain, respectively [115]. A detailed analysis of the bovine hsc70

ATPase crystal structure shows that it has 2 lobes separated by a deep cleft, at the base of which ATP can bind. The two lobes are called lobe I and II, and their upper and lower domains are named A and B. Domain IA is located at residues 1-39 and 116-188. They are followed by domain IB, from residues 40 to 115. Domain IIA is found at residues 189 to 228 and 307 until approximately 385 (somewhere near the end of the ATPase domain). Finally, domain IIB goes from residue 229 to 306 [116]. A cartoon of hsc70 is shown in figure 5.

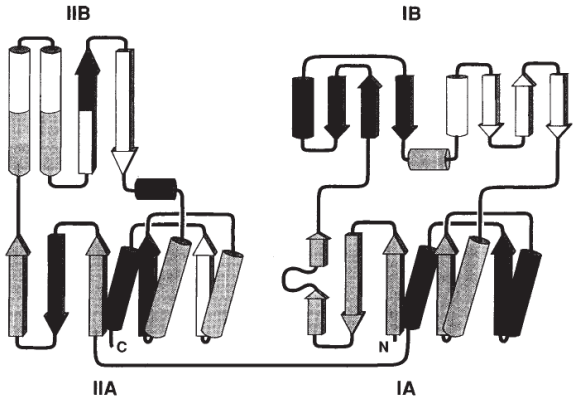

Figure 5: Scheme displaying the organization of hsc70, based on its crystal structure. Domains IA, IIA, IB and IIB are shown with the N and C terminal ends. Adapted from Flaherty *et al.* 1990 [116].

The major difference between hsp70 and hsc70 is that hsc70 is constitutively expressed in cells, even under normal growth conditions. It is generally assumed that hsc70 is not at all, or only slightly upregulated upon heat stress [3]. For example, as mentioned above, results have shown that it is not heat inducible [104], whereas others state that although the hsc70 gene carries highly homologous to heat-inducible DNA sequences, it was shown to only be induced 2

fold during heat stress [108]. Furthermore, from all the Hsp70 family members tested, hsc70 mRNA was the first to be found in the early and middle S phase of serum-induced proliferation of CV1 cell. This sort of induced proliferation is similar to a viral infection [117], demonstrating hsc70's importance in human health. Other data also suggest that hsc70 mRNA levels vary from organ to organ. Higher expression was found in skeletal muscle, liver and kidney, when compared to lungs, brain, spleen and heart. Furthermore, treatments with azetidine, a proline analog, or MG132 (a proteasome inhibitor), can increase hsc70 mRNA production up to 8 and 10 folds, respectively, in both mouse and human cells [118]. Despite a few differences, both hsc70 and hsp70 contribute to proper folding of nascent proteins, they prevent aggregate formation, send proteins to degradation or restore the conformation of denatured proteins, affect protein complex formation and disassembly, and participate in the trafficking of molecules through membranes ([119], and references therein). Overall, hsc70 is an essential protein that constantly takes part in proper cell function.

1.4. The Nucleolus

The nucleoli are somewhat circular intranuclear regions (Figure 6). There seems to be an inconsistency as to when, and by whom, was the nucleolus first observed/discovered. Some references point to Felice Fontana [120], dated to 1770 ([121], and references therein) or 1781, the year when Fontana published his observations [122], while Lo *et al*., (2006), suggest that the discovery of the

nucleolus happened in 1835. It is only in 1839 that the term "nucleolus" was used for the first time ([123] and references therein).

The number of nucleoli per nucleus can vary between 1 and 5, and their size is also variable, ranging between 0.5 μm to 5 μm in diameter [124, 125]. Their density, in *Xenopus* oocytes, was measured as 0.215 g/cm^3, compared to a surrounding nucleoplasm of 0.106 g/cm^3 [126]. The nucleolus does not have any membranes, but does have a well defined segmented structure (Figure 6). It is considered as a highly dynamic and multifunctional organelle [121, 124, 127-129]. As a main physiological function, the nucleolus is involved in the synthesis of ribosomes, and it is where rRNA production occurs concurrently with its movement from one nucleolar segment to another. Although the exact location is still controversial, it is believed that rDNA transcription, carried out by RNA polymerase I [130, 131], occurs between the fibrillar centers (FCs) and the dense fibrillar component (DFC), which is surrounding the FCs. As the pre-ribosomes are assembled, they extend from the DFC and enter the granular component (GC), which engulfs both the DFC and FC. During this movement between the three compartments, ribosomal assembly occurs, following processing and modification of the pre-ribosomes. In humans, dense chromatin also surrounds the nucleolus, which can reach into the FCs ([124] and references therein). Furthermore, it is estimated that human cells contain about 400 ribosomal genes [132], and over 4500 proteins were identified in the human nucleolus [133]. Well known nucleolar proteins are fibrillarin, nucleolin and B23, also called nucleophosmin (NPM). It is also interesting to note that the nucleolus is the compartment where

telomerase assembly occurs, and where it is sequestered when not needed [134]. This highlights the involvement of the nucleolus in many fundamental physiological processes.

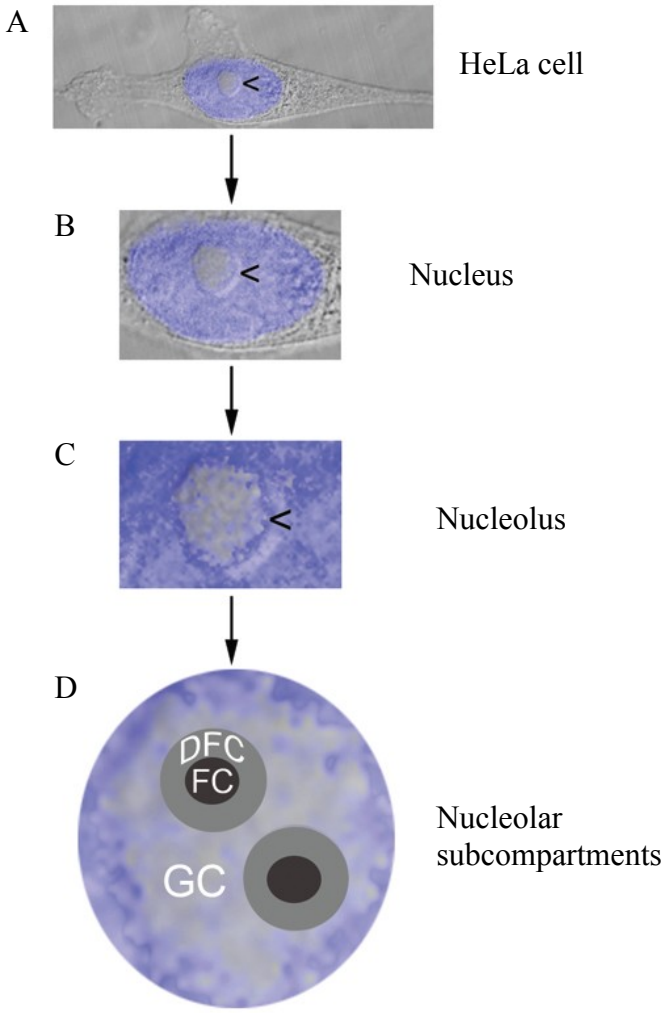

Figure 6: The nucleolus. **A**: DIC image of a cell showing the nucleolus and the nucleus stained with DAPI (4',6-diamidino-2-phenylindole, blue). **B**: Close-up of the nucleus in A. **C**: As shown in A and B, the nucleolus displays low DAPI-staining. **D**: The nucleolus is divided into well defined subcompartments: FC,

DFC and GC. In parts A-C, the arrowheads show the nucleolus. Adapted from Bański *et al.* (2011) [100].

Although a well described consensus sequence targets molecules to the nucleus, no such well defined consensus nucleolar localization sequence (NoLS) has been identified [127]. Nevertheless, several protein sequences were shown to target reporter proteins to the nucleolus [64, 135, 136]. For example it was possible to target pyruvate kinase to the nucleolus by attaching it to portion 250-267 of hsp70 [135]. In a quest to identify a consensus NoLS, Mekhail *et al*. (2007) screened the UniProtKB/SwissProt database [137]. As a result, a predictive nucleolar detention signal has been identified. More precisely, a nucleolar detention signal regulated by $[H^+]$ ($NoDL^{H+}$) has been identified as: $\{[RR(I/L)X_3r]_{(n,n\geq1)}+[L(\Phi/N)(V/L)]_{(n,\ n>1)}\}$; where R is a critical arginine, X is any amino acid, r is a less crucial arginine, n is the number of sequence elements, and Φ is any hydrophobic residue. The $[RR(I/L)X_3r]$ segment is defined as the subnuclear targeting arginine domain (STAD), and $[L(\Phi/N)(V/L)]$ is the subnuclear targeting hydrophobic domain (STHD); at least one of each of these segments should be present in a protein sequence to allow its nucleolar retention in response to a high extracellular hydrogen ion concentration [138]. It is nonetheless important to mention that although certain proteins contain clusters of basic residues, they do not migrate to nucleoli, which means that the presence of basic residues does not guarantee nucleolar accumulation [139]. As described in Bański *et al.* (2010), this suggests that the NoLS will target proteins to the nucleolus only when it is embedded in the proper context [64]. Moreover, because

of the amount of, and overlap between, the various signals that determine the subcellular localization of molecules, and the number of possible ways to get to the nucleolus (binding to nucleolar-bound molecules, trafficking to the nucleolus with other nucleolar-targeted proteins or binding to ribosomal RNA or proteins), it can be quite difficult to identify the NoLS [140]. Furthermore, proteins can diffuse through the nucleoplasm and through the nucleolus freely. It was calculated that nucleolar proteins stay in the nucleolus for few tens of seconds, and that non-nucleolar proteins reside there for at least one order of magnitude shorter. Finally, nucleolar proteins can be retained in the nucleolus due to their functional interactions with other nucleolar molecules, rather than sent/targeted to the nucleolus. Thus, the nucleolar targeting signal could be regarded as a retention signal more than one of targeting ([132, 140] and references therein).

1.5. The nucleolus in stress and disease

Several non-nucleolar proteins have been found in the nucleolus under varying conditions. Chaperones from the Hsp70 family, such as hsp70 and hsc70, accumulate in the nucleolus following stress [51, 64, 141, 142]. Co-chaperones and multitasking proteins are also found in the nucleolus under normal or stress conditions. In fact, it is possible that important chaperone-containing networks are present in the nucleolus, that contribute to cell survival under normal growth and stress conditions ([44, 100], and references therein). Moreover, nucleolar proteins can move out of the nucleolus in response to stress. An example of such a

nucleolar protein is fibrillarin, which has a role in ribosomal processing. Severe heat shock re-organizes fibrillarin into small dot-like structures, which are found in nucleoli immediately after heat shock. Following 20 hours of recovery, these structures disperse into the nucleoplasm [143]. Furthermore, nucleolin, a nucleolar protein, was shown to localize in the nucleoplasm following certain treatments, such as heat stress and ionizing radiation ([125] and references therein). This underlines the dynamic nature of the nucleolus and suggests that the nucleolus could play important roles in stress and human disease. This will be briefly described in the next sections.

Well established links between the nucleolus and cancer development are presented in multiple publications [121, 123, 127, 129, 134, 144-150]. The mechanism mainly occurs through cell cycle control, p53, $p14^{Arf}$, HDM2, and B23. B23 is a nucleolar protein that contributes to the biogenesis and proper maturation of ribosomes. $p14^{Arf}$ is a tumor suppressor gene present in the nucleolus ([145] and references therein). The steps through which $p14^{Arf}$ inhibits tumor formation start with the upregulation of $p14^{Arf}$ upon stress, which stabilizes p53 [123, 134, 148, 151] through the binding and inhibition of HDM2. HDM2 is capable of binding and blocking the action of p53 [152]. This can be seen as a way to sequester the HDM2 homologue in the nucleolus of mouse cells [123], although it is possible that the activity of HDM2 is blocked by $p14^{Arf}$ in the nucleoplasm ([148] and references therein). Nevertheless, $p14^{Arf}$ action prevents HDM2 from ubiquitinating p53 and targeting it for degradation. B23 is known to translocate to the nucleoplasm upon stress, and bind p53. This interaction prevents

the ubiquitination of p53 by the homologue of HDM2 in mouse cells [123]. The stability and activation of p53 are thereby increased, leading to cell-cycle arrest [148]. Interestingly, a study published in 2010 defines the nucleolar location of p53 as being unrelated to the site where ribosomal synthesis occurs, that it does not accumulate in FCs, and that p53 diminishes the transcription of rDNA by direct interaction with SL1, a polymerase I-specific transcription factor ([153], and references therein).

Nucleolar size and number is strongly linked to metabolic activity. Thus, the nucleolar size of cancer cells was directly correlated with tumor growth. This is explained through the constant division of cancerous cells, which necessitate massive ribosomal production ([134] and references therein) to sustain the high rate of protein turnover. Furthermore, in yeast cells, the nucleolus is involved in aging. In humans, no direct effect between aging and nucleoli has been established, although patients with Werner's syndrome, a disease characterized by premature aging, carry mutations in the WRN nucleolar helicase, which cause its inactivation ([121] and references therein). The nucleolus also plays roles in cell-cycle progression. For instance, p53-dependent postmitotic checkpoints are dependent on the nucleolus, as it plays an important role in their activation [154]. Furthermore, the nucleolus functions as a DNA damage ([155] and references therein) and stress sensor, contributes to the storage and assembly of molecules necessary for proper telomerase function, and is therefore considered a cellular health guardian [134, 148].

The nucleolus, which is now understood to be a multifunctional compartment, is also often involved in viral infections (reviewed in [123, 151]). As such, hepatitis D virus antigens have been shown to interact with nucleolin and B23, which modulate replication of the virus. Notably, 12 different RNA and DNA viruses utilize the nucleolus and subnuclear structures ([123], and references therein). Another example is the human immunodeficiency virus (HIV), which is one of the most studied viral-nucleolar interactions. Such interactions include the HIV-1 Rev protein that localizes to DFCs and GCs. Furthermore, the nucleolar morphology is modified in cells expressing Rev. This contributes to an abnormal nucleolar accumulation of B23, which colocalizes with Rev [156]. HIV-1 infection also correlates with cell-cycle deregulation and changes in overall nucleolar architecture ([140], and references therein). These are only some examples that demonstrate the important roles of nucleoli in proper cell function and growth. The nucleolus is currently the subject of intense research that aims to identify new nucleolar functions. This could lead to the targeting of the nucleolus for the treatment of devastating human diseases such as cancer and HIV.

1.6. Hsc70 and the nucleolus

Hsc70 shuttles between the cytoplasm and the nucleoplasm under normal growth conditions. Moreover, hsc70 accumulates in nuclei of heat shocked cells. When heat shocked cells recover, hsc70 moves to the nucleolus in a time-dependent manner [51, 64]. Although a 4°C treatment prevented hsc70 return to

the cytoplasm, it did not inhibit hsc70 nucleolar accumulation [51]. These data show that the nucleolar accumulation of hsc70 is controlled and occurs only under specific conditions. Furthermore, under normal growth conditions, steady state levels of hsc70 are present in the nucleolus, which is important as it emphasizes that hsc70 constantly interacts with nucleolar molecules. Interestingly, hsp70 also translocates to the nucleolus following its induction by stress [150, 157], indicating that hsc70 is not the sole Hsp70 family member capable of nucleolar accumulation.

There is a large number of different proteins that were identified in the nucleolus [133]. Some of these proteins are located permanently in the nucleolus while others accumulate there only under specific conditions, or move rapidly in and out of the nucleolus. It is thus difficult to identify the nucleolar anchors or targets of hsc70, which accumulates in the nucleolus during heat shock recovery [51, 64]. Hsc70 may accumulate in the nucleolus through various mechanisms [100], including a chaperone-dependent fashion, as it is believed to be the case for hsp70 [142]. In the nucleus of stressed cells, hsc70s colocalize and copurify with fibrillarin and with the ribosomal protein rpS6 [51]. One model explaining hsp70 nucleolar accumulation proposes that, following heat shock, the nucleolar accumulation of hsp70 accelerates the recovery of nucleolar morphology, as both the nucleolus and the ribosomal synthesizing and exporting machineries are affected by heat shock. This conclusion was made following the use of actinomycin D, a strong inhibitor of rRNA synthesis, on heat shocked and cells left at 37°C. When cells where treated with actinomycin D, nucleolar damage was

observed in the unstressed cells, whereas heat shocked cells did not show changes in nucleolar morphology [142]. These observations suggest that hsp70, which is activated in heat shocked cells, but not in unstressed cells, protects the nucleolus from damage.

Considering that nucleoli are sites of ribosomal biogenesis, ribonucleoproteins and rRNA are abundant in nucleoli. These molecules may also contribute to the nucleolar accumulation of hsc70. Moreover, chaperones assist the proper folding of RNA molecules and help them achieve their final, functional 3-D structures [158]. Other RNA-chaperone interactions have been identified. For instance, hsp70 directly binds to its own messenger RNA, which negatively affects its own production. This self-limiting mechanism prevents potential toxic effects that are observed when hsp70 synthesis is not regulated, thus leading to its overexpression [159]. Furthermore, hsp70 has the ability to bind certain mRNA sequences [160]; it is capable of associating with A + U rich RNA molecules, a process sensitive to ATP [161]. Hsc70 also associates with AUF1-containing complexes, in which case AUF1 serves as an A + U-rich element (ARE)-binding factor [162]. In the case of hsp70, the general conclusion is that the chaperone is able to stabilize transcripts that carry ARE domains, during heat shock. As the association of AUF1 and ARE induces mRNA degradation, it appears that hsp70 limits this effect ([160] and references therein). Moreover, hsp70, as well as hsc70, bind to AU rich mRNA and artificial RNA oligonucleotides [163]. Zimmer *et al.* (2001), showed the N-terminal ATPase domain of the chaperone to be sufficient for hsp70-RNA interactions, whereas the C-terminal domain controls the affinity with

which hsp70 binds to RNA. As such, a probe containing the IL-10 3' UTR 5XAUUUA sequence binds with more affinity than an artificial $(AUUU)_5A$ probe. Furthermore, the binding to an AU-rich 3' UTR RNA was significantly diminished when the C-terminal hsp70 domains were removed. Interestingly, when the N-terminal and C-terminal ends of hsp70 were prevented from interacting, the RNA-binding sequence specificity was lost [163]. Considering their high sequence similarity, it is conceivable that both hsp70 and hsc70 contribute to the rescue of nucleolar function and morphology. However it is at present not completely understood how hsc70 accumulates in nucleoli and what its nucleolar binding partners are. In this work, I provide new insights into the nucleolar accumulation of hsc70.

2. CHAPTER I

Nucleolar targeting of the chaperone hsc70 is regulated by stress, cell signaling, and a composite targeting signal which is controlled by autoinhibition

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Running head: Stress-induced accumulation of hsc70 in nucleoli.

2.1. Abstract

Hsc70s are constitutively synthesized members of the 70 kDa chaperone family; they are essential for viability and conserved among all organisms. When eukaryotic cells recover from stress, hsc70s accumulate in nucleoli by an unknown mechanism. Our studies were undertaken to characterize the signaling events and the targeting sequence required to concentrate hsc70 in the nucleoli of human cells. Here, we show that pharmacological inhibitors of phosphatidylinositol 3 kinase (PI3K) and MEK kinases as well as protein-tyrosine phosphatases abolished the stress-dependent nucleolar accumulation of hsc70. Furthermore, to identify the hsc70 nucleolar targeting sequence, green fluorescent protein-tagged fusion proteins with defined segments of hsc70 were generated and their subcellular distribution was analyzed in growing cells. These studies demonstrated that residues 225 to 297 serve as a heat-inducible nucleolar targeting signal. This segment directs green fluorescent protein to nucleoli in response to stress, but fails to do so under nonstress conditions. Fine mapping of the nucleolar targeting signal revealed that it has two separable functions. First, residues 245 to 287 direct reporter proteins constitutively to nucleoli, even without stress. Second, segment 225 to 244 functions as an autoinhibitory element that prevents hsc70 from concentrating in nucleoli when cells are not stressed. Taken together, PI3-kinase and MEK kinase signaling as well as tyrosine dephosphorylation are essential for the accumulation of hsc70 in nucleoli of stressed cells. This process relies on a stress-dependent composite targeting signal that combines multiple functions.

2.2. Introduction

Heat shock cognate proteins 70 (hsc70) are members of the 70 kDa family of chaperones, which are conserved among all organisms. Unlike cytoplasmic hsp70s, hsc70s are constitutively synthesized and essential for cell survival [164]. Hsp/hsc70s are involved in a large number of cellular processes: they contribute to the proper folding of nascent polypeptides, refolding of denatured proteins, and targeting of damaged proteins to the proteasome (reviewed in [13, 165, 166]). Hsp/hsc70s are essential for protein sorting to organelles and play a protective role in many human diseases and pathophysiologies, including Alzheimer and Huntington disease or ischemia of the heart and brain (reviewed in [167, 168]). Furthermore, defects in chaperone function have been linked to human disease and aging [167, 169-171].

Members of the hsp/hsc70 family are organized into three domains; the 44 kDa N-terminal ATPase domain is followed by a protein binding segment of 18 kDa and a 10 kDa variable domain at the C-terminal end [13, 165-168]. Under normal growth conditions, hsc70s shuttle between the nucleus and the cytoplasm; however, hsc70s shuttling is inhibited upon stress [51]. Following heat shock, hsc70s initially accumulate in the nucleoplasm, where they associate with chaperone substrates that require refolding [51, 69]. When cells recover from stress, hsc70s transiently concentrate in nucleoli possibly to restore nucleolar morphology and function [142].

The nucleolus is a specialized compartment within the nucleus, where pre-rRNA synthesis and processing take place. Together with ribosomal proteins and the assistance of multiple processing factors, these rRNAs are assembled into ribosomal subunits [123, 129, 172-174]. Other functions have been ascribed to the nucleolus as well, including the assembly of signal recognition particle, the coordination of stress responses, virus replication, and the control of cell cycle progression and apoptosis (reviewed in [127-129, 140, 175, 176]). Recent proteomic studies revealed the complex composition of the mammalian nucleolus that may contain several thousand different proteins [133, 177]. Importantly, the composition of nucleoli is dynamic and modulated by changes in cell physiology [178, 179]. With respect to heat shock proteins, nucleoli are of particular interest, because they possibly harbor a specialized network composed of chaperones and multitasking proteins that cooperate to support cell survival under normal and stress conditions [44].

In response to stress or other stimuli, cells activate kinase signaling cascades to initiate appropriate physiological reactions. Depending on the type of stress, different non-overlapping pathways are activated, although cross-talk between these routes exists. In particular, signaling through the PI3-kinase→Akt and MEK→ERK1/2 pathways promotes cell survival, stimulates growth as well as ATP production [180-182]. Stress-induced kinase activation alters the post-translational modification of a large number of proteins; these modifications include the phosphorylation of serine, threonine and tyrosine residues. Many such stress-induced modifications are dynamic, and a balance between phosphorylation

and dephosphorylation is required to respond properly to changes in growth conditions.

Despite the vast amount of information available for heat shock proteins and the stress response, little is known about the targeting of hsc70 to nucleoli and the signaling pathways involved in hsc70 trafficking. To address these questions, we used pharmacological tools and demonstrate that PI3-kinase and MEK kinase cascades as well as tyrosine dephosphorylation are essential for the proper accumulation of hsc70 in the nucleoli of stressed cells. Moreover, our studies define a region of hsc70 that mediates stress-dependent targeting to the nucleolus and contains two separable functions. Specifically, we identified a constitutive nucleolar targeting signal that is controlled by an autoinhibitory element which prevents nucleolar accumulation in the absence of stress.

2.3. Experimental Procedures

Generation of GFP-tagged Reporter Proteins— A derivative of pHM830 [183] lacking the *β-galactosidase* gene was used as vector. Different segments of the hsc70 coding sequence followed by a stop codon were amplified by PCR and fused in frame to the 3'-end of the *GFP* gene. Alternatively, oligonucleotides were inserted into the vector to generate GFP-fusions with shorter segments of hsc70. For comparison, plasmids were generated that encode GFP fused to the complete hsc70 coding sequence or hsc70 lacking codons 225-297. The expression of

fusion genes is driven by a cytomegalovirus promoter. Site-directed mutagenesis was carried out to mutate residue Thr^{265}. The correctness of all constructs was verified by DNA sequencing.

Growth, Transfection and Heat Shock Experiments— HeLa cells where grown to ~70% confluency, for no more than 12 passages. 24 h after transfection [51] cells were exposed to 1 h heat stress at 45.5°C and subsequently allowed to recover at 37°C for the times indicated.

Pharmacological Tools— The following inhibitors were dissolved in DMSO and used at the final concentrations indicated: 25 μM PD98059 (inhibitor of MEK, thereby preventing the activation of MAPKs ERK1/2 and ERK5), 0.1-0.6 μM phenylarsine oxide (PAO; inhibitor of protein-tyrosine phosphatases) and 50 μM LY294002 (inhibitor of PI3-kinase). LY294002 was purchased from Cell Signaling (Danvers, MA); other inhibitors were from Calbiochem (San Diego). In all experiments DMSO concentrations were identical (0.13%), and control cells were treated with DMSO only. Cells were incubated with protein kinase inhibitors 1 hour prior to heat shock, and drugs were present throughout heat shock and recovery periods. PAO was added following heat shock to analyze endogenous hsc70. For reporter proteins that can diffuse across the nuclear envelope, PAO was added immediately before heat shock.

Immunofluorescence and Detection of GFP-tagged Proteins— Immunolocalization of endogenous hsc70 was carried out essentially as described

[51]. Samples were incubated with 7.5 µg/ml of fluorescein isothiocyanate (FITC)- or 0.4 µg/ml Cy3-conjugated secondary anti-rat antibodies for 2 h (Jackson, ImmunoResearch, West Grove, PA); all other steps followed published procedures [51]. To visualize GFP-tagged reporter proteins cells were washed in phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) and samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Images were acquired for 0.7-µm slices with a Zeiss LSM510 inverted microscope using a ×63 oil-immersion objective with 1.4 NA and processed in Adobe Photoshop 11.0.

Quantification of Fluorescence Signals in Nucleoli and Statistics— Pixel intensities located in nucleoli were measured with the multiwavelength cell scoring module [184]. Pixel intensities/area were calculated and nucleolar/nuclear values were determined for each nucleus. For cells with more than one nucleolus, the sum of nucleolar pixel intensities/sum of the nucleolar area was used for further calculations. At least three independent experiments were carried out for each condition. Nucleolar accumulation of endogenous hsc70 was scored for a minimum of 55 cells in each experiment. For every experiment, all test results were compared with untreated control samples. Data in figure 9 are shown as mean ±S.E. (standard error). Student's *t* tests (two-tailed) for unpaired samples or one-way analysis of variance (ANOVA) was carried out to identify significant differences. For transient transfections, at least three different experiments were performed, each set with appropriate controls. Cells were visually inspected, and

reporter proteins displayed similar distribution in replicate experiments. Quantification was carried out for a representative experiment, and pixel intensities were measured for 42 to 74 cells for each data point. Results are shown as mean ± S.D. (standard deviations).

Western Blot Analysis and Quantification of ECL Signals— Quantitative Western blotting followed published procedures [73]. Antibodies against phospho-Akt473 (1:2,000; Santa Cruz), phospho-Akt308 and total-Akt (1:1,000 and 1:2,500; Cell Signaling), hsc70 (1:5,000; Stressgen), GFP (1:500, Clontech) and actin (1:100,000; Chemicon) were used at the dilutions indicated.

2.4. Results

Hsc70 and GFP-hsc70 Accumulate in Nucleoli during Recovery from Stress— Under normal growth conditions members of the hsp/hsc70 family shuttle between the nuclear and cytoplasmic compartments, but concentrate transiently in nuclei upon exposure to stress [51, 69]. Upon heat shock, endogenous hsc70 initially accumulates in the nucleoplasm. However, during recovery from stress the chaperone concentrates in nucleoli [51], a process which is not understood at the molecular level. To begin to define these events we used HeLa cells to monitor the nucleolar accumulation of endogenous hsc70 in response to heat stress (Figure 7). After a 1-h heat shock and subsequent incubation at 37°C, hsc70 nucleolar accumulation peaked at 3 and 4 h of recovery (Figure 7). At 5 h of

recovery hsc70 remained concentrated in the nucleoli of some cells, whereas after a 15-h recovery period the distribution was similar to unstressed cells [51]. Like endogenous hsc70, GFP-hsc70 in transiently transfected cells was nuclear and cytoplasmic under normal growth conditions and concentrated in the nucleus in response to heat shock. Furthermore, GFP-hsc70 accumulated in nucleoli when cells recovered from heat stress (Figure 7); the GFP tag did not concentrate in nucleoli under any conditions (data not shown). Thus, the analysis of GFP-tagged hsc70 is a valid approach to study the nucleolar accumulation of the chaperone.

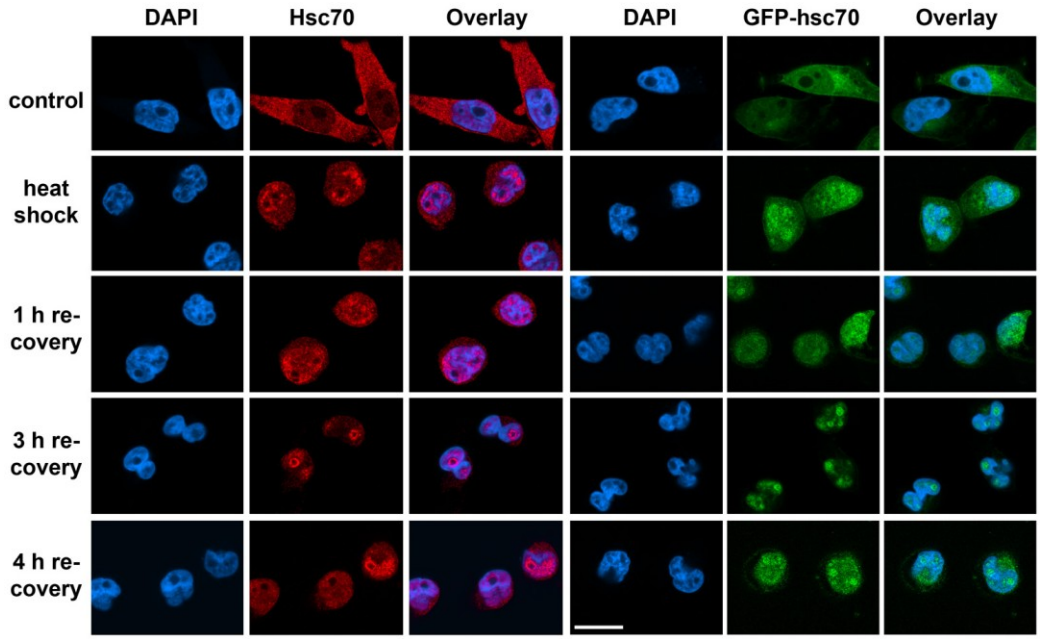


Figure 7: Stress-induced nucleolar accumulation of endogenous hsc70 and GFP-tagged hsc70. HeLa cells were exposed to heat shock and allowed to recover at 37°C. At the times indicated cells were fixed and endogenous hsc70 was detected by indirect immunofluorescence. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). HeLa cells transiently synthesizing GFP-hsc70 were fixed

at different time points and visualized with 4',6-diamidino-2-phenylindole (DAPI). Fusion proteins were detected by fluorescence microscopy. *Size bar* is 20 μm.

Heat Shock Activates PI3-Kinase→Akt and MEK→ERK1/2 Signaling— Stress can activate different signaling pathways, and PI3-kinase→Akt and MEK→ERK1/2 signaling cascades in particular play a role in cell survival [180-182]. Quantitative Western blotting revealed that Akt phosphorylation on Thr^{308} and Ser^{473} as well as dual ERK1/2 phosphorylation increased transiently when cells were exposed to heat shock (Figure 8). [Dual phosphorylation of Akt on residues Thr^{308} and Ser^{473} is necessary to fully activate the kinase ([182] and references therein).] As expected, levels of the constitutively synthesized hsc70 did not change drastically in response to heat shock and up to 4 h of recovery (Figure 8).

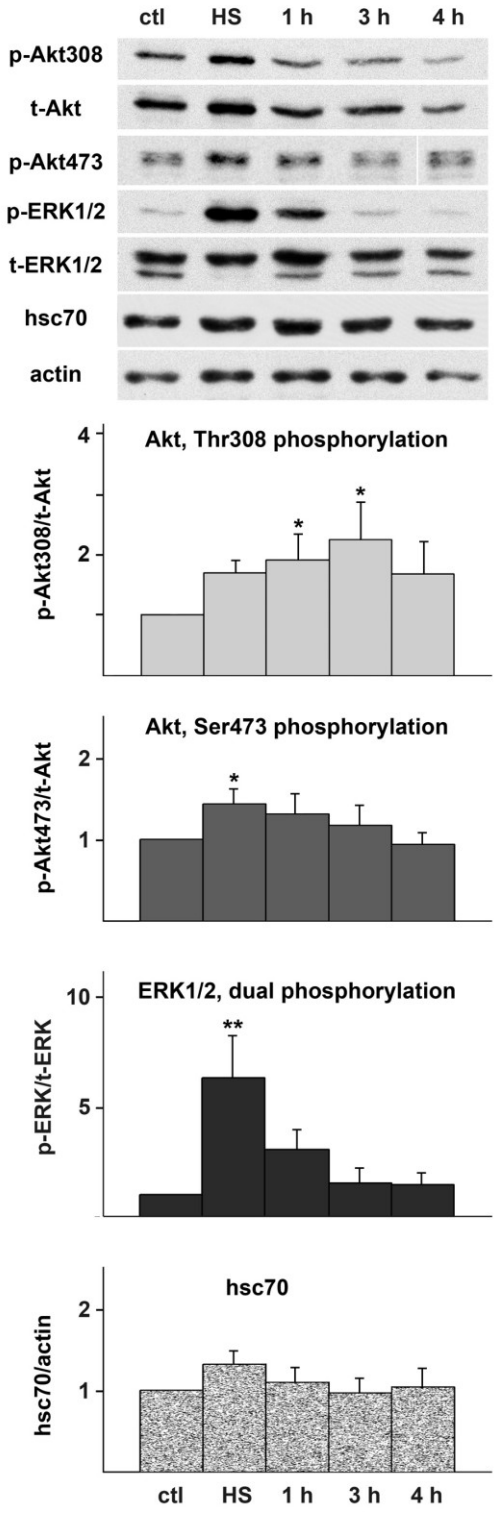
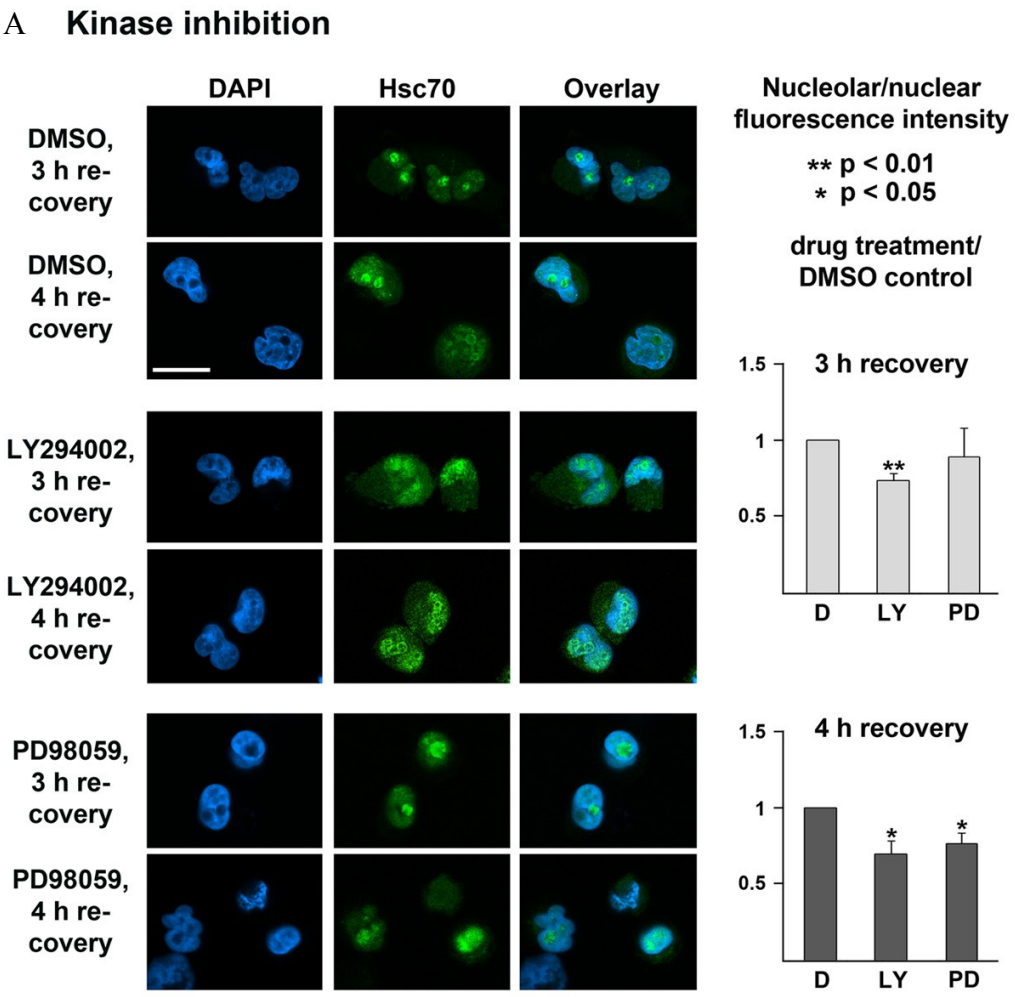

ctl
HS
1 h
3 h
4 h
p-Akt308
t-Akt
p-Akt473
p-ERK1/2
t-ERK1/2
hsc70
actin
Akt, Thr308 phosphorylation
p-Akt308/t-Akt
4
2
*
*
Akt, Ser473 phosphorylation
p-Akt473/t-Akt
2
1
*
ERK1/2, dual phosphorylation
p-ERK/t-ERK
10
5
**
hsc70
hsc70/actin
2
1
ctl
HS
1 h
3 h
4 h

Figure 8: Heat shock activates signaling through PI3-kinase and the MEK→ERK1/2 pathway. Crude extracts prepared from control (ctl), heat-shocked (HS) and recovering cells were analyzed by quantitative Western blotting with the different antibodies indicated in the figure. The relative phosphorylation of protein kinases (phospho-kinase/total kinase) or hsc70 levels (hsc70/actin) in unstressed cells was defined as 1. Data represent at least three independent sets of experiments. One-way analysis of variance (ANOVA) identified significant differences; unstressed control cells served as reference. *, $p<0.05$; **, $p<0.01$.

Inhibition of PI3-Kinase and MEK Activation Reduces the Nucleolar Accumulation of Hsc70— In stressed cells, the increase in *nuclear* levels of hsc70 is followed by *nucleolar* accumulation of the chaperone when cells recover from the insult. Therefore, we tested whether pharmacological kinase inhibitors that do not interfere with hsc70 nuclear import ([69] and data not shown) affect the concentration of hsc70 in *nucleoli*. PI3-kinase inhibitor LY294002 and MEK inhibitor PD98059 were used at concentrations that prevent Akt modification on Thr^{308} and ERK1/2 dual phosphorylation [67]. In these experiments, kinase inhibitors were present throughout the experiment. Following a 1-h preincubation, HeLa cells were heat-shocked, allowed to recover for 3 or 4 h, and monitored for hsc70 distribution. Nucleolar accumulation was quantified by measuring pixel intensities/area in nucleoli and the nucleus. Data are depicted as the nucleolar/nuclear ratio of fluorescence intensity. Results obtained for DMSO-treated control cells were defined as 1 (*D* in Figure 9A). A ratio <1 indicates that hsc70 is less abundant in nucleoli when compared with DMSO-treated controls.

Figure 9A demonstrates that with LY294002 (LY) hsc70 nucleolar accumulation was reduced modestly but significantly to 87 and 85%, respectively, after 3 and 4 h of recovery. Similarly, PD98059 (PD) inhibited nucleolar accumulation at 3 and 4 hours (to 95 and 89%), albeit with higher variability. These data support the idea that both PI3 kinase and MEK-dependent signaling pathways in stressed cells are important to concentrate hsc70 in nucleoli.



B

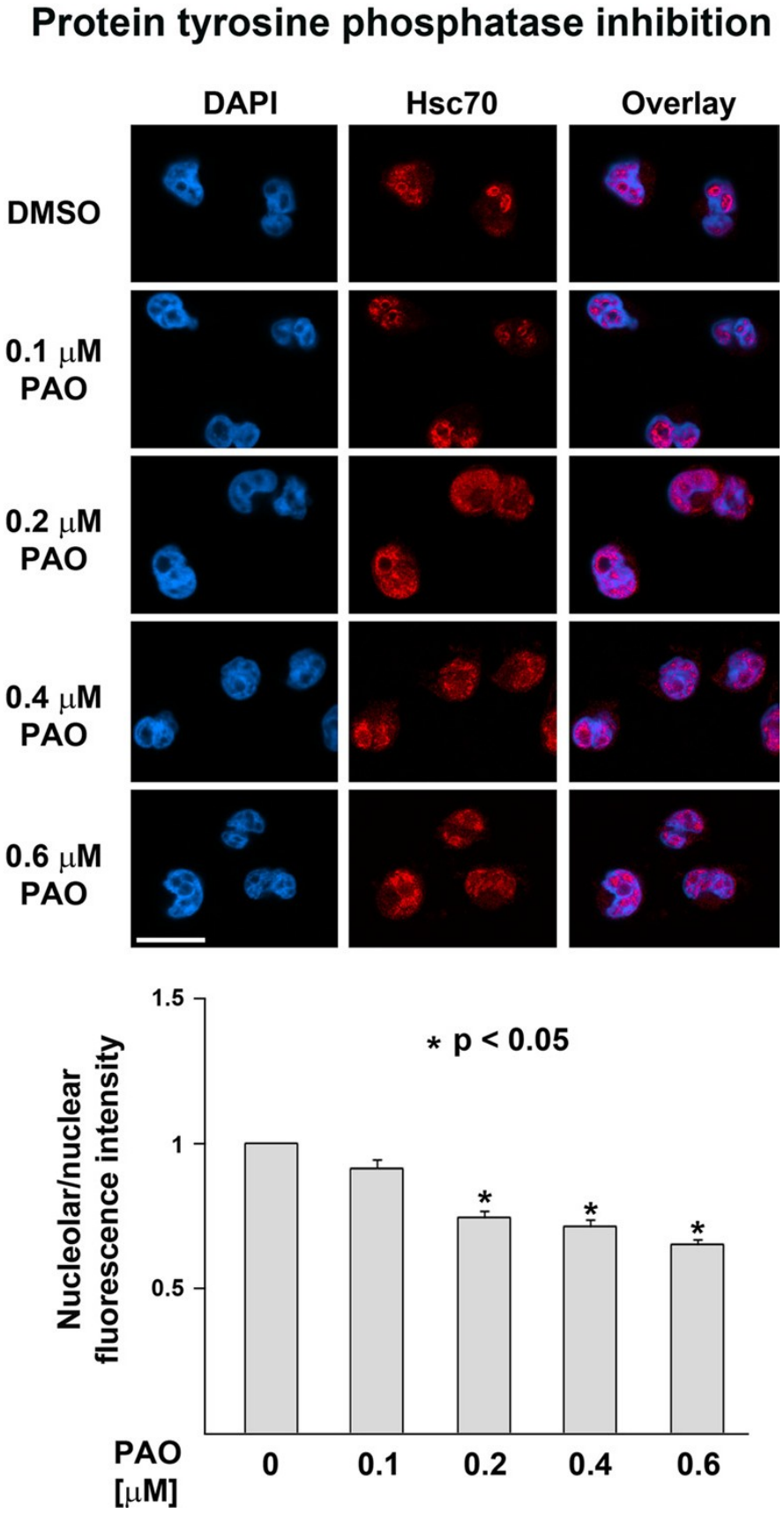

Protein tyrosine phosphatase inhibition
DAPI
Hsc70
Overlay
DMSO
0.1 μM PAO
0.2 μM PAO
0.4 μM PAO
0.6 μM PAO
Nucleolar/nuclear fluorescence intensity
1.5
1
0.5
* p < 0.05
*
*
*
PAO [μM]
0
0.1
0.2
0.4
0.6

Figure 9: Pharmacological inhibition of PI3-kinase and MEK kinases or tyrosine phosphatases alters hsc70 nucleolar accumulation during recovery from stress.
(A) HeLa cells were incubated with DMSO, LY294002 (*LY*, PI3 kinase inhibitor) or PD98059 (*PD*, MEK inhibitor) using previously established conditions [67]. Endogenous hsc70 was localized by indirect immunofluorescence and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Nucleolar accumulation was quantified after 3 and 4 h of recovery. Data are depicted as nucleolar/nuclear fluorescence intensity; results for DMSO-treated controls (D in the figure) were defined as 1. Three sets of experiments were carried out, between 57 and 86 cells were scored for each data point in every set. The graph shows mean ± S.E. Student's *t*-test was used to determine significant differences. *Size bar* is 20 μm.
(B) HeLa cells were treated with increasing concentrations of PAO (tyrosine phosphatase inhibitor) and endogenous hsc70s were located after 3 h of recovery as described for A. Nucleolar accumulation was quantified for different PAO concentrations; the ratio of nucleolar/nuclear fluorescence of DMSO-treated control samples was defined as 1. Three sets of experiments were performed, between 55 and 106 cells were evaluated for each data point in every set. Mean ± S.E. are depicted. One-way analysis of variance (ANOVA) was used for the statistical analysis. *Size bar* is 20 μm.

In control experiments we tested the effect of pharmacological inhibitors on the phosphorylation of Akt on Thr^{308} and the dual phosphorylation of ERK1/2, respectively (Figure 10). LY294002 completely abolished the phosphorylation of Akt residue Thr^{308}, whereas PD98059 drastically diminished the heat-induced

changes in ERK1/2 dual phosphorylation. These results support the idea that the inhibitors reduced the stress-dependent activation of Akt and ERK1/2. Importantly, the decreased activation of kinases correlated with a reduction in hsc70 nucleolar accumulation.

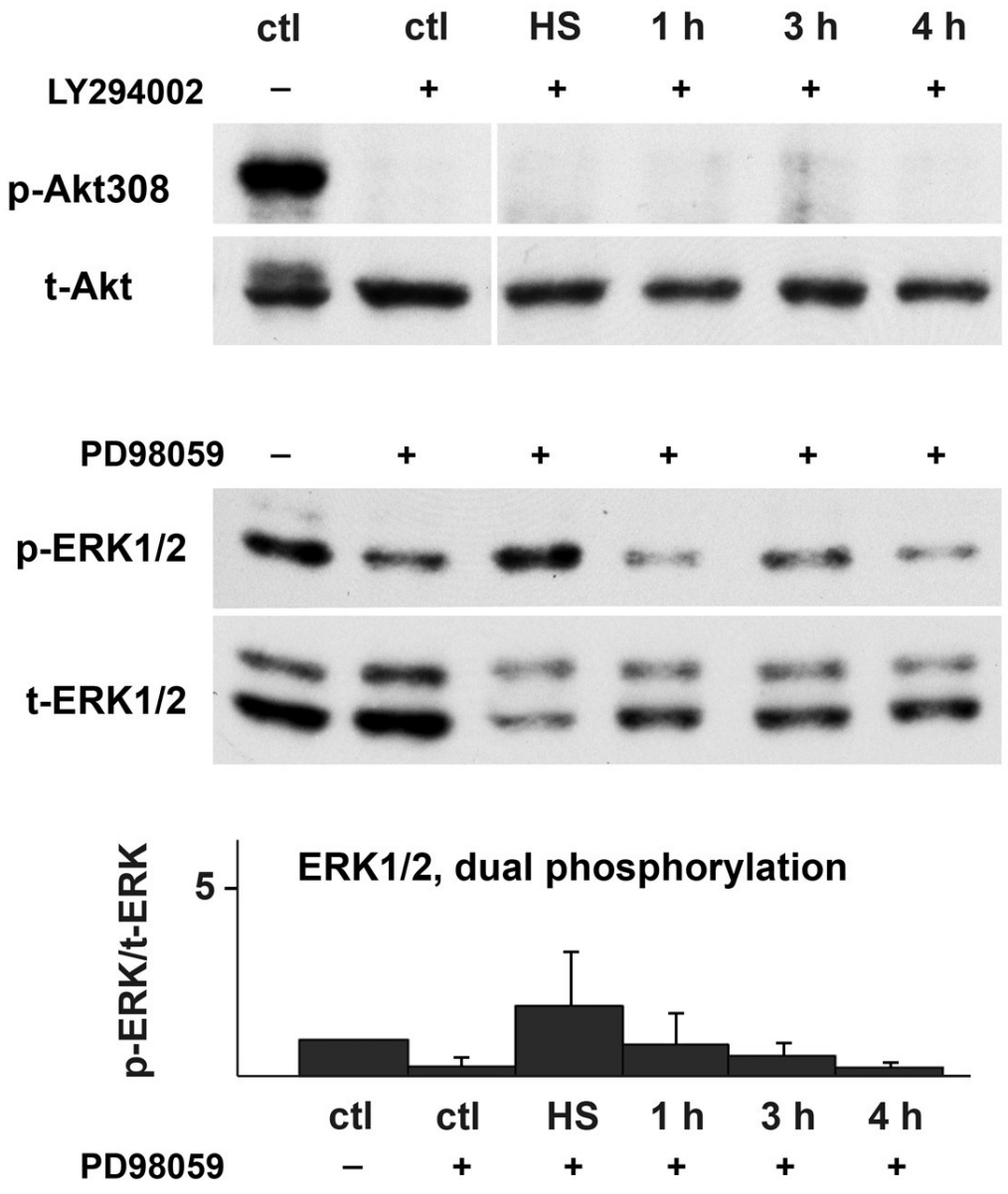
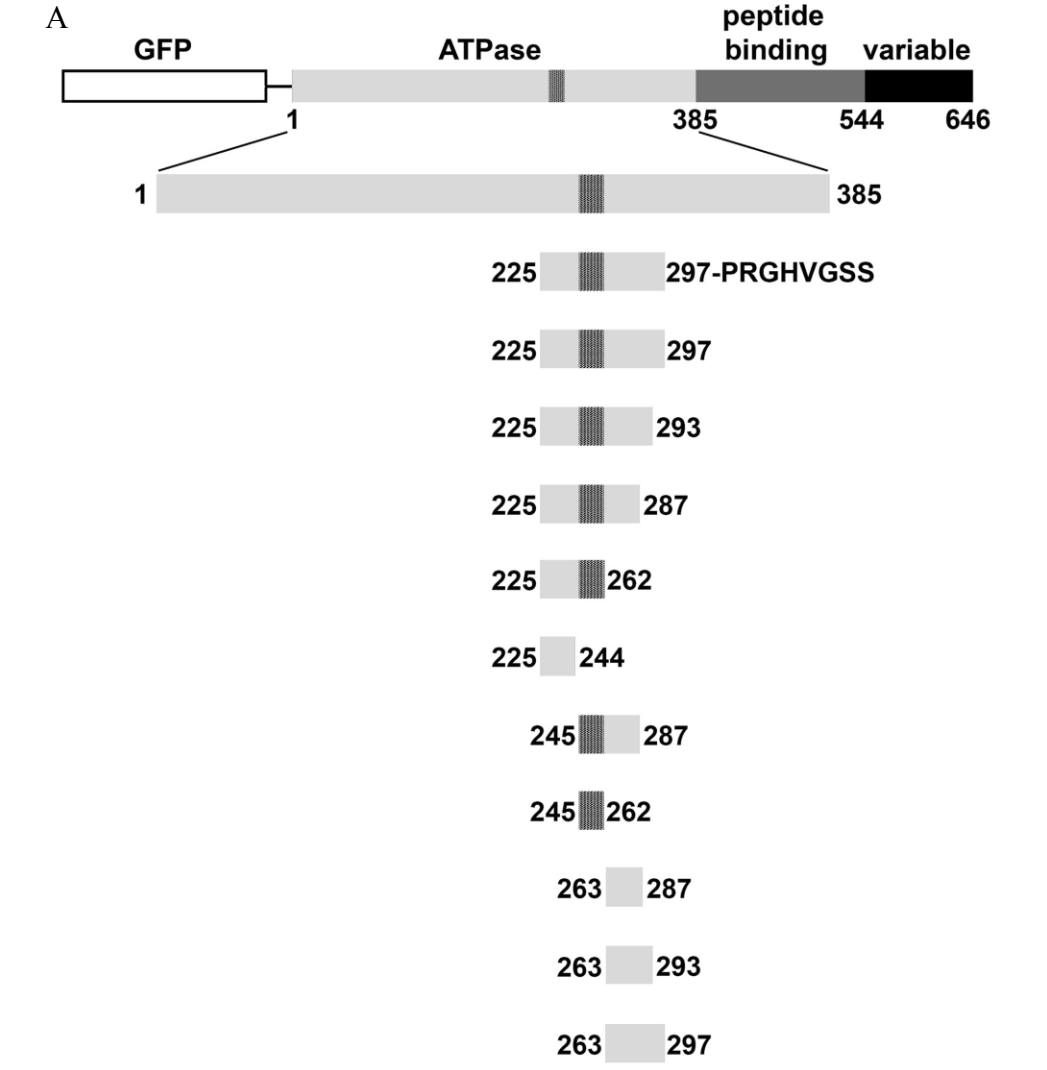


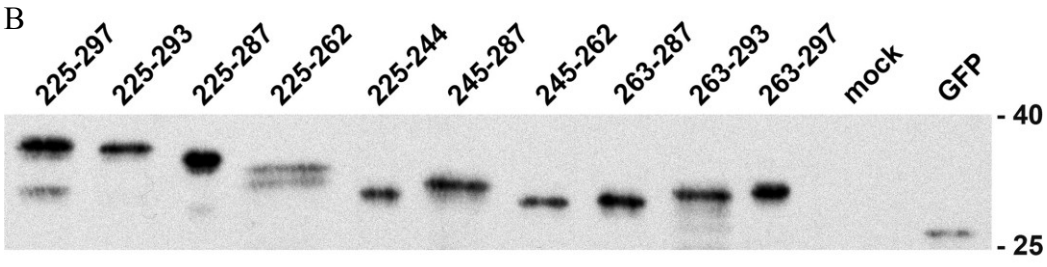
Figure 10: Treatment with LY294002 and PD98059 diminishes the heat-induced activation of Akt and ERK1/2. HeLa cells were pretreated with LY294002 or PD98059 as described in Experimental Procedures. Crude extracts were prepared at the times indicated. The presence of p-Akt308, t-Akt, dually phosphorylated ERK1/2 and t-ERK1/2 was determined by Western blotting, and the relative phosphorylation of ERK1/2 was quantified. Note that as compared to samples without inhibitors (Figure 8) incubation with drugs drastically reduced the stress-dependent activation of the kinases.

Protein-tyrosine Phosphatases Regulate the Nucleolar Targeting of hsc70— Our previous studies demonstrated that inhibition of tyrosine phosphatases abolishes heat-induced hsc70 nuclear import in HeLa cells [69]. However, it is not known whether tyrosine phosphatases also play a role in nucleolar targeting. To address this question, the tyrosine phosphatase inhibitor PAO was added to cells after heat shock, and nucleolar accumulation of endogenous hsc70 was analyzed after a 3-h recovery period. The nucleolar/nuclear fluorescence intensity in cells incubated with the solvent DMSO was defined as 1 (Figure 9B; 0 μM PAO). Except for 0.1 μM PAO, all of the treatments significantly reduced hsc70 nucleolar accumulation when compared with control cells. These results implicate the dephosphorylation of modified tyrosine residues in nucleolar targeting of the chaperone during recovery from stress.

Residues 225 to 297 of Hsc70 Are Sufficient to Promote Stress-induced Nucleolar Accumulation— Analysis of hsc70 nucleolar accumulation is

complicated by the fact that the 70 kDa chaperone exceeds the diffusion channel of the NPC. Thus, treatments that interfere with hsc70 nuclear import will also affect the subsequent concentration in nucleoli. To avoid this difficulty, we generated reporter proteins that are small enough to diffuse across the NPC. Fusion of defined hsc70 segments to a 28 kDa GFP tag produced reporter proteins with a molecular mass less than ~40 kDa (Figure 11A, B). In the following, with the exception of the deletion mutant GFP-hsc70(Δ225-297), numbers in parentheses denote the residues of hsc70 that were fused to GFP. Figure 11A shows the domain organization of hsc70 and the chaperone segments present in different constructs. Western blot analysis of crude extracts revealed that reporter proteins migrated as expected during SDS-PAGE (Figure 11B and C).


A
GFP
ATPase
peptide binding
variable
1
385
544
646
1
385
225
297-PRGHVGSS
225
297
225
293
225
287
225
262
225
244
245
287
245
262
263
287
263
293
263
297


B
225-297
225-293
225-287
225-262
225-244
245-287
245-262
263-287
263-293
263-297
mock
GFP
- 40
- 25

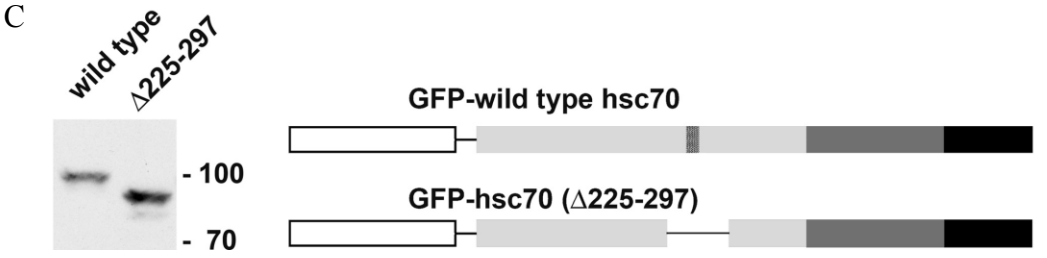
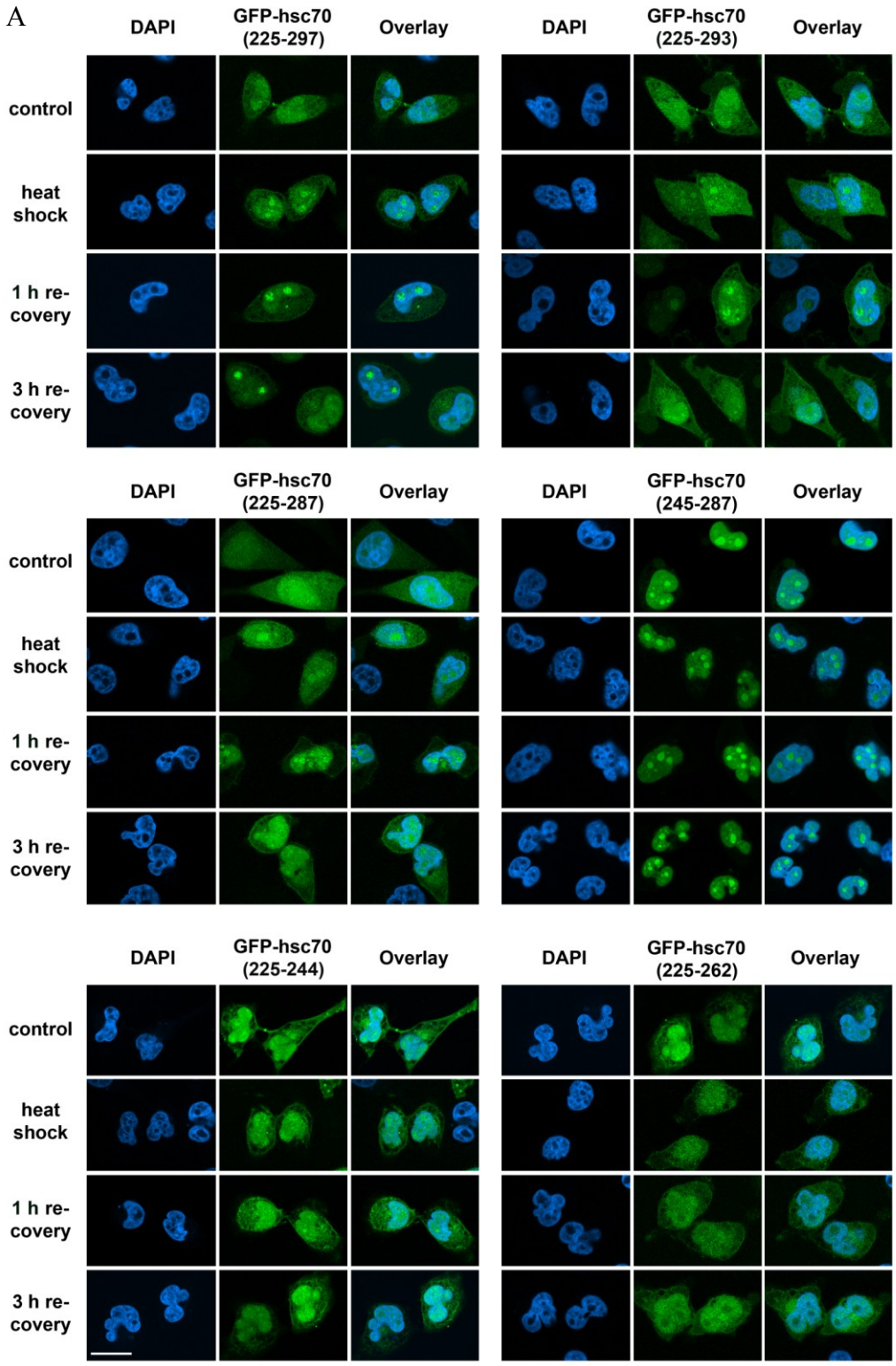
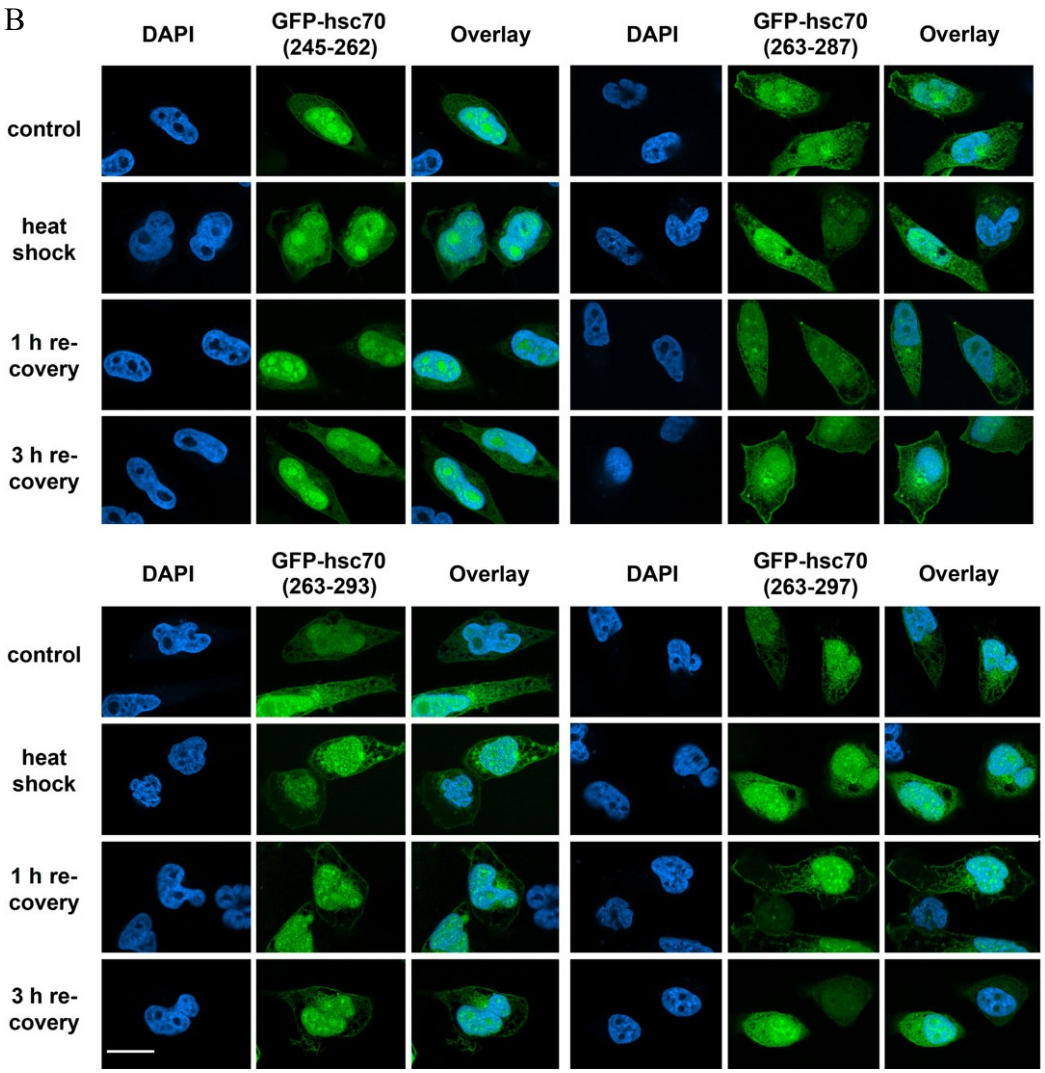

C
wild type
Δ225-297
- 100
- 70
GFP-wild type hsc70
GFP-hsc70 (Δ225-297)

Figure 11: Reporter proteins used in this study. (A) The domain organization of hsc70 and the fusion proteins analyzed are shown. *Numbers* denote the first and last amino acid residues of the hsc70-derived fragment. For each construct the last codon is followed by a stop codon. A segment within the ATPase domain that fits the consensus for a bipartite nuclear localization sequence (residues 245-262) is marked with *black stripes*. (B) HeLa cells were transiently transfected with plasmids encoding different reporter proteins that carry the indicated hsc70 residues. Crude extracts generated for transfected cells were analyzed by Western blotting. (C) GFP was fused to full-length hsc70 (wild type) or a deletion mutant lacking residues 225-297. In B and C fusion proteins were detected by Western blotting with anti-GFP antibodies. Molecular masses of marker proteins ($\times 10^3$) are indicated at the *right margin*.

In Figure 12, A-C, we analyzed the distribution of GFP-tagged fusion proteins in transiently transfected cells under different growth conditions. To this end, nucleolar and nuclear fluorescence intensities were quantified, and the ratio of nucleolar/nuclear fluorescence was calculated for each time point. Results for GFP-hsc70(225-297) under control conditions served as a reference in these studies. In each experiment, data were normalized to the nucleolar/nuclear ratio obtained for GFP-hsc70(225-297) in the absence of stress; this ratio was defined as 1.


A
DAPI
GFP-hsc70 (225-297)
Overlay
DAPI
GFP-hsc70 (225-293)
Overlay
control
heat shock
1 h re-covery
3 h re-covery
DAPI
GFP-hsc70 (225-287)
Overlay
DAPI
GFP-hsc70 (245-287)
Overlay
control
heat shock
1 h re-covery
3 h re-covery
DAPI
GFP-hsc70 (225-244)
Overlay
DAPI
GFP-hsc70 (225-262)
Overlay
control
heat shock
1 h re-covery
3 h re-covery


B
DAPI
GFP-hsc70 (245-262)
Overlay
DAPI
GFP-hsc70 (263-287)
Overlay
control
heat shock
1 h re-covery
3 h re-covery
DAPI
GFP-hsc70 (263-293)
Overlay
DAPI
GFP-hsc70 (263-297)
Overlay
control
heat shock
1 h re-covery
3 h re-covery

C **Nucleolar/nuclear fluorescence intensity**

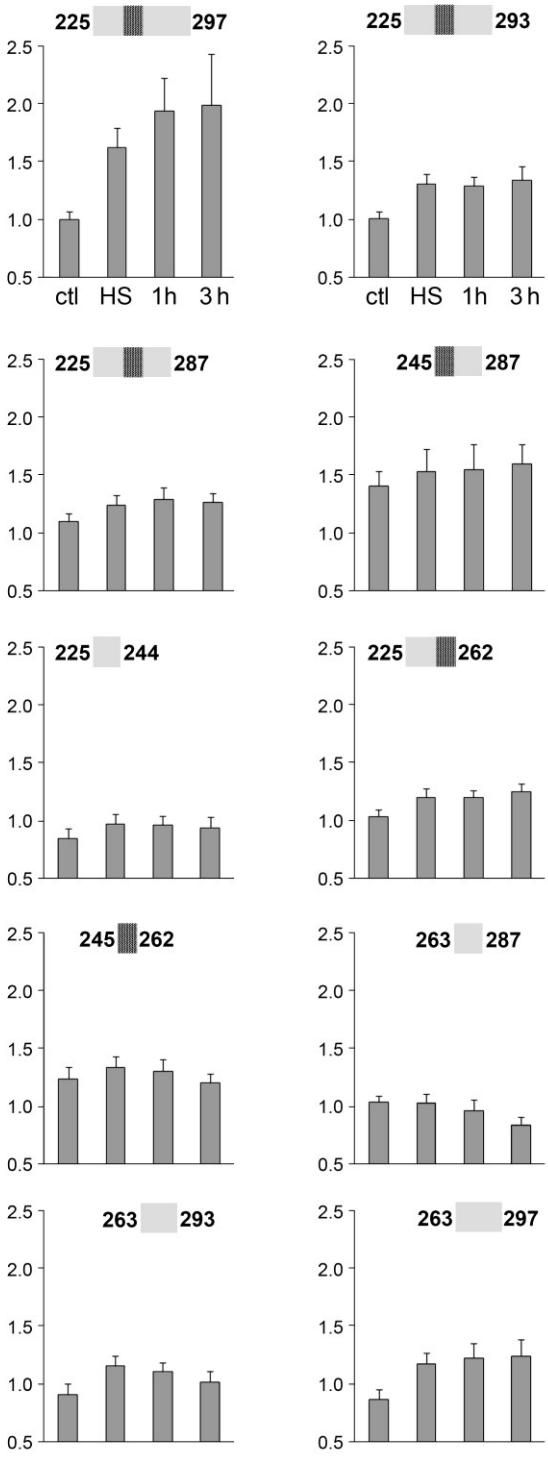


Figure 12: Multiple parts of segment 225 to 297 of hsc70 control the stress-induced nucleolar accumulation of reporter proteins. (A) and (B) GFP-tagged proteins containing different segments of hsc70 were exposed to heat stress followed by recovery for the times indicated. Fusion proteins and 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei were detected by fluorescence microscopy. *Size bar* is 20 μm. Note the constitutive nucleolar accumulation of GFP-hsc70(245-287) and GFP-hsc70(245-262) even in the absence of stress. (C) Fluorescence signals in nucleoli and nuclei were quantified for all constructs at different time points. Between 50 and 74 cells were examined for each data point. Results are shown as nucleolar/nuclear pixel intensities. Data were normalized to results obtained for GFP-hsc70(225-297) under nonstress conditions, which was defined as 1.

Figure 12, A-C demonstrates that, with the exception of GFP-hsc70(245-287) and GFP-hsc70(245-262), in the absence of stress all of the reporter proteins were present in the cytoplasm and nucleus, but not concentrated in nucleoli. Following heat shock, we observed nucleolar accumulation for several of these constructs, albeit with different efficiencies. The most prominent stress-dependent redistribution was observed for GFP-hsc70(225-297); the nucleolar/nuclear ratio increased from 1 in unstressed cells to ~1.8 at 1 h and 1.9 at 3 h of recovery (Figure 12, A and C). Addition of unrelated amino acid residues PRGHVGSS to the C-terminal end of the hsc70 fragment 225-297, referred to as GFP-hsc70(225-297aa), did not interfere with its nucleolar targeting function (see below and data not shown). Taken together, our results are consistent with the idea that residues

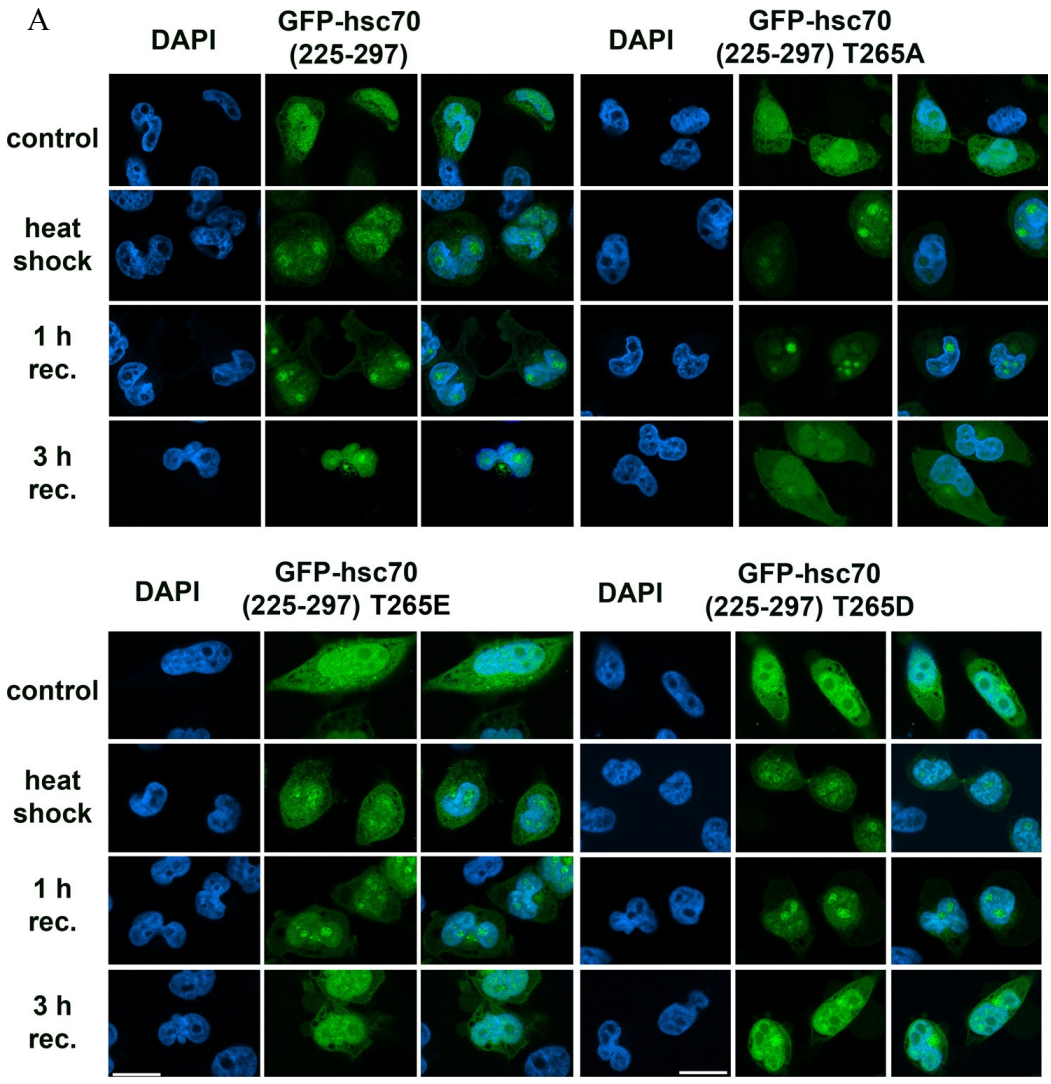
225-297 of hsc70 are sufficient to promote nucleolar targeting in response to heat shock. Thus, hsc70 residues 225-297 provide a stress-dependent nucleolar localization signal (NoLS).

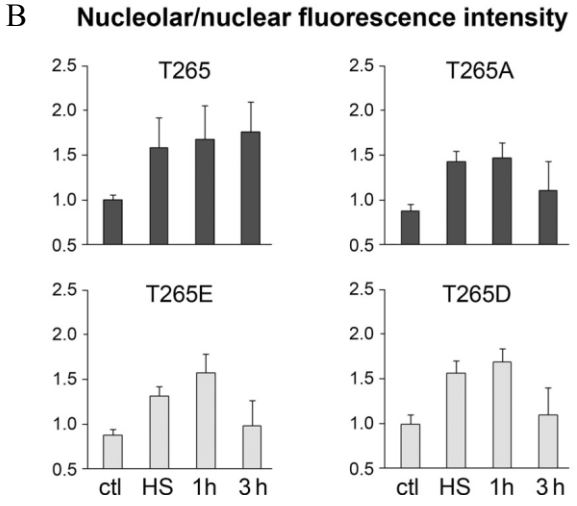
It should be noted that for all hsc70 segments that mediated targeting to nucleoli accumulation in the nucleolar compartment was observed at an earlier time point when compared with endogenous hsc70 or the full length GFP-tagged chaperone. This is most likely due to the fact that the hsc70 segments analyzed by us do not contain the peptide binding domain and therefore will not be retained in the nucleoplasm by chaperone/client interactions. We have shown earlier that the hsc70/client association contributes to hsc70 retention in the nucleoplasm of stressed cells [51].

C-terminal Truncations of the Hsc70 NoLS Diminish the Stress-induced Nucleolar Localization of Reporter Proteins— To gain further insight into the functional organization of the hsc70 NoLS, we generated a panel of different truncations. Under nonstress conditions, GFP-hsc70(225-293) and GFP-hsc70(225-287) displayed a distribution that was similar to GFP-hsc(225-297); however, GFP-hsc70(225-293) and GFP-hsc70(225-287) concentrated only 1.2- to 1.3-fold in nucleoli upon heat shock or during recovery (Figure 12, A and C). This suggests that the C-terminal portion of the hsc70 NoLS contributes to the heat-dependent nucleolar accumulation of reporter proteins, an interpretation supported by other reporter proteins (see below).

Residues 245 to 287 Promote Constitutive Nucleolar Targeting Which Is Negatively Controlled by Residues 225 to 244— Additional constructs were analyzed to characterize the hsc70 NoLS. A fragment containing residues 245-287, but not 225-244, targeted GFP to nucleoli, even under nonstress conditions. The nucleolar/nuclear ratio for GFP-hsc70(245-287) was ~1.4 under nonstress conditions, which increased to 1.5-1.6 during heat shock and recovery. This constitutive stress-independent nucleolar accumulation was also observed, but less pronounced, for the reporter protein GFP-hsc70(245-262), a fragment rich in lysine and arginine residues (Figure 12, B and C). By contrast, reporter proteins containing segments 225-262, 263-287, 263-293 or 263-297 showed no nucleolar accumulation in unstressed cells. After heat shock or during recovery, there was a slight nucleolar accumulation (1.1- to 1.2-fold) for segments 225-262, 263-293 and 263-297 (Figure 12 C). Together, these data support the model that residues 225-244 provide an autoinhibitory regulatory element for segment 245-287. This regulatory element prevents the concentration in nucleoli when cells have not been stressed. This is revealed by the fact that nucleolar targeting via residues 245-262 or 245-287 is abolished by the negative regulator under normal conditions. In addition, the C-terminal portion of the NoLS provides a weak stress-dependent nucleolar targeting activity on its own. These data are consistent with results described in the previous section; e.g. removal of residues 288-297 from the NoLS diminished the stress-induced nucleolar accumulation of reporter proteins.

Mutation of Thr^{265} in the NoLS Reduces the Nucleolar Accumulation in Stressed Cells— As discussed above, residues 245-287 are sufficient to regulate in a stress-independent fashion nucleolar accumulation of the hsc70 NoLS. This portion of the chaperone contains a potential site for Akt phosphorylation at position Thr^{265}. Indeed, mass spectrometry demonstrated the phosphorylation of Thr^{265} in hsp/hsc70 *in vivo* [138]. Because PI3-kinase signaling and possibly Akt activity are important for endogenous hsc70 nucleolar accumulation, we mutated this residues in the reporter GFP-hsc70(225-297) to alanine, glutamic acid or aspartic acid (Figure 13). Subsequently, the distribution of mutant reporter proteins was quantified in transiently transfected cells. Interestingly, mutating Thr^{265} to alanine, glutamic acid or aspartic acid had a similar effect on localization. When compared to the wild type segment, no drastic changes were detected immediately following heat shock or after 1 h recovery. However, upon a 3-h recovery period, each of the mutants displayed a reduced nucleolar/nuclear ratio. Control experiments showed that all of the reporter proteins migrated at the same position during SDS-PAGE (Figure 13 C).


A
DAPI
GFP-hsc70 (225-297)
DAPI
GFP-hsc70 (225-297) T265A
control
heat shock
1 h rec.
3 h rec.
DAPI
GFP-hsc70 (225-297) T265E
DAPI
GFP-hsc70 (225-297) T265D
control
heat shock
1 h rec.
3 h rec.


B
Nucleolar/nuclear fluorescence intensity
T265
T265A
T265E
T265D
2.5
2.0
1.5
1.0
0.5
ctl
HS
1h
3 h

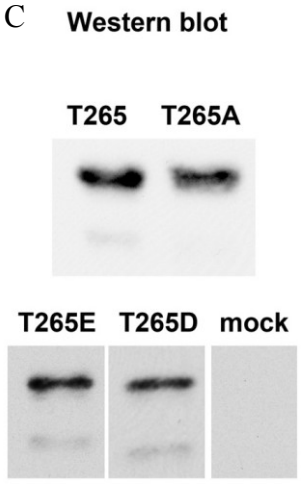
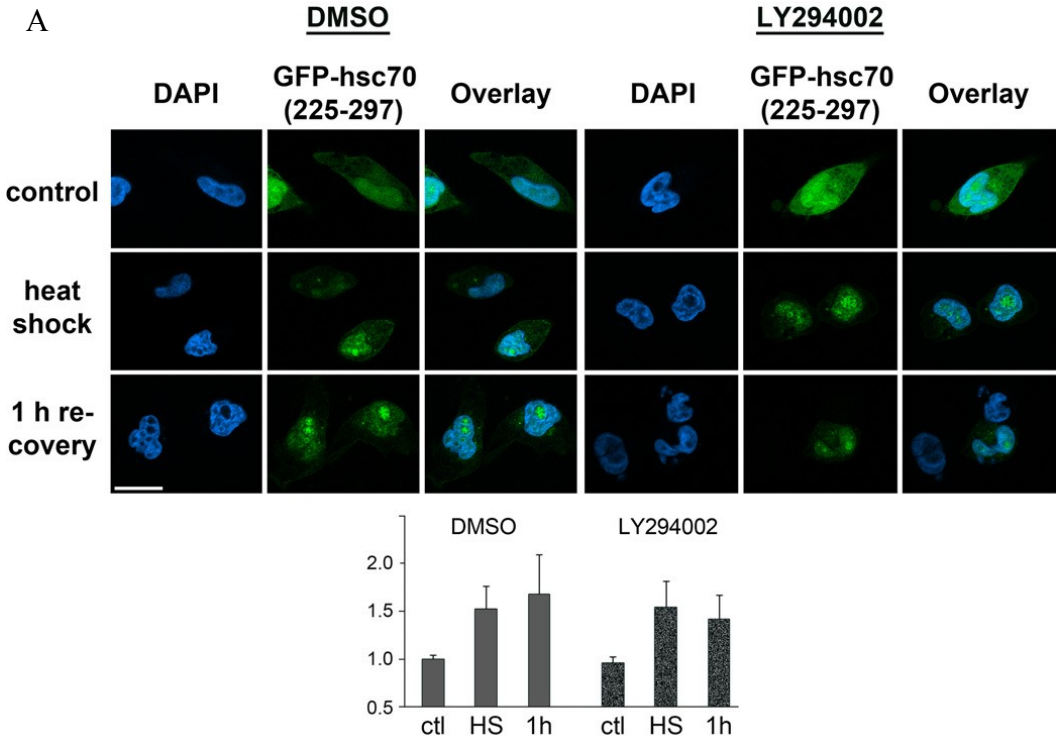

C
Western blot
T265
T265A
T265E
T265D
mock

Figure 13: Mutation of Thr^{265} alters the distribution of GFP-hsc70(225-297) during recovery from stress. In GFP-hsc70(225-297) residue Thr^{265} was mutated to alanine, glutamic or aspartic acid. (A) and (B) In transiently transfected HeLa cells the nucleolar/nuclear distribution was quantified for different reporter proteins under normal and stress conditions. For each data point pixel values for 42 to 69 cells were determined. Results are depicted as nucleolar/nuclear pixel intensities. Data were normalized to GFP-hsc70(225-297) under control conditions, which was defined as 1. Note that nucleolar accumulation is reduced in all mutants at 3 h of recovery. *Size bar* is 20 μm. (C) Western blot analysis of transiently synthesized reporter proteins. *DAPI*, 4',6-diamidino-2-phenylindole.

Inhibition of PI3-kinase Interferes with Nucleolar Accumulation Mediated by Hsc70 Segment 225-297— Since PI3-kinase signaling and tyrosine phosphatases play a role in the nucleolar accumulation of endogenous hsc70, we next examined the effect of pharmacological inhibitors on GFP-hsc70(225-297) nucleolar accumulation in heat-stressed cells. When compared with DMSO-treated controls, incubation with LY294002 did not alter the nucleolar/nuclear ratio following heat shock. However, the ratio was reduced to 84% after a 1-h recovery period (Figure 14A). This effect was similar to what was observed for endogenous hsc70 (Figure 9).


A
DMSO
LY294002
DAPI
GFP-hsc70 (225-297)
Overlay
DAPI
GFP-hsc70 (225-297)
Overlay
control
heat shock
1 h re-covery
DMSO
LY294002
2.0
1.5
1.0
0.5
ctl
HS
1h
ctl
HS
1h

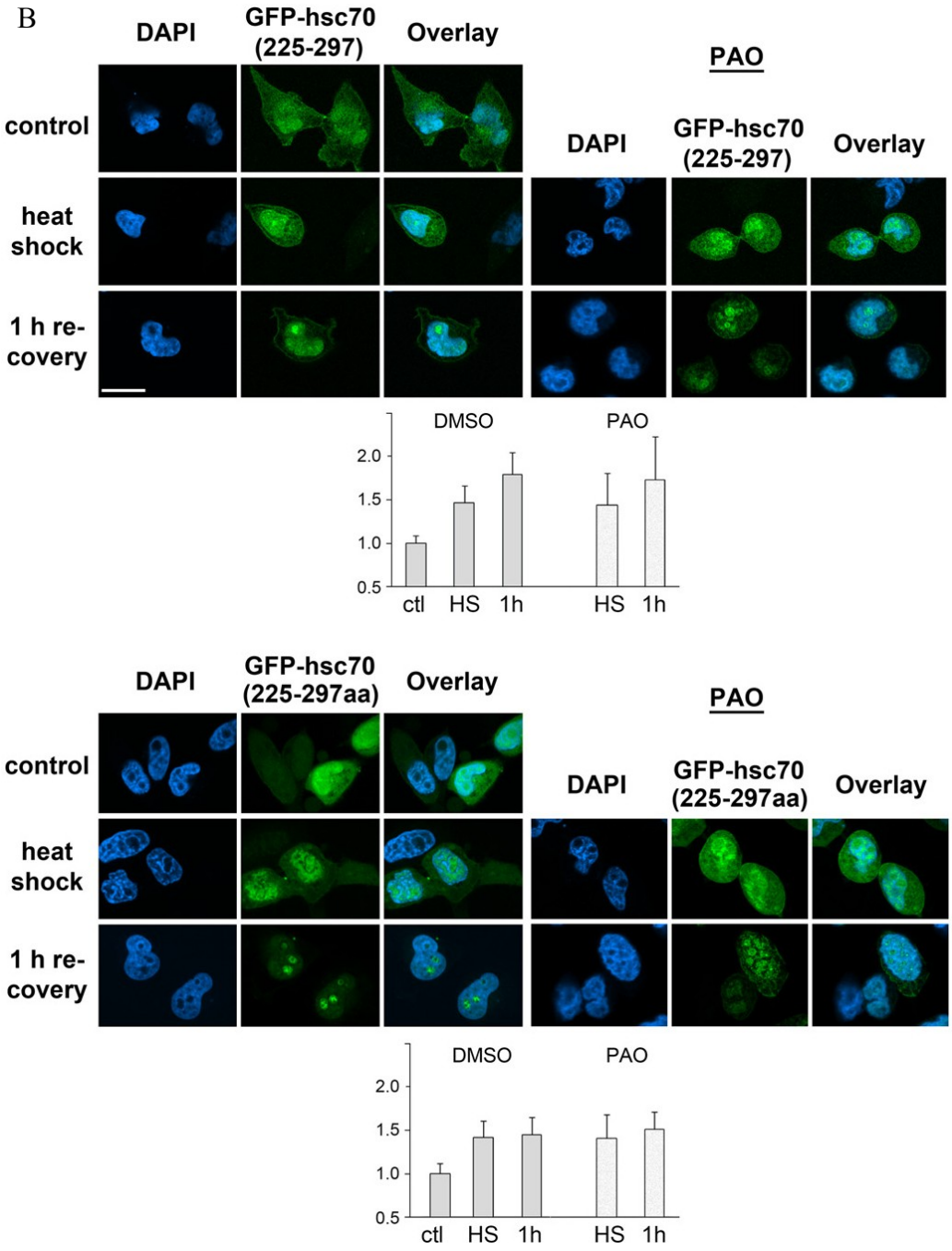


Figure 14: Effect of PI3-kinase signaling and protein tyrosine phosphatases on NoLS-mediated nucleolar accumulation of reporter proteins. (A) Treatment with LY294002 reduces the nucleolar accumulation of GFP-hsc70(225-297) in heat-stressed cells. Transiently transfected cells synthesizing GFP-hsc70(225-297) were incubated with DMSO or the PI3-kinase inhibitor LY284002 ("Experimental

Procedures"). (B) PAO does not inhibit the nucleolar accumulation of GFP-hsc70(225-297) and GFP-hsc70(225-297aa). Transiently transfected HeLa cells were incubated with DMSO or PAO as described under "Experimental Procedures." In A and B reporter proteins were detected in unstressed cells, after heat shock and upon 1 h recovery. *Size bar* is 20 μm. Quantification of nucleolar/nuclear pixel intensities was carried out as described for Figure 12. *DAPI*, 4',6-diamidino-2-phenylindole.

In an additional set of experiments we tested whether PAO alters the distribution of GFP-hsc70(225-297) and GFP-hsc70(225-297aa). Because GFP-tagged fusion proteins do not have to be imported into nuclei and show some nucleolar accumulation immediately after heat shock, we added PAO before stressing the cells. Under these conditions, PAO did not diminish the nucleolar/nuclear ratio of GFP-hsc70(225-297) or GFP-hsc70(225-297aa) when compared to DMSO-treated cells (Figure 14B).

Taken together, our results support the idea that PI3-kinase signaling contributes to the regulation of nucleolar accumulation of the reporter protein GFP-hsc70(225-297). By contrast, tyrosine phosphatases do not seem to play a significant role in this process.

Residues 225 to 297 Are Necessary, but Not Sufficient, for Nuclear Import of Hsc70— Given the importance of fragment 225-297 in hsc70 nucleolar accumulation, we next determined the role of residues 225-297 in hsc70 nuclear

import. A GFP-tagged reporter protein lacking residues 225-297 (GFP-hsc70(Δ225-297)) did not concentrate in nuclei under control or heat shock conditions, demonstrating that removal of this segment interferes with heat-induced nuclear accumulation (Figure 15).

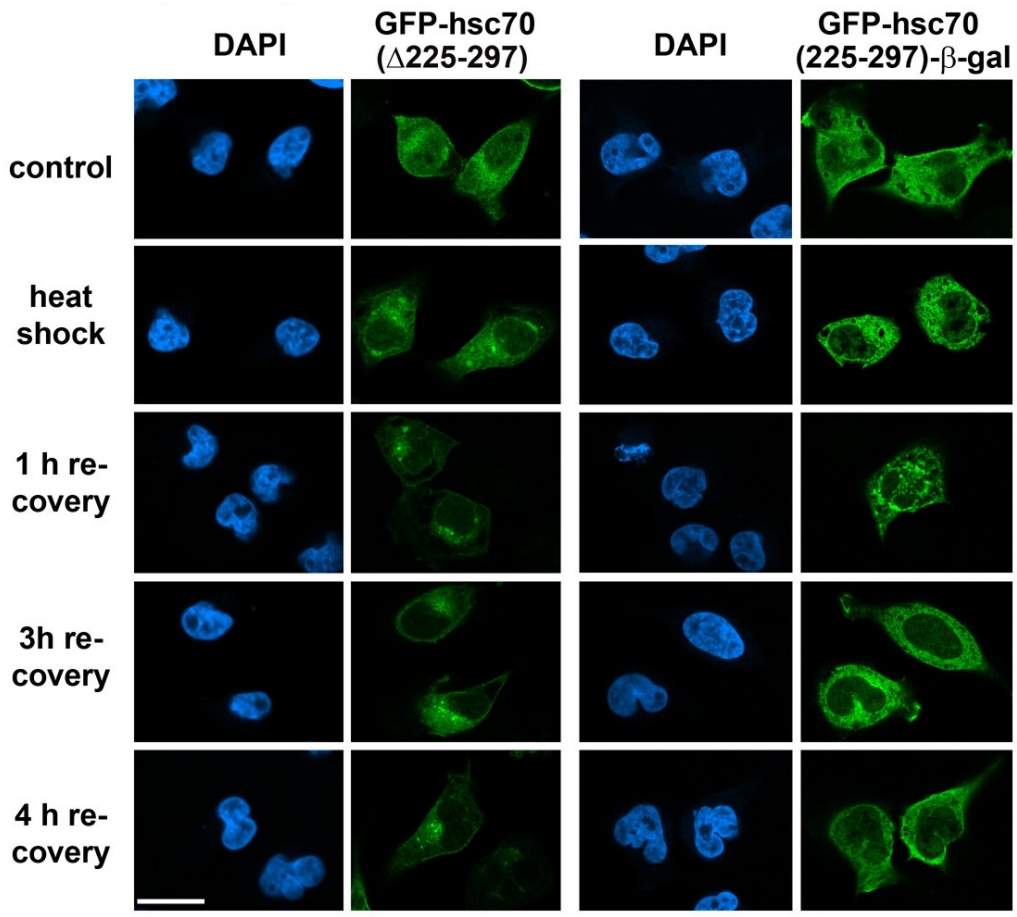


Figure 15: Residues 225 to 297 of hsc70 are necessary, but not sufficient, for nuclear import. (A) Transiently transfected HeLa cells synthesizing the reporter protein GFP-hsc70(Δ225-297) were localized under control conditions, upon heat shock and during recovery. (B) The distribution of GFP-hsc70(225-297)-β-galactosidase was analyzed in transiently transfected cells under normal and heat stress conditions. Nuclei were stained with 4',6-diamidino-2-phenylindole

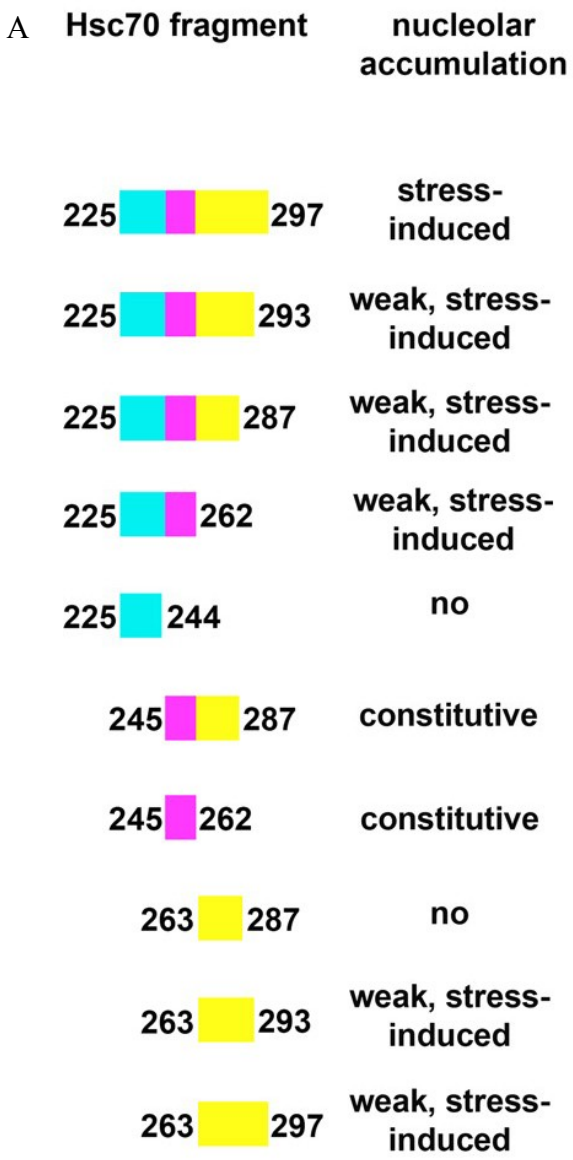
(DAPI). *Size bar* is 20 μm. Note that GFP-hsc70(Δ225-297) and GFP-hsc70(225-297)-β-galactosidase do not accumulate in nuclei or nucleoli under normal and stress conditions or during recovery.

Fragment 225 to 297 contains clusters of positively charged amino acid residues that fit the consensus sequence of a classical NLS [185]. To test whether this segment promotes transport into the nucleus, we generated GFP-hsc70(225-297)-β-galactosidase, a reporter protein that contains GFP-hsc70(225-297) fused to β-galactosidase. With a molecular mass of >150 kD for the monomer, this reporter protein cannot diffuse across the nuclear envelope. GFP-hsc70(225-297)-β-galactosidase did not accumulate in nuclei, neither in unstressed cells nor following heat shock (Figure 15), suggesting that at least in the context of this construct residues 225 to 297 do not mediate import into the nucleus. Thus, fragment 225-297 does not function as a constitutive or heat-inducible NLS.

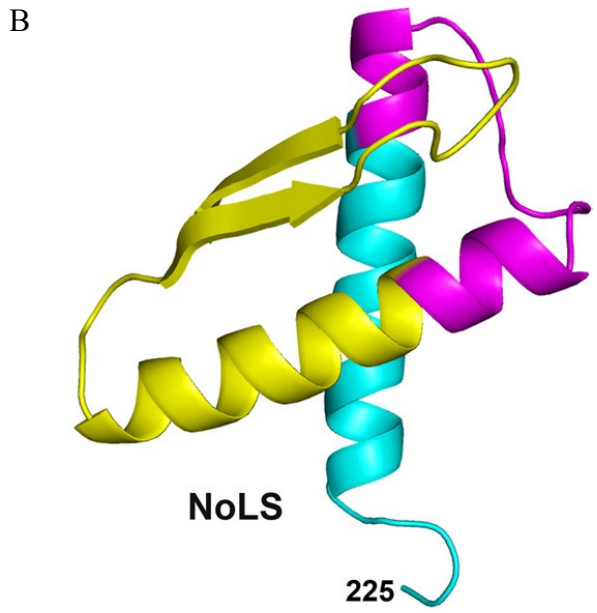
2.5. Discussion

The experiments described here characterize the mechanisms that underlie the nucleolar targeting of hsc70 in cells recovering from stress. Our studies define the minimal region of hsc70 that promotes nucleolar accumulation upon heat shock. In particular, we demonstrate that residues 225-297 are sufficient to concentrate the passenger protein GFP in nucleoli. Importantly, a prerequisite for this nucleolar accumulation is the exposure to stress. Taken together, our data show

that residues 225-297 function as a NoLS, which contains all the information required to promote stress-regulated nucleolar targeting (summarized in Figure 16). Within the tertiary structure of the N-terminal hsc70 ATPase domain, this segment is located in the well-defined domain B of lobe II, a region that comprises residues 229-306 [116, 186].



B



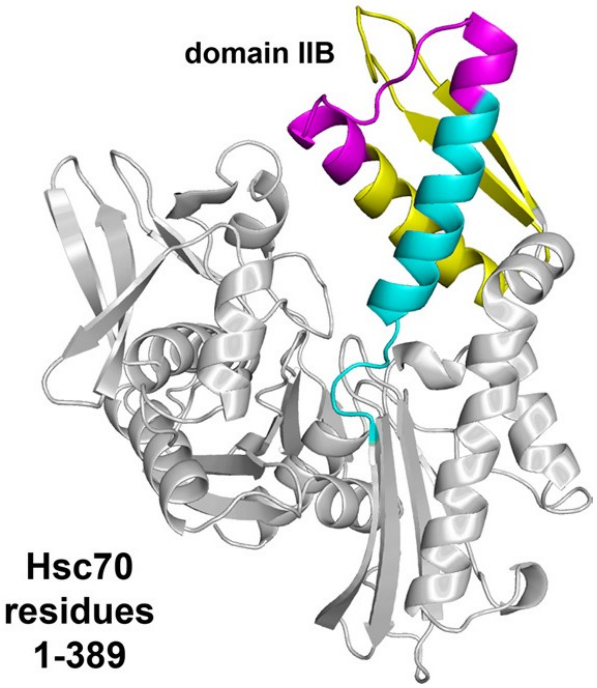


Figure 16: Simplified model for the hsc70 NoLS. (A) Results obtained for the nucleolar localization of GFP-tagged fusion proteins are summarized. (B) The secondary structure of the N-terminal residues 1-389 of hsc70 and different segments of the NoLS are shown (generated with Polyview-3D; [187]). Within the N-terminal ATPase domain the NoLS is located in domain IIB of lobe II [116, 186]. The autoinhibitory region (residues 225-244) is depicted in *blue*, segment 245-262 is *magenta* and the C-terminal portion including the enhancing region (residues 263-297) is *yellow*.

We further dissected the function of hsc70 fragment 225-297 and identified residues 245-287 as a constitutive signal that promotes nucleolar targeting even in the absence of stress (Figure 16A). The same applies to residues 245-262; however, nucleolar targeting by segment 245-262 was always less pronounced when compared with residues 245-287. This suggests that residues 263-287 enhance nucleolar localization, although they have no nucleolar targeting activity *per se*.

With our systematic analysis of the hsc70 NoLS we demonstrate that segment 225-244 diminishes the constitutive nucleolar targeting function that is provided by residues 245-287. This negative control operates in unstressed cells, thereby preventing the association with nucleoli under normal conditions. Interestingly, residues 225-244 interfered with the constitutive nucleolar targeting mediated by residues 245-262, even under stress conditions (Figure 16). This indicates that segment 263-287 plays a crucial role in overcoming the autoinhibitory effect of

region 225-244 upon stress, but is not sufficient on its own to promote nucleolar accumulation. Furthermore, the quantitative analysis of truncated forms of the NoLS revealed that residues 263-297 also contribute to the stress-induced nucleolar accumulation promoted by the NoLS.

On the basis of the results described here we propose a simplified model for the nucleolar targeting mediated by fragment 225-297 of hsc70 (Figure 16). Stress-dependent nucleolar accumulation requires the combined action of different parts of this fragment. Under normal growth conditions, nucleolar targeting is prevented by the autoinhibitory element located in residues 225-244. During recovery from stress, this negative regulator will be inactivated, and the nucleolar targeting function of residues 245-287 prevails. Furthermore, segment 263-297 provides an additional, albeit weak, stress-inducible signal for nucleolar targeting.

Interestingly, within the hsc70 ATPase domain all of the functional components of the hsc70 NoLS are present in domain B of lobe II [116, 186]. Domain IIB consists of two α-helical regions that are followed by two β-strands. Residues 245-287 of domain IIB, sufficient for constitutive nucleolar targeting (Figure 16, *magenta* and *yellow*), contribute to two α-helical regions that are connected by a loop (Figure 16, *magenta*, and Figure 17). The constitutively active NoLS is preceded by its negative regulator, located within residues 225-244 (Figure 16, *blue*). The NoLS enhancing portion, 263-297, gives rise to a helical region followed by a segment, predicted to be organized in antiparallel β-

strands. Several segments of domain IIB are conserved [116], and the C-terminal portion of the first β-strand in particular is highly conserved. Notably, this segment is part of the positive regulator identified by us.

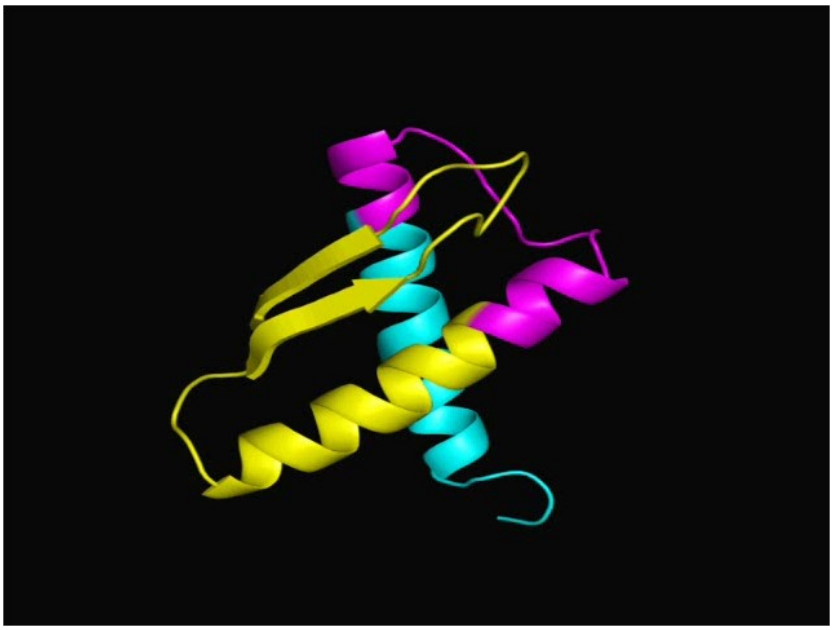


Figure 17: Animated view of the hsc70 NoLS (please visit http://www.jbc.org/content/suppl/2010/05/10/M110.117291.DC1/jbc.M110.117291-6.wmv). The 3-dimensional organization of the hsc70 NoLS is shown in motion (generated with Polyview-3D; [187]). The autoinhibitory sequence (segment 225-244) is depicted in *blue*, part 245-262 in *magenta* and the enhancing region (residues 263-297) is represented in *yellow*.

It is tempting to speculate how the hsc70 NoLS controls stress-dependent nucleolar localization. We propose that this process combines separate functions contributed by different parts of the NoLS. In one scenario, stress might trigger conformational changes in the NoLS, thereby masking sites on hsc70 that interact

with components in the nucleoplasm. Such masking of interacting sites would promote the release of hsc70 from anchors in the nucleoplasm. Alternatively, stress could expose sites on hsc70 that are recognized by binding partners in the nucleolus, but hidden under non-stress conditions. In this case, nucleolar retention of the chaperone would be favored. Given the complex contributions of different parts of the NoLS to nucleolar accumulation, we favor the idea that the combination of multiple interactions, possibly with different binding partners, determines the ultimate distribution of reporter proteins. At the same time, it is likely that not only hsc70, but also its binding partners, both in the nucleolus and the nucleoplasm, are regulated by stress. Ultimately, these associations could be altered by post-translational modification of hsc70 and/or its interacting components. It is feasible that these modifications are regulated by kinases and phosphatases that depend on signaling through PI3-kinase and MEK kinase pathways.

Our results for mutations introduced at Thr^{265} are in line with this speculation. Thr^{265} has been shown to be phosphorylated *in vivo*; it is also a potential target for the protein kinase Akt. Because mutations to alanine as well as glutamic or aspartic acid reduced the nucleolar accumulation of reporter proteins, it is feasible that Thr^{265} cycles between the unmodified and phosphorylated state. Both forms might interact with different binding partners, all of which could regulate hsc70 nucleolar accumulation. Future experiments will have to address these questions.

The complex regulation of hsc70 nucleolar targeting is consistent with recent studies for other components of the nucleolus. For example, the movement of individual proteins or ribosomal subunits between nucleoli and the nucleoplasm occurs in a highly organized fashion ([128, 129, 173] and references therein). The same applies to the intracellular trafficking of hsc70, which is, in part, dictated by its interactions with polypeptides that need to be folded. In support of this model, we demonstrated earlier that in heat-stressed cells binding of hsc70 to chaperone substrates controls its retention in the nucleoplasm [51]. However, chaperone/client interactions are not the sole contributor to hsc70 *nucleolar* accumulation. Our previous results showed that hsc70 binding to nucleoli is at least partially ATP-insensitive and thus likely to be unrelated to chaperone function [51]. This idea is further substantiated by our current study, as the reporter proteins used here offer the advantage that they do not carry the peptide binding domain. Consequently, interactions with chaperone clients did not interfere with their localization and enabled us to dissect the functional organization of the NoLS.

One of the limiting factors for the analysis of nucleolar targeting is that molecules larger than the diffusion channel of the NPC require active nuclear import. As transport in and out of the nucleus is sensitive to stress ([51, 72] and references therein), we used reporter proteins that are small enough to diffuse across NPCs. Interestingly, fragment 225-297, when fused to GFP and β-galactosidase, did not support nuclear import, neither under normal nor heat shock conditions. This suggests that the stress-dependent hsc70 NoLS does not operate

as an NLS. Our results are in line with Tsukahara and Maru [115], who reported that the protein binding domain of hsc70 plays a role in hsc70 nuclear import. These results, however, do not explain why GFP-hsc70(Δ225-297) failed to accumulate in nuclei of heat-shocked cells. One possibility is that two regions present in separate domains of hsc70 are simultaneously required to import hsc70 into the nucleus. Alternatively, deletion of residues 225-297 might generate a mutant protein, whose altered structure prevents an NLS located in the peptide binding domain from functioning.

Dang and Lee [135] previously described that residues 250 to 267 in hsp70 are similar to an NLS. This portion of the chaperone, when fused to pyruvate kinase, promoted constitutive nuclear and nucleolar localization in unstressed cells. Another study described a nucleolar retention signal for hsc70, comprised of nine mostly basic residues of the chaperone [138]. When fused to hydrophobic repeats, which are not present in authentic hsc70, nucleolar targeting of a GFP reporter protein was observed at pH 6.3. Collectively, these data support our idea that a short segment of hsp70 or hsc70, when removed from its normal context, could serve as a nucleolar targeting signal. However, the complex regulation of NoLS function is likely to be revealed only within the proper domain organization of the chaperone.

The experiments presented here analyzed for the first time the signaling events that participate in the nucleolar accumulation of hsc70. We showed that PI3-kinase and MEK kinases as well as protein-tyrosine phosphatase activities play a

part in the heat-dependent concentration of endogenous hsc70 in nucleoli. Importantly, PI3-kinase controls also the distribution of a reporter protein that contains only the NoLS. By contrast, tyrosine phosphatases have an effect on the nucleolar targeting of full length hsc70, whereas the NoLS is not affected. This points to the possibility that additional residues, located outside of the NoLS, participate in the regulation of hsc70 nucleolar accumulation.

Taken together, our studies defined the signaling events implicated in hsc70 nucleolar targeting and identified a novel stress-inducible NoLS which is composed of multiple functional elements. In particular, the combination of a constitutive nucleolar targeting signal with an autoinhibitory segment provides a sophisticated mechanism to control the localization of hsc70 under normal and stress conditions.

2.6. Acknowledgements

This work was supported in part by grants from Canadian Institutes of Health Research (CIHR), Natural Sciences and Engineering Research Council of Canada (NSERC), and Heart and Stroke Foundation of Canada (HSFC) (to U.S.). PB is supported by a fellowship from Fonds de la Recherche en Santé Québec (FRSQ)/Heart and Stroke Foundation of Canada (HSFC). MK is a recipient of a fellowship from the Heart and Stroke Foundation of Canada (HSFC).

The abbreviations used are: Hsc70, heat shock cognate protein 70; PI, phosphatidylinositol; DMSO, dimethyl sulfoxide; GFP, humanized green fluorescent protein; NPC, nuclear pore complex; NLS, nuclear localization sequence; NoLS, nucleolar localization sequence; PAO, phenylarsine oxide; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase.

The results presented in chapter I demonstrate that the nucleolar accumulation of hsc70 is mediated by a NoLS provided by residues 245-262. The NoLS's efficiency and function is modulated by upstream and downstream segments. My experiments also provide some insight into the possible pathways that contribute to the nucleolar accumulation of hsc70 during heat stress recovery. As such, although it is not always true for the GFP construct, a general phosphotyrosine phosphatase inhibitor was able to significantly decrease the number of cells showing nucleolar accumulation of hsc70. Similarly, inhibitors of PI3-K and MEK kinase reduced the nucleolar accumulation of hsc70.

Because of the interest in the nucleolar accumulation of hsc70 and the binding partners involved in this process, we reviewed the current knowledge of how do chaperones and multitasking proteins interact in the nucleolus. The next chapter therefore focuses on the possible generation of chaperone networks in the nucleolus. This work is based on studies performed on the nucleolar proteome under normal growth and stress conditions.

3. CHAPTER II

Chaperones and multitasking proteins in the nucleolus: networking together for survival?

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3.1. Abstract

The nucleolus has emerged as a key player that regulates cell growth, survival and recovery from stress. Progress in proteomics made it possible to sequence the nucleolar proteome under different physiological conditions. Together with other research, this work revealed the presence of multiple chaperones and co-chaperones in the nucleolus. Molecular chaperones are components of a larger network that promotes protein homeostasis, thereby providing continuous adaptation to a changing environment. Recent studies suggest that the cellular chaperone network is divided into individual branches which orchestrate specific functions. Input from separate branches is then combined to 'fine-tune' the cellular proteostasis network. Based on the latest developments in nucleolar and chaperone biology, we speculate that a unique network comprising chaperones, co-chaperones and multitasking proteins is located in nucleoli. This network supports and regulates fundamental biological processes, including ribosome biogenesis, cell signaling, and the stress response.

3.2. Organization and functions of the nucleolus

Eukaryotic cells are characterized by membrane-bound organelles as well as compartments that provide internal environments designed for specialized functions. The most prominent and largest eukaryotic organelle is the nucleus which coordinates a large number of essential cellular processes. Within the nucleus, the nucleolus represents the most conspicuous compartment, which has emerged as a key player that regulates many aspects of cell biology ([127-129, 140, 174-176, 188-190], Box 1). The functional diversity of nucleoli is reflected by many different proteins present in this compartment [121, 133, 191, 192]. As such, the nucleolar proteome is likely to comprise several thousand distinct proteins, a large number compared with the <1,500 proteins identified for mitochondria [193]. Due to their contribution to numerous biological processes, the improper function of nucleolar components is associated with many human diseases and pathologies, including various types of cancers [129, 134, 190]. Importantly, the protein profile of nucleoli is not static because it is modulated by changes in cell physiology [178]. Thus, the composition of nucleoli varies during cell cycle progression, and can be altered by stress, tumor development, signaling events and viral infections [179, 188, 190].

We believe that the functional organization of nucleoli relies on a network of chaperones, co-chaperones and multitasking proteins located in this compartment. The network provides the foundation to maintain nucleolar activities under normal conditions, and adapts these functions to environmental changes that are

elicited by stress or disease. We discuss the potential roles of the proposed network by dissecting the interactions between individual network components and their clients. These interactions implicate the network in nucleolar organization and a large number of essential functions. Here, we focus on pre-rRNA processing, ribosome biogenesis, signaling, cell cycle control and protein turnover.

3.3. Chaperone networks

Molecular chaperones are defined as proteins that support the folding or unfolding of biological molecules; chaperones (together with their co-chaperones) contribute to all aspects of cell biology. In particular, heat shock proteins are implicated in a wide range of cellular activities, as exemplified by protein folding and translocation, refolding or degradation of damaged proteins, regulation of transcription and signal transduction [5, 194-196]. In addition to chaperones whose clients are proteins, RNA chaperones are committed to RNA quality control [197]; these proteins are of pivotal importance in the nucleolus, where they play an essential role in ribosome biogenesis [198]. The significance of molecular chaperones for human health is emphasized by the numerous links to aging, diseases and pathologies. These include, but are not limited to, neurodegenerative and protein folding diseases, cancer, rheumatoid arthritis, type-1 diabetes and arteriosclerosis [171, 179, 194, 195, 199].

Many of the chaperone-controlled processes require the cooperation between multiple heat shock proteins, co-chaperones and additional factors. To this end, chaperones and their co-factors are frequently organized into networks in which they are ideally positioned to regulate cellular activities [103, 200-202]. Such networks promote efficient interaction with clients and ensure that protein homeostasis is preserved if environmental or disease-related changes are encountered [103, 171, 200-202]. Owing to these challenges, chaperone networks are dynamic, and the concentration and/or location of network components can adapt to the physiological state of a cell [103, 202].

Heat shock protein 70s (HSP70s) and HSP90s are key components of chaperone networks that cooperate to support protein homeostasis. Together with other folding factors, these chaperones are essential for protein quality control because they ensure the structural and functional integrity of polypeptides, maintain protein homeostasis, and protect cells from proteotoxicity [171, 203]. HSP70s bind newly synthesized proteins early during the folding process, whereas HSP90s associate with their substrates at a later point [204]. Among the important clients for HSP90s are multiple kinases whose activities rely on the continuous presence of HSP90s and their co-factor cell division cycle 37 (CDC37). For some HSP90 clients, persistent interaction with HSP90-CDC37 is necessary to keep them in a state in which they are ‘poised’ for activation [205-208].

Interestingly, separate chaperone networks with distinct functions have been identified in eukaryotes. For example, different networks are dedicated to *de novo* protein folding and the refolding of clients that have been damaged by stress [209]. Moreover, a specific mitochondrial chaperone network plays a critical role in the survival of cancer cells [210, 211]. As part of this network, HSP90 and the chaperone TRAP1 are present in tumor cell mitochondria, where they preserve mitochondrial integrity and thereby interfere with cell death [211].

3.4. Can a network of chaperones and multitasking proteins be built in the nucleolus?

Several research teams have analyzed the nucleolar proteome in HeLa cells or *Arabidopsis thaliana* [121, 133, 191, 192]; and studies on HeLa cell nucleoli build the foundation for the most comprehensive nucleolar protein database [http://lamondlab.com/NOPdb3.0/]. With >4,500 different entries, this database contains a growing number of nucleolar polypeptides. Moreover, quantitative changes in the nucleolar proteome induced by the transcriptional inhibitor actinomycin D have been determined [178]. Other experiments show that the abundance of chaperones and co-chaperones in nucleoli is regulated by cell physiology, however quantitative data for changes in abundance are not always available [51, 212, 213]. Members of all heat shock protein families, together with other factors involved in protein and RNA folding, have been detected in nucleoli by proteomics or other studies [133] (Table 1). Some of these factors are proteins

with multiple functions that not only participate in folding or processing events, but also contribute to other biological activities. Here, we refer to these factors as ‘nucleolar multitasking proteins’ (NoMPs) to distinguish them from conventional chaperones and co-chaperones. Notably, some NoMPs shuttle between the nucleolus and the nucleoplasm, and might even localize to the cytoplasm [214].

Table 1: Multiple chaperones and other factors involved in the folding of proteins or RNA in nucleoli. The list has been compiled from http://lamondlab.com/NOPdb3.0/; alternative names and major functions of the factors are depicted.

Factors involved in the folding of proteins and RNA	**Alternative name**	**Major function**	**Reference**
Members of the HSP70 family			
HSPA1L	Heat-shock protein 70 kD, homolog	Chaperones, protein folding, intracellular trafficking, signaling	[215-218]
HSPA4L	Osmotic-stress protein 94		
HSPA1	HSP72, HSP70-1		
HSPA2	Heat shock-related 70 kD protein 2		
HSPA4	HSP70		
HSPA5	BIP, GRP78		
HSPA6	HSP70B		
HSPA7	HSP70B		
HSPA8	HSC70		

HSPA9B	HSPA9, GRP75		
Members of the HSP40 family DNAJ A1 DNAJ A2 DNAJ B1 DNAJ C5 DNAJ C8 DNAJ C9 DNAJ C10 DNAJ C11 DNAJ C14 DNAJ C19	 HDJ2 DNJ3 HSP40 Cysteine string protein, CSP Splicing protein SPF31 DNAJ protein SB73 ER-resident protein ERDJ5 Dopamine receptor interacting protein TIM14	 Chaperones, associate with and are critical for, the function of HSP70s	 [215-218]

Members of the HSP90 family HSP90 AA1 HSP90 AA2 HSP90 AA4P HSP90 AA6P HSP90 AB1 HSP90 AB2P HSP90 AB3P HSP90 AB4P HSP90 AB6P HSP90 B1 HSP90 B2P TRAP-1	 HSP90A, HSP90 beta HSP90 beta HSP90BB HSP90Bf GRP94 HSP75, TNFR-associated protein 1	 Chaperones, protein folding, trafficking of steroid hormone receptors, signaling	 [215-218]
HSP110	HSP105, HSPH1	Protein folding	[215-218]

TCP1 subunits CCT1 CCT2 CCT3 CCT4 CCT5 CCT6A CCT7 CCT8	 TCP1, CCT alpha CCT beta CCT gamma CCT delta CCT epsilon CCT zeta CCT eta CCT theta	Protein folding	[215-218]
HSP60	HSPD1 (GroEL)	Protein folding, apoptosis, regulation of immune response, signal transduction	[215-218]
HSP10	HSPE1, CPN1 (GroES)	Protein folding	[215-218]

Co-chaperones and heat shock binding proteins AHSA1 AHSA2 BAG2 HOP STUB1	 AHA1 AHA1 homolog 2 BCL2-associated athanogene 2 STIP1, STI1 HSPABP2	 Activator of HSP90 ATPase Activator of HSP90 ATPase BAG family chaperone regulator Cofactor for HSP70 and HSP90	 [215-218]
TAH1 PIH1		HSP90 adaptor HSP90 adaptor	[219, 220]
FKBP52 FKBP10	FKBP4 FKBP65	Protein prolyl *cis-trans* isomerases	[215-218]

FKBP1A Cyclophilin A	FKBP12 CYP A	(PPIases)	
PDIA1 PDIA3	PDI ERp57	Protein disulfide isomerases	[215-218]
Calreticulin Calnexin	CRTC, CALR CNX	Calcium-binding protein, chaperone Protein folding	[215-218]
Ubiquitin activating enzyme E1 Ubiquitin-conjugating enzyme E2 D2 Parkin 2, Component of E3 ubiquitin ligase complex	UBA1, UBE1 UBC4, UBE2D2 PARK2	Protein degradation	[215-218]
ASF1A	HSPC146	Histone chaperone	[215-218]
PSMG3	PAC3	Proteasome assembly	[215-218]

		chaperone	
NAC A	Nascent polypeptide associated complex, alpha subunit	Protein folding	[215-218]
Prefoldin 2	PFD2	Protein folding	[215-218]
RNA chaperones			
IMP3	U3 snoRNP protein 3, Imp3p in yeast	Ribosome biogenesis, rRNA processing, physical interaction with Cct6p, Imp4p and Utp8p in yeast	[198, 221, 222]
IMP4	U3 snoRNP protein 4 homolog,	rRNA processing, physical interaction	[198, 222]

	Imp4p in yeast	with Imp3p in yeast	
YB-1	Y-box transcription factor	Cold shock domain-containing protein with transcription factor activity; interacts with FUS	[197]
		RNA-binding protein, interacts with YB-1	
FUS	RNA-binding protein FUS	RNA-binding protein, spliceosomal catalysis	[197]

U2AF65 Utp8p	U2 snRNP auxiliary factor large subunit	Yeast protein required for nucleolar export of tRNA. Maturation of rRNA in small ribosomal subunit. Regulation of RNA polymerase-dependent transcription, interacts with yeast HSP70s, HSP90s and Imp3p	[197] [222, 223]
B23	Nucleophosmin, Numatrin	Regulates apoptosis, ribosome biogenesis and trafficking, stress response, histone	[145, 214-218, 224, 225]

		chaperone activity	
Nucleolin	C23, Protein C23	Might assist in rRNA folding, histone chaperone, involved in chromatin remodeling	[215-218, 224]

In line with the idea that some cellular organelles and compartments have particular requirements to maintain protein homeostasis, budding yeast nuclear and nucleolar proteins interact with numerous chaperones [226]. Based on these observations and because discrete chaperone networks with specialized tasks have been identified in eukaryotes, we propose that nucleoli harbor a chaperone/NoMP network committed to compartment-specific functions. To begin to test this idea, we assembled a possible network that depicts interactions among folding factors that are present in nucleoli (Figure 18). Because many of the biological activities in nucleoli rely on extensive RNA processing; therefore, we incorporated RNA chaperones into our network (Figure 18, Tables 1 and 2). For simplicity, our network does not include the large number of potential nucleolar chaperone clients as exemplified by pre-rRNA processing factors, protein kinases and phosphatases.

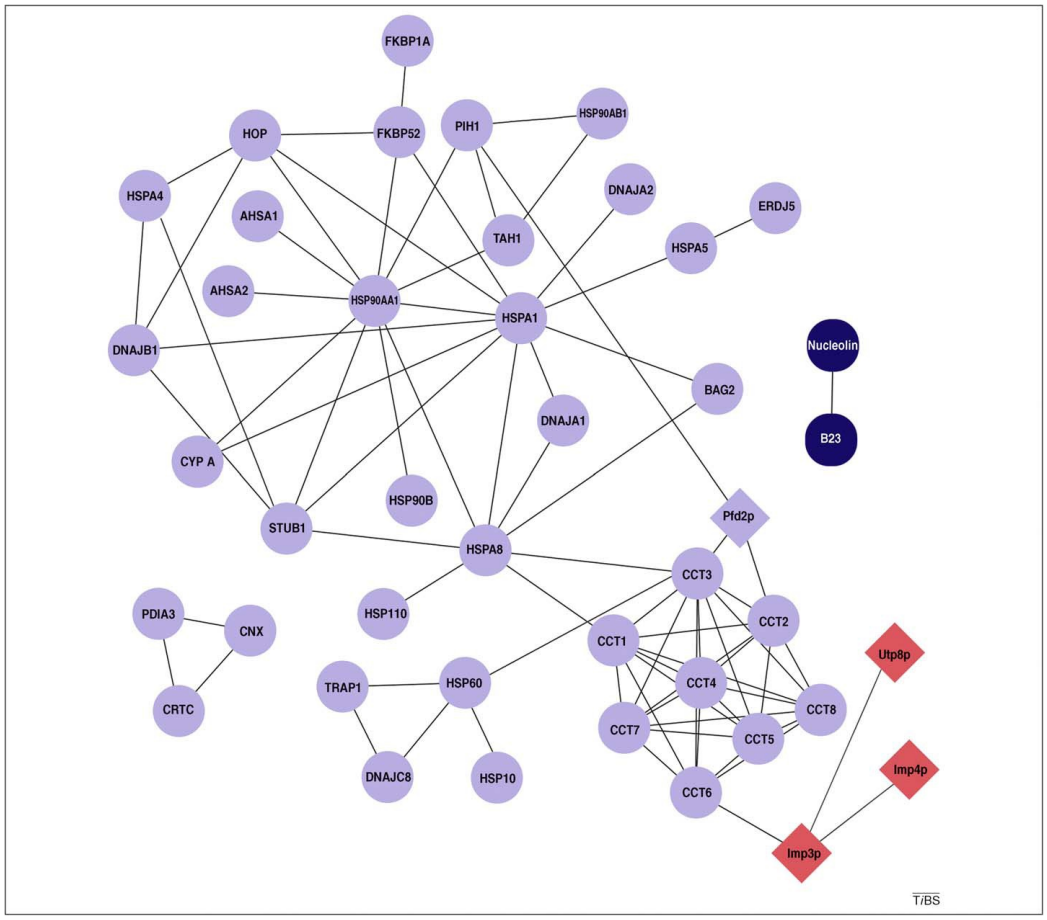

Figure 18: Hypothetical nucleolar chaperone/NoMP networks. We propose that chaperone/NoMP networks are located in nucleoli, where they can regulate a large number of compartment-specific functions, many of which are essential to cell viability. All of the chaperones, co-chaperones and NoMPs have been located in nucleoli by proteomics and/or other methods (e.g. immunostaining) [51, 121, 133, 191-193, 212, 213]. Most of the predicted interactions depicted in the diagram are based on data obtained for human cells as they are accessible in different databases [216-218, 226], circles]. We also included several interactions which have been detected in the budding yeast *Saccharomyces cerevisiae* [SGD project (http://www.yeastgenome.org/); diamonds]. Factors involved in protein folding

and/or assembly are shown in blue, RNA chaperones in red, and NoMPs in dark blue.

Table 2: Conventional and RNA chaperones participate in the assembly of ribosomal subunits. Yeast proteins that are involved in ribosomal biogenesis, their function and human orthologs are shown.

Yeast protein	**Function**	**Human orthologs**	**References**
Ecm10p	Protein folding	HSPA9B (hsp70 family)	[215-218, 222]
Ydj1p	Protein folding	DNAJA1, DNAJA2	[215-218, 222]
Gim4p	Protein folding	Prefoldin 2	[215-218, 222]
Imp3p	RNA chaperone	IMP3	[215-218, 222]
Imp4p	RNA chaperone	IMP4	[215-218, 222]
Tah1p	HSP90 adaptor	TAH1	[215-218, 222]
Pih1p	HSP90 adaptor	PIH1	[215-218, 222]

Notably, our nucleolar chaperone/NoMP network is not a simple copy of cytoplasmic folding factors. Indeed, nucleoli are enriched for some factors (B23 and nucleolin), whereas others are missing or underrepresented (e.g. HSP70 interacting protein (HIP), multiple BCL2-associated athanogene (BAG) family members, small HSPs). Technical difficulties (e.g. low abundance, unstable

association with nucleoli) preclude the detection of some folding factors, however we believe that a nucleolar chaperone/NoMP network is composed of a unique set of folding factors.

We expect the chaperone network (Figure 18) to be dynamic in order to modulate nucleolar activities in response to changes in cell physiology. This could be accomplished by adjusting the concentration of network components or by the recruitment/removal of individual constituents or modules [202]. HSPA8 (HSC70), DNAJ B1 and cyclophilin 40 are prominent examples for such a stress-dependent redistribution because nucleolar levels increase for all of the proteins when cells are exposed to heat shock [51, 212, 213]. Importantly, these stress-induced changes are not limited to heat shock, and quantitative analyses demonstrate that inhibition of transcription with actinomycin D increases the concentration of chaperonin containing TCP1, subunit 2 (CCT2), but reduces the levels of B23, nucleolin, HSP60 and HSP10 in nucleoli [178].

3.5. Possible roles of a nucleolar chaperone/NoMP network

Our proposal of a chaperone/NoMP network dedicated to nucleoli implies that this network contributes to functions that are unique for this compartment. Thus, we believe that the specific combination of clients in nucleoli requires the coordinated action of a designated group of chaperones, their co-factors and NoMPs. Such interactions, organized around nucleolar chaperones, could support

the maintenance of cellular homeostasis as well as the structural and functional organization of the nucleolus (Figure 19). To illustrate these processes, we connected chaperones to their potential nucleolar clients. Although all of the associations that we describe have been demonstrated *in vivo* and/or *in vitro* [215-218], it is presently not known how many of these interactions occur in nucleoli.

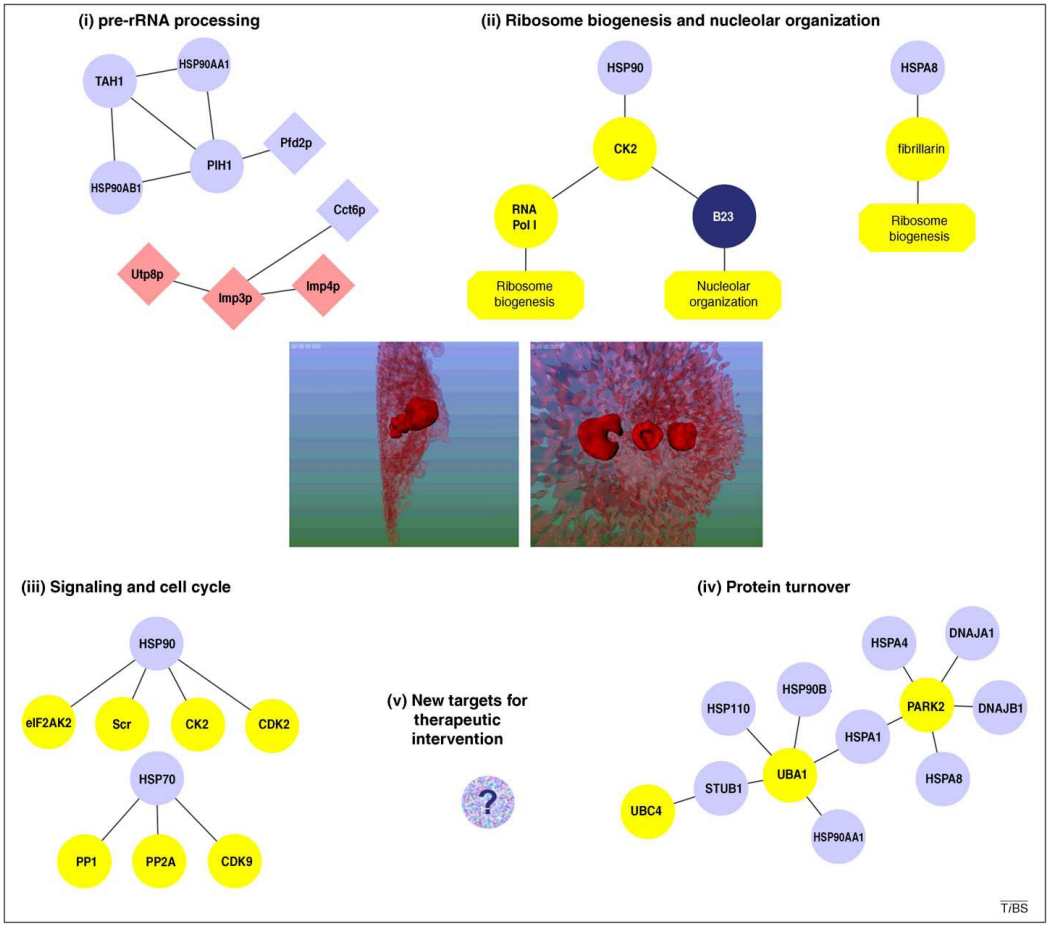

Figure 19: Several branches of the nucleolar chaperone/NoMP network contribute to cell survival by regulating essential biological processes. We speculate that fundamental cellular activities in nucleoli (red spheres in 3D reconstructions of HeLa cells) rely on a chaperone/NoMP network located in this compartment. All of the interactions depicted in the figure have been established

experimentally *in vivo*, *in vitro* or both. Some of the associations have been shown in *S. cerevisiae* (diamonds), but many of them also take place in higher eukaryotes (circles). The occurrence of these interactions in nucleoli is, to a large extent, hypothetical. (i) Pre-rRNA processing. Multiple conventional and RNA chaperones cooperate to promote proper processing of pre-rRNA, which is essential for the assembly of ribosomal subunits. This processing requires the coordinated action of conventional (blue) and RNA chaperones (red) [223]. (ii) Ribosome biogenesis and nucleolar organization. We propose that HSP90 controls the function and organization of the nucleolus by targeting nucleolar CK2. The downstream events (yellow) controlled by HSP90 *via* CK2 include RNA polymerase I-dependent transcription, which is directly linked to ribosome biogenesis. CK2 activity is critical for the correct organization of the nucleolus into its sub-compartments FC, DFC and GC, whereas the interaction between HSPA8 (HSC70) and fibrillarin could help re-establish nucleolar functions after stress. (iii) Signaling and cell cycle. The association in nucleoli between HSP90 family members or HSP70 family members and protein kinases or phosphatases might regulate signaling as well as cell cycle progression. (iv) Protein turnover. Multiple interactions mediated by a nucleolar chaperone network could contribute to compartment-specific protein degradation. (v) New targets for therapeutic intervention. Knowledge of a nucleolar chaperone network could provide a unique repertoire of drug targets that are relevant to disease and aging in humans.

One of our proposed nucleolar networks includes several conventional and RNA chaperones that are implicated in ribosome biogenesis [219-221, 226, 227]

(Figure 19i). In *Saccharomyces cerevisiae*, protein complexes involved in pre-rRNA processing frequently contain heat shock proteins; in particular, members of the HSP70 and DNAJ families are important for the efficient processing of pre-rRNA [226]. Orthologs of several of these factors are also present in nucleoli of higher eukaryotes (Table 2), where they might serve similar functions. In addition to HSP70s and DNAJs, other heat shock proteins are necessary for the efficient assembly of ribosomal subunits. For instance, HSP90, together with its adaptors TAH1 and PIH1, contributes to this process by modulating the biogenesis of small nucleolar ribonucleoproteins (snoRNPs) [219, 226]. Aside from these factors, the RNA chaperone activities of Imp3p and Imp4p play an important role in ribosomal biogenesis [198]. Interestingly, Imp3p also interacts with the chaperonin Cct6p and can therefore be linked to the TriC complex (also called CCT) [228]. Collectively, these data support our hypothesis that a chaperone network composed of multiple modules ensures the coordinated function of nucleolar processes as exemplified by the assembly of ribosomal subunits in nucleoli.

Another hypothetical set of interactions is organized around HSP90, its co-chaperones and NoMPs (Figure 19ii). HSP90, in conjunction with CDC37, places multiple protein kinases, including casein kinase 2 (CK2), in a position to be activated. Although CDC37 is not present in the nucleolar proteome databases, this co-chaperone is known to form complexes with fibrillarin [133, 217], a *bona fide* nucleolar marker, which is concentrated in this compartment under normal growth conditions. Thus, in the nucleolus, HSP90 might control CK2 activity,

which is important for several aspects of nucleolar function and organization. Specifically, we propose that the HSP90–CK2 link regulates the activity of RNA polymerase I and, subsequently, ribosome biogenesis [221, 229]. However, the role of HSP90–CK2 goes beyond the assembly of ribosomal because CK2 is also required to re-establish the proper organization of the nucleolus after its sub-compartments fibrillar center (FC), dense fibrillar component (DFC) and granular component (GC) have been segregated by stress [230]. The NoMP B23 is at the center of these activities, as it is modified by CK2 and required for proper nucleolar organization [230]. With respect to the chaperone/NoMP network, B23 is of particular importance because it functions as a stress sensor that has a role in cell survival [225]. In addition, we propose that the chaperone HSPA8 (HSC70) participates in the functional organization of nucleoli (Figure 19ii). Specifically, HSPA8 could restore nucleolar functions after stress by transiently associating with fibrillarin [51], a protein involved in pre-rRNA processing. Other biological processes could depend on a nucleolar chaperone network (Figure 19iii). For instance, the presence of protein kinases and phosphatases in nucleoli that form complexes with members of the HSP90 (e.g. cyclin-dependent kinase 4 [CDK4], CK2, Src, eukaryotic translation initiation factor 2 alpha kinase 2 [eIF2AK2]) or HSP70 families (e.g. CDK9, protein phosphatase 1 [PP1], PP2A) suggests that chaperones might participate in the regulation of cell cycle progression and signaling events controlled by the nucleolus. Alternatively, these proteins might post-translationally modify HSP90 and/or HSP70 to modulate nucleolar chaperone function. Furthermore, a potential contribution of the nucleolar chaperone network to protein turnover can be inferred from links between the

network and the ubiquitin proteasome system (Figure 19iv). For example, parkin 2 (PARK2; a constituent of an E3 ubiquitin ligase complex), ubiquitin-activating enzyme UBA1 and ubiquitin-conjugating enzyme UBC4 interact with multiple members of the nucleolar chaperone network.

3.6. How can a nucleolar chaperone/NoMP network promote survival?

Here, we propose that several biological processes in nucleoli depend on the coordinated action of chaperones, co-chaperones and NoMPs located in this compartment. Many of these processes, including ribosome biogenesis, assembly of signal recognition particle (SRP), and control of cell cycle progression, are vital under all growth conditions. A nucleolar chaperone/NoMP network would provide the assistance to complete these complex tasks in a timely and organized fashion. In our opinion, such a nucleolar network will become even more important when cells encounter stress. Several lines of evidence suggest that changes in cell physiology, brought on by the environment or disease, could alter the demand for chaperone or NoMP activities in this compartment. For example, the increase in concentration of multiple chaperones and their co-factors in nucleoli after heat shock points to a reorganization of the local network. Likewise, viral infections can induce the nucleolar accumulation of chaperones (HSP70) or loss of NoMPs (B23, nucleolin) [231]. Together, these data imply that nucleoli are positioned at the center of essential cellular activities that are particularly sensitive to stress and therefore require special attention from chaperones.

The importance of nucleolar functions and their dependence on chaperone activity support the idea that the nucleolar chaperone/NoMP network could provide unique opportunities for therapeutic intervention (Figure 19v). Indeed, many cancer cells show upregulated ribosome biogenesis due to an increase in rRNA synthesis. Recent studies demonstrate that compound CX-3543 (a small molecule that interferes with nucleolin–rDNA binding) relocates nucleolin to the nucleoplasm, concomitant with the inhibition of RNA polymerase I-dependent transcription and apoptosis [232]. Given the selective action of CX-3543 and the possibility to specifically inhibit HSP90 in the mitochondrial chaperone network [211], we expect that in the future other components of the nucleolar chaperone/NoMP network will be targeted by drugs to alter the physiological state of diseased or aging cells.

3.7. Concluding remarks

Chaperones and their co-factors make many contributions to numerous cellular functions. Recent studies support the idea that the cellular chaperone network is divided into individual branches which orchestrate particular functions. Input from separate branches is then combined to 'fine-tune' the cellular proteostasis network. One of the current challenges is to understand how the action of different branches regulates client function and the downstream events that rely on client activity. Here, we speculate that a specialized chaperone/NoMP network is located in nucleoli where it supports and regulates a unique set of tasks.

Thus, we propose that nucleolar chaperones, together with their co-factors and NoMPs, provide 'pillars' for a compartment-specific proteostasis network. Such a nucleolar network will be ideally suited to coordinate a large number of diverse functions, ranging from ribosome biogenesis to cell cycle control. In concordance with other studies on chaperone networks, we predict that the nucleolar chaperone/NoMP network is dynamic in order to adapt nucleolar activities to changing growth conditions. In particular, we propose that cell survival is regulated by the recruitment and/or loss of selected network components to nucleoli, where they contribute to the control of many downstream events. On the basis of recent progress in chaperone and nucleolar biology, we propose a model of a nucleolar chaperone/NoMP network that provides a focal point to modulate cellular functions under normal, stress and disease conditions. Our model and the questions it provokes (Box 2) will provide a starting point to obtain a new perspective on the functional organization of nucleoli.

3.8. Acknowledgements

This work has been supported by grants from CIHR, NSERC and HSFC (US). PB and MK were supported by fellowships from FRSQ/HSFC and HSFC. We thank Cory Glowinski for help with 3D reconstructions. PB and MK contributed equally to the manuscript.

3.9. Annex

3.9.1. Nucleolus

Nucleoli provide specialized compartments for numerous diverse cellular processes, some of which are pivotal to cell viability and the stress response. The nucleolus is organized around multiple copies of rDNA genes; they are transcribed by RNA polymerase I and processed into 28S, 18S and 5.8S rRNA [129, 190]. A large number of factors promote the processing of pre-rRNAs which, together with ribosomal proteins, are assembled into ribosomal subunits [129, 174]. Light and electron microscopy revealed that mammalian cells have tripartite nucleoli containing fibrillar centers (FC), surrounded by dense fibrillar components (DFC). Both of these structures are embedded in the granular component (GC). In mammalian cells, the size (and possibly number) of nucleoli is related to rDNA transcriptional activity, and a higher demand for ribosomal biogenesis correlates with the increased size of nucleoli [134, 190]. In addition to the biogenesis of ribosomal subunits, other functions have been ascribed to the nucleolus in recent years. For instance, nucleoli coordinate stress responses, participate in signal recognition particle (SRP) assembly, contribute to virus replication, control cell cycle progression and apoptosis, and regulate the activities of telomerase as well as p53 [127-129, 140, 175, 176]. In dividing cells of higher eukaryotes, nucleoli are disassembled in prophase when rDNA transcription ceases. They are re-assembled during telophase in parallel with the reinstatement of rDNA transcription [128, 129, 174]. Nucleoli are dynamic

compartments, and morphological changes in their organization can culminate in the separation of FC, DFC and GC, a process referred to as 'nucleolar segregation'. Although the three subcompartments remain in proximity of each other, the concentric organization is lost during nucleolar segregation. These changes can be induced by drugs such as actinomycin D that interfere with RNA polymerase I-dependent transcription [174, 189].

3.9.2. Future directions and questions

Our knowledge about the presence of chaperones and their co-factors in nucleoli is to a large extent based on groundbreaking proteomics research. It is now mandatory to obtain independent evidence by other methods, which have the potential to identify additional factors that are only loosely associated with nucleoli. We are only beginning to understand the role of chaperones in nucleoli. As listed below, there are many questions that have to be addressed in the future. How is a specific nucleolar chaperone/NoMP network generated and maintained? Which chaperones are essential to build a nucleolar network? Are chaperones recruited individually or as modules to nucleoli? Is the network required to disassemble and reform nucleoli during mitosis? Aside from stress, what regulates the composition and activity of the nucleolar chaperone/NoMP network? How does the nucleolar network communicate and integrate its function with chaperones outside of the nucleolus? Which targets of the nucleolar chaperone/NoMP network can be exploited for therapeutic intervention?

3.9.3. Glossary

B23: also called nucleophosmin and numatrin. This protein is located in the nucleolar GC sub-compartment, where it is involved in many interactions. B23 shuttles between the nucleus and cytoplasm, controls the organization of nucleoli, and participates in the stress response, ribosome assembly and ribosome transport. B23 regulates apoptosis, serves as a histone chaperone, and is implicated in cancer [145, 214].

Chaperones: promote the proper folding of *de novo* synthesized or damaged polypeptides. Moreover, chaperones participate in protein unfolding, and their activities are frequently required for the assembly or disassembly of complex macromolecular structures. Chaperone substrates are also referred to as 'clients'. In some cases, client proteins depend on the persistent presence of chaperones to remain active. We call chaperones that fit these criteria 'conventional' to distinguish them from RNA chaperones and nucleolar multitasking proteins.

Co-chaperones: Many chaperones require co-factors to optimize their biological functions. For example, several co-chaperones regulate the ATPase cycle of HSP70s or HSP90s. Moreover, some co-chaperones connect different chaperone modules, whereas others contribute to the selection of clients.

Heat shock proteins: The synthesis of many (but not all) heat shock proteins is upregulated when cells are exposed to various forms of stress, as exemplified

by heat shock. Members of the different heat shock protein families are involved in all aspects of cell biology under normal and stress conditions. Heat shock proteins are grouped into different families according to their molecular mass

Nucleolar multitasking proteins, NoMPs: in addition to functioning as chaperones for proteins and RNA, some nucleolar proteins also contribute to the processing of pre-rRNA, trafficking of macromolecules in and out of the nucleolus, or other functions. Based on their involvement in different processes that are related to nucleolar function and organization, we refer to these factors as nucleolar multitasking proteins, or NoMPs. A prominent example is B23.

Nucleolin: also called C23. This RNA-binding protein participates in rRNA processing, chromatin remodeling, and functions as a histone chaperone [224].

Protein homeostasis: also known as proteostasis, this term refers to the preservation of the proper concentration, distribution and function of proteins. This balance is achieved by a combination of protein synthesis, folding, degradation, aggregation, disaggregation and transport or proteins [201].

Ribosomal RNA: Ribosomes contain four different RNA molecules, 28S, 18S, 5.8S and 5S rRNA. Whereas 18S rRNA is present in the small ribosomal subunit, the large subunit contains 28S, 5.8S and 5S rRNAs. RNA polymerase I transcribes rDNA in the nucleolus and generates a 45S precursor molecule;

processing of this precursor produces 28S, 18S and 5.8S rRNA. By contrast, 5S rDNA is transcribed outside of the nucleolus by RNA polymerase III [190].

RNA chaperones: are a diverse group of protein families that engage in RNA quality control [197]. These chaperones can rearrange the RNA structure in a site-specific fashion. The maturation of pre-rRNA requires extensive RNA processing, therefore RNA chaperones play an important role in ribosome biogenesis [198].

Signal recognition particle (SRP): a protein–RNA complex that contains six proteins and a non-coding 7S RNA. SRP is critical for the targeting of nascent polypeptides to the endoplasmic reticulum; nucleoli are crucial for SRP assembly [129, 176, 190].

Small nucleolar RNPs (snoRNPs): protein–RNA complexes required for the maturation of ribosomal RNAs.

Chapter II of my thesis focused on the possible chaperone networks present in the nucleolus. This allowed me to introduce the idea of "multitasking nucleolar proteins", for proteins that function as chaperones, but also contribute to the proper biogenesis of ribosomes and trafficking in and out of the nucleolus. In this work, we reviewed the chaperones that were identified in the nucleolus, multitasking proteins, and chaperones that transiently move to the nucleolus in response to changing growth conditions. Chapter I and II set the stage for chapter III, in which we further examined the interactions that occur between hsc70 and the nucleolus under stress and normal growth conditions.

4. CHAPTER III

The chaperone hsc70 and its nucleolar localization sequence associate with RNA

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Department of Physiology McGill University, Montreal, PQ, Canada.

4.1. Abstract

Background

Hsc70 is a constitutively synthesized chaperone that associates with macromolecules in the nucleus and cytoplasm. The intracellular distribution of hsc70 is regulated by changes in cell physiology, and the chaperone accumulates in nucleoli when cells recover from heat stress. Within the N-terminal ATPase domain of hsc70, the nucleolar localization sequence (NoLS) is sufficient to promote the stress-induced nucleolar targeting of the chaperone. At present, the molecular mechanisms regulating the nucleolar localization of hsc70 are not fully understood. It is also not known whether the NoLS engages in other interactions.

Methodology/Principal Findings

To obtain a better understanding of the cellular interactions that engage hsc70, we analyzed the chaperone in growing mammalian culture cells. Specifically, we examined the interaction of hsc70 with RNA and identified multiple links between hsc70, stress, and RNA metabolism. Specifically, we demonstrated that in stressed cells, RNase treatment liberates a portion of hsc70 from nucleoli. Furthermore, the inhibition of RNA-polymerase I, an enzyme that transcribes rDNA in the nucleolus, increased and prolonged the nucleolar accumulation of hsc70 and of a reporter protein carrying the NoLS. Additional experiments demonstrated that the hsc70 NoLS is sufficient to bind polyA^{+}.

Conclusions/Significance

Our studies provide several lines of evidence that link the chaperone hsc70 to RNA metabolism. First, hsc70 association with RNA helps to retain the chaperone in nucleoli of stressed cells. Second, *de novo* RNA synthesis in the nucleolus of stressed cells controls the residence of hsc70 in this compartment. Third, the hsc70 NoLS, which targets hsc70 to nucleoli upon stress, interacts with polyA$^+$ RNA in unstressed cells. Together, our work suggests a complex relationship between RNA metabolism and hsc70 in different cellular compartments.

4.2. Introduction

Heat shock proteins (hsps) are chaperones that participate in numerous cellular functions; these include nascent protein folding, trafficking of macromolecules, stress recovery and aging [13, 164-166]. Hsc70 (heat shock cognate protein 70 kD) is a member of the hsp70 family of molecular chaperones that is constitutively synthesized and conserved among pro- and eukaryotes [13, 165]. Unlike other members of the hsp70 family, hsc70 is essential for the viability in human cell lines [164].

Members of the hsp70 family are organized into different domains whose collaboration is necessary for regulated chaperone function [165]. In particular, the 44 kD N-terminal ATPase domain cooperates with the 18 kD peptide-binding domain to refold chaperone clients; a variable C-terminal 10 kD segment interacts with several co-chaperones [165]. Under normal growth conditions, hsc70 is located in both the nucleus and the cytoplasm. However, this distribution is dynamic, as the chaperone shuttles between both compartments [51]. Heat shock inhibits hsc70 nucleocytoplasmic shuttling, causing the chaperone to accumulate in the nucleus [51]. Hsc70 concentrates in the nucleoplasm of heat-stressed cells due to binding of client molecules that require re-folding. Upon removal of stress, cells recover and hsc70 associates with nucleoli [142].

Since the interaction between hsc70 and clients is sensitive to ATP, incubation with ATP releases hsc70 from chaperone/client complexes [51]. This strategy was

efficient to liberate hsc70 from binding partners in the nucleoplasm of heat-shocked cells. By contrast, only a portion of the hsc70 present in nucleoli can be released with ATP, suggesting that the interactions in nucleoli go beyond the formation of chaperone/client complexes [51]. At present, these interactions have yet to be characterized.

Within the nucleus, the nucleolus is a specialized compartment, where RNA polymerase I transcribes the 45S precursor of 18S, 28S and 5.8S ribosomal RNA [129, 148]. Pre-rRNA is processed in a complex series of reactions that require the cooperation of small nucleolar RNAs (snoRNAs) and enzymes in the nucleolus. As an end product, small and large ribosomal subunits are produced in the nucleolus, exported to the nucleoplasm and ultimately to the cytoplasm [233, 234]. Aside from its role in the biogenesis of ribosomal subunits, the nucleolus is critical to many other biological processes, including cell cycle control, assembly of signal recognition particle and the stress response. As a multifunctional compartment, the nucleolus is linked to a growing number of human diseases and pathologies [44].

Although it is well established that members of the hsp70 family accumulate in nucleoli when cells recover from stress, the underlying molecular mechanisms are not fully understood. It was proposed that hsp70s concentrate in nucleoli to rescue rDNA transcription and the assembly of ribosomal subunits. Notably, all of these processes are compromised by stress [142, 235, 236]. At the same time, hsp70s might associate with nucleoli to sustain ribosomal DNA quality and

overall nucleolar integrity [237, 238]. In support of this idea, it was shown that the inducible hsp70 family member hsp72 binds to PARP1 (poly[ADP-ribose] polymerase I) and XRCC1 (X-ray repair cross complementing group) in the nucleus and possibly nucleolus of heat-shocked cells [237]. Other studies suggest that members of the hsp70 family can interact with RNA in growing cells and *in vitro* [163, 239]. As such, it was reported that several hsp70s bind to AU-rich sequences located in the 3' untranslated regions (3' UTRs) of some mRNAs ([163] and references therein). Hsp70 binds to RNA through its ATPase domain [163], and this RNA-binding capability is conserved among hsp70 family members from diverse organisms, including DnaK in *E. coli* [163]. Collectively, these studies suggest that chaperones and their function are connected to different aspects of nucleic acid function and metabolism.

In our previous work, we examined hsc70 localization under different physiological conditions and demonstrated that residues 225-297 provide a stress-inducible NoLS [64]. The NoLS is located in the N-terminal ATPase domain of hsc70 and participates in the interaction with several co-chaperones [29, 32, 240]. Here, we built on our studies to define the possible mechanisms that provide a link between hsc70 and RNA under normal and stress conditions. Our experiments show that the stress-dependent nucleolar concentration of hsc70 is in part sensitive to RNase treatment and that the pharmacological inhibition of RNA polymerase I prolonged the stress-induced nucleolar residency of hsc70. Moreover, we demonstrate that the hsc70 NoLS promotes the association with

polyA$^+$ RNA in growing cells. Collectively, our studies identify multiple pathways that connect hsc70 to RNA biology.

4.3. Results

RNase Releases Hsc70 from Nucleoli of Stressed Cells

In previous studies we showed that hsc70 concentrated in nucleoli can only be in part released by incubation with ATP [51], suggesting that associations different from chaperone/client interactions retain hsc70 in the nucleolus. Possible candidates for these interactions are RNA and/or RNA-binding proteins. If this is the case, one would predict that RNase treatment releases a fraction of the heat shock protein from nucleoli. In figure 20, we tested this hypothesis with heat-shocked cells that had recovered for 3 hours from stress. At this time point, hsc70 accumulates in nucleoli [51, 64]. When unfixed cells were incubated with the detergent NP-40 (see Materials and Methods), hsc70 was only partially liberated. However hsc70 was released from nucleoli when samples were treated with a combination of NP-40 and RNase (Figure 20). Since hsc70 RNA binding is sensitive to ATP [161], we excluded ATP from our buffers. Together, our data indicate that different types of interactions support hsc70 nucleolar localization; these include the binding to RNA and/or components associated with RNA.

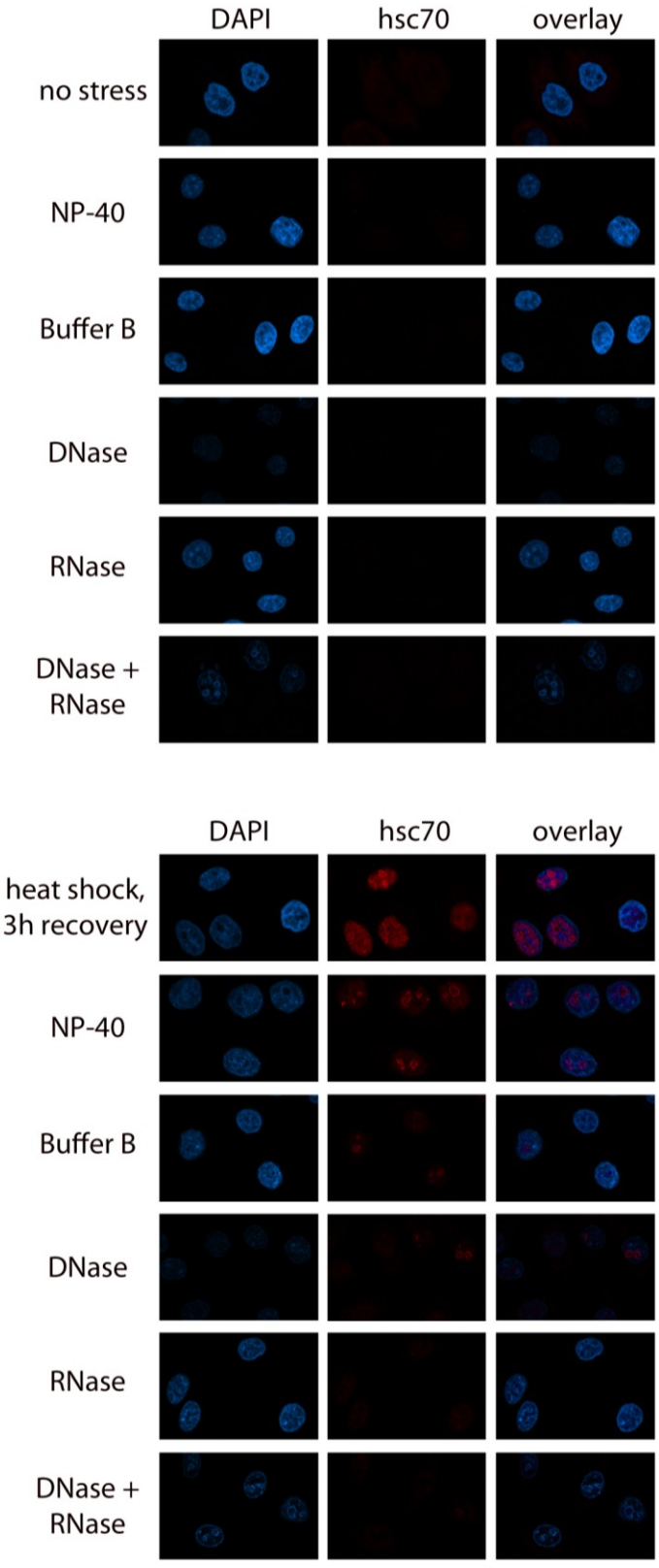

DAPI
hsc70
overlay
no stress
NP-40
Buffer B
DNase
RNase
DNase +
RNase
DAPI
hsc70
overlay
heat shock,
3h recovery
NP-40
Buffer B
DNase
RNase
DNase +
RNase

Figure 20: Release of endogenous hsc70 from nucleoli of heat-stressed cells. HeLa cells were exposed to heat shock and extracted with detergent, DNase and RNase as detailed in Materials and Methods. All images were acquired with a LSM510 confocal microscope with identical settings. In the absence of stress, hsc70 was easily solubilized with the buffers. By contrast, upon recovery from stress, hsc70 was retained in nucleoli. This retention was drastically reduced by RNase treatment.

Inhibition of RNA polymerase I Prolongs the Nucleolar Accumulation of Hsc70 in Cells Recovering from Stress

Actinomycin D (ActD) is a commonly used inhibitor of RNA polymerase I dependent transcription; this compound also leads to a reorganization of the nucleolus [241-243]. Here, we examined the effect of ActD on endogenous hsc70 in control and heat-stressed cells (Figure 21). To this end, ActD, or the vehicle DMSO, was added immediately before heat shock. ActD did not drastically alter the nucleocytoplasmic distribution of hsc70 in unstressed cells (not shown), but changed the nucleolar accumulation when cells were exposed to heat. Thus, ActD modulated the kinetics of hsc70 nucleolar accumulation and prolonged the chaperone association with nucleoli (Figure 21).

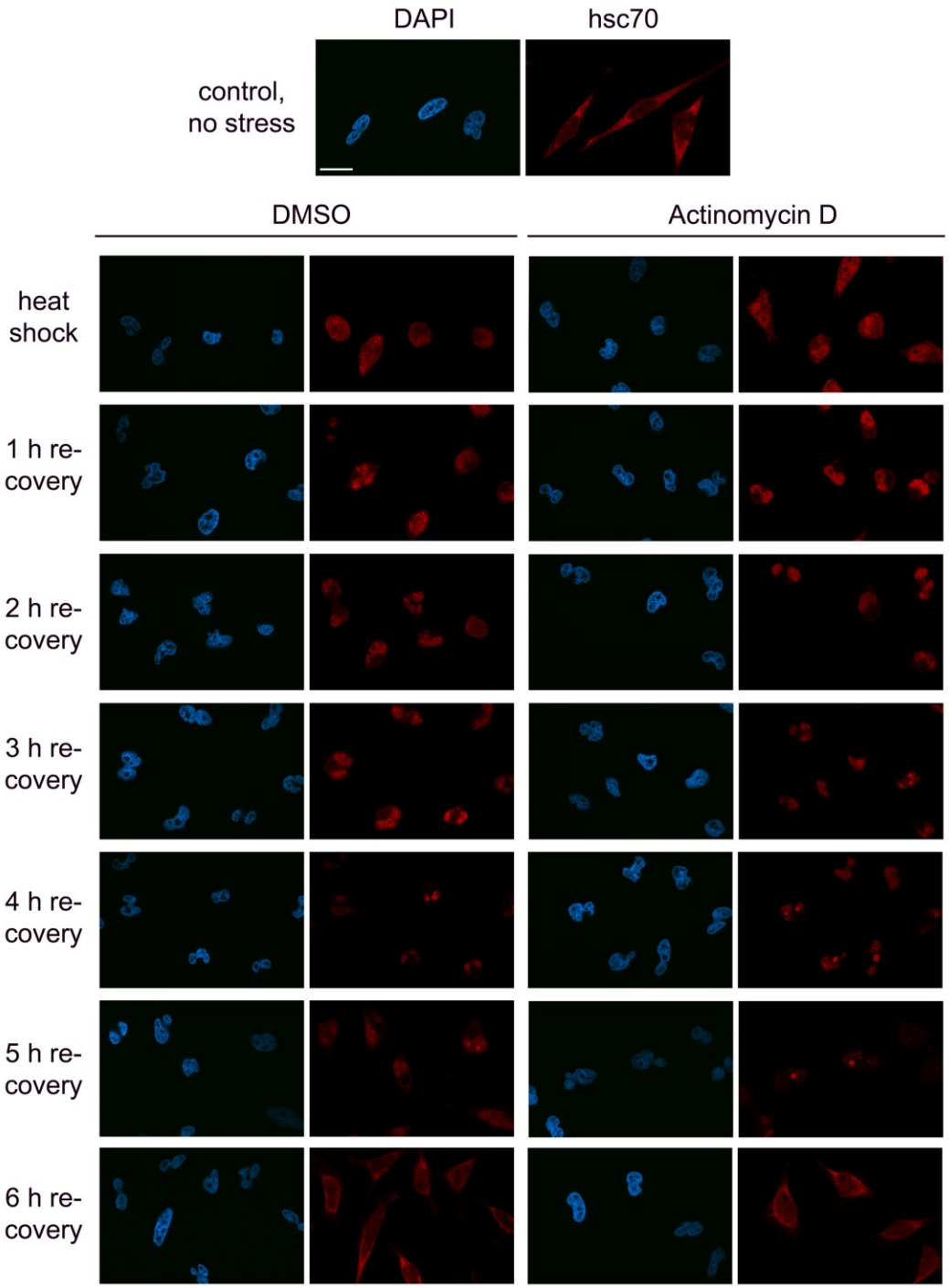
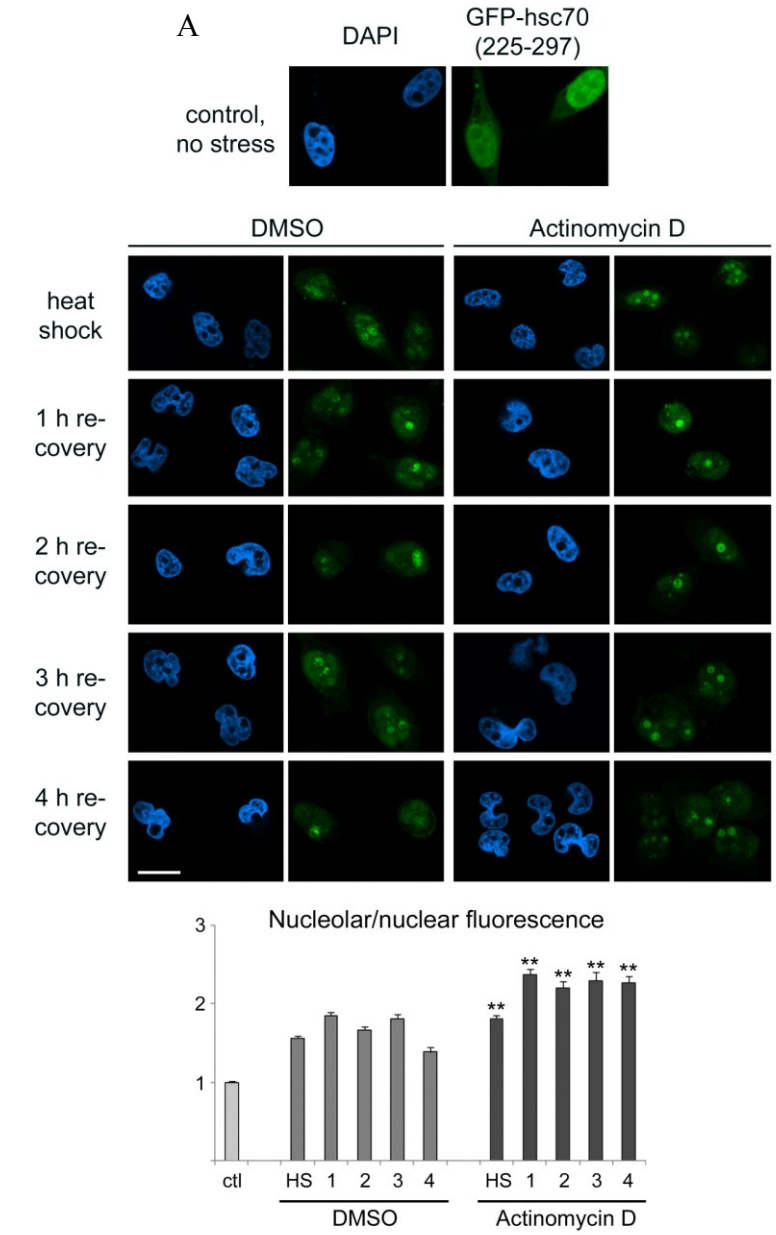


Figure 21: Effect of Actinomycin D treatment on the nucleolar accumulation of endogenous hsc70. HeLa cells were treated with ActD or the vehicle DMSO and hsc70 was located by indirect immunofluorescence. Cells were incubated for 5 hours at 37°C (controls) or heat-shocked for 1 hour and allowed to recover at

37°C. Note that there is no nucleolar accumulation with or without ActD when cells were not heat-shocked. Size bar is 20 μm.

Actinomycin D Increases the Heat-induced Nucleolar Accumulation of the Hsc70 NoLS

Our earlier studies identified residues 225-297 as the hsc70 NoLS that is sufficient for stress-induced nucleolar accumulation [64]. Since ActD changed the stress-induced nucleolar accumulation of endogenous hsc70, we wished to gain detailed information on how the transcription inhibitor affects GFP-hsc70(225-297) distribution. To this end, cells transiently synthesizing the reporter protein were heat-treated in the presence of ActD or the vehicle DMSO. Samples were fixed at different time points and analyzed by confocal microscopy (Figure 22A). To detect subtle changes in the distribution of GFP-hsc70(225-297), fluorescent signals in nucleoli were quantified following established procedures [64, 241]. Nucleolar/nuclear ratios were calculated for the fluorescence intensities of individual cells and data were normalized to unstressed control samples. Results in figure 22 support the idea that ActD prolonged and increased significantly the nucleolar accumulation of GFP-hsc70(225-297) in stressed cells. Control experiments demonstrated that the fluorescent tag GFP did not concentrate in nucleoli of heat-shocked cells upon ActD treatment (Figure 22B).


A
DAPI
GFP-hsc70 (225-297)
control, no stress
DMSO
Actinomycin D
heat shock
1 h recovery
2 h recovery
3 h recovery
4 h recovery
Nucleolar/nuclear fluorescence
3
2
1
**
ctl
HS 1 2 3 4
HS 1 2 3 4
DMSO
Actinomycin D

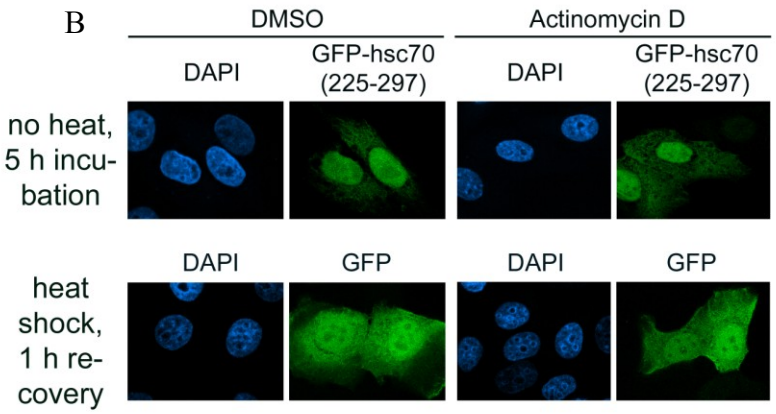

B
DMSO
Actinomycin D
DAPI
GFP-hsc70 (225-297)
DAPI
GFP-hsc70 (225-297)
no heat, 5 h incubation
DAPI
GFP
DAPI
GFP
heat shock, 1 h recovery

Figure 22: Actinomycin D increases the nucleolar accumulation of GFP-hsc70(225-297). HeLa cells were heat-shocked for 1 hour in the presence of DMSO or ActD and incubated at 37°C for the time indicated. (A) Nucleolar localization of GFP-hsc70(225-297) was quantified by calculating the ratio of nucleolar/nuclear fluorescence for the sum of nucleoli in each nucleus. The ratio of nucleolar/nuclear fluorescence of control cells (top panel) served as a reference and was defined as 1. Results +SEM are shown for controls (ctl), heat shock [244] 1, 2, 3, and 4 hours of recovery. Student's ttest identified significant differences between DMSO and ActD- treated cells. **, $p<0.01$; size bar is 20 μm. (B) In control experiments unstressed cells were incubated with ActD for 5 h. Note that GFP-hsc70(225-297) did not accumulate in nucleoli under these conditions. Additional controls examined the fluorescent tag GFP in heat-stressed cells after 1 h recovery in the absence or presence of ActD.

As for endogenous hsc70, incubation of unstressed cells with ActD failed to concentrate GFP-hsc70(225-297) in nucleoli (Figure 22B), in line with the idea that heat shock is a prerequisite for ActD-induced changes in localization.

The NoLS of Hsc70 Binds to PolyA$^+$-RNA

The data discussed above support the idea that the functions of hsc70 and its NoLS are linked to RNA metabolism. Others have shown that members of the hsp70 family bind to RNA, both *in vitro* and *in vivo* [239, 245]. Notably, the ATPase domain of some hsp70 family members is sufficient to mediate RNA-

binding, but other parts of the protein provide additional support for the association with RNA [245].

To obtain a better understanding of hsc70 interactions with mRNA, we investigated its binding to oligo-dT cellulose. This procedure isolates polyA$^+$ RNA as well as material associated with the RNA. Proteins that co-purify with polyA$^+$ RNA can then be detected by Western blotting. We used this assay with whole cell extracts generated for transiently transfected cells that synthesized different GFP-tagged reporter proteins. The extracts were incubated with oligo-dT beads and bound material was probed with antibodies against GFP. Figure 23A shows that a portion of GFP-hsc70 (GFP-fused to full length hsc70) associated with polyA$^+$ RNA (Figure. 23A; E = elution). Similar to the full length protein, GFP-hsc70(225-297) also bound polyA$^+$ RNA. This interaction was specific, as it was not observed for the GFP-tag alone (Figure 23A). Thus, the hsc70 NoLS is sufficient to promote the interaction with mRNA. Control experiments verified that endogenous hsc70 and the RNA-binding protein HuR [246, 247] co-purified with polyA$^+$ RNA under these conditions (Figure 23B).

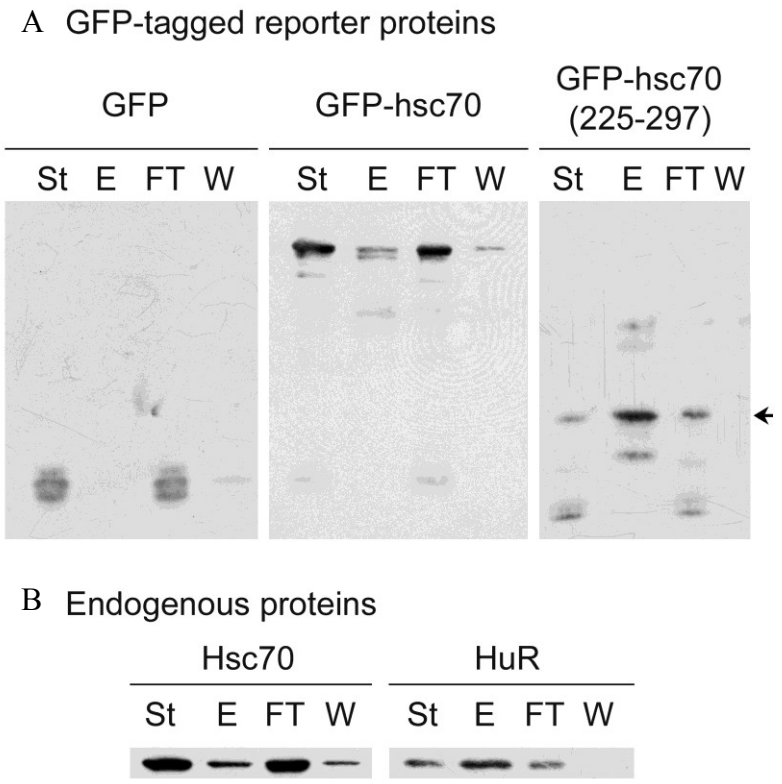


Figure 23: The hsc70 NoLS associates with polyA$^+$ RNA in growing cells. (A) GFP-tagged reporter proteins. Crude extracts were generated for HeLa cells synthesizing GFP, GFP-hsc70 (full length protein) and GFP-hsc70(225-297). Extracts were incubated with oligo-dT cellulose as described in Materials and Methods. Aliquots of the starting material (St), elution (E), flow through (FT) and wash (W) were analyzed side-by-side with antibodies against GFP. The position of GFP-hsc70(225-297) is marked by an arrow. (B) Endogenous proteins. Crude extracts depicted in part A were probed with antibodies against hsc70 and HuR.

Collectively, our results demonstrate that in unstressed cells hsc70 and its NoLS associate specifically with RNA. Therefore, the NoLS can interact with polyA$^+$-containing RNA in the absence of other parts of the chaperone.

4.4. Discussion

Experiments presented here address several questions regarding the association of hsc70 and RNA under different growth conditions. In particular, we demonstrated that RNA and/or RNA-containing complexes contribute to the nucleolar accumulation of hsc70 in cells recovering from heat stress. Furthermore, inhibition of RNA polymerase I in stressed cells prolonged and increased the association of hsc70 and its NoLS with nucleoli. Finally, the NoLS was sufficient to bind polyA$^+$ RNA in growing cells.

At present, it is not known whether the different hsc70 interactions with RNA or protein/RNA complexes occur independently. Alternatively, it is conceivable that these processes are linked and regulated by changes in cell physiology, such as stress. In support of this idea, heat shock interfered with rRNA transcription *in vitro* and *in vivo* [241, 248], and hsp70 overexpression increased transcription two and four hours after severe heat treatment [238].

It is tempting to speculate that the NoLS is recruited to mRNA by interacting with co-chaperones that are part of a larger mRNA/protein complex. Interesting in this context is the ability of hsc70 to stabilize mRNAs containing AU-rich elements in their 3'-UTR, and the binding of purified hsc70 to AU-rich sequence elements [163, 239]. As such, hsc70 associates with polyA$^+$ RNA, and together with co-chaperones hsp40 and Hip, hsc70 is believed to stabilize Bim mRNA [239]. Although the interaction with hsp40 or Hip could, in principle, explain hsc70 binding to polyA$^+$ RNA, this interpretation does not apply to the hsc70 NoLS. We based this assumption on the fact that major interaction sites for hsp40 are in the C-terminal portion of hsc70; thus outside of the NoLS ([249] and references therein). By contrast, our results are compatible with the idea that hsc70 and its NoLS are recruited to polyA$^+$ RNA by binding the co-chaperone hsp110. Hsp110 interacts with multiple hsc70 residues, some of which are located in the NoLS; this includes R258, R262, E283, D285 and D292 [240]. Moreover, research on hsp70, hsc70 and hsp110 revealed that all three of these proteins can interact with RNA [161]. Given that these mRNAs are located in the nucleoplasm and cytoplasm, the question arises which RNAs bind hsc70 or the NoLS in the nucleolus. Candidate RNAs in the nucleolus have to be present in this compartment during the recovery from heat shock, and it is possible that they stay in nucleoli after ActD treatment. A potential candidate for these interactions is U8 snoRNA, which is not released from nucleoli by treatment with ActD [250]. Notably, human U8 snoRNA contains several stretches of AU-rich regions that might provide binding sites for hsc70 [251-253]. However, not all RNA-binding proteins remain in nucleoli upon ActD treatment. For example, fibrillarin a

nucleolar protein that associates with snoRNPs [254] and binds hsc70 [51], relocates from nucleoli when cells are treated with ActD [241]. Whether fibrillarin also relocates in heat-shocked cells treated with ActD is currently not known.

On the basis of our work, we propose that hsc70 nucleolar accumulation results from a combination of events that are linked to stress, and one of these events is the inhibition of transcription in the nucleolus. Importantly, inhibition of RNA polymerase I alone is not sufficient to trigger hsc70 concentration in nucleoli, because both ActD and heat stress are necessary for nucleolar accumulation. With respect to intracellular trafficking and RNA binding, the NoLS is of particular interest. This segment is not only sufficient to bind polyA$^+$ RNA, but nucleolar accumulation of the NoLS is also sensitive to ActD. Thus, the NoLS provides a sequence element that engages in multiple interactions, including the association with different types of RNA.

Taken together, our results indicate that the nucleolar accumulation of hsc70 is a complex event in which hsc70 may interact with the nucleolus in part through its RNA binding capabilities. The precise mechanisms that contribute to the RNA-chaperone interaction, as well as the specific types of RNA, are not known at this point.

4.5. Materials and Methods

Growth, transfection and heat shock experiments

HeLa S3 cells where grown to ~70% confluency, for no more than 12 passages. 24 hours after transfection [51], cells were exposed to 1 hour heat stress at 45.5°C and subsequently allowed to recover at 37°C for the times indicated.

Pharmacological inhibitors

HeLa S3 cells were grown on coverslips in 6-well plates. Actinomycin D (final concentration 2 µg/ml [255]) or the vehicle DMSO was added immediately before heat shock.

In vitro Cell Extraction

At different stages of the procedure, specimens were fixed and processed for immunostaining; a schematic representation of the extraction protocol is depicted in figure 24. Cells were grown on coverslips to ~70% confluency, heat-shocked for 1 hour at 45.5°C and then incubated at 37°C for recovery. Following recovery, cells were rinsed on ice once with buffer B plus DTT (20 mM Hepes, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, pH 7.3, protease inhibitors (Roche), 2 mM DTT). Samples were then extracted with 40 µg/ml digitonin in buffer B for 5 min and washed twice with buffer B. All subsequent steps were carried out at room temperature. Specimens were treated with buffer B containing 5 mg/ml BSA and 0.05% Nonidet P-40 (NP-40). Samples were rinsed once with buffer B without DTT and EGTA, and incubated

for 30 min at 37°C with buffer B, omitting DTT and EGTA. For this step, both DNase (50 µg/ml) and/or RNase (50 µg/ml) were added. Aliquots of the start, eluted, unbound material and wash were analyzed by western blotting with antibodies against GFP (Santa Cruz; sc-8334l 1:1,000), hsc70 (StressGen; SPA815; 1:5,000) and HuR (Santa Cruz; sc-5261; 1:2,000). HRP-coupled secondary antibodies (1:2000) were used and signals were detected by enhanced chemiluminescence [64].

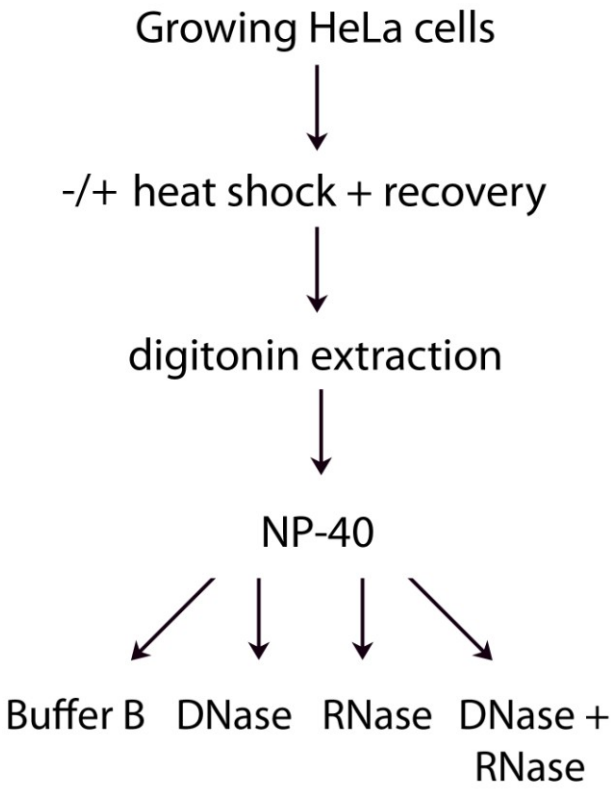


Figure 24: Extraction protocol. Scheme demonstrating all the different steps for the extraction of HeLa cells. All steps were carried out on HeLa cells attached to poly-L-lysine coated coverslips.

Oligo dT cellulose and transfected cells

Cells were transfected with plasmids encoding GFP only, GFP-hsc70(225-297) and GFP-hsc70(wild type) as described above. Unstressed cells were grown to about 70% confluency in a 100 mm dish, and rinsed once with PBS then frozen at -70°C for future use or scratched off with 500 µl of binding buffer (100 mM KCl, 25 mM Tris-HCl, 10 mM EDTA, 0.1% Triton X-100, pH 7.4; supplied with 1X protease inhibitor and 10 mM VAC, in DEPC treated water). Cells were lysed by passing 4 times through a 26.5 gauge needle, and extracts were incubated with 30 U of DNase for 15 minutes at 37°C after which 5 mM DTT was added to the sample. One fraction was saved as a starting control. Beads where equilibrated with binding buffer (3 washes) and equal volumes of proteins where added to the equilibrated beads. The extract was incubated with the beads for 30 minutes at room temperature (RT). Following centrifugation at 10 000 rpm for 30 seconds, the flow through fraction was kept and frozen at -70°C. Beads where then washed 2 times with binding buffer and the wash fractions where frozen at -70°C. Bound material was eluted with final sample buffer. Final sample buffer was also added to all other fractions. All samples where heated 15 minutes at 95°C and centrifuged for 2 minutes at 13000 rpm. Equal amounts of the samples where loaded on 15% SDS-polyacrylamide gels. Western blots were blocked in 5% milk in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 8.4), and probed with anti-GFP (Santa Cruz; sc-8334l 1:1,000), anti-HuR (Santa Cruz; sc-5261; 1:2,000), and anti-hsc70 (StressGen; SPA815; 1:5,000) antibodies. HRP-coupled secondary antibodies (1:2000) were used to detect proteins by enhanced chemiluminescence [64].

Immunofluorescence, Image Acquisition and Processing

Immunostaining and detection of GFP-tagged reporter proteins were performed essentially as described [64]. Images were acquired with a Zeiss LSM510 confocal microscope and a 63x objective (NA = 1.4), and processed with Adobe Photoshop CS4 version 11.0.2. Nucleolar fluorescence was quantified following published procedures [64, 241].

4.6. Acknowledgements

This research was supported by grants from NSERC (Natural Sciences and Engineering Research Council of Canada), FQRNT (Fonds de recherche Nature et technologies Québec) and HSFQ (Heart and Stroke Foundation of Quebec) to US.

5. CONCLUSIONS

Hsc70 is an important member of the Hsp70 protein family. It is a house-keeping chaperone that constantly maintains the proper functioning of proteins, that plays important physiological roles such as clathrin uncoating, and that contributes to the degradation of permanently damaged proteins. Hsc70 is linked to human disease such as heart and brain ischemia, neurodegenerative disease oxidative damage and cancer. The overall role of hsc70 is to assist proper protein folding and sustain cell survival at all times. Furthermore, hsc70 was shown to redistribute to the nucleolus during heat stress recovery, a mechanism that is believed to rescue nucleolar function and ribosomal synthesis. My PhD studies aimed to further the understanding of this important protein. By generating constructs of various hsc70 portions tagged with the GFP reporter protein, we were able to identify a stress inducible NoLS found at position 225-297. We also identified specific parts of this peptide as either increasing or negatively regulating the nucleolar accumulation of hsc70. Moreover, we showed that the nucleolar accumulation of hsc70 not only relies on this 225-297 segment, but is also modulated by the context in which the NoLS is embedded. Thus, as presented in Bański *et al.*, (2010), segment 225-297 of hsc70 can function as an NLS, and is necessary but not sufficient to target the chaperone to the nucleus. As such, when portion 225-297 was truncated from the GFP-hsc70 wild type construct (GFP-hsc70(Δ225-297)), the nuclear accumulation of GFP was lost. GFP-hsc70(Δ225-297) has a size that requires active transport through the NPC, as opposed to the GFP-hsc70(225-297) construct that can passively diffuse between the two

compartments, suggesting that residues 225-297 have the role of an NLS in the context of the wild type protein. A different scenario was observed for the hsc70(225-297)-β-galactosidase construct that also has a size that exceeds the limit to passively traffic between the cytoplasm and the nucleoplasm. In this context, portion 225-297 of hsc70 failed to target GFP to the nucleus, confirming that the NLS role of residues 225-297 is effective only under certain conditions. Further experiments could be carried out with more constructs containing point mutations to narrow down the crucial residues for the NoLS function.

The concept of protein networks, including those generated by chaperones, is well established, but protein-protein interactions under different physiological conditions remain extremely complex due to the high number of variables. My work proposes new links between the nucleolar proteome, chaperones and NoMPs. By combining and analyzing data from multiple databases, we were able to bring forward the networks that chaperones could form in the nucleolus under stress and normal growth conditions. We proposed that these networks, in the nucleolus, play important roles for cell survival.

The last chapter of my thesis focused on the nucleolar binding partners of hsc70. Results show that hsc70 nucleolar accumulation is sensitive to RNase treatment. Furthermore, actinomycin D increased the nucleolar accumulation of hsc70 constructs and prolonged the nucleolar retention of the chaperone. Moreover, experiments done on transfected cells and total RNA extracts show that portion 225-297 of hsc70 binds to polyA$^+$ RNA. Endogenous hsc70 depicts a

similar behavior. Together, these results show that hsc70 is involved in the metabolism of RNA in nucleoli. Experiments carried out with the same settings for the RNA extracts, but on heat shocked cells, and for the RNase treatments on transfected cells, could provide additional information regarding the interactions between hsc70 and its nucleolar anchors. Pull-downs of tagged proteins from purified nucleoli could also provide additional insights into the nucleolar binding partners of hsc70. Furthermore, generating stable cell lines with inducible gene expression may facilitate the analysis through the automation of confocal image acquisition and by reducing the variability of fluorescent signals and stabilizing the binding affinity between the nucleolar anchors and hsc70.

Taken together, this work contributes to our general knowledge on hsc70 intracellular trafficking and the mechanisms that underlie these events. I defined the sequence of the hsc70 NoLS, and propose the presence of important chaperone networks in the nucleolus under normal and stress conditions. Finally, I showed that hsc70 binds to the nucleolus and that this binding is related to RNA metabolism. The importance of hsc70 for cellular functions under normal and stress conditions is well established. I expect that the detailed understanding of its function will contribute to the advancement of medicine and perhaps to the discovery of new treatments for human pathologies. These include, but are not limited to, heart and brain ischemia, neurodegenerative diseases, and cancer. Furthermore, the concept of intracellular networks could be expanded to many protein sets, and advance the development of medical intervention. In my opinion, one of the limiting factors to improve the therapy of highly complex and multi-

factorial diseases like cancer is our inability to assemble the pieces of a puzzle, as exemplified by network components such as hsc70. Defining the roles of these components will ultimately provide the knowledge to integrate them into complete and functional models, which can be applied to the treatment and cure of human disease.

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