Bases moléculaires du syndrome de l'X Fragile :Identification d'un nouvel ARNm cible de FMRP et établissement d'un nouveau mécanisme d'action

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Thèse présentée pour obtenir le grade de

Docteur de l'Université Nice-Sophia Antipolis

Sciences du vivant : Aspects moléculaires et cellulaires de la biologie

par

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Soutenue publiquement le 7 Mars 2008 devant le jury :

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Mme. Le Docteur Annette Schenck	Rapporteur externe
M. Le Docteur Stefan Hüttelmaier	Rapporteur externe
M. Le Docteur Hervé Moine	Examinateur
M. Le Docteur Enzo Lalli	Examinateur
Mme. Le Docteur Barbara Bardoni	Directrice de thèse

Ce travail a débuté à l' Institut de Génétique et de Biologie Moléculaire et Cellulaire à Strasbourg pour finir à l'Institut de Pharmacologie Moléculaire et Cellulaire à Nice en passant pas la Faculté de Médecine de Nice. Je remercie le Pr. Jean Louis Mandel de m'avoir accordé la chance d'intégrer son équipe et d'entamer le projet « pré-thèse ».

Ce projet a été accompli grâce à l'aide financière de l'Association GIS Maladies rares, la Fondation Jérôme Lejeune et la Fondation de la Recherche Médicale auxquelles j'adresse mes remerciements les plus sincères pour la confiance qu'elles ont accordée à ce projet.

Je tiens à remercier les membres du Jury d'avoir accepter d'évaluer mon travail de thèse.
Thank you Dr. Schenck for all the discussions we had since I started my Master. Thank you for your advices and support.
Another warm thank to Dr. Hüttelmaier for all the fruitful discussions we had, thank you for your help and support during the last two years. Collaborating with you was a tremendous motivation for me.
Great thanks to Dr. Nahon for preparing our "arrival" to the IPMC and for all his help and support to the Bardoni's team.

Octobre 2003: Rencontre avec Dr. Bardoni.
Mars 2008: soutenance de thèse & Elias est toujours vivant....
Presque cinq années Barbara où tu surveillais de trop près ce travail. D'abord en DEA et puis en thèse. La confiance que tu m'as accordée était un motif assez important pour poursuivre ce projet. Je te remercie Boss d'avoir été si dynamique, si motivée et motivante, si proche et si attentive. Je te remercie de m'avoir écouté et de m'avoir permis de t'embêter toutes les deux minutes. Merci de n'avoir jamais fermée la porte de ton bureau Boss et d'avoir toujours été là pour les discussions. Merci de m'avoir fait assez confiance pour m'envoyer présenter les résultats dans des congrès internationaux et merci de m'avoir laissé la chance de faire les manipes qu'il fallait là où il fallait. Merci d'avoir su diriger ce projet et de m'avoir montré que la science est un challenge quotidien avec des hauts et des bas qu'il faut savoir contrôler. Merci de m'avoir accordé cette liberté de travail dans une ambiance assez chaleureuse et humaine. Merci d'avoir accepté mon sens d'humour, même si parfois lourd. Merci de m'avoir montré qu'on peut travailler en étant de bonne humeur et qu'on peut surtout travailler en rigolant. Merci de m'avoir témoigné une tolérance envers certaines différences et conceptions. Je te remercie Boss de m'avoir laissé partager des moments de familles avec Alessandro & Valentina. Merci pour tous les repas qu'on avait partagés, je n'oublierai jamais : *«t'as intérêt à être sage avec ta sœur pour mériter ton cadeau, car ta maman ne l'est pas trop avec ses étudiant(e)s!»*..... Merci Boss pour tout !

Pendant ces quatre dernières années j'ai eu une chance exceptionnelle de collaborer et travailler avec 2 chercheurs qui ont su me transmettre leur passion pour la recherche. J'adresse mes remerciements les plus chaleureux au Pr. Khandjian qui m'a initié au monde des polyribosomes. Merci pour toutes les discussions, toute votre compréhension, et merci pour l'accueil que vous m'avez offert à Québec et au sein de votre laboratoire. Je n'oublierai jamais votre attention et dévouement.
Je remercie également le Dr. Moine qui m'a introduit au monde de l'ARN et surtout à l'architecture de ces molécules. Merci Hervé car tu as su me transmettre

brillement ton savoir faire, eh oui Hervé, ces grands gels que j'arrive rarement à couler correctement ont bien trouvé leurs places dans mon quotidien. Merci pour toutes les discussions, ta disponibilité, ton accueil et surtout ta patience !
Dr. Lalli ! Par où je dois commencer ?! A part le fait que tu sois le mari de ma chef, je tiens à te remercier en mettant au clair certains points: Merci pour ta façon de voir la science en général et la recherche en particulier. Ton ouverture d'esprit et les horizons assez réels et proches m'ont été un repère assez solide. Merci pour tous tes conseils et les discussions qu'on a eues ; Merci d'avoir écouté et supporté mes « sens » et « nonsens ». Merci d'avoir témoigné et « approuvé » que la science est un combat chronique et vivant. Merci pour tous les attributs dont tu m'as peint. Merci pour tous les midis et soirées *dégustation* et d'avoir surtout apprécié ma cuisine ! (à bientôt *«Le Resto»*, non ?)

Mon passage à l'IGBMC reste toujours animé et d'actualité, merci à tous ceux qui ont contribué à me mettre à l'aise chaque fois que j'y retourne, et ceux que j'ai connus pendant ma période de résidence *«permanante»* à commencer par Marie Castets, ton aide et soutien pendant mon DEA m'étaient assez cruciaux pour continuer ce projet.
Tom, Delphine, Maria, Aurora, Stéphane, Anna, Jocelyn, Karine, Eric, André, Claudine, Aline, Léa, Dom et Didier, Patricia et tous ceux que j'ai oubliés....
Grand merci à Fabrice, Chantal, Myriam, Aurélien, Yvon, Anne-Sophie, et Stéphane pour votre amitié et soutien... Merci d'avoir témoigné que les temps d'incubation et les PCR sur Nice ne se passent pas au bord de la plage !!!! Un grand merci pour votre soutien et toutes les soirées billards avec *«attention à vos balles»* , les soirées fléchettes avec les *«action-réaction»* les meilleures soirées dansantes du *C4* et les fou rires du labo .....
Solange, je te remercie énormément d'avoir supporté mes aigus quand il faisait beau et d'avoir subi mes » insistances « ces dernières années. Merci pour l'écoute et l'aide que tu m'as offertes ces derniers temps....
Mon passage à la faculté de Médecine de Nice m'était un enrichissement sur le plan humain, je tiens à remercier tous ceux qui ont contribué à mon épanouissement pendant les deux années et demie de séjour !

Ce travail de thèse fût enrobée d'une présence féminine assez marquée dans l'équipe Bardoni. Je tiens à remercier chacune pour ce qu'elle m'a apporté

Josiane, je vais essayer de trouver les mots pour te remercier mais je pense que les mots ne suffiront pas pour exprimer toute ma reconnaissance envers une personne dont la présence m'était un équilibre quotidien. Merci pour toutes les discussions, tous tes conseils, merci pour ton écoute et surtout merci pour ton soutien et tes encouragements....

Mounia, ton calme, ton rire, ta discrétion et ton engagement dans ton projet m'ont toujours été un exemple pour ne pas baisser les bras quand pour la nième fois la manipe ne marche pas.... Je te remercie Mounia pour ton soutien, merci pour tous les *fou rires «* évidemment *»* et surtout merci d'avoir supporté sagement et sans aucun désaccord *Rafaella et les autres...* Merci d'avoir accepté d'écouter mes musiques et de subir mes *opéras* et ma caméra ! Merci pour ton amitié et ton soutien ininterrompu !

Laetitia, d'un « Back to Back bench and desk » à une fournisseuse officielle d'accessoires qui ont servi à l'aboutissement des différents personnages adoptés par Elias durant sa thèse....Merci pour ce que tu es... Merci pour ton amitié et ton

soutien sans limites... Merci de m'avoir coatché dans les moments de dérapage incontrolables.Merci de m'avoir fait rire avec tes "*j'en ai plein.....*" et tes acharnements conflictuels avec les appareils *parlants*. Merci pour les soirées dégustation et autres. Merci les *chup*s de m'avoir apporté cette attitude de relativiser et de regarder la vie d'une façon plus sereine... Merci d'avoir su réimplanter cette confiance en moi dans les moments les plus durs. Et Merci pour ce que vous êtes !

Mireille, les paillasses de la salle de TP n'étaient qu'un petit entraînement pourque tu envahisses la mienne en thèse ! Merci d'avoir supporté tous mes états d'âme. Merci pour ta patience et ton savoir faire. Merci pour ton calme qui, même si certaines fois me rendait fou, en réalité il m'était un équilibre assez important pour remettre les pendules à l'heure et me permettre de continuer à avancer comme il faut. Merci pour tes encouragements et ton soutien ininterrompus, merci pour ton sourire et ta façon de voir la vie. Merci d'avoir survécu à mes aigues et mes graves sans vraiment te plaindre. Merci d'avoir été là quand j'avais besoin et merci d'avoir su replacer les railles au bon endroit. Merci de m'avoir montré que certaines choses ne sont pas forcément évidentes pour tout le monde. Merci pour ta douceur et ta joie de vivre qui m'ont comblé durant ces dernières années.

Ma collaboration avec le Dr. Lalli m'a aussi confronté à une présence féminine assez marquée. Je tiens à remercier les filles pour leur accueil et l'organisation de notre arrivée. Merci d'avoir *supporté* mes délires et un grand merci pour votre soutien lors de l'impression de ce manuscrit.

Janette & Georges m'ont offert 5 sœurs et un frère ; Illkirch et les Héliotropes m'ont re-offert 5 sœurs et un frère que je tiens à remercier particulièrement et en détail pour ce qu'ils m'ont apporté :

Gretta, « l'eau s'infiltre chez les voisins et les murs passent à l'autoclave », c'était juste une petite parenthèse pour remercier Marj d'avoir participé à mon installation...Je me demandais souvent à quoi pensait l'architecte de l'IGBMC pour avoir eu cette ingénieuse idée de mettre un mur entre nos paillasses. Merci d'avoir remis les pendules à l'heure plusieurs fois où le temps ne semblait point savoir s'arrêter. Merci d'avoir bouleversé l'aboutissement de certaines de mes idées arrêtées par l'ouverture de ton esprit, tes principes et tes convictions inébranlables ! Merci Gretta d'être restée fidèle à notre amitié. Merci pour tout !

Hala, l'élégance incarnée et la douceur dans toute son ampleur ! Merci pour ton amitié, ton écoute sans limite et tes conseils. Merci pour ton soutien et ta confiance. Merci d'avoir su placer les bons mots au bons moments rétablissant ainsi un ordre fluide et continu dans un « désordre » chronique et sans fin ! Merci pour ta générosité et ton esprit de partage. Merci de m'avoir soutenu jusqu'au bout et d'être toujours restée disponible même aux moments les plus durs. Merci pour toutes les soirées Pizza aux 17 fromages « light » et tous les « Writing Enhancer Suppressors » !
Mazen, alias Mazzunti, ton soutien sans limites m'était une source où je puisais certaines forces afin de pouvoir continuer. Merci pour tout, d'être un élève assidu et sérieux à mes cours de danse ! Merci d'avoir supporté mes interventions « hors sujets » au beau milieu des discussions assez sérieuses.

Laure , Merci d'avoir adoucit le froid de Strasbourg par ton sourire, ton rire communicatif et ta vitalité. Merci de m'avoir montré que même si le chemin du combat humain est toujours bordé d'épines, les bourgeons du Laurier pourront éclore au moment inattendu ! Merci pour ta sincérité, ton intégrité et ta spontanéité. Merci d'avoir écouté toutes les histoires censées et insensées. Merci de m'avoir partagé tes chagrins, tes soucis et ton bonheur. Merci d'avoir toujours surfé sur la même longueur d'onde à tout moment. Merci pour toutes les soirées avec et sans « arguilé », avec et sans appéro, avec et sans salade !!!!

Nadine, De la porte 104 pleinement envahie par des mèches multicolores, à une imitation en parapluie rouge pour un anniversaire ! Neddo, tu as assisté à tous mes délires et tu as approuvé mon *«exigence»* ! Merci Neddo pour ta serviabilité, ton sens de partage et ta générosité. Merci pour ta sincérité et ton amitié. Merci de m'avoir délicatement prouvé que, même si les voiles de la vie semblaient tourbillonner sans arrêt, les souffles du courage de la patience et de la confiance sont capables de retracer l'acheminement de notre voyage....

Joelle, la *«mini»* du groupe ! ta discrétion ton calme et ton objectivité m'ont été un ancrage solide quand la réalité semblait vouloir m'échapper ! Merci pour ton amitié et ton soutien ininterrompus ! Merci d'avoir fait confiance aux séances *«Jus-coiffure-aspirateur»*. Merci d'avoir supporté ma *façon de qualifier les êtres humains!* Plein de courage pour le reste !

Mon arrivée sur Nice a été facilitée par la simplicité et la douceur de Danielle. Merci *«ya bénét»* pour tout ce que as fait discrètement pour me rendre l'existence assez simple. Merci pour tes coups de fils, tes encouragements sans cesse, merci pour toutes les sorties, les soirées et surtout merci pour ton amitié assez sincère et fidèle. Je te dis juste que la *15ème glorieuse d'Elias* pour cette année sera : *Je resterai assis sagement pendant la soirée de thèse de Danielle, until a Drin comes and proposes a fantastrophic danse!*

Et un Merci assez profond à Rana. La personne qui, entre Poitiers, Toulouse, Paris et Barcelone a su rester fidèle à cette amitié. Merci Rana pour tout ce que tu as pu et su m'apporter toutes ces années. Ton écoute, tes conseils et ta façon de voir la vie ont fondamentalement participé à la mise en place d'un système de quiétude et de tranquillité dans ma vie.... Merci beaucoup !

Depuis mon arrivée en France, j'ai été guidé par deux personnes qui ont suivi ma progression durant toutes ces années. Je vous remercie du fond du cœur Véronique et Antoine pour tout ce que vous m'avez offert comme soutien et encouragement ces dernières années, je vous remercie pour votre amitié assez solide et fidèle, je vous remercie car grâce à vos conseils et support j'ai pu arriver jusqu'ici.

Un grand merci à tous les « *Nancéens-Strasbourgeois»,* Marie-Cécile & Sebastien, Sabrina & Marco, Audrey & Fred, Nathalie & Gaetan, pour toutes les soirées, toutes les sorties et discussions... Merci pour votre amitié et « *au suivant(e) »!*

Encore un grand merci aux Nancéens, la famille Halisso pour toute leur amitié et la famille Zingraff, merci Noëlle pour ton affection et ton attention, merci de m'avoir appris à préparer les *«bonnes Pizzas»,* merci pour ce que tu es. Ça y est ton *«fils adoptif»* a réussi !

Pas finis les remerciements des Nancéens !!!! Sonia et Sabrina, *de l'arrêt Placieux, au Placieux même, au Vélodrome, à Strasbourg puis à Nice!!!!* on dirait qu'il a fait un grand détour ce bus !!!! *Vire* tiens à présenter ses remerciements les plus chaleureux et affectueux à *dégage & fous le camp!* Merci pour tout les filles, votre fidèle amitié et soutien m'on été un encouragement et un suivi assez crucial afin que je puisse continuer. encore une fois Merci pour tout !

Une autre *«envoie express»* de remerciements pour le *«groupe A»* !!! Merci pour cette amitié fidèle qui a su perdurer malgré la distance et *«le vieillissement»* !!! Merci d'être restés fidèles aux repas de *Jennou* ainsi que ses *engueulades* !
Ma deuxième famille, les *«Badr »*, je vous remercie pour tout ce que vous m'avez apporté comme soutien, encouragements et affection. Courage pour Nadine et Christian !

Un énorme merci pour l'amitié de Pascal (s), Will, Dumé, Rob, Fla, Julien, Steven, Greg, Yves, qui ont supporté d'abord mes week-ends labo. Merci à Olfa et Slim (u're the next very soon !!! courage !) d'avoir su me rendre la vie plus simple sur Nice. Et surtout merci à vous tous pour vos encouragement durant la rédaction !
Un tendre *«merci»* à Pascal qui a pu trouver les moyens de m'encourager et me soutenir depuis Octobre 2000 ! Merci Franck pour ce que tu es et pour l'aide que tu m'a offerte, merci pour ta serviabilité et ta façon de faire qui rend les choses beaucoup plus simple. Merci Linda pour tous les moments qu'on a partagés autour d'un café au petit matin. Merci pour ton rire et ta joie de vivre qui me motivait depuis que j'arrivais au labo !!! Merci pour ton amitié et tes encouragements.

Another warm thanks to Holger & Verena. Thanks for your friendship, support, care and attention through the last years. Thanks for being true friends !!!

Avant de finir, j'ai l'impression que les mots commencent à fuir, je me perds dans les souvenirs, et j'entends le souffle de vos voix me caresser tendrement. Septembre 2000 – Mars 2008, je ne sais pas si le temps est passé vite mais si je me projette un peu dans le passé je trouve que c'était une éternité pour moi, et je n'ai aucune idée comment faire pour rattraper le temps perdu loin de vous ! Je ne sais pas quoi dire pour vous remercier, car les mots ne suffisent point. Vous avez été toujours là, à m'encourager à me soutenir et à me faire rire. Votre amour et dévouement m'ont comblé et constituait une énergie *auto - renouvelable* et inépuisable. *B7ébbkoun ktir w iza fi chaghlé wé7dé bi hal 7ayét béndam 3layya hiyyé énnou kén kél hal wa2ét b3id 3ankoun.... Kam marra kénét éw3a w étmanna énnou choufkoun éddémé awésma3 sawtkoun aw éntour énnou néz3moul ahwé? kam marra kéntou t7éwlou tfarjouné énnou l7ayét ba3da 7élwé w btéts7é2 énnou ténzéch raghém kéll elli 3am bi sir? kam marra kénét ébké ba3éd ma sakkir él khatt ba3éd ma é7kikoun la énnou ktir chta2ételkoun? kam marra kén yétla3 3a bélé énnou bass 7éss béwjoudkoun la énnou kénét méchta2élkoun ktir? iza b2élkoun énnou intou a7san ahél w a7san ékhwét bi hal déné ma bi kaffé w bkoun 3am assér bi 7a2kkoun ktir... éntou akbar w aktar mén heik bé ktir... akbar bé ktir mén énnou ténwajad kélmé 3al aréd towsouf élli b7éss fi tijéhkoun...bétloub mén rabbé énnou yé3tini él 3omor ta é2dir échkérkoun léil w nhar 3ala kéll chi rabbaytouni 3léy... haydé él thèse hiyyé la 2élkoun la énnou éntou éllé shértou 3layya, éntou élli 3ataytouné él défi3 énnou kaffé... haydé él thèse hiyyé la 2élkoun!*

A toi mon bébé, j'ai trouvé ces paroles :

If I caught the world in a bottle and everything was still beneath the moon
Without your love would it shine for me? If I was smart as Aristotle
And understood the rings around the moon. What would it all matter if you loved me?
Here in your arms where the world is impossibly still. With a million dreams to fulfil. And a matter of moments until the dancing ends. Here in your arms when everything seems to be clear. Not a solitary thing would I fear. Except when this moment comes near the dancing's end.....
If I caught the world in an hourglass. Saddled up the moon so we could ride.
Until the stars grew dim, Until...
One day you & I'll meet a stranger. And all the noise is silenced in the room
You & I'll feel that you're close to some mystery. In the moonlight and everything shatters.
You feel as if you & I've known her all your life. The world's oldest lesson in history...
Until.....

Your reason and your passion are the rudder and the sails of your seafaring soul.
If either your sails or our rudder be broken, you can but toss and drift, or else be held at a standstill in mid-seas.
For reason, ruling alone, is a force confining; and passion, unattended, is a flame that burns to its own destruction.
Therefore let your soul exalt your reason to the height of passion; that it may sing;
And let it direct your passion with reason, that your passion may live through its own daily resurrection, and like the phoenix rise above its own ashes.

Jibran Khalil Jibran

À Jennou & Abou Fadi

Abbreviations

ACEs	A/C-rich ESEs
AGS	audiogenic seizures
ALS	Amyotrophic lateral sclerosis
AMPARs	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
APP	amyloid precursor protein
APRA	Antibody Positioned RNA Amplification
ARE	AU-Rich Elements
ClC-1	Chloride Ion Channel 1
CPEB	cytoplasmic-polyadenylation-element-binding protein
CUG-BP1	CUG Binding Protein 1
CYFIP1/2	Cytoplasmic FMRP Interacting Protein1/2
DM	Myotonic Dystrophy
DMPK	dystrophia myotonica-protein kinase
dsRBD	double-stranded RNA Binding Domain
EAAT2	excitatory amino-acid transporter 2
eEF1A	eukaryotic translation elongation factor 1A
eIFs	Eukaryotic Initiation Factors
eRF	eukaryotic release factors
ESEs	exonic splicing enhancers
ESSs	exonic splicing suppressors
FMR1	Fragile X Mental Retardation 1
FMRP	Fragile X Mental Retardation Protein
FXR1	Fragile X-Related 1gene
FXR1P	Fragile X-Related 1Protein
FXR2	Fragile X-Related 2 gene
FXR2P	Fragile X-Related 2 Protein
FXS	Fragile X syndrome
FXTAS	Fragile X-associated tremor/ataxia syndrome
GTP	Guanine Triphosphate
GTPases	Guanine Triphosphatases
GPx	glutathione peroxidase
HGMD	Human Gene Mutation Database
hnRNP	heterogeneous ribonucleoprotein particle
IQ	Intellectual Quotient
IR	Insulin receptor
IRES	Internal Ribosome Entry Site
ISEs	intronic splicing enhancers
ISSs	intronic splicing suppressors
ITAF	IRES *Trans* Acting Factors
Kc1	kissing complex
KH	K-homology
KO	Knock out
LTD	long-term depression
LTP	long-term potentiation
MAP	microtubule associated protein
MAP1B	Microtubule Associated Protein 1B
MBNL	muscleblind-like
mGluR1	metabotropic glutamate receptor 1

miRNAs	microRNAs
mRNA	messenger RiboNucleic Acid
NDF	N-terminal Domain of FMRP
NFTs	neurofibrillary tangles
NGD	No-Go decay
NES	nuclear export signal
NLS	nuclear localisation signal
NMD	nonsense-mediated decay
NMDARs	N-methyl-D-aspartic acid receptors
NO	nitric oxide
NOVA	neuro-oncological ventral antigen
NSD	nonstop decay
NUFIP	Nuclear FMRP Interacting Protein
PABP	poly(A)-binding protein
POF	Premature ovarian failure
PSD95	Post Synaptic Density 95
PTB	polypyrimidine tract binding protein
PTCs	premature termination codons
RBDs	RNA binding domains
RBPs	RNA binding proteins
REMD	ribosome extension-mediated decay
RISC	RNA-Induced Silencing Complex
RNA	RiboNucleic Acid
RNP	RiboNucleoprotein Prticles
ROS	reactive oxygen species
RP	retinis pigmentosa
RRM	RNA recognition motif
SLA	amyotrophic lateral sclerosis
SLBP	stem-loop-binding protein
SMA	Spinal muscular atrophy
snoRNAs	Small nucleolar RNAs
snRNA	small nuclear RNA
snRNPs	small nuclear Ribonucleoprotein Particles
Sod1	Super Oxide dismutase 1
SR proteins	serine/arginine-rich proteins
SSLIP	*Sod1* Stem Loops Interacting with FMRP
TIA-1	T-cell intracellular antigen 1
TIAR	TIA-1-related protein
TNFα	tumor necrosis factor alpha
tRNA	transfer RNA
U2AF	U2 auxiliary factor
UTRs	untranslated regions
ZBP1	Zipcode Binding Protein 1
ZNF	zinc finger
40S	small ribosomal subunit
60S	large ribosomal subunit
82-FIP	82 KDa FMRP Interacting Protein

Contents

List of figures and tables

Introduction

Introduction

1. From the nucleus to different locations : following the mRNA in its mission.

Following the lives of eukaryotic mRNAs from their birth in the nucleus until they are processed by gangs of exonucleases lying in wait in dark recesses of the cytoplasm will be the main interest of this chapter.

Throughout their lifetimes, mRNAs are escorted by a host of associated factors, called RNA binding proteins (RBPs), some of which remain stably bound while others are subject to dynamic exchange so that mRNAs may be shuttled to and from or anchored at specific subcellular locations, be temporarily withheld from the translation apparatus, have their 3' ends trimmed and extended, fraternize with like-minded mRNAs encoding proteins of related function, and be scrutinized by the quality-control police.

Although some of these processes were originally thought to affect only select mRNA populations or be largely limited to highly specialized cell types like germ cells and neurons, recent work suggests that the majority of mRNAs in multiple cell types are subject to a diverse array of regulatory activities affecting essentially every aspect of their lives. It is the unique combination of factors accompanying any particular mRNA, as well as their relative positions along the transcript, that dictates almost everything that happens to that mRNA in the cytoplasm.

RBPs are able to recognize and bind to specific RNA sequences and structures through their RNA binding domains (RBDs). In humans, more than 40 different RBDs are found, providing an exhaustive regulation of diverse pathways. Two RBDs, the RNA recognition motif (RRM) which is represented in nearly 500 different human genes and the heterogeneous nuclear ribonucleoprotein K-homology (KH) domain, are present in almost all RBPs for single stranded RNA recognition (Messias and Sattler 2004). Other motifs as the double-stranded RNA binding domain (dsRBD), zinc fingers, RGG boxes, and the Pumilio homology domain in PUF proteins are also found (Sontheimer 2005) (Stefl et al. 2005).

RBPs orchestrate and regulate several coordinated post-transcriptional pathways, leading to a normal development and cellular homeostasis. Given the critical importance of RBPs in the dynamic gene expression system, it is not surprising that a deficiency in the

normal function of RBPs will disrupt RNP organization and cause a number of clinical disorders. Once born, the pre-mRNA is captured by specific hosts leading it to "splicing"

1.1. Splicing

Gene regulation can be monitored by the splicing process which appears to be especially common for genes expressed in the nervous system. The identity, properties and types of neurons are a reflect of different protein isoforms generated by this mechanism.
Splicing occurs when a precursor mRNAs is processed into the mature forms found in the cytoplasm via the precise joining of exonic sequences and the removal of introns that can be hundreds of kilobases long. The borders of pre-mRNA exons and introns are delineated by the 5' splice site at the upstream end of the intron and the 3' splice site at the downstream end. The spliceosome, a large ribonucleoprotein complex, is able to identify bone fide exons, ignore pre-mRNA segments that resemble exons (pseudoexons), join contiguous exons without inadvertent skipping and appropriately regulate alternative splicing to meet the physiological requirements of cells and tissues.

1.1.1. The spliceosome and constitutive splicing

The core of the splicing machinery is essentially composed of five small nuclear ribonucleoproteins (snRNPs) U1, U2, U4, U5, U6 and more than hundred other auxiliary proteins. Each snRNP results from the association of single uridine-rich small nuclear RNA (snRNA) with multiple proteins. The assembly of this complex is highly dynamic involving rearrangements of RNA:RNA, RNA:protein and protein:protein interactions (Du and Rosbash 2002) (Lallena et al. 2002). Both the recognition of the intron/exon boundaries and the catalysis of the cut-and-paste reaction are mutually orchestrated by both *cis* elements as the short and degenerate splice sites consensus and the exonic and intronic splicing enhancers and suppressors (ESEs, ISEs and ESSs, ISSs) and the *trans* acting factors. Enhancer elements promote the inclusion of an exon, and silencers promote its skipping or exclusion from the final mRNA. Establishment of a correct network of interaction between *cis* elements and *trans* factors permits exon recognition (Figure 1). In addition to the classical splice site sequences, recognition of constitutive exons also depends on different sets of auxiliary

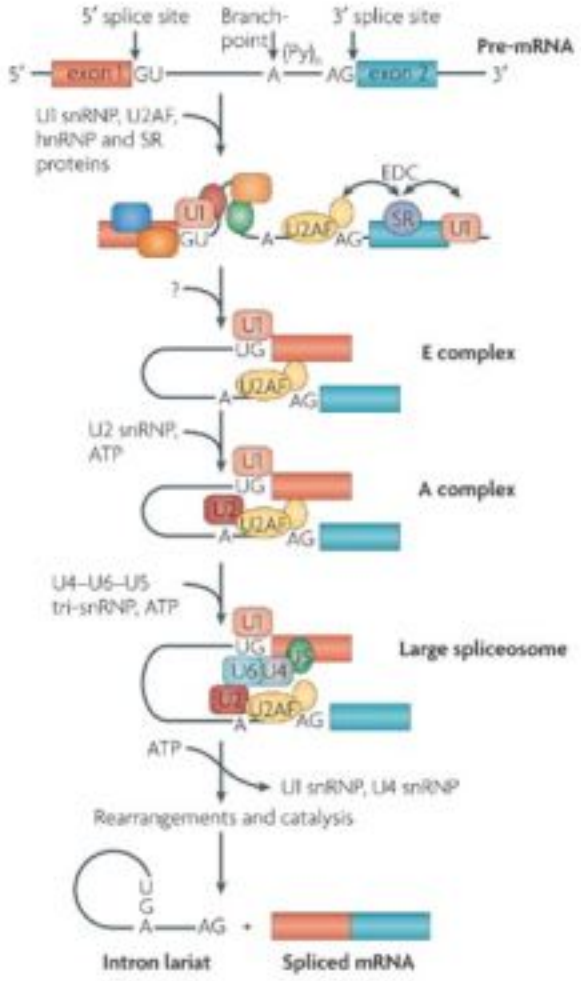

Figure 1 : Schematic diagram of the assembly of spliceosome.

U1 binds to the 5' splice site while U2AF binds to its 3' splice site, together with other proteins, the complex E get formed which bridges the intron and brings the splice sites into juxtaposition. U2 gets recruited and therefore the A complex is constituted. Several steps of dynamic protein-protein and protein-RNA interaction leads to formation of the large spliceosome that excises the intron. (Li et al., 2007)

elements. Spliceosome assembly and splice site choice can be altered by transcription elongation rates and the secondary structure of a pre-mRNA that blocks the access to the splice sites. However, the best understood alterations in splicing are determined by proteins that bind to the pre-mRNA and enhance or repress spliceosome assembly at various steps. Small changes in the relative rates of spliceosome assembly at different splice sites can lead to large changes in the choice of splicing pattern for a transcript (Matlin et al. 2005). A large number of diseases are correlated to mutations either masking or creating novel *cis* elements and generating an abnormal splicing pattern.

1.1.2. Exonic Splicing Enhancers and Suppressors (ESE and ESSs)

Based on nucleotide composition, two classes of ESEs have been defined: purine-rich and A/C-rich. The purine-rich ESEs are recognized by a conserved family of serine/arginine-rich (SR) proteins that recruit spliceosome components (such as U2 auxiliary factor, U2AF) to the splice sites (Blencowe 2000). The A/C-rich ESEs (ACEs) bind the cold-box protein, YB-1, and promote splicing. Both can enhance splicing by inhibiting adjacent ESSs (Zhu et al. 2001). Combining results obtained using functional systematic evolution of ligands by exponential enrichment (SELEX , (Liu et al. 2000)) and computational analysis of human genomic sequence lead to the identification of additional ESEs (Fairbrother et al. 2002). An ESE prediction program was then developed (http://exon.cshl.org/ESE) and used to identify ESE mutations causing exon skipping by disrupting binding sites of SR proteins leading pathogenic splicing abnormalities (Cartegni et al. 2002).

1.1.3. Alternative splicing

Almost all cells in the organism use this process to permit a gene to encode multiple protein isoforms with diverse and even antagonistic functions. The primary source of human proteomic repertoire is strongly due to this mechanism that controls the inclusion of a particular exon or the shifting of an individual splice site. Splicing pathways can be modulated according to cell type, development stage, gender or in response to external stimuli. Intonic repressor and activator elements, distinct from the classical splicing sequence, play a major role in the regulation of cell-specific alternative splicing. Cell specificity emerges from two features: first, the repression of splicing in the inappropriate cell type is

combined with activation of splicing in the appropriate cell type; and, second, combinatorial control is exerted by multiple components involving cooperative assembly of activation and/or repression complexes on the *cis*-acting elements surrounding the regulated splice sites (Smith and Valcarcel 2000). These complexes serve to enhance or inhibit recognition of the classical splice sites by the basal splicing machinery. Activating and repressing activities coexist within cells, and it remains unclear why activation dominates in one cell type whereas repression dominates in another. Importantly, mutations that perturb this balance can result in aberrant regulation of alternative splicing, causing the expression of protein isoforms that are inappropriate for a cell type or developmental stage. Recently, combined microarray and computational analyses have identified subsets of commonly regulated splicing events that share cis-acting elements that seem to be associated only with alternative splicing. These elements are bound by factors that are not generally associated with the spliceosome, such as FOX proteins, CUG-BP- and ETR-3-like (CELF) proteins, muscleblind-like (MBNL), the neuro-oncological ventral antigen (NOVA) proteins and TIA1. Regulation of alternative splicing in vertebrates involves a dynamic interplay of antagonistic regulatory factors; for example, between the SR and hnRNP protein families (Black 2003) and between pairs of proteins including NOVA–polypyrimidine tract binding protein (PTB), CELF–PTB, CELF–MBNL, TIA1–PTB, and PTB–FOX (for review (Blencowe 2006))

1.1.4. Alternative splicing during neuronal development

Alternative splicing is highly abundant in brain relative to other tissues (Blencowe 2006) where the production of particular protein isoforms helps to determine the properties of the many different types of neurons. It also regulates many aspects of neurophysiology through spatial and temporal alterations in proteins that comprise ion channels and membrane-bound receptors involved in neurotransmitter storage and release (Lipscombe 2005). In almost every step of neuronal development, splicing events are key actors for establishing a homogenous network of communications. It affects the pattering of the neural tube, the cell fate determination, the axon guidance and many features of synaptogenesis (for review (Li et al. 2007)) .

1.2. Translation control

The final step in the gene expression pathway is the translation of mRNA into protein. Fine tuning gene expression by the regulation of translation is a mechanism used in a wide range of biological situations. Emerging from early embryonic development to cell differentiation and metabolism, translation is regulated in both time and space (Wikens et al., 2000). There are two general modes of translation control: the global control, in which the translation of most mRNAs in the cell is regulated and occurs by modification of translation-initiation factors; and the mRNA-specific control where the translation of a specific subset of mRNAs is modulated without affecting general protein biosynthesis; the principal actors of the latter mechanism are regulatory protein complexes that recognize particular elements present in the 5' and/or the 3' untranslated regions (UTRs) of the target mRNA (Figure 2A).

1.2.1. Translation initiation

Two crucial events that take place during the initiation of translation are the recruitment of mRNA to the ribosomal complex, and the selection of the AUG initiation codon. In eukaryotes this complex process is assisted by more than 25 polypeptides (for review (Pestova et al. 2001)). The small (40S) ribosomal subunit initially binds to the 5' end of the mRNA and scans it in a 5'- 3' direction until the initiation codon is identified. The large (60S) ribosomal subunit then joins the 40S subunit at this position to form the catalytically competent 80S ribosome (Figure 2B).

The small ribosomal subunit, together with other factors, forms a 43S pre-initiation complex that binds to the mRNA. This 43S assembly contains the Eukaryotic Initiation Factors (eIFs) 3, 1, 1A and 5, and a ternary complex, which comprises the methionine-loaded initiator tRNA that will recognize the AUG codon during initiation and eIF2 that is coupled to GTP. At least in mammals, binding of the 43S pre-initiation complex to the mRNA is thought to involve bridging interactions between eIF3 and the eIF4F protein complex, which associates with the 5' cap structure of the mRNA (Lamphear et al. 1995).

The eIF4F complex contains several proteins, which include: eIF4E, which physically binds to the m7GpppN cap structure; eIF4A, a dead box RNA helicase and eIF4G that interacts with the poly(A)-binding protein (PABP), and the simultaneous interaction of eIF4E and PABP with eIF4G is believed to circularize the mRNA, which brings the 3' UTR in close

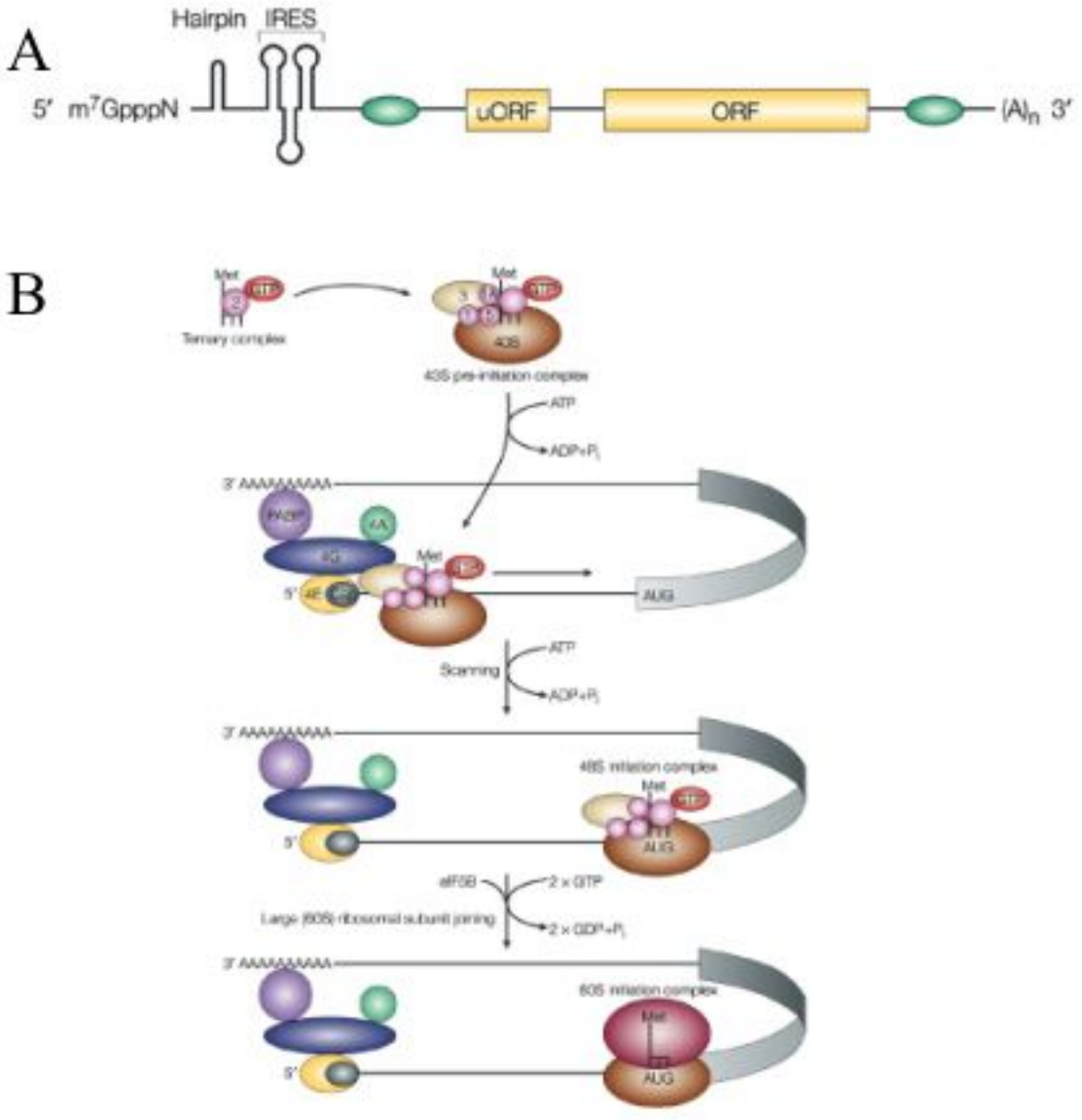

Figure 2 : Mechanisms of translational initiation control.

A- *cis* acting factors that regulate translation initiation; the m7GpppN cap, hairpins, IRES, and poly A tail.

B- schematic representation of the assembly of translation initiation factors

proximity to the 5' end of the mRNA (Wells et al. 1998). This provides (at least conceptually) a spatial framework in which the 3'-UTR-binding factors can regulate translation initiation. In fact, most known regulatory sequences are found within the 3' UTR, even though translation begins at the 5' end of the mRNA, which highlights the functional connection between the mRNA ends during translation.

The global control of protein synthesis initiation is achieved by changes in the phosphorylation state of initiation factors or their interacting regulators. Phosphorylation of the α-subunit of eIF2 by several known kinases inhibits global mRNA translation but can also result in translation activation of specific mRNAs.

1.2.2. Other mechanisms

Specific RNA binding proteins regulate the translation of specific mRNA by several mechanisms. Steric blockage, observed with the Iron Regulatory Protein that binds a stem loop motif in the 5'UTR of the ferritin mRNAs blocking therefore the recruitment of the 43S complex (Muckenthaler et al. 1998). Another well known mechanism is provided by the cytoplasmic-polyadenylation-element-binding protein (CPEB) that binds to uridin rich sequence located in the 3'UTR of target mRNAs. CPEB binds to Maskin that competes with eIF4G for binding to eIF4E preventing the EIF4F complex formation (Stebbins-Boaz et al. 1999).

It has been admitted that translation initiation can also be regulated in a cap-independent manner. The discovery of Internal Ribosome Entry Site (IRES) in a vast repertoire of mRNAs lead to the establishment of their role in the translation initiation and their regulation by *trans* factors ITAF (IRES *Trans* Acting Facotrs) (for review (Jackson 2005)). Another mechanism of translation regulation is provided by small RNA molecules of around 22 nucleotides in length known as microRNAs (miRNAs). They hybridize by incomplete base pairing to target mRNAs and regulate their translation at several levels affecting the initiation (Mathonnet et al. 2007), the elongation and/or termination (Filipowicz et al. 2008).

1.3. Quality control

RNA quality-control mechanisms are known to target aberrant RNAs for degradation by a few conserved nucleases (for review (Houseley et al. 2006)). Over the past five years, most of the enzymes involved in mRNA decay have been identified and the complex regulation that determines the path and rate of mRNA degradation is beginning to be unraveled. Furthermore, the recent discovery that there are specific cytoplasmic sites, known as mRNA-processing (P) bodies, where mRNA turnover occurs, was quite unexpected.

Eukaryotic mRNAs are created with two integral stability determinants - the 5' 7-methylguanosine cap and the 3' poly(A) tail - that are incorporated co-transcriptionally. These two structures interact with the cytoplasmic proteins eIF4E and the poly(A)-binding protein (PABP), respectively, to protect the transcript from exonucleases and to enhance translation initiation. To initiate decay, either one of these two structures must be compromised or the mRNA must be cleaved internally by endonucleolytic attack.

In eukaryotes, the bulk of mRNAs undergo decay by a pathway that is initiated by poly(A)-tail shortening. This first step in the turnover pathway is unique in that it is reversible; transcripts that bear the correct signals can be readenylated and return to polysomes. Nevertheless, once the cell determines that an mRNA must be destroyed, one of two irreversible routes is taken. Either the 5' cap is removed by a process known as decapping, which allows the mRNA body to be degraded in the 5'-3' direction by the XRN1 exoribonuclease, or the unprotected 3' end is attacked by a large complex of 3'-5' exonucleases known as the exosome. These two pathways are not mutually exclusive and the relative contribution of each mechanism remains a subject of debate. In Saccharomyces cerevisiae, knocking out components of either the 3'-5' or the 5'-3' pathway had minimal effects on the transcriptome, which implies redundancy (He et al. 2003). Results obtained by using a sensitive assay to detect decay intermediates indicate that both 5'-3' and 3'-5' pathways are involved in the decay of unstable ARE-containing mRNAs in mammalian cells (Murray and Schoenberg 2007). It therefore seems that the precise pathway of mRNA decay might be flexible.

In the nucleus, the exosome plays the major role in RNA quality control, although a paralog of Xrn1p, termed Xrn2/Rat1p in yeast, may also affect nuclear RNA degradation (Bousquet-Antonelli et al. 2000; Danin-Kreiselman et al. 2003). In the yeast nucleus, the core exosome complex also associates with an additional 3’-5’ exonuclease called Rrp6p (Allmang

et al. 1999; Burkard and Butler 2000), whereas in mammals the Rrp6p ortholog, PM/Scl100, is observed in both the cytoplasm and nucleus (Lejeune et al. 2003).

In the cytoplasm, several quality-control mechanisms degrade eukaryotic mRNAs that have abnormalities in translation. An emerging principle is that aberrant mRNAs can be distinguished from normal mRNAs by adaptor proteins that interact with the translation machinery and direct the aberrant mRNAs into a degradation pathway referred to as nonsense-mediated decay (NMD) (Figure 3).

1.3.1. Nonsense-mediated decay.

NMD generally eliminates the production of mRNAs that prematurely terminate translation and occurs, although by varying mechanisms, in every eukaryotic cell. NMD appears to have evolved to protect cells from the potentially deleterious effects of routine abnormalities in gene expression that result in the premature termination of translation. For example, transcription initiation upstream of the proper site could generate an mRNA harboring a nonsense codon upstream of or within the usual translational reading frame if translation initiates upstream of the normal site. As another example, inefficient or inaccurate pre-mRNA splicing could result in an mRNA harboring an intron-derived nonsense codon or a nonsense codon downstream from the site of missplicing. NMD detects and degrades transcripts that contain premature termination codons (PTCs). PTCs can arise from mutations, frame-shifts, inefficient processing, leaky translation initiation and extended 3' UTRs. These transcripts, if translated, could produce truncated proteins with aberrant functions. The NMD pathway has been found in all eukaryotes and the core proteins of the NMD complex, UPF1, UPF2 and UPF3, are highly conserved (Conti and Izaurralde 2005). Beyond this conservation, however, the detection of the PTC and the method of decay of the transcript seem to have diverged. Nevertheless, a common thread is the recognition of an aberrant mRNP conformation.

During a normal translation-termination event, the stop codon is recognized by the peptide-release factors eRF1 and eRF3 leading to peptidyl-transfer RNA (tRNA) hydrolysis. When termination occurs at a PTC, peptide release is delayed and the ribosome remains stalled at the stop codon. This allows the binding of UPF1 and the SMG1 kinase to the release factors, forming a complex, known as SURF, on the stalled ribosome (Kashima et al. 2006). At this point, through binding to UPF2, UPF1 interacts with the EJC, which lies at an

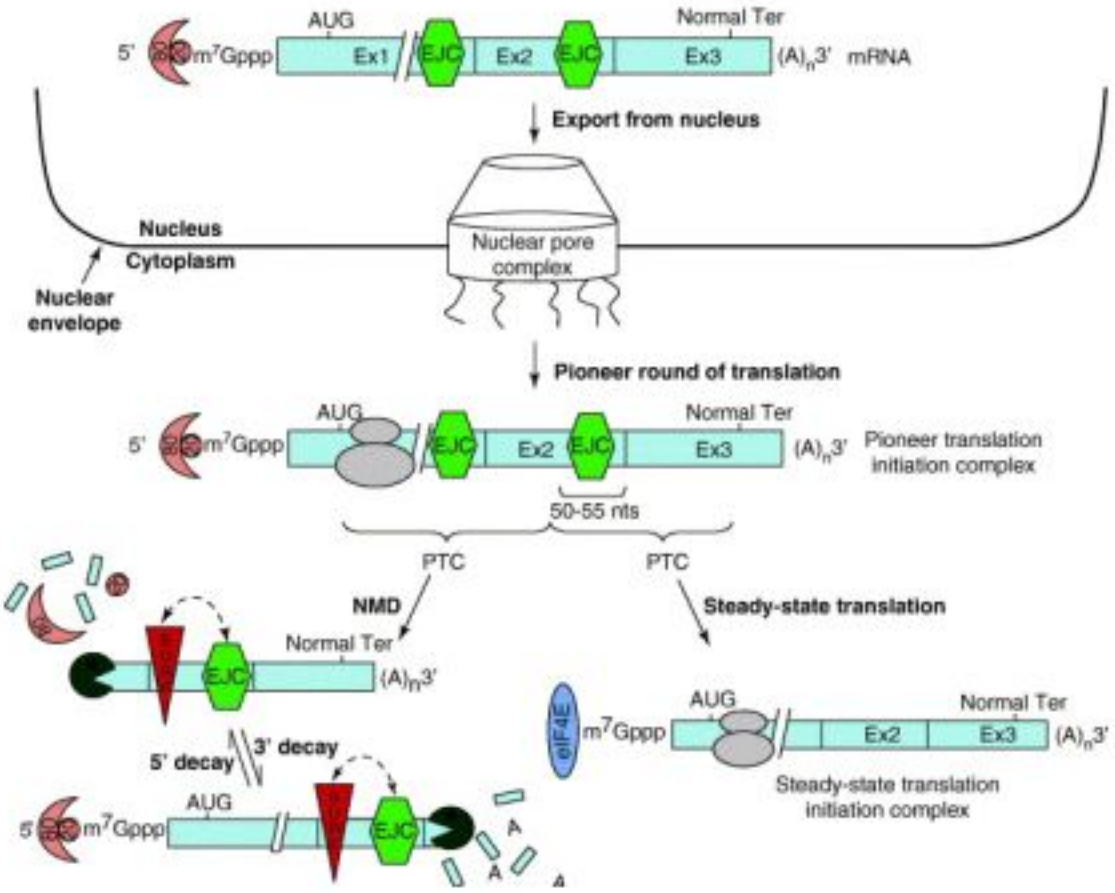

Figure 3 : the Nonsense Mediated Decay

Newly synthesized, spliced mRNAs are exported to the cytoplasm through nuclear pore. If a premature termination codon (PTC) resides more than 50–55 nucleotides (nts) upstream of an exon–exon junction (EJC) then the UPF1 component of the SURF complex will interact with the UPF2 constituent of the EJC and trigger NMD. (Kuzmiak et al., 2006)

exon–exon junction downstream of the termination site. Formation of this bridge between SURF at the ribosome and the EJC leads to phosphorylation of UPF1 by SMG1 and dissociation of the release factors (Kashima et al. 2006). Finally, SMG7, a 14-3-3-related protein, associates with the complex and triggers decay (Unterholzner and Izaurralde 2004).

NMD pathway is not solely reserved for the decay of aberrant transcripts, but has an important role in the regulation of normal gene expression. Microarray analysis of the transcriptome in UPF1-depleted mammalian cells identified numerous upregulated normal transcripts, some of which contained upstream ORFs, 3'-UTR introns, frameshifts or other features that mimic a PTC. Furthermore, mRNAs that apparently lack any feature resembling a PTC, namely histone mRNAs and the ARF1 mRNA, degrade in a UPF1-dependent manner. Intriguingly, in these cases, UPF1 is recruited to the transcript through interaction with a factor bound to the 3' UTR- the stem-loop-binding protein (SLBP) in the case of histone mRNAs and Staufen in the case of ARF1. Neither of these mechanisms requires UPF2 or UPF3, which indicates that these pathways are distinct from classic NMD.

1.3.2. Non Stop Decay, No-Go Decay, and Ribosome Extension-Mediated Decay

In a process referred to as nonstop decay (NSD), ribosomes that have reached the end of mRNAs lacking translation termination codons recruit the exosome through the action of Ski7p-a paralog of the eEF1A (eukaryotic translation elongation factor 1A) and eRF3 (eukaryotic release factor 3) proteins, which interact with the ribosomal A site during elongation or termination, respectively (Frischmeyer et al. 2002; van Hoof et al. 2002). This suggests that Ski7p recognizes the empty A site produced when a ribosome reaches the 3’ end of an mRNA.

Similarly, when mRNAs have strong pauses in elongation, the mRNA is targeted for endonucleolytic cleavage in a process referred to as No-Go decay (NGD) (Doma and Parker 2006). NGD is promoted by the Hbs1 and Dom34 proteins, which are paralogs of the translation termination factors eRF3 and eRF1 (eukaryotic release factor 1) and presumably interact with the stalled ribosome.

Finally, when ribosomes inappropriately translate and then terminate within the 3’UTR at least some mRNAs are destabilized in a process referred to as ribosome extension-mediated decay (REMD) (Inada and Aiba 2005; Kong and Liebhaber 2007).

1.4. RNA transport and localization.

A wide range of cell types and eukaryotic organisms restrict local protein synthesis via localization of mRNA. This phenomenon is crucial for establishment of an efficient asymmetric distribution of cytoplasmic factors leading therefore to what is known as polarity of a cell. Destination of mRNAs is often attributed by signals (localization elements or zipcodes) generally found in their 3' UTR. Zipcodes recruit *trans* factors, a panoply of RNA binding proteins that recognize sequence and/ or structure of these signals, resulting in mRNP formation. These travelling mRNPs rely on active motor-protein dependant transport that occurs both along actin and microtubules filaments.

Zipcode Binding Protein 1 (ZBP1) is one of the most studied *trans* factors involved in the localization of β-actin mRNA within the cell. ZBP1 contain 2 RRM and 4 KH motifs allowing both recognition and binding to β-actin mRNA zipcode. Mutations affecting the zipcode sequence were shown to reduce the interaction ZBP1/β-actin mRNA (Ross et al. 1997). In mature neurons, it was shown that ZBP1/β-actin mRNA resides in dendritic shafts and spines (Tiruchinapalli et al. 2003).

The microtubule depending transport of localized RNAs was analyzed in several organisms and cell types. In drosophila oocytes and embryonic development, it was demonstrated that *oskar* mRNA localization, determinant for posterior pole plasm, depends on kinesin I whereas, *bicoid* mRNA, determinant for anterior structures development, relies on dynein as a microtubule-associated motor (for review (Palacios and St Johnston 2001)).
In neurons, isolation of complexes containing localized mRNAs and their associated motor proteins shed new lights on the molecular composition of these mRNPs (Kohrmann et al. 1999; Mallardo et al. 2003). As a component of motile RNP particles that move within dendrites using kinesin motors, the authors isolated enriched Staufen-containing RNP complexes and were able to demonstrate that these RNPs contained Staufen, kinesin heavy chain together with BC1and Ca^{2+}/calmodulin-depenedant kinase as dendritic RNAs.

Another study of Staufen RNPs revealed that these particles also contain Purα and the Fragile X Mental Retardation Protein (FMRP) (Ohashi et al. 2002). The components of the Staufen containing RNPs were therefore demonstrated to be subjects of dynamic remodelling (Barbee et al. 2006). Recently, Davidovic and colleagues showed that FMRP interacts with a neurospecific kinesin KIF3C, to act as a molecular adaptor between RNA granules and the microtubule linked motor KIF3C (Davidovic et al. 2007) (Figure 4)

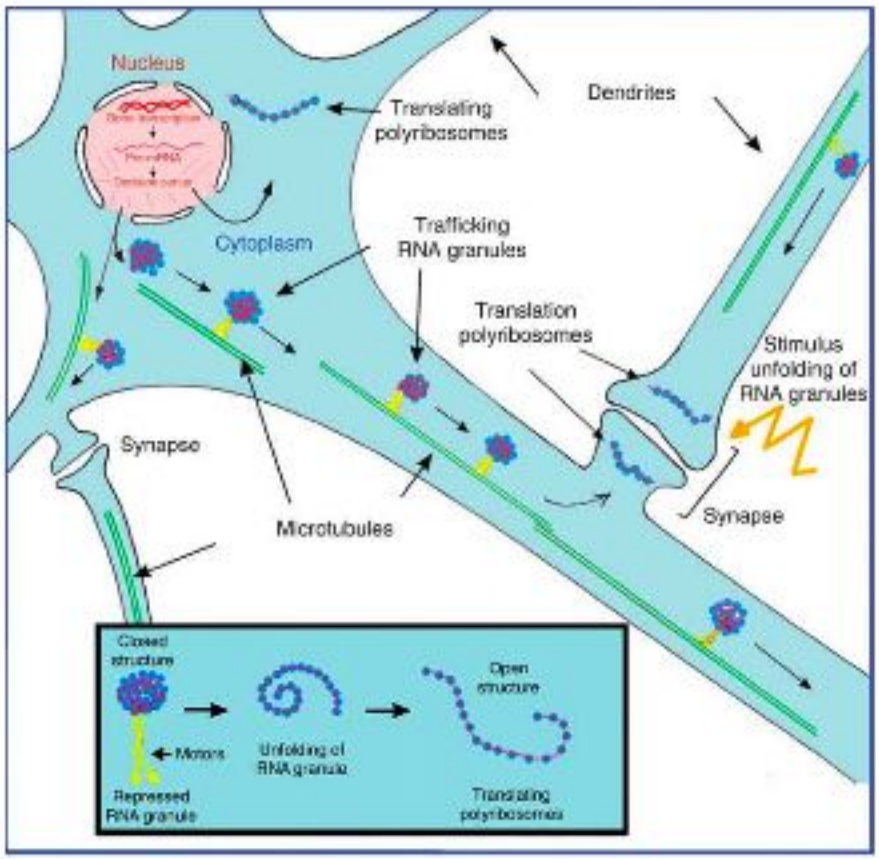

Figure 4 : Schematic representation of RNA granules sorting and transport.

In neurons, after mRNP assembly, granules are transported along the microtubules using motor proteins to reach their final destination. (*kindly provided by Davidovic.L and khandjian.E.W)*

2. RNA, RNA Binding Proteins and related neurologic disorders

It has been widely admitted that misregulation of mRNAs lead to specific disorder, however, correlation between human diseases and defects in RNA binding proteins was assessed for only few of them. In this chapter , I will give a general overview about human diseases and defects in RNA processing and I will focus the next chapter on the analysis of the Fragile X Mental retardation Protein (FMRP), an RNA binding protein whose absence results in the Fragile X Syndrome.

2.1. Splicing and diseases

Several criteria have been used to classify splicing mutations that can disrupt a splicing *cis*-element affecting therefore the expression of a single gene. Other mutations would disrupt a *trans* component of the splicing machinery or the splicing regulatory complex altering the expression of several genes. On the other hand, some mutations create either unnatural splicing patterns or aberrant regulation of splicing by disrupting choice of alternatively splice sites. A list of splicing mutations is available via the Human Gene Mutation Database (HGMD, http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html).

2.1.1. *Cis*-acting splicing disorders and Neurologic diseases

Exonic mutations are assumed to cause disease by affecting only the coding potential but 50% of point mutations resulting in human disease are caused by *cis*-acting defects in pre-mRNA splicing. These mutations may weaken or strengthen splicing enhancer and silencer elements, and may create or destroy splice sites altering therefore the splicing of constituve or alternative exons. Ideally, a detailed analysis of mRNA linear structure for correct splicing and mRNA steady-state levels is the definitive test to correlate a splicing defect with a disease. RNA from the affected tissue should be analyzed because *cis*-acting splicing mutations can have cell specific effects (Slaugenhaupt et al. 2001).

2.1.1.1. Frontotemporal dementia and Parkinsonism linked to Chromosome 17 (FTDP-17)

A well characterized neurodegenerative disease due to mutations in the Tau gene. FTDP-17 patients exhibit behavioral, cognitive and motor disturbances and frontotemporal atrophy associated with gliosis and the formation of intraneuronal Tau containing deposits. The Tau gene generates six major protein isoforms via alternative splicing highly regulated spatially and developmentally (D'Souza et al. 1999). Tau is a microtubule associated protein (MAP) thought to promote and stabilize microtubule association and contribute to neuronal vesicular transport in a microtubule dependant manner and highly enriched in axons of mature and growing neurons. Tau interacts with microtubules via its C terminal domain through repetitive microtubule binding motifs (R) encoded by exon 9 through 12. Inclusion or skipping of exon 10 produces protein isoforms containing four R or three R respectively. Two molecular mechanisms were proposed for FTDP-17 depending on the mutation. Missense mutations, but not all, impair Tau function by altering its interaction with microtubules. A substitution N279K within the exon 10 has no effect on Tau binding to microtubules, however, patients carrying this mutation exhibit a two fold increase in the 4R/3R ratio that leads to the formation of neurofibrillary tangles (NFTs) and neurodegeneration. Misexpression of Tau isoforms due to mutations within exon 10 could affect its alternative splicing.

Exon trapping and minigene experiments in cultured cell lines were used to test the effect of several exon 10 and intron 10 mutations on exon 10 splicing efficiency. These mutations disrupt the formation of a stem loop structure present at the 3' boundary of exon 10 which normally functions to restrict spliceosome assembly by preventing efficient interaction of the U1 snRNA with the 5' splice site (Grover et al. 1999). N279K significantly increases the activity of a purine rich ESE by changing TAAGAAG to GAAGAAG favourizing the inclusion of exon 10 and an increase of the 4R/3R ratio. A missense mutation within exon 10, L284L, result in almost complete exon inclusion.

A Δ280K mutation results in complete exon skipping in a minigene construct, presumably because it weakens an ESE (D'Souza et al. 1999). This mutation also decreases the 4R-tau protein function in vitro. Other intronic mutations also alter the hairpin structure in the 5'splice site leading to the inclusion of exon 10 (Hutton et al. 1998). Alterations in splicing of exons 2, 3 and 6 of the Tau protein were also involved in other diseases including

gliopathy, spinal cord degeneration, Alzheimer's disease and myotonic dystrophy (Andreadis 2005) (Leroy et al. 2006).

2.1.2. *Trans*-acting mutations

An efficient function of the spliceosome is accomplished by a dynamic association of several kinases, phosphatases, helicases and many other mRNA export factors and transcription factors (Jurica and Moore 2003). Despite the expectation that dysfunction of the basal splicing machinery should be lethal regardless of cell type, mutations that disrupt the assembly or function of spliceosomal snRNPs are responsible for two human diseases in which two different subsets of neurons are affected : Spinal muscular atrophy (SMA) and retinis pigmentosa (RP). These two neurospecific diseases affect different subsets of neurons and this cell specificity may be due either to the wide range of sensitivities to deficiencies of essential splicing factors exhibited by different exons, or to specific subset of pre-mRNAs affected in either disease by deficiencies of the splicing factors.

2.1.2.1. Spinal muscular atrophy (SMA)

SMA, one of the most common causes of childhood mortality, is an autosomal recessive neurodegenerative disorder characterized by progressive degeneration of the spinal cord motor neurons. SMN gene falls in a complex inverted repeat of around 500Kb on chromosome 5q13 resulting in telomeric SMN1 and centromeric SMN-2 copies. SMN1 is deleted (93% of cases), interrupted (5.6%) or mutated in nearly all SMA patients (Lefebvre et al. 1995). SMN1 and SMN2 genes are remarkably similar, differing only in five nucleotides in their 3' region.

SMN1 produces full length SMN protein, but SMN2 produces a similar protein lacking exon 7 due to alternative splicing caused by a C-T transition of the sixth nucleotide of SMN-2 exon 7. This substitution disrupts an ESE and causes complete exon 7 skipping and the SMNΔ7 has a limited ability to complement the deficiency of the full length protein (Lorson et al. 1999). SMN is a ubiquitous protein with particularly high levels in motor neurons. It shuttles between the cytoplasm and the nucleus where it is found in dot-like structures that colocalize with cajal bodies creating the Gemini of cajal bodies. SMN is a part of a large complex consisting of at least six other interacting proteins (Gemins 2-7). This

complex is believed to play a role in the assembly of snRNPs in the cytoplasm and the regeneration of snRNPs during the splicing cycle in the nucleus, promoting high fidelity interactions between RNA binding proteins and their target sequences (for review (Monani 2005)).

Involvement of SMN in SMA was contrastingly reported by two different schools of thought. The first one suggests that SMA is a direct consequence of a disruption in SMN's role in snRNP biogenesis and pre-mRNA splicing based on observations from both SMA patients where snRNP assembly is compromised. The other school thought claims that SMA is a consequence of a motor neuron-specific function of the SMN protein, based on observations of both in vitro and in vivo experiments demonstrating that SMN accumulates, in axons and growth cones, in granules that associate with microtubules and exhibit bidirectional movement between the cell body and the growth cone. These observations lead to the conclusion that SMN has specific role in neuronal cells and even more specific one in motor neurons (Monani 2005).

SMN interaction with β-actin mRNA mediated by hnRNP-R together with observations from primary motor neurons of SMA mice models that exhibit reduced growth cone size, β-actin levels, and shorter neuritis support the hypothesis that SMN might be constituent of a different RNP complex involved not just in mRNA transport and localization but also in regulating translation in neuritis. Recently, it has been shown that SMN complex is associated with the Fragile X Mental Retardation Protein (FMRP) suggesting that both proteins may have a complementary effect of the actin neurofilament organization and strengthening the role of SMN in localized translation and actin metabolism (Piazzon et al. 2007). A more detailed work aiming to the identification and characterization of specific RNAs associated with SMN granules in growth cones and the association of SMN to polyribosomes in axons may be critical to better understand the function of SMN protein.

2.1.2.2. Amyotrophic lateral sclerosis (ALS)

ALS is an adult onset, progressive disorder characterized by degeneration of cortical motor neurons and spinal/bulbar motor neurons. Oxidative stress is thought to contribute to motor neuron degeneration in a large proportion of ALS cases, either through mutations in SOD1 (found in 15–25% offamilial ALS cases) or through glutamate-mediated excitotoxicity. A decrease in the excitatory amino-acid transporter EAAT2 has been observed in around two-

thirds of patients with sporadic ALS. EAAT2, which is expressed by astrocytes, pumps glutamate from the extracellular space into cells, where it is metabolized.

Motor neurons are particularly susceptible to excitotoxicity as a result of exposure to high levels of glutamate, and decreased EAAT2 levels might therefore account for their selective vulnerability in ALS. Interestingly, the level of EAAT2 RNA in the motor cortex is unchanged in ALS patients. The selective loss of EAAT2 protein appears to be the result of aberrant splicing of EAAT2 pre-mRNA in the specific regions of the brain affected in ALS (Lin et al. 1998). The basis of this splicing misregulation is still unknown. The striking cell-specificity of the pre-mRNA affected suggests that there is not a general disruption of the splicing machinery but, rather, one or few cell-specific auxiliary splicing factors are functioning aberrantly. A precise analysis of additional RNAs whose splicing is similarly affected and whether these RNAs have regulatory sequences in common with EAAT2 will bring new lights on the mechanism that govern the EAAT2 splicing.

2.1.3. *Trans* effects : mutations that affect regulators of alternative splicing.

In this kind of mutations, aberrant splicing of pre-mRNA occurs indirectly as a result of dysfunction in another gene which leads to a secondary splicing defect. Some repeat expansion mutations present in RNAs act as dominant negative sinks for splicing regulatory factors. When amplified by disease causing mutations, these repeats bind to and sequestrate splicing regulatory factors which leads to altered splicing of pre-mRNAs that would normally be regulated by these factors (Ranum and Cooper 2006). Another involvement of RNA dominant diseases is observed in the Fragile-X-Associated Tremor/Ataxia syndrome (FXTAS) a late-onset neurologic disorder that will be detailed on the next chapter, is also due to an RNA gain-of-function mutation. A similar molecular mechanism as DM was proposed, resulting from observations revealing the presence of two RNA binding proteins, hnRNP A2 and MBNL1 in intranuclear inclusions isolated from FXTAS brain specimens (Iwahashi et al. 2006) features and characteristics of this disease will be discussed in the next chapter.

2.1.3.1. Myotonic Dystrophy (DM)

DM, a dominantly inherited neuromuscular disorder, is characterized by progressive skeletal muscle weakness, myotonia, cardiac conduction defects, dilated cardiomyopathy, endocrinopathy, alterations in smooth muscle function, cognitive impairment and behavioral disturbances. Two types of DM were identified. DM1 is caused by CTG trinucleotide expansion in the 3' UTR of the dystrophia myotonica-protein kinase (DMPK) gene on chromosome 19. Severely affected individuals have more than 1500 repeats whereas in unaffected individuals the CTG repeats range from 5 to 38.

DM2 results from a CCTG expansion in the intron 1 of the zinc finger 9 (ZNF9) gene on chromosome 3 where the repeats can reach more than 11.000. Both diseases are clinically similar, however, differences such as the absence of congenital form in DM2 where proximal muscles are affected. However, in DM1, distal muscles are affected and an overall increased severity of both muscular dystrophy and the CNS symptoms allow to distinguish between both forms of DM (for review (Ranum and Cooper 2006)).

At a molecular level, DM is due to misregulation of alternative splicing that can affect essentially MAPT together with NMDAR1 and APP genes in brain, and the Insulin receptor (IR), the Chloride Ion Channel (ClC-1) in skeletal muscles. Mouse model expressing CUG repeats in the 3'UTR of the muscle skeletal alpha actin protein showed alterations in the splicing of *ClC-1* pre-mRNAs revealing the effect in *trans* of the repeats. Co-transfection experiments of CUG repeat-containing mRNA with splicing reporter minigenes resulted in the same splicing pattern observed in DM patients, confirming the *trans* acting effect of the repeats (Wang et al. 1995).

The mechanism by which these repeats induce a *trans* dominant effect on splicing is still unclear but an explanation could be raised out from the sequestration of splicing regulators by these repeats resulting in nuclear depletion and loss of function of these regulators. Two RNA binding proteins, involved in several steps of RNA processing, were identified to bind the CUG repeats, CUG-BP1 (CUG Binding Protein 1) member of the CELF family, and MBNL (muscleblind like protein) (Lu et al. 1999; Timchenko et al. 1999). A growing list of evidences link an altered function of these two proteins with DM. MBNL binds the CUG repeats and localizes in nuclear Foci of CUG or CCUG repeats containing RNAs, in vivo experiments showed that MBNL is able to bind directly some intronic elements of the IR gene and the cTNT gene, whose splicing is misregulated in DM. Finally, a mouse knockout model of *MBNL* exhibits a DM-like phenotype.

CUG-BP1 binds short single stranded CUG repeats but unlike MBNL, it doesn't bind proportionally to the length of the repeat RNA and doesn't colocalize with CUG or CCUG RNA repeats foci. CUG-BP1 regulates the splicing of ClC1, IR, and cTNT genes mostly affected in DM. The expression level of CUG-BP1 is significantly increased in tissues and cell cultures from individuals with DM (Miller et al. 2000).

Put together, all these evidences prove that both MBNL and CUG-BP1 antagonistically regulate the splicing of affected genes where a loss of function of MBNL, thought to be the dominant cause of DM, and a gain of function of CUG-BP1 are observed. Nevertheless, another alternative model suggests that the loss of activity of MBNL is not sufficient to explain the splicing abnormalities in DM. This model is based on co-transfection experiments of RNA-containing CUG repeats with cTNT and IR minigenes , to analyze the splicing pattern of these minigenes when depleting MBNL by siRNA. The results of these assays lead the authors to conclude the importance of the direct effect of CUG-BP1 binding to its target pre-mRNAs altering their splicing pattern in a MBNL independent manner.

2.2. NMD and diseases

Recently NMD was linked to Mental retardation by Tarpey and colleagues. In a large screening aiming to identify novels genes involved in mental retardation, they found three truncating mutations in UPF3B gene in three different families. Two of these mutations were deletions within the exons resulting in frameshift and truncated protein while the third mutation was nonsense mutation, all of them leading to a PTC.

The authors checked whether this PTC in the UPF3B mRNA would be targeted by NMD. Given that UPF3B is involved in this pathway, mutations affecting this protein would lead to an alteration in the NMD. Quantitative analysis showed that UPF3B mRNA level from affected individuals was significantly lower than that of normal individuals suggesting that targeting of NMD for UPF3B mRNA degradation was not altered in the absence of UPF3B protein. They also checked alteration of the classical and alternative NMD pathways and demonstrated that only the classical NMD pathway is affected whereas the alternative pathway is intact (Tarpey et al. 2007).

2.3. RNA binding proteins in Neurologic diseases

The neuron-specific inclusion or skipping of alternative exons is primarily achieved by tissue-specific expression of particular RBPs. Over a dozen neuron-specific RBPs have been identified, the Nova and the Hu protein families that regulate a wide range of target gene transcripts, encoding proteins involved in cytoskeletal rearrangement, vesicular transport, cell adhesion, signal transduction pathways, and synaptic activity.

2.3.1. Paraneoplastic encephalomyelitis

Paraneoplastic encephalomyelitis is a neurological disorder associated to certain types of tumors, with predominantly small cell carcinoma of the lung expressing a particular Hu antigen. Indeed, Hu proteins were first identified as the targets of autoantibodies found in affected patients. This neuroimmune disorder results from the ability of these antibodies to cross the blood brain barrier. Hu proteins are the human homologues of Drosophila ELAV, an RNA-binding protein whose deletion results in an **e**mbryonic **l**ethal **a**bnormal **v**ision phenotype in flies. Neuronal specific members of the Hu family, HuB, HuC and HuD are thought to be one of the earliest markers of neuronal differentiation showing a unique pattern of spatial and temporal expression, while the fourth member, HuA is expressed in other tissues (for review (Deschenes-Furry et al. 2006) and references theirin). Hu proteins harbour three RRM similar to those observed in splicing regulators, however, the best characterized function of "Hu" is in the stabilization of specific messenger mRNAs.

2.3.2. Paraneoplastic opsoclonus myoclonus ataxia (POMA)

Nova proteins form the second family of RNA binding proteins associated with the paraneoplastic opsoclonus myoclonus ataxia (POMA). This disorder is mostly characterized by cerebellar ataxia, dysphasia, mutism, and irritability. Nova 1 and 2 have been identified as the two members of the nova protein family. Nova1 and Nova2, are complementary expressed in brain regions and contain three KH domains involved in recognition and binding to specific RNA targets (Lewis et al. 1999; Lewis et al. 2000). The authors showed that a UCAUY (Y can be any pyrimidine) element in the context of a stem loop is recognized by the protein.

This UCAY sequence bound by the KH3 domain was used to identify putative targets for Nova1. Involvement of Nova1 in both splicing and neuronal survival emerged from Nova1 null mice that showed apoptotic death of the ventral spinal cord motor neurons and alteration in the splicing pattern of the glycine receptor (GlyRα2) (Jensen et al. 2000). Recently, affymetrix microarrays analysis showed that the splicing pattern of a small set of exons was affected in Nova1$^{-/-}$ and Nova2$^{-/-}$ mice brains suggesting therefore that Nova forms one of the multiple factors that regulate splicing of these exons (Ule et al. 2005).

Another neurological disorder related to RNA binding proteins is the Fragile X syndrome that will be detailed in the next chapter.

3. The Fragile X syndrome: An unstable repeat expansion disorder

3.1. The Fragile X mental retardation gene

3.1.1. discovery of the *FMR1* gene

When the chromosome sets of mentaly-retarded patients were studied, a peculiar constriction was observed at the tip of the long arm of the X chromosome at position q27.3. Under specific culture conditions, this constriction appeared sensitive to low folate concentrations. This particular site turned out to undergo a local instability of chromatin, and was so designated as 'fragile'. This fragile X signature was confirmed through studies of families with X-linked mental retardation and the term of Fragile X was adopted. FXS affects in average 1/4000 males and 1/7000 females. In 1991, the *Fragile X Mental retardation 1* (*FMR1*) gene was mapped on the X chromosome at position Xq27.3 and spans around 40 kb (Verkek et al. 1991). Footprint analysis revealed the existence of many *cis* elements in its 5'UTR (GC boxes, E-box and palindrome sequence) of *FMR1*, that are able to bind several transcription factors such as Sp1, USF1 and USF2 regulating therefore its expression (Hwu WL 1993; Kumari D 2001). In addition CGG repeats present int the 5'UTR of the gene contribute to the recruitment of CGG binding proteins and other components of the transcriptional machinery that control *FMR1* gene expression. The human *FMR1* gene is transcribed in a 3.9 kb mRNA constituted by 17 exons. Alternative splicing play a crucial role in the generation of several transcript isoforms by affecting the inclusion or skipping of exons 12 and 14 in one hand and the choice of the splicing acceptor sites in exons 15 and 17 (Verkek et al. 1993).

The 5'-untranslated region of *FMR1*, that controls its normal expression, is located at this particular fragile site and shown to contain a stretch of unstable CGG repeats (Figure 5). FXS was therefore classified among the 14 documented trinucleotide repeat expansion disorders that affect humans (summarized in Table 1). All these disorders cause neurological dysfunction and involve a dynamic mutation affecting the number of repetitions of a nucleotidic triplet: CAG (that codes for glutamine (Q)) in the case of polyQ diseases such as Huntington's disease, and other repeats in non-polyQ disease such as FXS, myotonic distrophy or Friedreich's ataxia.

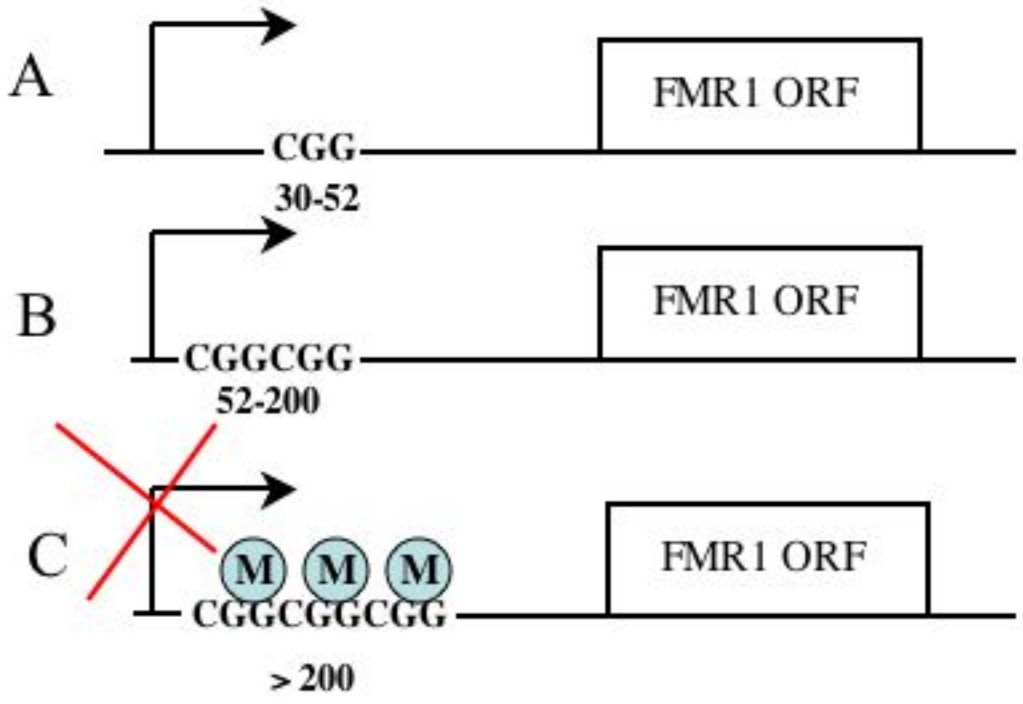

Figure 5 : Schematic representation of FMR1 alleles

A- Normal individuals carry between 30-52 CGG repeats in the 5'UTR of FMR1. In these individuals the gene is entirely transcribed.

B- The case of premutation appears when the CGG repeats number is between 52-200. The gene is transcribed and the protein product is detected in these individuals.

C- Full mutation occurs when the repeats exceed 200, the promoter region becomes hypermethylated and the gene is completely silenced.

The exact molecular mechanism for the expansion of CGG repeats in the 5'UTR of the *FMR1* gene is still poorly understood, however several studies suggest that a DNA polymerase slippage occur during replication due to the hairpin structures adopted by the CGG repeats. The presence of the AGG that interrupt the CGG repeats would act as a "switch off" for the formation and the dimerization of hairpins, stabilizing therefore the repeats.

3.1.2. Mutations in the CGG repeats induce abnormal *FMR1* expression

3.1.2.1. The premutation and FXTAS

The 5'UTR of the *FMR1* gene of normal individuals carries between six and fifty four CGG repeats, often interrupted by one or two AGG. Intermediate sizes ranging from 52 to 200 have been detected in parents of children affected with FXS, and since these parents did not express any clinical or cytogenetic phenotype, they were considered as 'carriers' of a premutation. This premutation is unstable during oogenesis and is associated with a significant risk of further expansion, upon maternal transmission, to a full mutation in succeeding generations. Initially, the premutation was believed to be medically and psychologically benign, however, the existence of phenotypic features specific to the premutation condition had been recently reported. First, premature ovarian failure (POF), defined by cessation of menses prior to age 40, occurs in 20% of females with the premutation as compared to 1% of the general population (Sherman 2000). Second, a fragile X-associated tremor/ataxia syndrome (FXTAS) occurs in a significant proportion of older males and in occasional older females with the premutation. It had been also shown that premutation carriers present reduced hippocampal volume and gray matter density. The primary features of FXTAS include intention tremor, ataxia, brain atrophy with white matter disease, autonomic dysfunction including high blood pressure and impotence, sensory neuropathy in a stocking distribution in the lower extremities, and cognitive decline beginning with memory deficits. This neurodegenerative phenotype is entirely distinct from FXS.

In these individuals, *FMR1* mRNA level had been shown to be particularly elevated up to five to ten folds above the normal range. This increase was suggested to be the cause of toxic gain of function in neurons leading to inclusion formation, neuronal cell death, brain atrophy, and white matter disease (Hagerman PJ 2004). However, the downstream effects of these molecular mechanisms on psychiatric/psychological functioning have not yet been fully elucidated.

Disease	Mutation/ repeat unit	Gene name (protein product)	Putative function	Normal repeat length	Pathogenic repeat length
Diseases that are caused by loss of protein function					
FRDA	$(GAA)_n$	*FRDA* (frataxin)	Mitochondrial iron metabolism	6–32	200–1,700
FRAXA	$(CGG)_n$	*FMR1* (FMRP)	Translational regulation	6–60	>200 (full mutation)
FRAXE	$(CCG)_n$	*FMR2* (FMR2)	Transcription?	4–39	200–900
Diseases that are caused by altered protein function					
SCA1	$(CAG)_n$	*SCA1* (ataxin 1)	Transcription	6–39	40–82
SCA2	$(CAG)_n$	*SCA2* (ataxin 2)	RNA metabolism	15–24	32–200
SCA3 (MJD)	$(CAG)_n$	*SCA3* (ataxin 3)	De-ubiquitylating activity	13–36	61–84
SCA6	$(CAG)_n$	*CACNA1A* ($CACNA1_A$)	P/Q-type α1A calcium channel subunit	4–20	20–29
SCA7	$(CAG)_n$	*SCA7* (ataxin 7)	Transcription	4–35	37–306
SCA17	$(CAG)_n$	*SCA17* (TBP)	Transcription	25–42	47–63
DRPLA	$(CAG)_n$	*DRPLA* (atrophin 1)	Transcription	7–34	49–88
SBMA	$(CAG)_n$	*AR* (androgen receptor)	Steroid-hormone receptor	9–36	38–62
HD	$(CAG)_n$	*HD* (huntingtin)	Signalling, transport, transcription	11–34	40–121
Diseases that are caused by altered RNA function					
DM1	$(CTG)_n$	*DMPK* (DMPK)	RNA-mediated	5–37	50–1,000
DM2	$(CCTG)_n$	*ZNF9* (ZNF9)	RNA-mediated	10–26	75–11,000
FXTAS	$(CGG)_n$	*FMR1* (FMRP)	RNA-mediated	6–60	60–200 (premutation)
Diseases of unknown pathogenic mechanism(s)					
SCA8	$(CTG)_n$	*SCA8* (transcribed/ untranslated)	Unknown	16–34	>74
SCA10	$(ATTCT)_n$	Unknown	Unknown	10–20	500–4,500
SCA12	$(CAG)_n$	*PPP2R2B* (PPP2R2B)	Phosphatase regulation	7–45	55–78
HDL2	$(CTG)_n$	*JPH3* (junctophilin 3)	PM/ER junction protein	7–28	66–78

Table 1 : inheritance pattern and clinical features of unstable repeat expansion disorders. (Gatchel et al., 2005)

3.1.2.2. The full mutation and silencing of the FMR1 gene

When the number of repeats exceeds two hundred, the CGG expansions create CpG islands which are targets to hypermethylation (Verkek et al. 1991). This increased methylation coupled with decreased histone acetylation (Coffee B et al. 1999) results in the inactivation of the FMR1 gene. These events affecting chromatin structure are thought to be at the basis of silencing of the *FMR1* gene in patients, eventhough which of these two mechanisms occur first is still in debate. It is known that methylated CpGs lead to the recruitment of several repressive complexes which compel the chromatin to adopt structures that are inaccessible for transcriptional activators. In another hand it has been shown that acetylation of histones H3 and H4 is decreased in fragile X cells where lysine 9 of histone H3 (H3-K9) is methylated, reflecting a pattern observed in inactive genes. Both observations lead to conclude that methylation of CpG islands and chromatin condensation in FXS patients prevent the transcription factors to bind to the *FMR1* promoter and provoke its silencing.
Since the discovery of the *FMR1* gene, its silencing mechanisms and the establishment of the clinical features of FXS patients, several other mutations were reported in patients exhibiting the FXS traits but not showing any abnormal CGG repeat expansions.

3.1.2.3. Other mutations affecting *FMR1* gene

Eventhough the vast majority of Fragile X patients bear abnormal expansions of the CGG repeats in the 5'UTR of FMR1 gene, several point mutations in the coding sequence of the gene have been reported. First, Lugenbeel and colleagues reported that one patient showed the absence of an adenosine residue in position +373 in exon 5, resulting in a frameshift and predicting a premature STOP codon. A second patient showed a two base pairchange (GG-TA) in the splice acceptor site of exon 2 resulting in two RT-PCR products, one reflected the skipping of exon 2 while the other reflected the exclusion of both exon 2 and 3 in the mRNA. Exclusion of exon 2 creates a frameshift and a premature STOP codon, while the absence of both exon 2 and 3 doesn't disrupt the reading frame but removes 49 amino acids from the protein. In both patient, FMRP was not detected suggesting that their *FMR1* mRNA product is unstable and may be degraded by the NMD (Lugenbeel et al. 1995).

Wang and colleagues performed a large scale screening and reported a point mutation in three FXS patients modifying a C into T in position 14 of intron 10. This substitution affects the splicing pattern of the *FMR1* mRNA causing both the exclusion of exon 10 and a

frameshift after exon 11 creating a premature STOP codon. Furthermore, the authors showed that one of the three patients presents another substitution in exon 15 where a G is substituted by an A leading to an Arginine to Histidine shift in amino acid position 546. They concluded that this mutation would have no significance in FXS formation, simply due to its existence downstream of the STOP codon created by the abnormal splicing pattern in this patient {Wang, 1997 #1557}. The authors didn't examine the protein level in these patients where it's probably absent due to degradation of the mRNA with premature STOP codons. It's of a great interest to mention that Arg546His substitution falls in an active functional domain of FMRP. A more detailed analysis of this substitution at a protein level would enlighten its importance and predict if it results in any alteration of FMRP function.

Another missense mutation was described by De Boulle and colleagues, a T to A conversion leads to substitution of an isoleucine by an asparagine in amino acid position 304 (Ile304Asn). This mutation was found in a patient severely affected by FXS where FMRP is detected but presents functional alterations (De Boulle et al. 1993). This mutation and its consequence on the protein function will be discussed later.

Other deletions affecting the *FMR1* gene were also described (for review (Bardoni and Mandel 2002)). All the described mutations affecting directly the *FMR1* gene allowed to confirm its role as an exclusive cause for FXS manifestation.

3.2. Clinical features of Fragile X syndrome

3.2.1. Phenotypic hallmarks of the FXS

Mental retardation, the "hallmark" of the FXS is also accompanied by some behavioral phenotype including hyperarousal, social anxiety and withdrawal, social deficits with peers, abnormalities in communication, unusual responses to sensory stimuli, stereotypic behaviour (mood swings, self injury, aggression), autism spectrum disorders, inattention, impulsivity, and hyperactivity. All these characteristics vary among patients, some exhibit mild mental retardation with an IQ average around 50 while others can be more severely affected (Hagerman and Cronister 1996).

In addition to impaired higher cognitive functions, various physical features are now recognised, such as facial dysmorphism with large and inverted ears, long face and prominent jaws, often accompanied by flat feet and large testicles (macro-orchidism) in FXS male patients. Some forms of connective tissue dysplasia, such as hyperextensible joints, flat feet

and hand calluses can also be observed. FXS is actually considered as a complex syndrome, since symptoms are multiple and highly heterogenous, also varying in severity from one patient to another.

3.2.2. Neuroanatomic features

Selective changes in brain size of FXS patients had been observed by neuroimaging and magnetic resonance studies. Most importantly, a reduction of both the posterior cerebellar vermis and the temporal lobe volume occurs with an age-dependant increase in hippocampal volume and enlarged caudate nucleus and thalamus and parietal white matter (for review (Kates WR 2002)). Furthermore, a detailed analysis of the alteration of the integrity and connectivity of the white matter, suggested a relative involvement of the white matter in both frontostriatal pathways and parietal sensory motor tracts (Barnea-Goraly N 2003), however, the size difference remain subtle.

At the cellular level, the only abnormality detected in postmortem microscopic observations of FXS patients was the dendritic spines (Irwin SA 2001). Dendritic spines are membranous protrusions emanating from dendrites that constitute the postsynaptic compartment of major excitatory synapses in brain. FXS patients exhibit higher density of spines, with increased number of long and thin immature spines together with a decreased number of short and mature spines in the temporal and visual cortex (Figure 6). These spine abnormalities are a general phenomenon in disorders related to mental retardation, and are thought to be at the basis of the alterations of higher cognitive functions observed in fragile X patients. The role of FMRP in this context will be discussed in the following part.

Fmr1 KO mice, like FXS patients, display phenotype traits including enlarged testes, hyperactivity and a mild spatial learning impairment (Bakker et al. 1994), characteristics of the phenotype of fragile X patients. They present dendritic spines that are longer, thinner and denser than the ones of wild-type mice (Comery et al. 1997; Irwin et al. 2000). (Figure 6). These mice display alteration of some forms of synaptic plasticity. Indeed, in mice hippocampal LTD is increased in the absence of Fmrp (Nosyreva and Huber 2006) and, conversely, cortical LTP is reduced in *Fmr1* null mice (Wilson and Cox 2007). These animals show an increased susceptibility to audiogenic seizures (AGS) at all ages (Musumeci et al. 2000) (Yan et al. 2004).

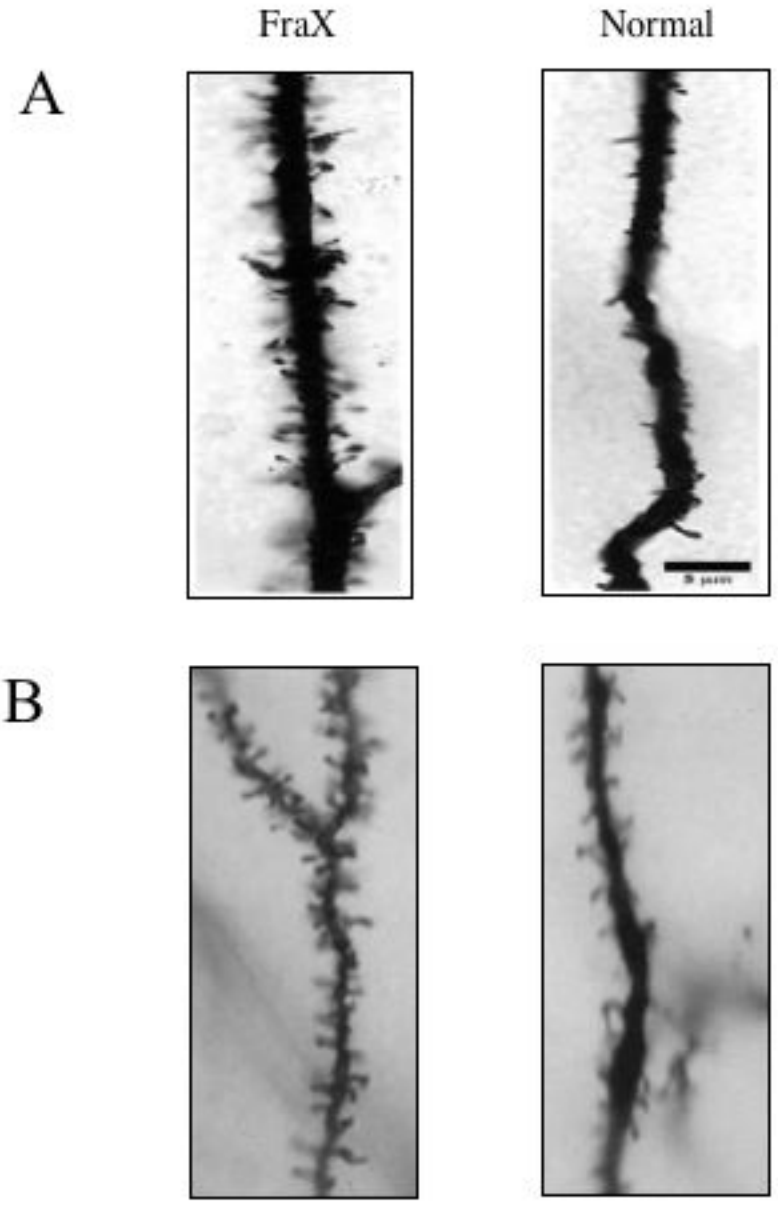

Figure 6 : Abnormal dendritic spines in Fragile X syndrome.

Morphology and density of dendritic spines in Fragile X patients (FraX) (A) and *fmr1 null* mouse (B) compared to their unaffected related (Normal). (Adapted from Comery et al., 1997 and Irwin et al., 2000)

3.2.3. Expression pattern of fmr1

Two major techniques were used to follow the expression of *FMR1* gene during embryonic development, *in situ* and immunohistochemistry in both human and mouse tissues. Results obtained using these experiments were confusing or even contradictory due to the probe used in the study. The probe was able to recognize the mRNA of FMR1 two homologues FXR1 and FXR2. Nevertheless, they have allowed to define a scheme of the dynamic expression of *FMR1* during development. Bakker and colleagues established a "time course " expression of FMRP in mice embryos. In These embryos, FMRP was detected after 2 days of gestation, its ubiquitous expression gets gradually elevated between days 2 and 14 especially in ectodermic derived tissues and testes. A more specific expression of FMRP is observed after day 14, till birth and kept in adult. Always high levels in testes and brain, FMRP expression decreases in heart and skeletal muscle (Bakker et al. 2000). Similar results were observed in human embryos where FMRP was highly expressed in testes and brain, especially in hippocampus and nucleus basalis magnocellularis, both regions involved in learning and memory (Abitbol M 1993; Tamanini et al. 1997).

In both human and mice adults, FMRP was not detected neither in heart , blood vessels nor in skeletal muscles, whereas it was highly expressed in the brain, testes and ovaries. More specifically, both hippocampal pyramidal neurons and cerebellum Purkinje cells showed a significant increase in FMRP expression. In adult testes, FMRP expression is restricted to spermatogonia (Devys et al. 1993).

The ubiquitous expression of FMRP reflects the function of this protein in several tissues and can be correlated with symptoms other than mental retardation in FXS patients. However, it's worth mentioning that both brain and testes, the most affected tissues in FXS patients, are sites where FMRP is highly expressed.

3.3. The Fragile X gene family

3.3.1. FXR1 and FXR2

The human *FMR1* belongs to a gene family which includes two other autosomal members that are not present on the X chromosome: *FXR1* and *FXR2,* that map at 3q28 and 17p13.1, respectively (Coy et al. 1995; Siomi et al. 1995).

3.3.1.1. FXR1

The FXR1 mRNA is highly subjected to alternative splicing, its pre-mRNA gives rise to several *FXR1* mRNA isoforms including or not exons 15, 16 and 17 with insertion or deletion of sequences belonging to exons 12 and 13 (Kirkpatrick et al. 1999). Thus, an affluent repertoire of FXR1P isoforms are produced and expressed in a tissue specific manner (Khandjian et al. 1998; Dubé et al. 2000) . Both aspects of FXR1P distribution and function will be discussed later. Interestingly, homozygous *Fxr1* $^{-/-}$ mice die shortly after birth most likely due to cardiac and/or respiratory failure. These animals showed a less well developed limb musculature and a reduced size of heart, lung, spleen and testes compared to WT animals (Mientjes et al. 2004). At a cellular level, a reduction of the cytoplasmic volume was observed in both cardiac and skeletal muscle accompanied by an alteration of contractile filaments and an increase in nuclei number. A deeper observation showed that costameric proteins such as vinculin, dystrophin and α-actinin were completely delocalized. The costameric regions are probably involved in translational control. For this reason, Mijentes and colleagues suggested a putative function of FXR1P similar to that of FMRP (Mientjes et al. 2004).

3.3.1.2. FXR2

As *FXR2* has not been correlated to any known pathology or defect in humans, its molecular function had been poorly investigated. Mahishi and colleagues analyzed the promoter region of *FXR2* and identified the transcription factors involved in the regulation of its activity. Interestingly, Sp1, Nrf1 and AP-2 were found to be bound by the promoter region. These factors were also shown to regulate the transcription of *FMR1* (Mahishi and Usdin 2006). FXR2 pre-mRNA is not subjected to alternative splicing, giving therefore a unique full-length transcript.

Mouse models were generated for behavioral tests. *Fxr2* null mice are viable and shown some behavioural phenotypes similar to those observed in *Fmr1* knockout mice, suggesting a role for FXR2 in the developing of cognitive functions (Bontekoe et al. 2002). *Fxr2/Fmr1* double KO mice were generated and analyzed by a battery of behavioral assays to assess multiple aspects of CNS function. Exaggerated behavioral aspects such as hyperactivity, a decreased acoustic startle response and decreased conditioned fear were

observed suggested a genetic interaction between *Fmr1* and *Fxr2* and the possibility that FXR2P may compensate for the absence of FMRP in FXS patients (Spencer et al. 2006).

3.3.2. Evolutionary conservation of the fragile X family

The fragile X gene family has been highly conserved during evolution, since members of this family have been found from the jellyfish to human. Vertebrates, such as human and mouse, as well as the zebrafish *Danio rerio* possess three members of the FXRP family, the frog *Xenopus laevis* has two, and the fruitfly *Drosophila melanogaster* only one. Even *Hydractinia*, an evolutionary relative of coral and jellyfish belonging to the Cnidarian phylum possesses an homologue of the *FMR1* gene that is expressed in its nervous cells. Since Cnidarians represent the most primitive alive metazoans possessing a nervous system, the surprising preservation of the FXRP family evokes the fascinating possibility that these proteins have been conserved by evolution to carry out an essential function relative to the nervous system.

3.3.3. Animal models

The evolutionarily conservation of the fragile X family has enabled the development of various animal models that essentially recapitulate the phenotypic traits observed in fragile X patients.

In addition to the mouse model (discussed above), a model for fragile X syndrome was also generated in zebrafish. Indeed, inactivation of *zFmr1* gene by morpholinos causes abnormal axonal branching of Rohon–Beard and trigeminal ganglion neurons together with guidance and defasciculation defects in the lateral longitudinal fasciculus. Interestingly enough, Tucker and colleagues described novel findings of abnormalities in the abundance of trigeminal ganglion neurons and of craniofacial abnormalities apparently due to dysmorphic cartilage formation. The authors suggested that these abnormalities may be related to a role for *zFmr1* in neural crest cell specification and possibly in migration (Tucker et al. 2006).

In flies, *dFMR*1 mutants have abnormal circadian rhythms and courtship behavior (Inoue et al. 2002; McBride et al. 2005). In the CNS mutant interneurons have abnormal neurite extension, guidance and branching (Morales et al. 2002). In addition, these flies showed developmental defects of mushroom body lobe morphogenesis, of which the most

common is a failure of b lobes to stop at the brain midline (Michel et al. 2004). Interestingly, this is an highly plastic brain region, known to have a role in multimodal sensory integration, olfactory associative learning and visual context generalization. However, also peripheral phenotypes have been observed including overgrown, dysfunctional motor neuron terminals (Zhang et al. 2001) and overgrown sensory neuron dendritic arborescences (Lee et al. 2003). The dFMR1 profile of expression is reminiscent of FXR proteins, since in addition to nervous system a high expression is also found in muscle and gonads (Schenck et al. 2002). While the eventual implication of dFMR in muscle development in fly was never investigated, in testis Zhang and colleagues reported that dFMR mutants lose specifically the central pair microtubules in the sperm tail axoneme. The frequency of central pair microtubule loss becomes progressively greater as spermatogenesis progresses, suggesting that dFMR regulates microtubule stability (Zhang et al. 2004).

The drosophila model provided a great input to understand the molecular function of FMRP and was determinant for the identification of Futsch (homologue of MAP1B), the interaction of dFMR with the RISC complex and the implication of dFMR in the Rac pathway.

4. The Fragile X Mental Retardation Protein (FMRP)

4.1. Structural and Functional architecture of FMRP

As discussed in a previous paragraph, at a molecular level, FXS results from either an absence of FMRP (in almost all cases of FXS patients) or from alterations in both structure and function of FMRP (rare cases). RT-PCR analysis revealed the existence of more than 20 splice variants of *FMR1* mRNA, which are translated in several FMRP isoforms, most probably ten, detected as four to six different bands by western blot ranging between 70 and 80 KDa. The longest isoform, Iso1, encoded by the 17 exons , comprises 632 amino acids while the most frequent isoform, Iso7, lacks exon 12 (Khandjian et al. 1995) (Figure 7).

FMRP is predominantly localized in the cytoplasm where it interacts with several other proteins, however a small proportion of FMRP was also found in the nucleus suggesting that FMRP could shuttle between the nucleus and the cytoplasm. Structural comparisons

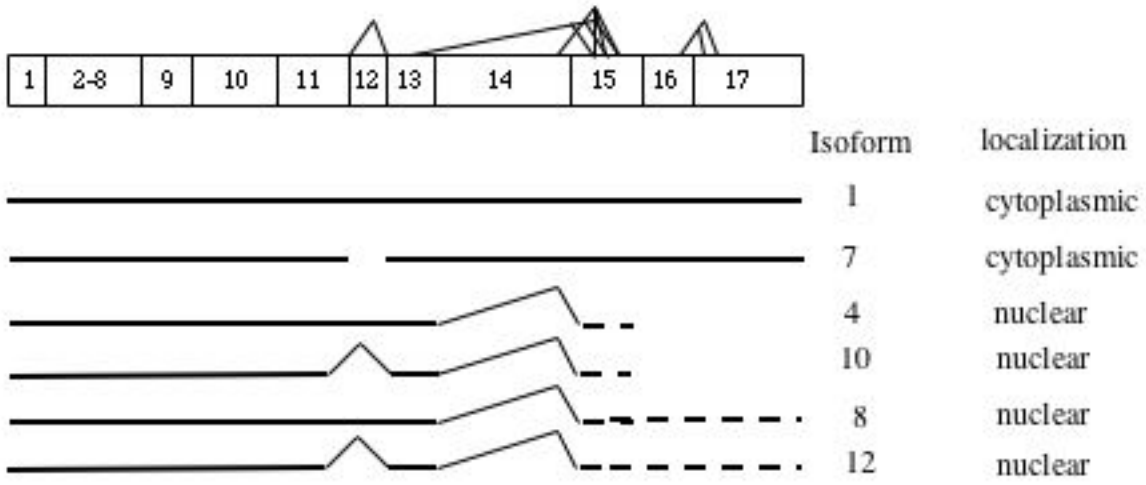

Figure 7 : Structure and localization of several putative FMRP isoforms (Sittler et al., 1996)

succeeded to predict the RNA binding capacities of FMRP and the major focus of this part is the analysis of the function of FMRP through its different structural domains.

4.1.1. The N-terminus region of FMRP : a trap for proteins and RNAs

4.1.1.1. Structural analysis of the N-terminus region of FMRP

Spanning the first 217 amino acids, this region shows a high affinity domain for protein-protein interaction. Adinolfi and colleagues have identified an independently folded domain within this region termed NDF (N-terminal Domain of FMRP) (residues 1-134) (Adinolfi et al. 2003). The structure of the NDF domain was investigated in details (Ramos et al. 2006). NDF is formed by two repeats NDF1 (residues 3-50) and NDF2 (residues 63-113), linked by an unstructured tail of 20 residues. The C- terminal domain of NDF is formed by residues 114-134, contains a single turn helix (α1 aa 122-126), but is unstructured. NDF2 is less compact than NDF1, nevertheless both NDF1 and 2 are folded in four stranded antiparallel β-sheet with a fifth strand serving to close the cavity of the sheet. Interactions between these two domains occur mostly assisted by Trp and Phe residues. The succession of these three structural motifs is essential for NDF stability, moreover mutations that destabilize the NDF fold can dramatically affect the subcellular localization of FMRP. Double mutation of amino acids 125 and 126 of the α1 helix of FMRP Iso 12 (Iso 12 was previously shown to distribute in the nucleus, mostly in perinucleolar region (Bardoni and Mandel 2002)) causes a complete diffusion of Iso12 in the nucleus where a small portion (2-6% of observed cells) remain perinucleolar reflecting the importance of the correctly folded NDF in the nucleolar localization of FMRP.

Maurer-Stroh and colleagues used a comparative sequence analysis to predict that the repeats observed in NDF domains share significant similarities with members of the “Royal” superfamily including Tudor and Agenet domains. A four β-strand fold is a common feature for the members of this family (Maurer-Stroh et al. 2003). When Ramos and colleagues established the structure of the NDF domain, they demonstrated that its tudor domains are highly similar to those observed in SMN protein even if domains of both proteins share low sequence identity (15%). Other similarities were found with the DNA binding domain of HIV-1 integrase and the transcription elongation factor NusG from Thermus thermophilus (Ramos et al. 2006). The hydrophobic pockets formed on the surface of NDF are mainly involved in protein-protein interaction, nevertheless the high plasticity and dynamic

exposures of hydrophobic cavities of NDF2 play a crucial role in the interaction with NUFIP and 82-FIP (Bardoni et al. 2003a; Bardoni et al. 2003b; Bardoni et al. 2003c). (Figure 8)

4.1.1.2. Exon 7 and the Protein-Protein Interacting Domain

The secondary structure of the region encoded by exon 7 was predicted by multilple alignment to fold in a helix-loop-helix motif . This region contains the dimerisation unit of FMRP. Dimerisation could occur through pairing of the two motifs resulting in a four helix bundle (Adinolfi et al. 2003). The domain encoded by exon 7 enables FMRP to interact with itself, its other two homologues FXR1P and FXR2P, as well as CYFIP1 and CYFIP2 (Cytoplasmic FMRP Interacting Protein 1/2) (detailed in next paragraphs)

4.1.2. NLS/NES condition the nucleocytoplasmic shuttling of FMRP

4.1.2.1. FMRP N-terminus contains a Nuclear Localisation signal

Exon six and seven encodes the second part of the N-terminus region of FMRP, corresponding to amino acids 135-217. This segment reveals two important function of the protein: a non classical nuclear localisation signal (NLS) and another platform for dimerisation and protein- protein interaction.

Two different studies demonstrated the existence of an NLS in this region. Eberhart and co-workers used truncated portions of the protein to better define the NLS. They were able to prove that the first 167 amino acids of FMRP are sufficient for its localisation in the nucleus. To strengthen their observations, they analysed the first 184 amino acids of FMRP and showed that this region contains clusters of Lysine and Arginine (K, R ; 24 residues) observed in most known NLSs (Eberhart et al. 1996). They suggested that FMRP may contain a novel NLS sequence as the one observed in the U1A protein (Kambach and Mattaj 1992). A more acute analysis performed by Bardoni and colleagues had refined the localisation of FMRP NLS reported by the pervious group. The authors pointed out that the NLS activity is localized in the region between residues 114-150, could be reinforced by residues 151-196 and that the cluster KK and KR in position 151-152 and 179-180 might positively modulate the NLS activity (Bardoni et al. 1997)

Both studies demonstrated the absence of known NLS consensus which usually consists of stretches of Lys-Arg/Lys-X-Arg/Lys where X could be lysine, proline, arginine,

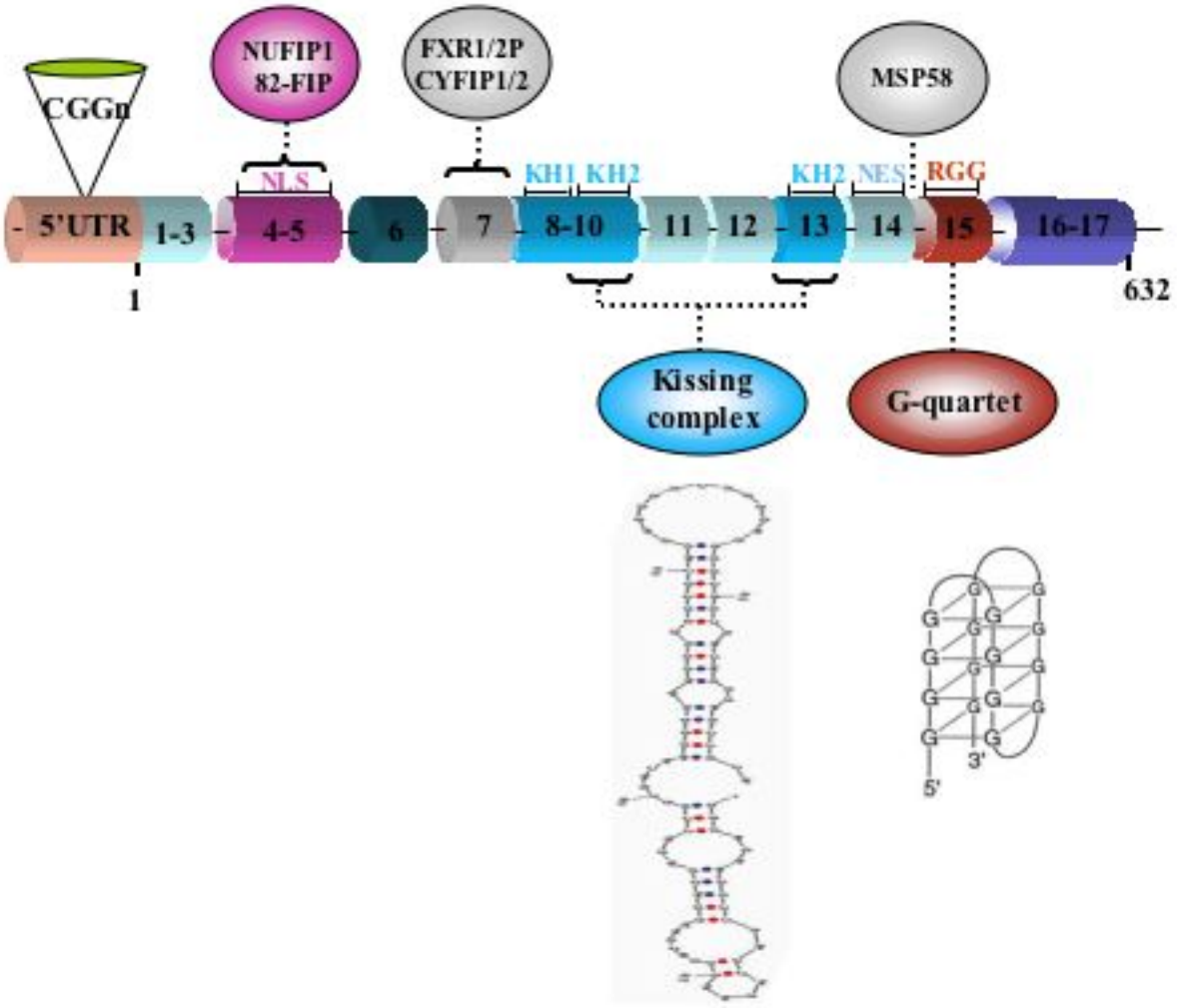

Figure 8 : Schematic representation of FMRP showing its functional domains, protein interactors and target RNA structures

valine or alanine (Chelsky et al. 1989). FMRP localization in the nucleus could be explained by a three dimensional (3D) signal interacting with a nuclear carrier. Either the identification of this partner or the destabilisation of the 3D fold by suitable mutations would better enlighten the mechanism by which FMRP enters the nucleus.

4.1.2.2. Exon 14 encodes NES

Immunofluorescence experiments on cells transfected with FMRP isoforms lacking exon 14 showed that the protein was completely captured in the nucleus (Sittler et al. 1996). These observations lead to the identification of a NES encoded by exon 14 (Eberhart et al. 1996). Standing at the C-terminal end of the KH2 domain, this region did not seem participate in the stabilization of KH2. The sequence involved in the nuclear export LRLERLQI is similar to that observed in the Rev protein. The fold adopted by the NES was predicted to be a coiled-coil which would not be possible due to its length and composition. It is more probable that the sequence encoded by the exon 14 forms a helix which may interacts with other regions of FMRP. The presence of both NLS and NES in the sequence of the protein suggested that FMRP is able to shuttle between the nucleus and the cytoplasm. This hypothesis was confirmed by Tamanini and colleagues who blocked the nuclear export by treating cells with leptomycin B. In these conditions, a clear accumulation of FMRP in the nucleus was observed (Tamanini et al. 2000). Using electron microscopy, Feng and colleagues showed that FMRP is present near the nuclear pores (Feng et al. 1997), this would suggest that some of FMRP are in their “transit” way from the nucleus to the cytoplasm.

4.1.3. The Phosphorylation domain : keep a low profile and execute silently

Analysis of the phosphorylation status of FMRP revealed that only one region could be affected (aa 483-521) with either one, two or three phosphoresidues. The 38 amino acid peptide was assigned as the phosphorylation domain of FMRP located between the NES and the RGG box (Ceman et al. 2003). The effect of this post translational modification on FMRP function was analysed by two different groups resulting in contradictory observations. In mouse, the phosphorylation does not affect the ability of FMRP to associate with RNA (Ceman et al. 2003) which was not the case observed in drosophila (Siomi et al. 2002).

Under steady-state conditions, the phosphorylation status of FMRP does not seem to play a role neither in the subcellular localization of FMRP nor in its association to polysomes

(Ceman et al. 2003). However, ribosome run-off experiments performed by this group showed that unphosphorylated FMRP (associated with actively translating polysomes) runs-off more quickly than phosphorylated FMRP, which appears resistant to run-off and may be associated with stalled polyribosomes.

Recently, it was shown that FMRP interacts with MSP58, an RNA binding protein, through its phosphorylation domain. The RNA binding characterization of MSP58 is the subject of publication 3.

4.1.4. The RNA-binding domains

4.1.4.1. A glance at the N-terminus region of FMRP as an RNA binding domain

Structural definition of the N-terminus domain established by Adinolfi and colleagues, showed an amino acids distribution typical of globular domains. A helical predominance, as a secondary structure element was predicted by multiple alignment suggesting that the first 217 amino acids of the protein should be able to fold, either alone, or with other region of the protein, into a globular domain. An α-helix structure was revealed by Circular Dichroism analysis but the three dimensional globular fold was not demonstrated.

The N-terminus region showed an affinity for RNA homopolymers at physiological salt concentrations, but this binding was completely abolished at high salt concentrations where only specific interactions occur due to high ionic strength. In the absence of any specific mRNA sequence bound by FMRP, the authors used the RNA homopolymers. They suggested that the disability of the N-terminus region to bind these stretches at high salt concentrations is only because they are recognized and bound to the protein fragment by electrostatic forces which are completely destabilized at high salt concentrations (Adinolfi et al. 1999). The authors did not define the motif responsible for this interaction nevertheless, they hypothesised that both the RNA binding site would act in competition with the NLS and that in the nucleus, the protein would bind to a mRNA (the specificity of this interaction is conferred by other regions of the protein) and to some other proteins. Once the complex is formed, the NLS is therefore masked and the complex is exported to the cytoplasm.

Four years later, the same group developed an extensive biochemichal and biophysical characterization of the N-terminus of FMRP and showed that the NDF is not only able to interact with NUFIP1 and 82-FIP but to homodimerize as well as to bind RNA

homopolymers even at high salt concentrations. The authors demonstrated that the NDF binds with high affinity to polyU and polyA homopolymers and that this affinity decreases progressively with the length of the fragment. Furthermore they showed different sequence specificities for the homoplymers when using different fragments of the N-terminus region suggesting that different binding sites could exist along the N-terminus and act cooperatively for the binding to RNAs (Adinolfi et al. 2003).

4.1.4.2. The KH homology domains of FMRP

The fascinating diversity of functions accomplished by FMRP as an RNA binding protein would suggest a large diversity in the structures that are responsible for the RNA recognition. However, like other RNA binding proteins, FMRP contains "multiple copies" of RNA binding domains in a various structural arrangements expanding its specificity while fishing its target mRNAs. The "multiple copies" of these modules allow any protein to bind RNA with high affinity and specificity than would be possible with individual domains which often binds RNAs with a weak affinity. The advantage of having multiple copies of the same domain as an interaction surface will result in high affinity and specificity by combining multiple weak interactions permitting a very dynamic assembly/disassembly of the protein/RNA complex when needed.

In addition to the tudor domains, FMRP also contains 2 KH domains spanning amino acids 216-404. Both of them present the characteristics of KH domains type I.

The heterogeneous nuclear (hn)RNP K-homology domain (KH domain) is around 70 amino acids with a functionally important signature sequence of (I/L/V)IGXXGXX(I/L/V) mostly located in the centre of the domain. A three stranded β-sheet packed against three α helices is the most common feature of all known KH domains.

KH are divided in two distinct types : type I KH domains fold in a βααββα topology with an antiparallel β-sheet that features β3 as the central strand , while type II domains have a αββααβ topology and a β-sheet in which β2 is the central strand that is parallel to β3 and antiparallel to β1. The two consecutive a-helices are connected by the so-called 'GXXG loop', which is part of the conserved sequence motif.. Four nucleotides are recognized in a cleft that is formed by the GXXG loop, the flanking helices, the β-strand that follows a2 (type I) or a3 (type II) and the variable loop between β2 and β3 (type I) or between a2 and β2 (type

II). This binding platform is free of aromatic amino acids; recognition is achieved instead by hydrogen bonding, electrostatic interactions and shape complementarity.

The crucial role(s) that the KH domains may play in FMRP functions were widely debated after the I304N mutation was reported. Indeed, while in almost all FXS patients FMRP is not detected, one patient presenting severe symptoms appeared to have a mutation in the KH2 domain were an Isoleucine in position 304 is replaced by an Asparagine (De Boulle et al. 1993). The effect of this mutation on FMRP function is dramatic, partial or complete abolishment of its RNA binding properties were reported (Siomi et al. 1994; Brown et al. 1998), nevertheless this property was very slightly affected when the binding of the mutant protein to EF-1A mRNA was analyzed (Sung et al. 2003). Its association to polyribosomes was also altered (Feng et al. 1997) and its presumed role in translation regulation of its target mRNAs was completely abolished (Laggerbauer et al. 2001). Same observations were reported in KH mutants in drosophila (Banerjee et al. 2007). In the absence of any three-dimensional structure, it had been suggested that the mutation would disrupt the folding of the domain. This hypothesis was issued from comparison with the a similar mutation in the KH domains of vigilin and Nova resulting in an unfolded domain (for review(Musco et al. 1996)). Another hypothesis was also given for the direct involvement of the mutated residue in RNA binding.

The mystery of this scavenging mutation was brought to light by Valverde and colleagues who provided for the first time the structure of the KH1-KH2 domain (Figure 9). Two major points were revealed by the authors. First they demonstrated that the KH1-KH2 domain is a monomer in solution by both gel filtration chromatography and sedimentation equilibrium ultracentrifugation measurements. Second, they showed that the Isoleucine 304 forms part of an extensive network of hydrophobic residues stabilizing the a1, a2 and a' on the b sheet of the KH2. This position is completely inaccessible, thus demonstrating that the Ile304 doesn't participate in direct contact with RNAs and the fact that it resides in a hydrophobic core, its substitution with an Asparagin would disrupt the core and destabilize the protein. In sum, this mutation causes a decrease in both the secondary structure of FMRP and its stability (Valverde et al. 2007).

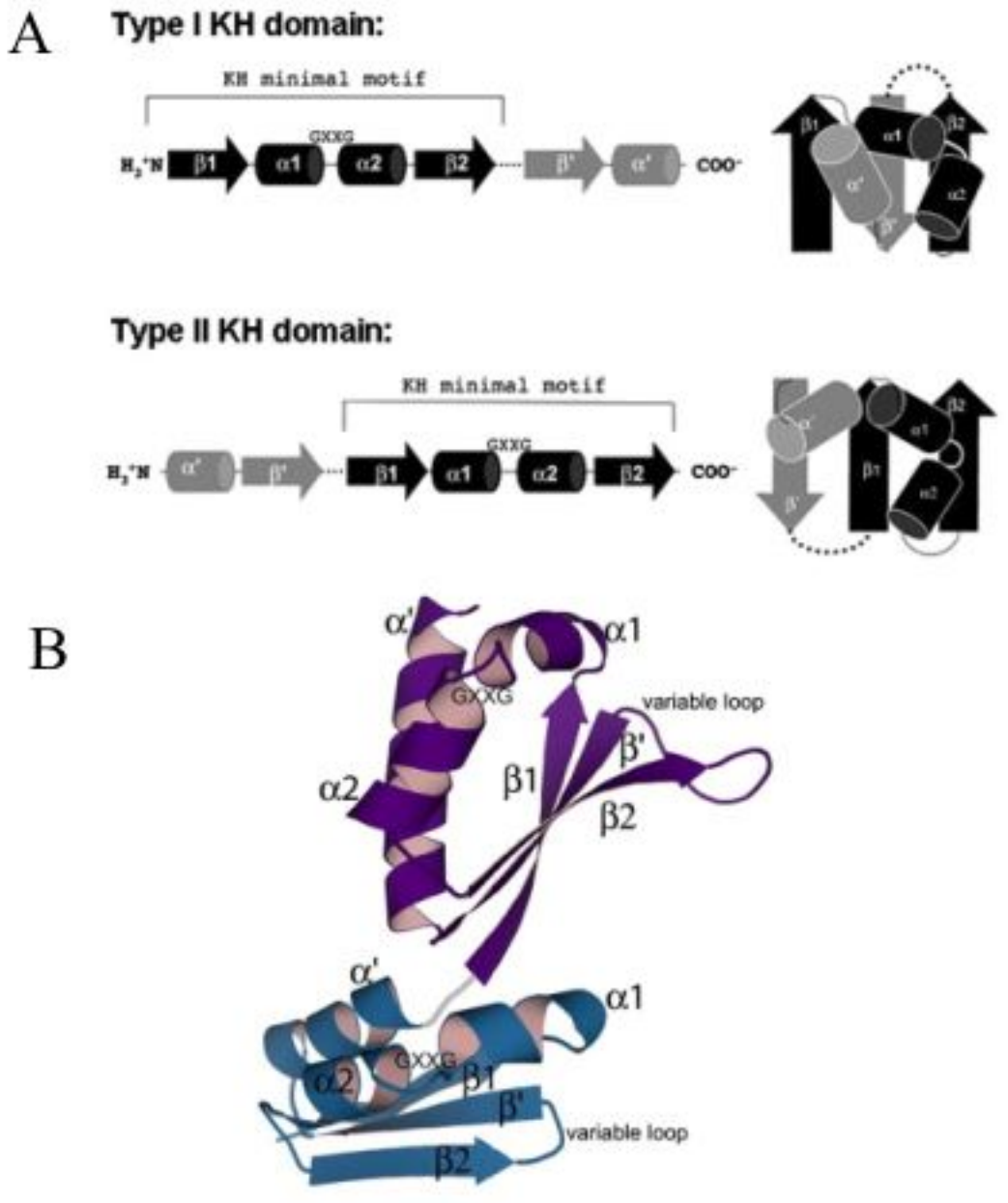

Figure 9 : KH fold and structure

A- Representation of type I and type II KH domains.

B- Crystal structure of hFMRP KH1-KH2. KH1 is blue whereas KH2 is in purple.

(Adapted from Valverde et al., 2007)

4.1.4.3. The RGG box : a real "hotspot" box trapping specifically mRNAs.

Despite their low homology degree in the C-terminus region, the three FXR family members contain clusters of Glycin and Arginin named RGG box which was first identified in hnRNPU protein. Many other RNA binding proteins were than shown to contain this motif (Kiledjian and Dreyfuss 1992). The Glycin-Arginin rich sequence is able to confer to its containing protein, the ability to bind RNA. It was also predicted that the interaction with the RNA occurs through electrostatic forces in an unspecific manner. The RGG box of FMRP spans residues 527 to 558, this fragment of the protein was shown to be unstructured when produced alone (Ramos et al. 2003), however it was used for *in vitro* binding assays and showed to be able to bind an RNA structure called "G-quartet"(Darnell et al. 2001). Biophysical analysis revealed that G-quartets are required but not sufficient for FMRP RGG box binding and that this interaction, when it occurs, should act on stabilizing the G-quartet structure (Zanotti et al. 2006). This domain was also shown to be necessary for driving FMRP to granules and its RNP complex assembly (Mazroui et al. 2002). The authors used truncated fragments of the protein and the question of the "intact" global folding of FMRP in the absence of some domains was not verified. The interaction RGG-G-quartet will be discussed later and was the aim of publication 2.

4.2. Protein interactors

4.2.1. FMRP is able to homo and hetero-dimerize

The ability of exon 7 to confer a platform for homo/heterodimerization was clarified by Adinolfi and colleagues. The authors used biophysical coupled to structural techniques claiming that previous studies were carried out with deletion mutants of the N-terminus region of FMRP based on exon boundaries without any structural indications. The major risk with such approach is that a negative result does not reflect necessarily the direct involvement of a region in the interaction but the loss of a region structurally essential. The authors suggested that NDF strongly mediates the dimerization and that it remains possible that other dimerization sites can cooperatively determine the dimer stability of the full-length protein. One candidate to such hypothesis is the helix-loop-helix encoded by exon 7 that could pack

either against other proteins or against distal regions of FMRP such as the KH domains(Adinolfi et al. 2003). Only a three dimensional structure of the full length protein would enlighten the potential of either hypothesis to fine tune the dimerization of the protein.

FMRP together with FXR1P and FXR2P share a high sequence homology degree that can also be reflected in their functional domains. The RNA binding domains especially the KH and the Tudor domains are very similar in all of the three proteins. Their ability to shuttle between the nucleus and the cytoplasm and their belonging to several RNPs especially the polyribosomes and their involvement in the translational control was extensively analyzed. By comparison, It was suggested that these proteins may have a similar or redundant function and that they could to some extent compensate the absence of FMRP (Figure 10), therefore explaining the heterogeneity of symptoms in FXS patients. Molecular function of FXR2P is still poorly investigated, nevertheless FXR1P started to arise attention.

4.2.1.1. FXR1P and FMRP : similar but so different.

FXR1P is widely expressed in all cell lines and mouse tissues analyzed. Seven distinct protein isoforms were detected among which, the two longest 82 and 84 KDa (Isoe and Isof) are only expressed in skeletal muscle and heart (Bakker et al. 2000; Dubé et al. 2000). FXR1P Isoe and Isof expressed at low level are sequestered in the nuclei of undifferentiated myoblasts, whereas they are associated with cytoplasmic mRNPs in myotubes. Moreover, accumulation of FXR1P Isoe and Isof coincides with expression of different myogenic markers (Dubé et al. 2000).

In testis, FXR1P showed a restricted expression in A-type spermatogonia in young animals (6 days). At day 14, an increased presence of FXR1P was observed in primary spermatocytes at early pachytene stage, whereas at day 20 a positive cytoplasmic signal was observed in almost all cells ranging from pachytene to round spermatids. In adult mice no staining was observed in lumen devoid of mature spermatozoa. FXR1P is localized in tail of mature spermatozoa associated to microtubules (Huot et al. 2001). Interestingly, the isoforms present in spermatozoa tail are Isog and Isoe, also found in adult muscle.

Involvement of FXR1P in several processes was reported. It was suggested to be a target for autoimmune response in humans. Patients affected by scleroderma or progressive systemic sclerosis (PSS) showed autoimmune IgGs to FXR1P (Bolivar et al. 1998).

In addition, FXR1P has been reported to bind the AU rich element (ARE) and, through the interaction with this element, to regulate the expression of the proinflammatory cytokine

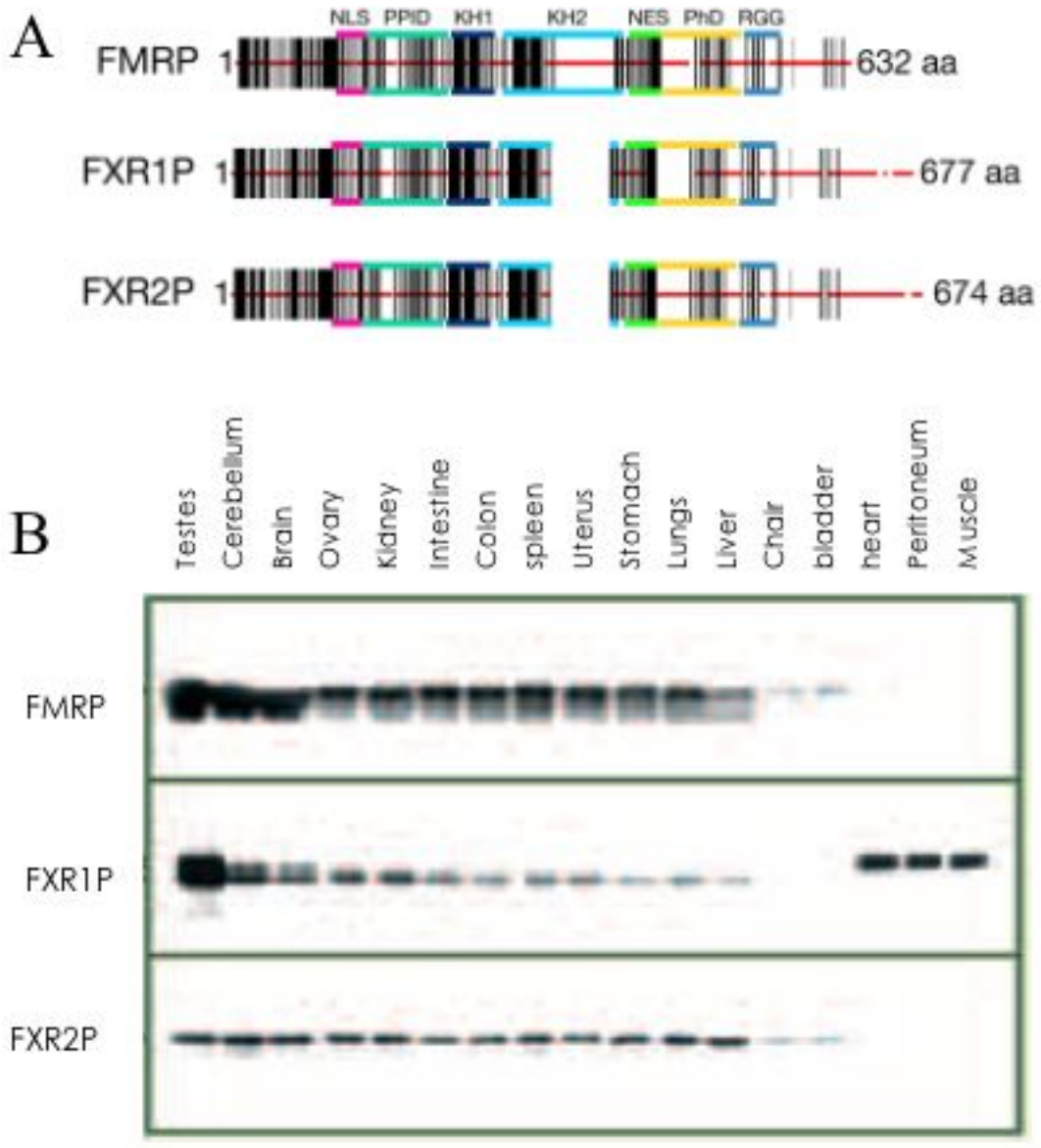

Figure 10 : Structural comparison of the FXR family members.

A- Sequence and functional domains homology are represented by vertical and horizontal lines respectively.

B- Tissue expression of FXR proteins in adult mice analyzed by western blot.

(Adapted from Davidovic et al., 2005)

tumor necrosis factor (TNFα) RNA in macrophages (Garnon et al. 2005). Starting from this observation, Vasudevan and Steitz have shown that the TNFα ARE upregulates translation in response to cell-cycle arrest in HEK293 cells and in THP-1 monocytes when induced by serum starvation or other treatment. Since this upregulation appears to have a physiological relevance because the cell-cycle accompanies the differentiation of monocytes into macrophages in vivo, the authors identified different ARE-associated proteins. They found that FXR1P and AGO2 associate with TNFα ARE and function as translation activators in response to serum starvation. Indeed the mechanism of translation activation by the FXR1P/AGO2 ARE complex seems to involve the recruitment of the TNFα mRNP to heavy polyribosomes upon serum starvation (Vasudevan and Steitz 2007).

Furthermore, they extended their analysis to better decipher the mechanism that leads to translation activation. They found that base-paring of the microRNA miR369-3 with two UAUUAUU sequences contained in the TNFα ARE may be at the basis of recruitment of microRNP that may mediate translational activation in G1/G0 phases of cell cycle (Vasudevan et al. 2007). On another hand, FXR1 was reported to be one of the most frequently overexpressed genes in the center of the amplified chromosomal domain in squamous cell carcinomas together with CLAPM1 and EIF4G (Comtesse et al. 2007).

Specific function of FXR1P in brain is still unknown, however, some emerging hypothesis about its key role in muscle development start to elucidate its molecular properties. Huot and colleagues used a Xenopus model and by microinjecting morpholino oligonucleotides targeting the *xFxr1* sequence, they were able to show that MyoD expression is disrupted, somitic myotomal cell rotation and segmentation are inhibited and an abnormal dermatome formation leading to a dramatic muscle specific effect during embryogenesis. Moreover, using microarray analyses, the authors demonstrated that genes whose expressions are altered by the inactivation of *xFxr1* are mostly involved in development of nervous system and myogenesis (Huot et al. 2005).

A better understanding of the molecular functional analogy between FMRP and FXR1P was the aim of publication 2 and will be discussed later.

4.2.1.2. FXR2P and FMRP : back to back or separate ?

Little is known about the function of FXR2P, its RNA binding properties were never investigated despite the high homology that it shares with FMRP in the functional domains. However, a pattern of expression was established form both human and mouse tissue sections.

FXR2P is expressed in organs affected by the FXS and the pattern of tissue distribution is highly similar with slight differences between the three members. A clear cytoplasmic expression of FXR2P in the Purkinje, cortex and brain stem neurons is found. However a stronger labelling of the FXR proteins was seen in the proximal dendrites of cortex and brain stem neurons. This distribution was not affected in the absence of FMRP in brain tissue section of FXS patients. In adult testes, FX2P is expressed in early spermatogonia and its expression is also detected at high levels in maturing spermatogenic cells (Bakker et al. 2000).

4.2.2. FMRP interacts with the nucleocytoplasmic RNA binding proteins NUFIP1 and 82-FIP

4.2.2.1. NUFIP (Nuclear FMRP Interacting Protein)

NUFIP1 is a 495 amino acid protein containing a C_2H_2 zinc finger motif. This motif is a folded domain that contain conserved cysteines and histidines coordinated to zinc. Proteins with such motifs can bind to either DNA or RNA basically by the same mechanism of recognition. Generally, A single C_2H_2 zinc finger, composed of a β-hairpin and an α-helix held together by a tetrahedrally coordinated zinc ion, will span a DNA or RNA sequence of three or four consecutive base pairs. Frequently, the contacts are made by the side chains of amino acids located at positions -1, +2, +3 and +6 of the α-helix.

NUFIP1 is able to bind RNA probably via another unknown motif. Mutations in the zinc finger domain of the mouse NUFIP1 did not alter its binding to RNA homopolymers *in vitro*. It possesses a NLS and a Nuclear Export Signal (NES) that allows it to shuttle between the nucleus and the cytoplasm where it is associated to polyribosomes and colocalizes with ribosomes in active synaptoneurosomes. In the nucleus, NUFIP1 is found in a micropunctuate pattern where it colocalizes with FMRP's nuclear isoforms. NUFIP1 is expressed in neurons of the cortex, the hippocampus and the cerebellum suggesting that NUFIP1/FMRP interaction is relevant for neuronal function *in vivo*. NUFIP1 interacts specifically with FMRP but doesn't reveal any interaction with neither FXR1P nor FXR2P despite the high homology degree that the FXR proteins share. Co-transfection experiments of both NUFIP1 and FMRP ISO12 modifies the distribution of the latter in a dot-like nuclear structure suggesting that NUFIP1/FMRP interaction in the nucleus may target the latter to specific subdomains relevant for its putative nuclear function (Bardoni et al. 1999).

NUFIP1 was previously reported to show a specific NUFIP1/BCRA1 interaction that stimulates specifically the activator-independent pol II transcription in the nucleus (Cabart et al. 2004). More recently, NUFIP was characterized as one of the four novel box C/D snoRNP biogenesis factors : BCD1, NOP17, NUFIP and TAF that are associated with U3 and U8 pre-snoRNP complexes and involved in their biogenesis. In the nucleolus, the eukaryotic rRNAs are cotranscribed as a large precursor RNA (pre-rRNA) which will be subjected to a complex series of processing and modification steps to generate the mature rRNAs. Small nucleolar RNAs (snoRNAs) are an evolutionarily conserved group of noncoding RNAs involved in the modification and processing of rRNAs. Two classes of snoRNA have been defined; the H/ACA and box C/D snoRNAs. NUFIP interacts with all four of the core box C/D snoRNP proteins and play a crucial role in the early stages of pre-snoRNP assembly, possibly in the initial recruitment of the box C/D core proteins. One of the proposed mechanism is that these biogenesis factors may form an extensive and potentially repeating scaffold around which the pre-snoRNP is assembled (McKeegan et al. 2007)

Taken together, all these observations may suggest that NUFIP1 may shuttle between different complexes linking transcription, biogenesis and assembly of snoRNPs and mRNA export from the nucleus.

4.2.2.2. 82-FIP (82 KDa FMRP Interacting Protein)

82-FIP is a 695 amino acids protein, its sequence shows no homology to any protein of known function. In neurons of the cortex, 82-FIP is distributed in both nucleus and cytoplasm, colocalizing with FMRP. Nevertheless, it shows a specific cytoplasmic pattern in neurons of the dentate gyrus, in the olfactive bulb, in the ependymal epithelium and in the granular layer of the cerebellum. 82-FIP is an RNA binding protein with high affinity for A-homopolymers, it binds specifically to FMRP but shows no affinity to neither FXR1P nor FXR2P. In the cytoplasm, it is associated to polyribosomes and this association is not altered in the absence of FMRP. Surprisingly, the distribution of 82-FIP is cell cycle dependant, being mostly cytoplasmic in the G2/M phase and strictly nuclear in G1 phase. These observations suggested the highly dynamics of FMRP containing mRNPs could be modulated by the cell cycle in growing cells (Bardoni et al. 2003a).

Both NUFIP1 and 82-FIP binds to FMRP via its NDF, partially overlapping the NLS. This would suggest, that FMRP is either driven to the nucleus with one or both of them or that it remains in the cytoplasm due to the interaction that might mask its NLS.

4.2.3. FMRP interacts with the cytoskeleton-linked proteins CYFIP1 and CYFIP2

4.2.3.1. Cytoplasmic FMRP Interactor Proteins

Identified by yeast two hybrid system, CYFIP1 and its homologue CYFIP2 showed a high homology degree (88%). Highly conserved among species, their sequences didn't match with any known functional motifs. In the cytoplasm, their pattern expression completely overlaps with that of FMRP and colocalize with the latter on ribosomes. They were also observed in dendrites. Both of them exhibited a direct interaction with FMRP via the exon 7 previously described as the dimerization motif, suggesting a role for CYFIP1/2 in the modulation of homo and heterodimerization and therefore affecting the RNA binding properties of FMRP, FXR1P and FXR2P. They didn't show any affinity for RNAs, however both proteins are linked with Wave complex involved in the regulation of actin nucleation via Rac1 (Schenck et al. 2001). Indeed, CYFIP2 interacts with FXR1P and FXR2P from one side and with WAVE-1 (member of Wiskott-aldrich syndrom protein) from another, this protein family is involved in connecting stimulatory signals to actin cytoskeletal organization. Whereas CYFIP1 interacts only with FMRP and show no affinity for neither FXR1P nor FXR2P, it also interacts with RhoGTPase Rac1 (component of RhoGTPases involved in Mental Retardation). The Drosophila CYFIP was shown to play a pivotal role in establishment of neuronal connectivity, defining interaction between Rac1 and FMRP (Schenck et al. 2003).

4.2.4. Other Interactors

Many RNA binding proteins were found to interact with FMRP. Beside those already discussed above, FMRP was shown to interact with IMP1, dicer and eIF2C2, and to be part of a large mRNP complex containing nucleolin, YB1/p50, purα, and Staufen. These interactions were only demonstrated by immunoprecipitation analysis and a direct interaction between FMRP and those partners still needs to be clarified. All these interactors would link FMRP to several pathways involved in mRNA metabolism.

4.3. FMRP RNA targets : fishing for specificity

4.3.1. FMRP and mRNA : Identification of *in vivo* putative targets

How does FMRP bind to its target mRNAs *in vivo* ? Does it act alone or assisted by its partners? What might be the fate of its ligands?

A critical step in the understanding of FMRP function is first, the identification of its mRNA targets and second, the characterization of the functional significance of each validated target. This would give new insight in global mechanism of mRNA translation regulation mediated by FMRP.

In order to identify "global" FMRP-related mechanisms of mRNA recognition, different methodologies have been developed and applied by several groups. Starting from mouse brains, a large screen with microarrays analysis was used by Brown and colleagues who were able to identify 432 mouse mRNAs co-immunoprecipitated with FMRP-RNP complex. In the same study, they analyzed polyribosomal fractions obtained from human lymhoblasts. They obtained 251 mRNAs differentially distributed along the polyribosomes in patients and normal individuals (Brown et al. 2001). Some mRNAs with an important neuronal function were identified, such as MAP1B, Semaphorin. Only eight of the idendified mRNAs were predicted to contain a G-quartet structure (Darnell et al. 2001).

Miyashiro and colleagues had developed an in situ approach, APRA (Antibody Positioned RNA Amplification) to identify direct mRNA binding to FMRP/ FMRP-associated RNP particles. 81 mRNAs were identified by this study and 60% were shown to be directly associated to FMRP. A subset of these mRNAs displayed slight changes in either expression levels and/or subcellular localization in *Fmr1* knockout mice brains. Important neuronal functions such as cytoskeleton structure and function, synaptic signaling and nuclear trafficking were the most relevant functions of proteins encoded by these mRNAs (Miyashiro et al. 2003). The Sod1 (Super Oxide dismutase 1), one of the identified mRNAs by this study, was analyzed in detail and is the subject of publication 1.

The weak overlap between the mRNA populations identified in both studies is mostly due to the microarrays where only a small overlap of probes was present and the distinct experimental procedures used by both groups.

A huge amount of data about putative FMRP mRNA targets was delivered but only a few specific interactions were analyzed and the functional significance of FMRP-mRNA interaction is still poorly investigated. In the next part I will overview the approaches used by several groups to analyze FMRP targets.

4.3.1.1. Targets (if any!) of the N-terminus

BC1 (Brain Cytoplasmic RNA 1) and BC200 (Brain Cytoplasmic RNA 200-nt) RNAs are small non coding RNAs (ncRNAs) transcribed by RNA polymerase III. Most ncRNAs associate with proteins and contribute to the assembly of RNP complexes. BC1 and BC200 share little sequence homology but predicted to form similar secondary structure (Figure 11). Both of these cytoplasmic RNAs are specifically expressed in neurons and are actively transported to dendrites (Tiedge et al. 1993a; Tiedge et al. 1993b). In cultured hippocampal neurons, the expression of BC1 is first detected at the onset of synaptogenesis, and the expression level is reversibly regulated by neuronal activity (Muslimov et al. 1998). Owing to their evolutionary origins, BC1 and BC200 have been subject to speculation that they participate in the regulation of protein synthesis in neuronal dendrites because BC1 arose from a tRNA and BC200 represents the Alu domain of SRP RNA, the domain involved in translation arrest. Both RNAs interacted with poly(A)-binding protein, a regulator of translation initiation. BC1 affected translation by inhibiting the formation of the pre-initiation complex, and the inhibitory effect was not restricted to particular mRNAs (Wang et al. 2002).

A very speculative model for RNA translation regulation by FMRP via its interaction with BC1 was proposed by Zalfa and colleagues. The authors used an extremely elegant analysis proving the direct interaction between FMRP and BC1 in both co-immunoprecipitation and band shift analysis at “high-stringent” salt concentrations (Zalfa et al. 2003). They claimed that BC1 binds to FMRP and associates with mRNAs thus bridging FMRP to mRNAs.

Two years later, the authors went further to analyze the specific interaction FMRP/BC1 and demonstrated that the N-terminus region of FMRP binds BC1/BC200 RNA. More precisely they delimited the interaction domain and sequence and concluded that the helix-loop-helix formed by the amino acids 181-214 binds to a stem loop formed by BC1/BC200 (Zalfa et al. 2005).

FMRP/BC1 interaction was controversially debated and many technical points achieved by Zalfa and colleagues were severely criticized.

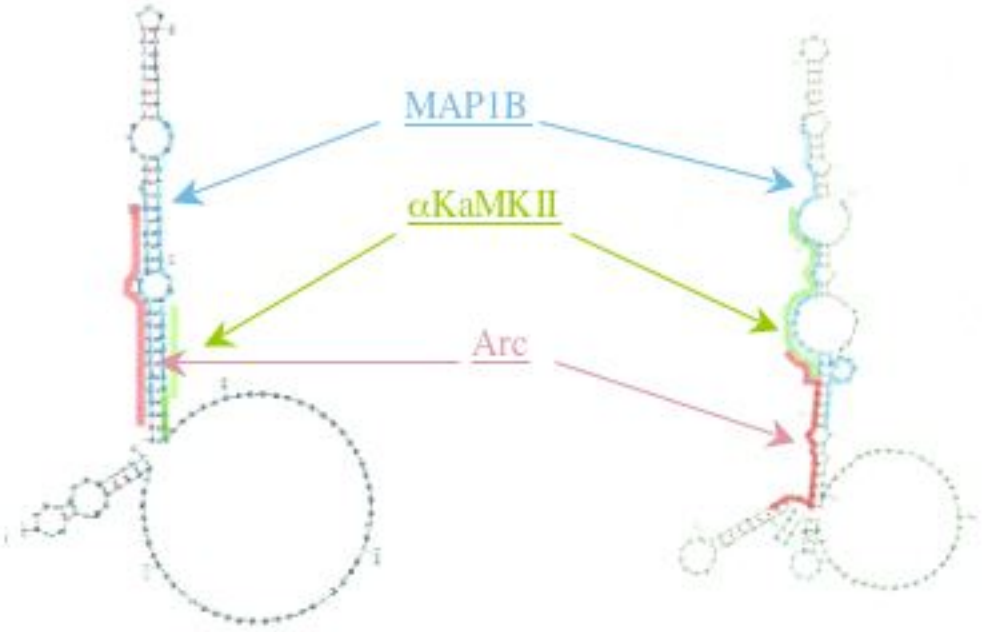

Figure 11 : Structure of BC1 and BC200

The secondary structure of BC1 (left) and BC200 (right) as predicted by the mfold program. Complementary regions to MAP1B (blue), αKaMKII (green) and Arc (pink) are represented in both RNAs.

The use of "non physiological conditions" together with a high molar excess of recombinant protein able to shift a small fraction of labelled BC1 in gel shift experiments. Recently, several laboratories have shown that any specific interaction between FMRP and BC1 could occur neither *in vitro* using gel shift and filter binding assays in presence of specific RNAs as competitors, nor *in vivo* proving that the "love story" FMRP/BC1 was an illusionary artefact (Iacoangeli et al. 2008).

In summary, the N-terminus domain of FMRP contains an RNA binding motif for whom no specific mRNA target was actually found.

4.3.1.2. KH2 and mRNAs : the Kissing complex, another genuine love story.

The clinical relevance of the I304N mutation had, to some extent, attributed to the KH2 domain a crucial role in FMRP functions. Up to date, any mRNA was shown to be specifically bound by this domain, however, using the SELEX technique, Darnell and colleagues provided a synthetic aptamer structure called the "Kissing complex" able to be recognized by the KH2 domain (Darnell et al. 2005). This kissing loop-loop interaction were first described in tRNAs and in most cases polycistronic messengers of diverse origins (retrovirus, yeast retrotransposon T*y*, several prokaryotes) are also affected. It occurs by the hybridyzation between two stem-loops which starts with the formation of base pairs between the complementary loop regions of two hairpins generating a kissing hairpin complex. They are known to be involved in protein expression and especially in the modulation of the translation rate of several messages (for review (Paillart et al. 1996)). Recently, Bindewald and colleagues have developed an algorithm predicting the putative formation of a kissing loop between two sequences (Bindewald et al. 2008). The kissing complex (Kc1) identified by Darnell and colleagues, is a 96 mer-RNA. It harbors two stem-loops, with a short single stranded region between them. Specifically recognized by the KH2 of FMRP when both stem-loops are present in the same molecule. The interaction KH2-Kc1 is coordinated by Mg^{2+} which is necessary for the folding and stabilization of the stem region and that pairing of loops occurs through 4 bases (Figure 12). The mutated KH2 domain (I304N) was not able to recognize the Kc1.

The I304N mutation was reported to disrupt the association of FMRP to polyribosomes, reflecting the importance of the KH2 domain in this phenomenon (Sung et al.

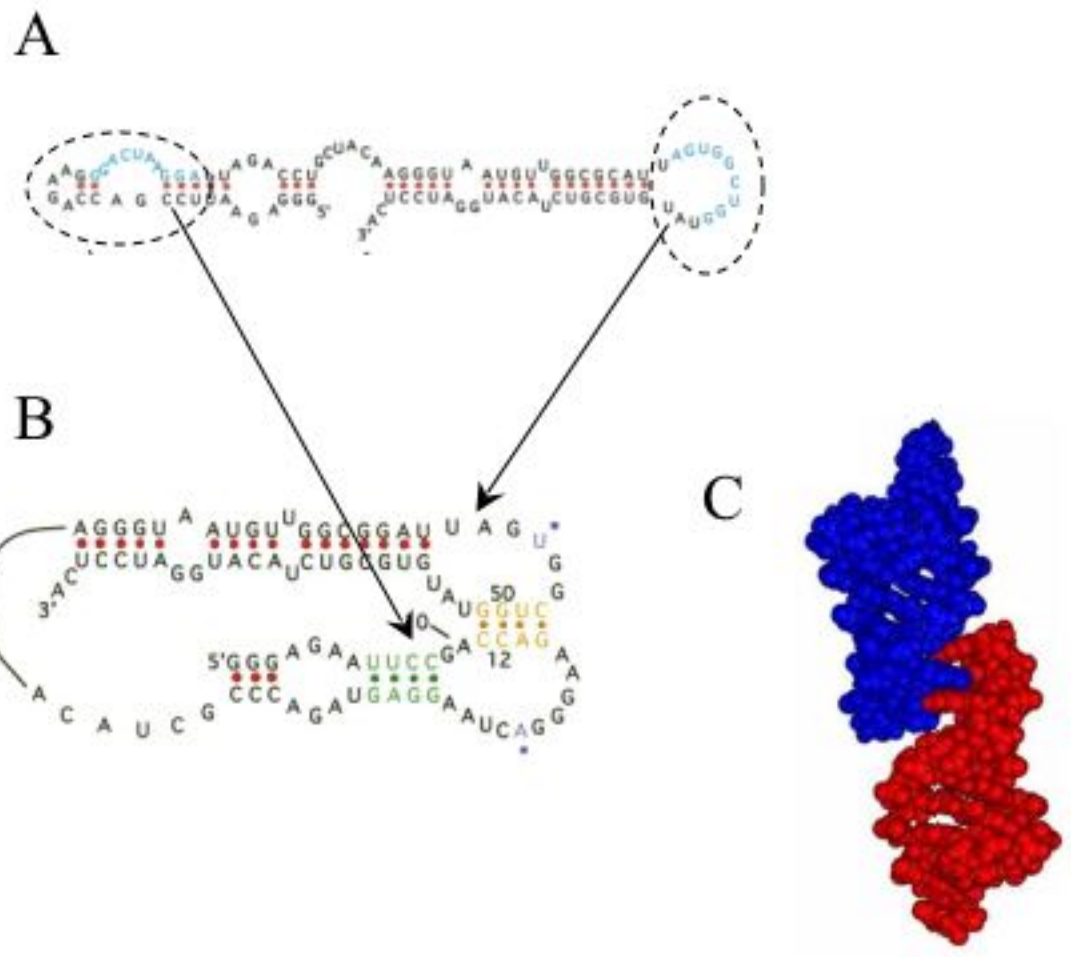

Figure 12 : Kissing complex structure

A- sequence and secondary structure of kc1 obtained by Darnell et al., 2005. The loop-loop interactions as suggested by chemical probing are represented in blue.

B- The predicted structure after loop-loop interaction of the kc1. Darnell et al., 2005

C- Tertiary structure formed by loop-loop interaction between identical hairpins. The conformation of such structure responds to mechanical forces that could render it more elastic or brittle

2003). Darnell and colleagues analyzed the effect of the kissing complex structure on FMRP association to polyribososmes. They incubated brain extracts with increasing amount of kissing complex and followed the distribution of FMRP on polyribosomes after ultracentrifugation. They were able to demonstrate that 100 nM of Kissing complex compete FMRP off the polyribosomes, leading to a general conclusion that such structures would mediate the association of FMRP to polyribosomes (Darnell et al. 2005). Still a nascent hope, using the algorithm developed by Bindewald and colleagues, to find *in vivo* kissing complex structures, analyze their physiological relevance of their interaction with FMRP and its putative involvement in the disease.

4.3.2. The G-quartets structure

4.3.2.1. The G-quartets : where are they? What do they do?

A very simplistic way to define these structures is:"G-quartets are nucleic acid structures in which four guanine residues are arranged in an planar conformation stabilized by Hoogsteen-type hydrogen bonds, several of theses structures can stack and be stabilized by different ions" (Keniry 2000). A number of techniques have been proposed to monitor G-quartet formation: NMR, crystallography, circular dichroism, Raman spectroscopy, gel electrophoresis, nuclease sensitivity, photocrosslinking, chemical probing, calorimetry and ultraviolet absorption. These structures are stabilized by the coordination of monovalent cations. G-quartets can be either intermolecular or intramolecular. There are several major differences between these two species. Intermolecular quartets involving four "independent" strands are usually parallel-stranded, and in many cases, all guanines are in the anti-conformation, they are stabilized by high Na^+ concentrations and form very slowly *in vitro*. Whereas, the intramolecular G-quartets fold rapidly adopting different conformations and stabilized by K^+ and completely destabilized by either Na^+ or Li^+. In cells, predominantly existing is the stable intramolecular structure due to high K^+ concentration (Figure 13).

Several bioinformatics programs were developed to search for sequences that may adopt a G-quartet structure (RNABOB and RNAMOT). Based on the use of a "canonical sequence": DDGG-N(0-2)-DDGG-N(0-2)-DDGG-N(0-2)-DDGG (D could be any nucleotide except C), such programs might be useful to some extent, nevertheless they do not take the purine quartets into account and predicting the presence of G-quartets needs to be confirmed experimentally.

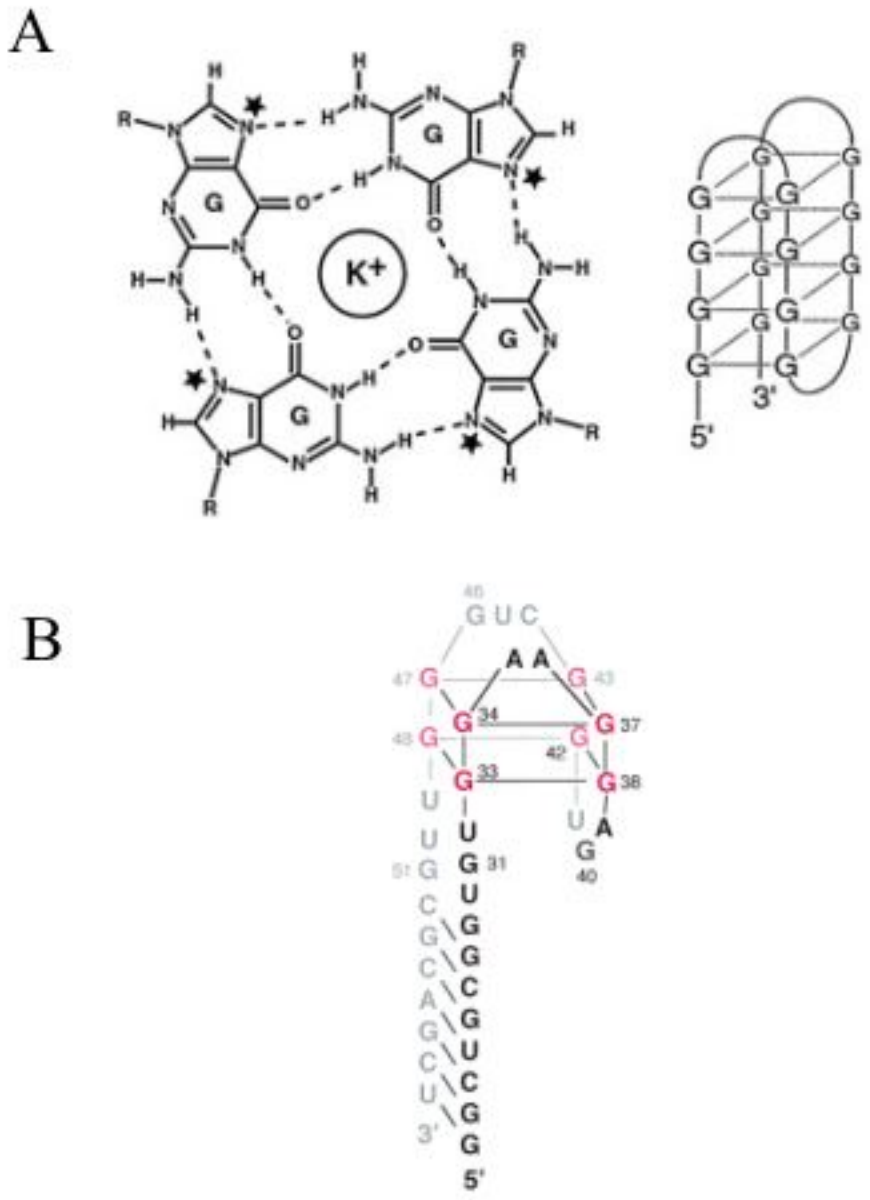

Figure 13 : G-quartet structure

A- Schematic representation of hydrogen bonds leading to the formation of Guanine tetrads stabilized by coordination of monovalent ion (K^+) adapted from Schaeffer et al., 2001

B- Structure of the Sc1 proposed to adopt a G-quartet structure (Darnell et al., 2001)

The major contribution of G-quartets at the DNA level is the stabilization of telomeres whereas several involvements of these structures were observed at the RNA level. They would affect the splicing simply by preventing the binding of splicing factors as observed in the case of human growth hormone (Cogan et al. 1997). Intermolecular G-quartets were also shown to inhibit the translation initiation of gene 2 of bacteriophage Fd (Oliver et al. 2000). Another aspect of translational control affected by the presence of G-quartets was observed in the fibroblast growth factor 2 FGF-2 where it mediates an IRES dependant translation that generates four FGF-2 isoforms.

4.3.2.2. RGG-G-quartet interaction : the hidden facet of FMRP predilection

In 2001 , two different groups demonstrated for the first time a specific interaction between FMRP and a G-quartet structure (Darnell et al. 2001; Schaeffer et al. 2001). Nevertheless, little is known about the structural features that rule this interactions. The first group established, using the SELEX technique, an aptamer (SC1) forming G-quartet and bound by the RGG box (Figure 13).

The SC1 was shown to have only two G-tetrads and its binding to the RGG box may be assisted by hairpin structures formed by the surrounding nucleotides establishing necessary contacts to stabilize the complex structure (Darnell et al. 2001).

Meanwhile, the second group came out with the “physiological” mRNA containing a G-quartet and specifically bound by FMRP (Schaeffer et al. 2001). Based on previous observations assuming that FMRP is able to bind its own mRNA (Ashley et al. 1993; Ceman et al. 1999). Schaeffer and colleagues proceeded by deletion and ladder selection experiments to define the precise binding site within the 3.69 Kb FMR1 mRNA. They were able to isolate a fragment of 100 bases corresponding to nucleotides 1557-1658 in the 3’ terminal part of the FMR1 mRNA coding region. Using a vast repertoire of chemical and enzymes for probing experiments combined to reverse transcription assays in presence of different ions, they showed that this fragment is able to fold in a G-quartet structure bound by FMRP with high affinity (1 nM). Two major conclusions were drawn from these observations, the authors relocated the binding site of FMRP in the coding region of FMR1 mRNA and not in its 3’UTR as previously reported (Brown et al. 1998), the binding occurs “specifically” with an

affinity higher at least 10 folds than that observed by Brown and colleagues who performed their binding assays in the absence of specific competitors (Brown et al. 1998).

To conclude, Schaeffer and colleagues subtly provided firm proofs about experimental procedures for both the detection of a G-quartet structure and the RNA binding affinities of FMRP.

Only two mRNAs were analyzed in detail and showed a G-quartet fold in their structure , FMR1 (Schaeffer et al. 2001) and PP2A (Castets et al. 2005a). However several other mRNAs were reported as putative G-quartet containing mRNAs. These reports were based on binding assay experiments in presence of different cations, MAP1B (Darnell et al. 2001), Semaphorin 3F (Darnell et al. 2001; Menon and Mihailescu 2007), or prediction by alignments with "canonical motif" in the absence of any structural experimental assay, PSD-95 (Todd et al. 2003), APP (Westmark and Malter 2007).

4.3.2.3. Functional relevance of FMRP-G-quartet interaction : "up close and so tight !"

It is very tempting, at some points, to simply speculate: "FMRP binds tightly G-quartets, keeps them along its travel and releases them just to obey cell laws." A large debate of "how could FMRP regulate its G-quartet containing mRNAs?" persists. I will detail thereafter the mRNA "containing" G-quartet recognized by FMRP.

MAP1B

The MAP1B (Microtubule Associated Protein 1B), homologue of the drosophila *futsh* protein, plays a microtubule stabilizing role and it is required for dendritic and axonal development. Futsch mutant flies show defects in microtubule loop domains similar to those appearing in collapsing growth cones. MAP1B is a scaffold protein that interacts with several other proteins such as gigaxonin that links microtubules and intermediate filaments and is involved in giant axonal neuropathy (Roos et al. 2000). MAP1B mRNA was predicted to have a putative G-quartet present in its 5'UTR, bound by FMRP *in vitro* (Darnell et al. 2001), co-immunoprecipitated with FMRP and its distribution along the polyribosomes was altered in the absence of FMRP (Brown et al. 2001). A further analysis of this mRNA in brain neuron development was reported by Lu and colleagues. In this study the authors confirmed the "so-called" direct interaction FMRP-MAP1B mRNA by immunoprecipitation experiments and stipulated that FMRP exerted a negative translational control on this mRNA (Lu et al. 2004).

To my knowledge, any experimental data was delivered proving the presence of a G-quartet in the MAP1B mRNA, moreover, the detection of an mRNA by immunoprecipitating FMRP reflects its belonging to the FMRP-RNP complex and, in any case, its direct interaction with a any specific protein of this complex.

Zhang and colleagues had previously reported the link dFMR-Futsch-control of synaptic structure and function (Zhang et al. 2001) suggesting that this mechanism could be evolutionary conserved. However, giving a deeper alignment analysis among different species of the region predicted to form a G-quartet in the MAP1B, it is clearly obvious that this region is not conserved between MAP1B and Futsch. Thus, a direct interaction FMRP-MAP1B via a G-quartet, if any, should be investigated in a more detailed analysis.

PSD95

Another mRNA, PSD95 (Post Synaptic Density 95) was also among the mRNAs predicted to form a G-quartet structure in its 3'UTR (Todd et al. 2003). This observation was also provided by alignment comparisons with the "canonical sequence". Any direct interaction was analyzed until Zalfa and colleagues scrutinized the FMRP-PSD95 interaction and showed that PSD95 mRNA does not form a G-quartet structure, nevertheless it binds in a sequence specific manner to the C-terminus part of FMRP. They also demonstrated that FMRP-PSD95 interaction is not affected by the presence of high Lithium salt concentrations excluding definitely the presence of a G-quartet in this mRNA. In this study the authors revealed that FMRP would positively regulate the stability of this mRNA.(Zalfa et al. 2007). Any information about the specificity and the affinity of this interaction was reported by the authors. As discussed above, the C-terminus region of FMRP recognizes mRNAs most probably via a "fit" mechanism thus it is more convincing that it is able to recognize a "structure" rather than a sequence. Once again, a more "appropriate" analysis is of great interest to better understand the FMRP/PSD95 direct interaction, and a more "appropriate" experimental procedure is needed to prove or exclude the presence of G-quartet in PSD95 mRNA.

APP

The most intriguing case in the FMRP-G-quartet story was provided recently by Westmark and colleagues. The authors showed that FMRP binds the amyloid precursor protein (APP) mRNA and negatively regulates its translation (Westmark and Malter 2007). APP plays crucial role in synapse formation in developing brain and is at the basis of senile

plaques found in brain patients affected by Alzheimer disease. The authors used the alignment with the canonical sequence and showed a perfect match, predicting the presence of a putative G-quartet formed by the nucleotides in position 951 to 972 within the coding region of APP mRNA. Nevertheless, this motif did not seem to be recognized by FMRP as shown in Ribonucleases (T1) digestion assays. Strikingly, FMRP recognized, bound and protected a G-rich sequence just upstream the putative G-quartet (699-796). This interaction did not affect the stability of the APP mRNA as shown for PSD95. FMRP-APP interaction should be revisited with a sharper approach to better define both domains (protein and RNA) contributing to this interaction.

FMRP-PP2A mRNA interaction will be the aim of publication 4.

5. FMRP functions in translational control

5.1.1. FMRP shuttling among several mRNPs

5.1.1.1. FMRP and Polyribosomes : where is FMRP's throne ?

Immunofluorescence and immunogold labeling of endogenous FMRP observations revealed that, in the cytoplasm, FMRP is distributed in granular pattern and it colocalizes with ribosomal structure especially at the perinuclear rough endoplasmic reticulum (RER) (Khandjian et al. 1996; Feng et al. 1997; Mazroui et al. 2002). Subcellular fractionation on sucrose gradient allowed to demonstate that FMRP is associated to polyribosomes and that I304N mutation completely abolishes this association as previously discussed (Khandjian et al. 1996; Corbin et al. 1997; Feng et al. 1997).

In brain, Zalfa and colleagues showed that FMRP is not associated to polyribosomes (Zalfa et al. 2003). This observation was reviewed by biochemical experts who demonstrated that using ionic detergent (Deoxycholate) is able to shift FMRP from polysomal fractions (Khandjian et al. 2004; Stefani et al. 2004). It is widely accepted that FMRP is associated to polyribosomes in cells and brain.

5.1.1.2. FMRP and Stress granules (SG) : " drag off " and protect.

Interestingly, it has been shown that, in cultured fibroblasts, FMRP moves from polyribosomes into stress granules during application of stress such as heat shock or arsenite (oxidative stress) (Mazroui et al. 2002). Stress granules (SG) are cytpoplasmic microdomains where the housekeeping mRNAs are sequestered. They contain two specific RNA-binding proteins, T-cell intracellular antigen 1 (TIA-1) and TIA-1-related protein (TIAR), that are not present in polyribosomes(Kedersha et al. 1999). Kim and colleagues showed that FMRP shifts into stress granules in response to oxidative stress, using arsenite to treat hippocampal tissue and to *in vivo* electrode insertion.

The authors showed that arsenite causes a shift of FMRP in addition to the shift of abortive preinitiation complexes. They have also examined the change of FMRP level in the polyribosome and stress granule compartments *in vivo*, in response to insertion of an electrode into the hippocampus. They demonstrated a bidirectional shift of FMRP after electrophysiological stress (Kim et al. 2006) suggesting that translocation of FMRP and its associated mRNAs into SG could result in partial translational arrest during stress, whereas the movement back into polyribosomes could be a mechanism for restarting baseline translation during recovery.

5.1.1.3. FMRP –Staufen-RISC- P bodies : continuous reshaping and remodelling

FMRP is suggested to be component of granules that are in a state of dynamic flux, in activity- regulated equilibrium with the surrounding translational control granules. In drosophila, it has been shown that dFmr1 is part of the RISC complex (RNA-Induced Silencing Complex) where it interacts with both Argonaute 2 and Dicer proteins (Caudy et al. 2002; Ishizuka et al. 2002). In vertebrates, FMRP-RISC association still occurs, Jin and colleagues demonstrated that FMRP associates with both eIF2C2, the vertebrate orthologue of drosophila AGO1 and AGO2, and Dicer (Jin et al. 2004; Lugli et al. 2005). It will be important to analyze the specific interaction FMRP-AGO and determine if the association of FMRP occurs with AGO1 or AGO2 due to their distinct role in mRNA processing.

Moreover, Barbee and colleagues recently reported that, in drosophila, Staufen and dfmr1 colocalized extensively but not completely, indicating that dfmr1 and Staufen exist substantially in the same granules but can also be observed in separate yet related particles. This colocalization can be increased by overexpressing either protein. They also showed that,

Me31B localizes to dfmr1-containing RNPs especially at neurite branch points in cultured drosophila neurons and that it coimmunoprecipitates with dfmr1 from drosophila head extract. This physical interaction links FMRP to P bodies as Me31B homologs in yeast and mammals have been shown to function in P body formation in somatic cells. Me31B acts, at least in part, within neurons to promote translation repression and/or mRNA degradation in response to miRNAs (Barbee et al. 2006).

5.1.2. FMRP and translation : more than a decade of "repression"

The ability of FMRP to shuttle among different RNPs suggested that it plays a role in translation of its target mRNAs.

Li and colleagues used a rabbit reticulocyte lysate and showed that FMRP is able to repress a large subset of mRNAs (Li et al. 2001) whereas Laggerbauer and colleagues demonstrated that FMRP represses translation in both Xenopus oocytes and *in vitro*. This effect was not observed with the mutant I304N (Laggerbauer et al. 2001).

In drosophila, the translation of futsch mRNA (discussed above) was shown to be derepressed in the absence of dfmr1(Zhang et al. 2001). In parallel, MAP1B mRNA (see above) distribution on polyribosomes shifted towards more active translating particles in the absence of FMRP (Lu et al. 2004). Two different analysis using transient transfection system showed that FMRP acts also as a translational repressor on the eEF1A and SIX3 mRNAs (Mazroui et al. 2002; Sung et al. 2003). All these data support the hypothesis that FMRP is a negative translational regulator *in vitro* and *in vivo* but how can such a function be compatible with its predominant presence in so-called actively translating polyribosomes? Although many hypothesis involving FMRP had emerged such as "stalled polyribosomes" or translational control by miRNA, a direct link of FMRP in these processes should be more clarified.

A lack of interpretation of the huge amount of results obtained from several groups would lead to a global misunderstanding of FMRP functions. While, only the population of mRNAs that were shifted towards heavy polysomes in the absence of FMRP was considered as a solid clue strengthening the "translation repression" dogma of FMRP, the other population of mRNAs (that shifts towards light polysomes fractions in the absence of FMRP) in the same study was not considered. Therefore should this observation stipulate that FMRP

is exclusively a translational repressor ? In another hand, overexpressing FMRP in a cell system would reflect, in any case, the endogenous behavior of the protein. As discussed in the previous paragraph, FMRP shuttles among several dynamic mRNPs, disturbing the stocheometry composition of these particles would have a dramatic effect on their belonging and fate.

FMRP was recently reported to act on the stabilization of the PSD95 mRNA, by binding to an undefined structure (Zalfa et al. 2007).

6. Neuronal functions accomplished by FMRP : shaping dendritic spines

The only observed abnormalities observed in the brain of fragile X patients and *Fmr1* KO mice concern the neuronal dendrites, the branched projections of neurons that conduct the electrical stimulation received from synapses established with upstream neurons. It was shown that the brain cortex of fragile X patients display a higher density of dendritic spines which are the membrane protrusions emanating from a dendrite and forming one half of a synapse. In addition, even in adult FXS patients' brain, these surnumerary spines appear longer, thinner and more tortuous than normal, looking alike immature, not fully differentiated spines. It is thought that, in the developing brain of FXS patients, a suboptimal development and maturation of dendritic spines is at the basis of mental retardation. But how does FMRP control dendritic spines development at the molecular level?

6.1. What are dendritic spines ?

Dendritic spines constitute the post-synaptic compartment of most synapses. They are typically between 0.5-2 μm in length and occur at a density of 1-10 spines per μm of dendrite length on principal neurons. Their shape had been categorized as "mushroom", "thin" or "stubby" but a continuum exists between these categories as shown by electron microscopy. Functional mature spines present a bulbous head receiving a single excitatory synapse and a constricted neck serving as connection with the parent dendrite and inspecting diffusion of molecules to and from the parent dendritic shaft. These structures constitute microcompartments in which biochemical changes leading to specific pathways activation can occur upon distinct receptor activity.

They are highly heterogeneous structures, showing dynamic motility especially during development, where a growing body of evidences links spine shapes and sizes to different development stages and/or altered strength of synapses (Kasai H 2003; Hayashi Y 2005). Imaging experiments showed that the volume of spine heads increases with stimuli that strengthen the synapse and decreases with those that weaken the synapse leading to both conclusions that the number, size and shape of the spines are subjected to plastic remodelling correlated with modifications of the synaptic strength and interneuronal connectivity, and the key role of the spine neck as a diffusion barrier controlled by neuronal activity (Bloodgood BL 2005; Hayashi Y 2005).

It is important to note that changes in dendritic spine density and shape underlie many brain higher cognitive functions, such as motivation, learning, and memory. In particular, the development and maturation of new dendritic spines participates in the reinforcement of neuronal pathways. By strengthening the connection between two neurons, the ability of the presynaptic cell to activate the postsynaptic cell is enhanced. This type of synaptic regulation forms the basis of synaptic plasticity and is crucial for the establishment of an optimal brain neuronal network during development. Strong synaptic connections are provided by dendritic spines with large heads which are generally stable and express large numbers of (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (AMPARs), they are called "memory spines". Whereas, small head spines contribute to weak synaptic connections, are less stable and preferentially undergo long-term potentiation (LTP) so that may represent the "plasticity spines". These small thin spines are more susceptible to induction of LTP, due to the geometry of their neck that shows greater Ca^{2+} increases mediated by activation of the (N-methyl-D-aspartic acid) receptors (NMDARs). They contain few AMPAR. The activation of NMDARs, conferring morphological changes in spines, may contribute to activity-dependent formation and elimination of synaptic connections. LTP-inducing stimuli cause formation of new spines and enlargement of existing spines, whereas long-term depression (LTD)-inducing stimulation is associated with shrinkage and/or retraction of spines (K. Okamoto 2004; U.V. Nagerl 2004).

Spine morphogenesis is controlled by a complex network of regulatory proteins, including the Rho-family GTPases which orchestrate actin cytoskeleton rearrangement. In addition, a highly-specialized localised protein synthesis occurs in dendritic spines and that directly control synapse growth and dendritic plasticity. As we will see in the next paragraphs, FMRP plays a crucial role in these processes.

6.2. FMRP and localized synaptic translation

6.2.1. FMRP regulates localized synaptic translation

Most dendritic spines contain smooth endoplasmic reticulum, which in the largest spines takes the form of a specialized organelle called the "spine apparatus". In addition, polyribosomes and protein translational machinery are often anchored at the base of spines including initiation and elongation factors, poly(A) binding protein, some microRNAs and brain specific small RNAs. These observations lead different group to suggest a local ”synaptic” translation (Rao A 1991; Torre ER 1992; Weiler and Greenough 1993) {Steward, 2003 #1233}(Schratt et al. 2006) demonstrated in either synaptosomal fractions or in dendrites physically separated from their cell body. This local translation enables neurons to quickly mediate mRNA-specific postsynaptic responses to signalling events and is at the basis of the long-lasting form of synaptic plasticity. This hypothesis was proven by Ostroff and colleagues who demonstrated a relocalization of the polyribosomes from the dendritic shaft into the dendritic spines after induction of LTP (Ostroff LE 2002).

In particular, stimulation of the metabotropic glutamate receptor (mGluR1) stimulation initiates a phosphorylation cascade, triggering rapid association of some mRNAs with translation machinery near synapses, and leading to protein synthesis. *Fmr1,* associates with translational complexes after mGluR1 stimulation of synaptosomes, correlated with an increased expression of FMRP after mGluR1 stimulation. Finally, FMRP is associated with synaptic polyribosomal complexes (Weiler et al. 1997) (Feng et al. 1997)and *Fmr1* KO mice display a reduced number of post-synaptic polyribosomes aggregates (Weiler et al. 2004) pointing out its critical role in the regulation of local translation.

6.2.2. FMRP is involved in the trafficking of RNA granules towards the synapse

Local postsynaptic protein translation relies on the presence of mRNAs at the base of dendritic spines, that are transported along microtubules of the neuronal arborization by mRNP complexes called RNA granules. RNA granules transport mRNAs with RNA-binding proteins, translation factors and ribosomal subunits to the site where the translation occurs. Among the RNA-binding proteins encountered in RNA granules lies FMRP, together with several of its interacting proteins : FXR1P, FXR2P, MSP58. A FMRP-GFP fusion allowed to visualize the movement of FMRP containing granules in neuronal cell lines. This movement

appeared microtubule-dependant and bidirectional (De Diego Otero et al. 2002). Another study, aiming to reveal the importance of kinesin 5 in the transport of RNA granules, identified several proteins including FMRP, FXR1P, FXR2P, Purα, and Staufen and a number of mRNAs {Kanai, 2004 #780). Moreover, Miyashiro and colleagues observed some subtle differences in some RNAs localization in the absence of FMRP (Miyashiro et al. 2003). These observations suggested a role of FMRP in transport and localization of some mRNAs. More recently, Davidovic and colleagues established a direct interaction between FMRP and the neurospecific kinesin KIF3C suggesting that FMRP acts as a molecular adaptor between RNA granules, providing new insight into FMRP functions in the transport of mRNAs (Davidovic et al. 2007). Finally, in *Fmr1* knockout brain, a significant decrease in the amount of mRNA granules is observed relative to WT mice (Aschrafi et al. 2005). All these data point out the important role of FMRP in RNA granules formation and transport. (See Figure 14 for neuronal function of FMRP).

6.3. FMRP modulates synaptic actin-cytoskeleton

6.3.1. Via regulating translation of actin-linked mRNA

6.3.1.1. MAP1B

The link FMRP-microtubule-actin was assessed in drosophila with Futsch protein (discussed above) which reshapes cytoskeletal loops for the period of synaptic bouton division. MAP1B light chain binds actin stress fibers *in vivo* (Togel et al. 1998). Increased Futsch expression leads to an overall increase in growth, branching and number of these boutons in dfmr1 null fly, whereas an overexpression of FMRP leads to an opposite effect (Gao 2002) suggesting that synaptic structure and function is regulated by dfmr1 repression of futsch mRNA. Another regulation of the cytoskeleton was provided by Castets and colleagues, demonstrating the link FMRP-Rac1 *via* PP2A. This aspect will be discussed in publication 4.

6.3.2. Via an interaction with the Rac1 pathway mediated by CYFIP

Another mode of regulation was proposed by the link FMRP-CYFIP1-Rac-1. Rac-1, plays a crucial role in dendritic spine maturation and maintenance by regulating the actin cyroskeleton, is constitutively active in the absence of FMRP (Schenck et al. 2003; Tashiro

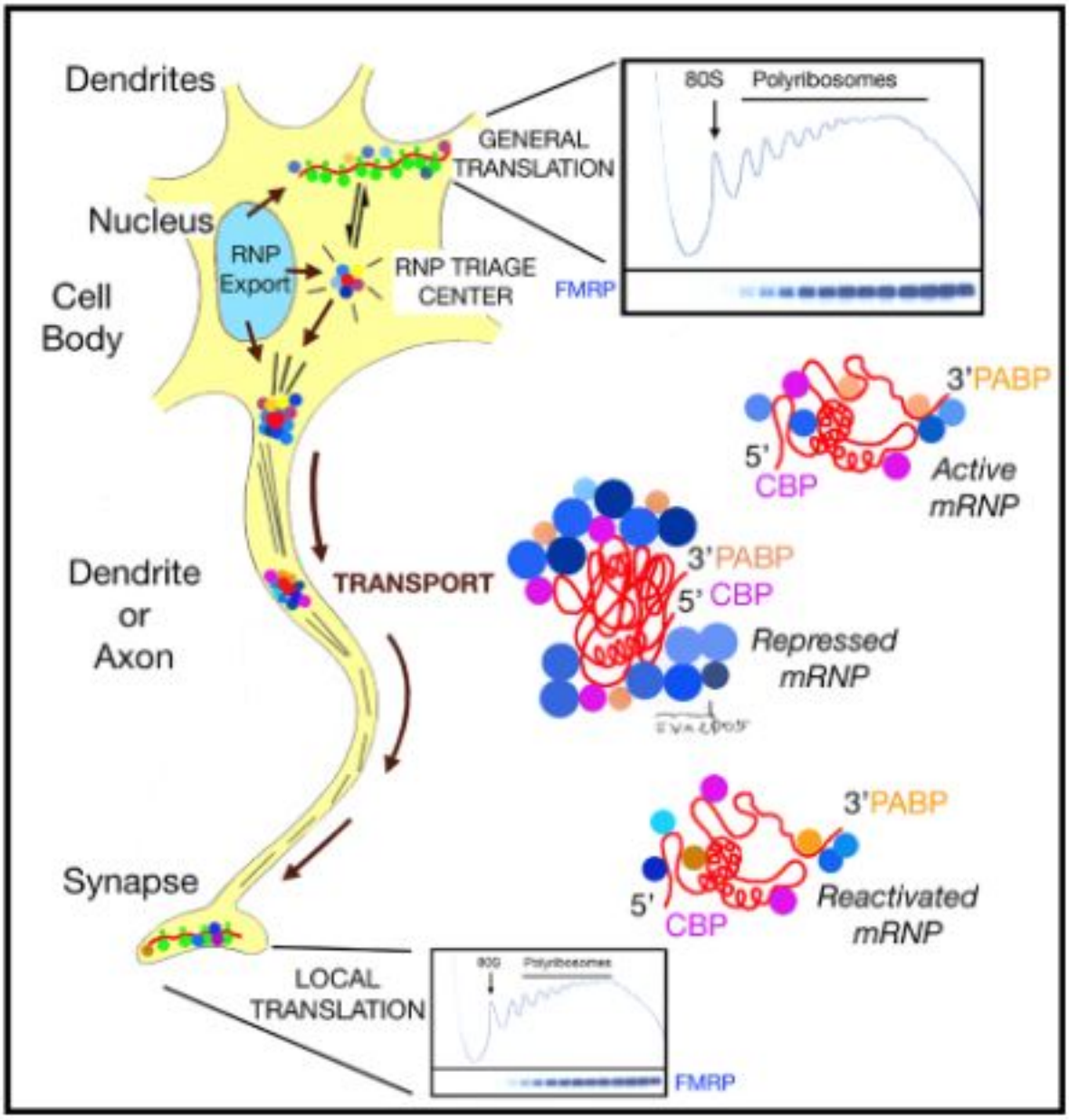

Figure 14 : Schematic model of FMRP neuronal functions representing especially the translational control and RNA trafficking by FMRP. (*Kindly provided by Davidovic., L and* Khandjian., E.W).

and Yuste 2004). Another confirmation for the involvement of FMRP in the regulation of actin cytoskeleton via Rac-1 pathway, was provided by Castets and colleagues (Castets et al. 2005a) and will be discussed in publication 4.

Furthermore, it has been shown that FMRP interacts with the human zipcode binding protein1 ortholog IMP1 (Rackham and Brown 2004) which binds to β-actin mRNA. Both proteins share the KH as RNA binding domain and are able to associate with each other independently of a bridging RNA. IMP1 binds to β-actin mRNA and associates with FMRP via protein-protein interaction, forming a complex and promoting formation of transport granules. If FMRP accomplishes its role as a translation repressor, when absent, the translation of β-actin would lower the efficiency of its dendritic localization, although net production would not be changed. The system used by the authors seemed to be very promising, nevertheless, it would have been of great interest to use an adequate cell model for such analysis.

Taken together, all these observations converge into the hypothesis of FMRP orchestrating the synaptic connections by modulating both the cytoskeleton and receptor activities. The absence of FMRP would lead to misregulated network of connections affecting both transport, localization, and translation of crucial mRNAs and resulting in abnormal spine morphologies that are the major “hallmark” in Fragile X patient brains.

Aim of my thesis

Understanding the molecular functions of FMRP, its ability to specifically bind mRNA targets and regulate their fate is a crucial step to better understand the Fagile X Syndrome. When I started my work, several groups had proceeded by a large screening looking for putative FMRP RNA targets. FMRP was shown to bind specifically its own mRNA *via* a G-quartet structure thus, the first question that raised :

Is FMRP able to recognize specifically any other mRNA structure ?

If so, what would be the functional significance of this interaction? And what would it enlighten in the physiopathology?

On another hand, FMRP has two other homologues FX1P and FXR2P with whom it interacts, since FXR1P and FXR2P were never correlated to any know disease, their molecular function was always suggested relying on the high homology degree that the three proteins share, and it is commonly hypothesized that FXR1P and FXR2P may compensate for the absence of FMRP. Here again, another question was crucial :

Do the three family members exhibit the same function, at least in their RNA binding properties ?

If a mechanism of compensation occurs, what could be the influence of either FXR1P or FXR2P on FMRP ?

Results

Publication 1

Several FMRP mRNA putative targets were described by many groups using a vast panel of experimental procedures, however, a specific interaction was shown for very few targets. As detailed in the introduction, Miyashiro and colleagues had developed a technique that allowed them to fish for *in vivo* FMRP targets. Based on their observations, we used several of their putative mRNA targets and screened them for the presence of a G-quartet

Aim : Among the provided list of putative FMRP mRNA targets, is there any novel mRNA structure bound specifically by FMRP ?

Procedure : To analyze the presence of any RNA harbouring a G-quartet structure, we used the binding assays in presence of potassium or sodium and to confirm the presence or the absence of a G-quartet we performed a reverse transcription assay in presence of either ion as described by Schaeffer and colleagues.
Once established, we therefore analyzed the functional significance of this interaction by determining the distribution of the mRNA on polyribosomes in the absence of FMRP and the effect that FMRP may exert on the translation of this mRNA.

Results : We were able to demonstrate that FMRP binds specifically to a 64 base fragment in the *Sod1* mRNA that folds in triple stem loops separated by short single strand region. FMRP/ *Sod1* mRNA interaction did not seem to affect the stability of the mRNA but it may regulate positively its translation.

Conclusion : The take home message from this study was FMRP binds specifically a novel RNA structure and depending on the structure features, FMRP might act as a negative or a positive regulator of translation.

FMRP positively modulates *SOD1* mRNA translation *via* SSLIP (Sod1 Stem Loops Interacting with FMRP), a novel RNA motif

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Abstract

*Background:*Fragile X syndrome, the most frequent form of inherited mental retardation, is due to silencing of *Fragile X Mental Retardation 1* (*FMR1)* gene, encoding FMRP (Fragile X Mental Retardation Protein). FMRP is an RNA-binding protein involved in several steps of RNA metabolism: nuclear export, trafficking in dendrites and axons, stabilization and translational control of a subset of mRNAs. Up to date, only two RNA motifs have been found to mediate FMRP/RNA interaction: the G-quartets and the «kissing complex». Interaction between FMRP and each of these RNA motifs induces translational repression via different mechanisms of action: prevention of ribosome scanning and retention of mRNAs in translationally inactive ribonucleoparticles, respectively. *Results:*We show here that *Super Oxide Dismutase 1* (*Sod1*) mRNA is specifically bound with high affinity by FMRP through a novel RNA motif, SSLIP (*Sod1* mRNA Stem Loop Interacting with FMRP), which is folded as three independent stem-loop structures. SSLIP/FMRP interaction improves the association of *Sod1* mRNA with polyribosomes, resulting in a decreased expression of Sod1 protein in *FMR1* null cells and brain. *Conclusion:* These data are pointing out that the RNA motif bound by FMRP determines its mechanism of action and FMRP can also positively regulates translation. In addition, we propose that the disregulation of Sod1 expression is at the basis of pathophysiology of the syndrome. Indeed, an increase of oxidative stress in brain of *FMR1* knock-out mice has been recently described and modifications of oxidative stress have been linked to anxiety, sleep troubles and autism: all phenotypic characters displayed by Fragile X patients.

Introduction

Fragile –X Mental Retardation Protein (FMRP) is an RNA binding protein whose absence causes the Fragile X Mental Retardation syndrome, the most frequent form of inherited mental retardation. In addition to mental retardation, the phenotype of patients includes facial dysmorphies, macroorchidism in post-pubertal boys, connective tissue dysplasia, hyperkinetic behavior, anxiety, sleep disorders, autism and epilepsy (Hagerman and Hagerman, 2001). *FMR1* null mice display enlarged testes, hyperactivity and a mild spatial learning impairment in the Morris water maze (Bardoni et al., 2006). The pleiotropic effect of the absence of FMRP suggests that it has a complex function and it reflects its involvement in the control of hundreds of mRNA targets via its different RNA-binding domains. Indeed, FMRP contains two KH domains and one RGG box domain, which can mediate RNA/protein interaction (Khandjian et al., 2005). While a specificity of binding for KH1 domain was not proved, the KH2 domain was shown to specifically bind a category of synthetic aptamers (« kissing complex ») a sequence-specific element within a complex tertiary structure stabilized by Mg++ concentration (Darnell et al., 2005). On the other hand, the RGG box domain is able to bind with high affinity the RNA G-quartets, a structure that is present in several FMRP RNA targets, such as *FMR1, MAP1B, PP2Ac, APP* ((Schaeffer et al., 2001); (Lu et al., 2004); (Castets et al., 2005); (Westmark and Malter, 2007)) and is stabilized by K+ ions, whereas smaller size cations (e.g. Na+ or Li+) cause their destabilization (Schaeffer et al., 2001). The subcellular localization of FMRP is consistent with that of a protein involved in several steps of metabolism and maturation of RNA. Indeed, FMRP is able to shuttle between nucleus and cytoplasm. In the cytoplasm, it is mostly associated to polyribosomes but was also found to colocalize with stress granules and PBodies (PBs), (Barbee et al., 2006; Bardoni et al., 2006; Khandjian et al., 2005). In addition, in neurons, FMRP is involved in RNA trafficking along dendrites and axons

being a component of RNA granules and functioning as a molecular adaptor between these complexes and the neurospecific KiF3C kinesin (Davidovic et al., 2007). Moreover, after traveling along neurites, FMRP associates to polyribosomes localized at the synapse to participate to the translational control of proteins synthesized in this compartment (Grossman et al., 2006).

Taking into consideration the results obtained from different laboratories, several mechanisms of action of FMRP have been proposed: i) Polysomal stalling for *MAP1B* mRNA expression regulation (Lu et al., 2004); ii) Retention of mRNAs in translationally inactive mRNPs via a specific RNA structure, as suggested by the effect of the kissing complex motif and the FMRP presence in these structures (Darnell et al., 2005); iii) Inhibition of translation preventing ribosome scanning via a G-quartet structure localized in the 5'UTR of a target mRNA, as for *PP2Ac* mRNA (Castets et al., 2005). In addition, iv) the interaction between FMRP and a G-rich sequence in the 3'UTR of *Post Synaptic Density 95* (*PSD95)* mRNA stabilizes this mRNA in the hippocampus (Zalfa et al., 2007). Here, we show that the interaction between FMRP with one of its known RNA targets, *Super Oxide Dismutase 1* (*Sod1*) mRNA, is mediated by a novel structure the *Sod1* Stem Loops Interacting with FMRP (SSLIP). FMRP specifically recognizes and binds this motif with high specificity and affinity, comparable to the one for G-quartets. The SSLIP/FMRP interaction improves the association of *Sod1* mRNA with polyribosomes, as shown by a decrease of the expression level of Sod1 protein in *FMR1* null cells and brain. The characterization of this novel interaction of FMRP sheds new light on the ability of FMRP to be associated with mRNAs and to function as a translational activator. In addition, we are pointing out the disregulation of Sod1 expression as one of the determinants of the of physiopathology of Fragile X syndrome.

Results

FMRP binds *Sod1* mRNA with high affinity via its C-terminal domain

The RNA binding specificity of FMRP is not completely understood. Up to date only a single structure, the G-quartets, was found to mediate the interaction of FMRP with several of its target mRNAs (Castets et al., 2005; Khandjian et al., 2005; Schaeffer et al., 2001). In addition a synthetic RNA with a specific structure, called the kissing complex, binds with high affinity FMRP but up to date is not found harbored by any natural mRNA, yet (Darnell et al., 2005; Khandjian et al., 2005). Furthermore, the 3'UTR of *PSD95* mRNA has been reported to interact with FMRP via a novel structure that was not precisely defined (Miyashiro et al., 2003; Zalfa et al., 2007). The specific sequence/region mediating the interaction of most putative mRNA targets with FMRP is poorly investigated (Khandjian et al., 2005; Miyashiro et al., 2003). Aiming to find novel mRNA structures specifically recognized by FMRP, we performed a systematic analysis of already identified FMRP mRNA targets, in order to exclude the presence of G-quartets and kissing complexes in these mRNAs. It is important to underline that the detection of G-quartets structure in *FMR1* and *PP2Ac* mRNAs was only possible in an experimental way and not by an *in silico* analysis (Schaeffer et al., 2001). Then, as a first step, we screened several FMRP RNA targets by their capacity to bind a recombinant FMRP protein by gel-shift analysis or filtration method in the presence of Na+, K+ or Mg++. Indeed, K+ ions stabilize the mRNA G-quartets structure leading to a solid interaction with FMRP, while Mg++ favours the interaction FMRP/Kissing complex (Darnell et al., 2005; Schaeffer et al., 2001).

Using the APRA (Antibody-Positioned RNA Amplification) technique *Sod1* mRNA was found to be a neuronal target of FMRP (Miyashiro et al., 2003). FMRP/*Sod1* interaction

takes place in the presence of K+ (Fig.1a) and is not affected by the presence of Na+ (Fig.1b), while, as expected, Na+ affects the binding of FMRP to the G-quartets containing N19 RNA, the portion of *FMR1* mRNA harbouring a G-quartets structure as we have previously characterized (Schaeffer et al., 2001). To definitely exclude the presence of a G-quartets structure in the *Sod1* mRNA, we performed a reverse transcriptase (RT) elongation reaction, as previously described (Schaeffer et al., 2001). In the presence of K+, G-quartets structures are very stable, blocking RT progression at its 3' edge and resulting in a truncated transcription product. Conversely, in the presence of Na+, G-quartets structures are destabilized and the RT can proceed to the end of the RNA. The RT elongation test on *Sod1* mRNA did not reveal any K+ dependant stop of the enzyme (Supplemental Figure), demonstrating that the *Sod1* mRNA is not able to form a G-quartets structure. Moreover, the FMRP/*Sod1* interaction was not dependent on the presence of Mg++, an ion that is necessary to stabilize the "kissing complex" RNA structure (data not shown). Taken together, these findings suggest that FMRP binds to *Sod1* mRNA *via* a novel sequence/structure.

In order to define the affinity of FMRP/*Sod1* mRNA interaction, we tested the ability of *Sod1* mRNA to compete the binding of FMRP/G-quartets forming RNA structure (Schaeffer et al., 2001). Indeed, 5 nM of unlabelled *Sod1* mRNA competed very efficiently (65%), with the previously identified FMRP binding site N19 in a gel-shift assay while a negative control, N8 RNA (corresponding to the mRNA region of *FMR1 1-654*), was not able to compete the same interaction (Fig.1c). To precisely define the region of *Sod1* mRNA interacting with FMRP, we generated 3 different constructs from *Sod1* encompassing its full-length cDNA: its 5'UTR and a portion of its coding region (*Sod1-5'*region), a stretch part of the coding region (*Sod1-mid* region) and, finally, a fragment overlapping the end of the coding region and the 3'UTR (*Sod1-3'* region) (Fig.2a). RNA

sequences corresponding to each fragment were produced and tested for their ability to interact with FMRP. Among the different RNAs synthesized, only the *Sod1*-5' region (spanning *Sod1* mRNA from –70 to +148) competed with N19 binding to FMRP with the same affinity as for full-length *Sod1* mRNA (3 nM of both cold probes compete 50% of N19/FMRP binding) (Fig.2b). To identify the sequence of *Sod1* mRNA that is recognized and bound by FMRP, we performed a site boundary determination (Schaeffer et al., 2001). In this experiment the 3'- or 5'- end labeled *Sod1-5'* RNA was treated by mild alkaline hydrolysis in order to generate a pool of smaller fragments. The RNA fragments retaining capacity to bind to FMRP were selected on immobilized GST-FMRP, as previously described (Schaeffer et al., 2001). Bound RNAs were analyzed by electrophoresis on a denaturing polyacrylamide gel (data available on request). The border positions were at -30 and +34 for 3'- and 5'-end labeled fragments, respectively. This technique allowed us to define a 64-base region spanning both sides the *Sod1* AUG start codon that is protected by FMRP. We subcloned this sequence and we synthesized its corresponding mRNA, generating the *Sod1*-64 RNA. This RNA was bound specifically by FMRP, since it was able to compete the FMRP/*Sod1* full-length mRNA interaction (Fig.2c). Interestingly, this interaction could be competed at the same extent by the G-quartets-N19-containing RNA (not shown).

To assess which portion of FMRP was able to interact with the *Sod1* mRNA, we produced protein fragments of the different RNA binding domains of FMRP (e.g. KH1, KH2, KH1/2 and RGG box-containing-C-terminal domains)(Adinolfi et al., 1999) as recombinant proteins in a bacterial system, and we used them in binding assays with the *Sod1*-64 RNA. Interestingly, we observed that only the C-terminal domain has the ability to interact with *Sod1*-64 RNA (Fig.2d). Indeed, the *Sod1*-64 region was not able to interact with any of the KH domains even at high protein concentration (Fig.2d). Since the C-terminal domain of

FMRP contains the RGG box that binds to the G-quartets RNA structure (Darnell et al., 2001), it is possible that this domain binds to different structures. Indeed, the G-quartets forming structure RNA and *Sod1* mRNA compete with each other to interact with it or alternatively FMRP recognizes similar structural features in both RNAs (e.g. specific atoms properly spatially oriented and/or in the correct electrostatic environment).

FMRP binding site on *Sod1*-64 RNA folds in three stem loops

To assess whether the *Sod1*-64 RNA binds FMRP in the same ions conditions that the full-length *Sod1* mRNA, we performed a binding assay of this RNA to FMRP in the presence either of K+ or Na+. As shown in Fig.2e, no differences were observed in the RNA/protein interaction in both conditions. To determine the secondary structure of *Sod1*-64 RNA, we performed the probing of the structure of this 64-base region in solution, using a panel of chemical and enzymatic modifications as described (See Methods) (Brunel and Romby, 2000). This technique is based on the reactivity of RNA molecules towards chemicals or enzymes that modify or cleave specific atomic positions in RNA respectively. The probing experiments were performed using unlabeled or radioactively end-labeled *in vitro*-transcribed RNAs (*Sod1-5'*region), which were subjected to statistical digestions with RNases T1, T2, V1 or chemical modifications with dimethyl sulfate (DMS) and a carbodiimide derivative (CMCT). RNase T1 cut after G residues present in single-stranded regions, RNase T2 cleaves after all single-stranded residues, but preferentially after. As, whereas RNase V1 cuts at doubled-stranded or stacked bases. DMS alkylates N1 position of As and N3 of Cs while CMCT modifies N1 of Gs and N3 of Us. The sites of cleavage or modification were then identified by primer extension with reverse transcriptase, using radiolabeled primer complementary to *Sod1*-5'region. Analysis of the resulting cDNAs was performed on sequencing polyacrylamide gels that were run together with the

corresponding RNA sequencing ladder to allow identification of the modified residues (Fig.3a). A secondary structural model was further derived by combining experimental data and free energy data calculated using the mFOLD program (http://helix.nih.gov/apps/bioinfo/mfold.html). The structure of *Sod1*-64 RNA appears as a succession of three independent stem-loop structures that are separated by short single stranded regions. (Fig.3b). We called this novel target of FMRP SSLIP for *Sod1* Stem Loops Interacting with FMRP.

Sod1 mRNA stability is not affected by the absence of FMRP

Recently, FMRP could stabilize *PSD95* mRNA by interacting with a G-rich sequence in its 3'UTR in hippocampal primary neurons (Zalfa et al., 2007). To investigate a putative role of FMRP to act the same way on the SSLIP structure, we explored *Sod1* mRNA decay in primary cultured hippocampal neurons after blocking transcription by Actinomycin D treatment. We did not observe any significant alteration in *Sod1* mRNA level in the presence or in the absence of FMRP, even after twelve hours of Actinomycin D treatment (Fig.4a). The same results were obtained in STEK cells expressing or not a *FMR1* transgene (not shown). Our results show that the interaction between FMRP and SSLIP has no role in regulating stability of *Sod1* mRNA.

Association of _Sod1_ mRNA to polyribosomes is reduced in brain and cells lacking FMRP

The association of FMRP to polyribosomes has been clearly established (Corbin et al., 1997; Khandjian et al., 2004), as well as its shuttling between different mRNP complexes (Barbee et al., 2006; Bardoni et al., 2006; Khandjian et al., 2005) and polyribosomes, revealing thereby its involvement in translational control. We asked whether the

SSLIP/FMRP interaction could influence the association of *Sod1* mRNA with polyribosomes. First we analyzed the level of *Sod1* expression in cytoplasmic RNA extracts from STEK cells (Castets et al., 2005), total mouse brain, hippocampus and cerebellum expressing or not the *FMR1* gene. Using quantitative (q) RT-PCR, the amount of *Sod1* mRNA was found to be equivalent in both wild type and *Fmr1* knock-out cells and tissues when compared to the level of *Hprt* mRNA (Fig.4b). Since no difference has been observed in *Sod-1* mRNA expression level between wild type and *FMR1*-null mice brains and in the STEK cells line expressing or not FMRP (Fig.4a), we analyzed cytoplasmic *Sod1* mRNA distribution on 15-45% sucrose gradients prepared with extracts of STEK cells expressing or not *FMR1* transgene and of whole brain of wild type and *FMR1* null mice (Khandjian et al., 2004). In the absence of FMRP, we observed a decreased level of *Sod1* mRNA in polyribosome fractions (light and heavy) obtained from fibroblasts as quantified by qRT-PCR (and using *Hprt* mRNA as standard) (Fig.4c) as well as in the same polyribosome fractions obtained from total brain (Fig.4d). We used the purification of polyribosomes previously described (Khandjian et al., 2004) since this method is based on the concentration of polyribosomal franctions, avoiding contamination of mRNP. These results suggest that the absence of FMRP plays a role in *Sod1* mRNA incorporation in the translating machinery.

Sod1 expression is impaired in *FMR1* null mice

To assess whether the reduction of the association of *Sod1* mRNA to polyribosomes impaired the expression of Sod1 protein in the absence of FMRP, we analyzed total protein extracts obtained from STEK cells expressing or not a *FMR1* transgene (Castets et al., 2005) and we observed that Sod1 protein expression is reduced around 40 % in *FMR1* null cells as compared to cells expressing FMRP (Fig.5a). Similarly, we observed a significant

decrease of Sod1 level in total protein extracts from total brain (Fig.5b), hippocampus (Fig.5c) and cerebellum (Fig.5d) of 12 days old from *FMR1* knock out mice as compared to wild type littermates. Sod1 levels were also reduced in *FMR1* null mice embryos at 10*dpc* (Fig.5e). We therefore concluded Sod1 levels are directly correlated to the reduced localization of its mRNA on active polyribosomes in *FMR1* knock out mice. In addition, our findings confirm a perturbed expression of Sod1 in the absence of FMRP and are not compatible with the sole proposed role of FMRP as a translational repressor.

Discussion

Primary function of FMRP is its ability to bind a subset of mRNAs. It is inferred that the functional significance of the FMRP/RNA interaction is the critical step to understand the molecular bases of Fragile X syndrome. Based on conclusions from several laboratories, it has been considered that FMRP behaves exclusively as a translational repressor (Laggerbauer et al., 2001; Schaeffer et al., 2001). However, recent results have pointed out to a more complex function of FMRP, possibly depending on the specific binding of its target RNAs or due to conformational changes in its structure or on the influence of FMRP-interacting proteins (Bardoni et al., 2006; Khandjian et al., 2005; Miyashiro et al., ; Zalfa et al., 2007). In this study, we dissected the mechanism of binding of FMRP to the *Sod1* mRNA that was identified as a target of FMRP in dendrites of cultured primary neurons by the APRA technique (Miyashiro et al., 2003). Indeed we show here that FMRP recognizes the *Sod1* mRNA via a novel motif, SSLIP, organized in three stem loops separated by short sequences. In the absence of FMRP, *Sod1* mRNA association to polyribosomes is reduced and Sod1 protein is less expressed in brain from the *FMR1* knock out mice. For the first time, our results clearly exclude the model that FMRP behaves exclusively as a translational repressor, since in the absence of FMRP the expression of one of its bound target mRNA, the *Sod1*, is reduced. Furthermore we suggest that, even in this novel function, the mechanism of action of FMRP is mostly dependent on the RNA structure to which it binds. Interestingly, we have precisely defined the secondary structure of the RNA motif that mediates the FMRP/ *Sod1* mRNA interaction. FMRP binds G-quartets RNA and SSLIP RNA trough its C-terminal region containing the RGG-box, even if in different ions concentrations. For this reason, we are tempted to speculate that the binding may occur in an alternative way depending on the local ion concentration (K+ vs Na+) and this possibility might be particularly intriguing at the synaptic level.

Interestingly, the FMRP homologue, Fragile X Mental Retardation Related Protein 1 (FXR1P) that by homology has been considered to have the same function that FMRP (Laggerbauer et al., 2001), was reported to functionas a translation activator when associated with the TNFα ARE and in response to serum starvation (Vasudevan and Steitz, 2007).

It has been shown that FMRP is associated in the cell to structures with different translational status. In this scenario FMRP might shuttle not only between nucleus and cytoplasm, but also between polyribosomes (translationally active) and translationally inactive structures, such as PBodies, RNA granules or stress granules. Our results help in a more precise definition of the mechanism of action of FMRP, since it appears that it can regulate the translatability of an mRNA favouring its association to polyribosomes. It is possible that the FMRP binding to other sequence determine its association to other cytoplasmic compartment, as suggested by its interaction with "kissing complex" motif. Unfortunately, no natural mRNA harbouring a kissing complex have been identified so far and it is impossible to performe comparatif studies with Sod1 expression level (as mRNA and protein) in the presence or in the absence of FMRP. In addition *Sod1* mRNA is present in dendrites and in axons meaning that is an axonally synthesized protein essential for axon development and axonal transport and implicating that FMRP may also regulate its neurites trafficking and it association to synaptic polyribosomes (Willis et al., 2005). Sod1 has anti-oxidative properties and gene mutations in *Sod1* can cause familial amyotrophic lateral sclerosis (SLA). Several *Sod1* mutants found in SLA patients perturb fast axonal transport (De Vos et al., 2007) and can cause motor axonopathy in zebrafish (Lemmens et al., 2007). Sod1 is also regulated by cellular stress, suggesting that the axonal localization of its mRNAs may provide a mechanism to locally respond to axonal injury.. Alterations of axons development have been described in the Fragile X models in zebrafish and

drosophila (Morales et al., 2002; Tucker et al., 2006) as well alterations of the white matter of frontostriatal in Fragile X patients (Barnea-Goraly et al., 2003). Modifications of oxidative stress have been linked to anxiety (Gingrich, 2005), sleep troubles (Cirelli, 2006), and autism (Ming et al., 2005): all phenotypic characters displayed by Fragile X patients (Hagerman and Hagerman, 2001). Alterations of oxidative stress have been proposed to exist in *FMR1* null flies since changes in the expression of proteins involved in redox reactions have been observed (1-cys peroxiredoxin in brain and peroxiredoxin and thioredoxin peroxidase in testis) (Zhang et al., 2005; Zhang et al., 2004) and a moderate increase of oxidative stress in brain of *FMR1* knock-out mice has been recently described (El Bekay and al., 2007). These data lead to the suggestion that abnormalities of Sod1 expression may contribute to the physiopathology of the Fragile X syndrome.

The facts that i) hundreds of putative target RNAs of FMRP have been identified, but only few of them have been validated (Miyashiro et al., 2003); ii) this protein contains several RNA binding domains that, as in the case of the C-terminal region, may have multiple (alternative?) capacity of binding (Darnell et al., 2001; Zalfa et al., 2007) and this study; iii) FMRP-interacting proteins can modify its ability to bind to RNA or compete for the same binding (Bechara et al., 2007; Davidovic et al., 2006), all suggest that the comprehension of the mechanism of action of FMRP is just in its infancy. Our study sheds new light on the physiopathology of the Fragile X syndrome, since that the alteration of Sod1 expression, we described here, probably generates the modification the cellular reduction/oxidation observed in Fmr1 KO mice (El Bekay and al., 2007) and may represent one of the molecular defects resulting in the complex phenotype of Fragile X patients.

Materials and methods

Cells culture

Primary cultures of hippocampal neurons were obtained from wild type and *FMR1* null mouse embryos at 18 days of gestation. STEK cells *FMR1* null and after reintroduction of *FMR1* cDNA (Rescued) (Castets et al., 2005) and 10 days *in vitro* cultured neurons were treated with 5 μM of Actinomycin D (Sigma) for 2, 4, 6 and 12 hours. Total RNAs was purified from actynomicin-treated cells using the Qiagen RNeasy Mini Kit and the RNA quality was verified on a 1% agarose gel and by O.D. measurement.

Plasmids constructions

Primer sequences used to amplify *Sod1* and *FMR1* cDNAs are summarized in Supplemental Table, in the "Supplemental Data" section.

Sod1 full-length (BC002066), and two of its deletion constructs (mid region and *Sod1-64*) were subcloned into the pGEM-T vector (Promega), *Sod1* 3'UTR construct was subcloned into the pCR2.1-TOPO (Invitrogen).

Sequences coding for KH1, KH2, KH1/2 and FCT domains were amplified by PCR from obtained from *FMR1* ISO7 cDNA (Adinolfi et al., 1999) using the appropriate primers. The PCR products were subcloned into the pET 151/DTOPO vector (Invitrogen) and the constructions verified by sequencing.

Table I

	Forward	Reverse
Sod1 full-length	5'CCCACGCGTCCGCTCGTC3'	5'CTCTTCAGATTACAGTTT3'
Sod1 mid-region	5'GGGTTCCACGTCCATCAGT3'	5'GGCCAATGATGGAATGCTC3'
Sod1 3'UTR	5'CCGTACAATGGTGGTCCAT3'	5'CTCTTCAGATTACAGTTTAAT3'
Sod1-64	5'CGCCGCGCGTCTTCCG3'	5'GACCGTCGCCCTTCAGC3'
FMRP-KH1	5'CACCGCTAGTAAGCAGCTGG3'	5'CTAAAATTCGAGAAAGCTTCTAG3'
FMRP-KH2	5'CACCGCTGAAGATGTAATACAAGTTC3'	5'CTATAAATAGTTCAGGTGATAATCCAA3'
FMRP-FCT	5'CACCGCTCCAACAGAGGAAGAGAG3'	5'CTAGGGTACTCCAATCACGAGTG3'

Immunoblot analysis

Protein extraction and immunoblot were performed as previously described (Castets et al., 2005). The antibodies used in immunoblot analyses were used at the following concentrations: anti-FMRP antibody 1C3 1:10000 (Castets et al., 2005), rabbit polyclonal anti-Sod1 antibody (Sod-100) (Stressgen) 1:5000, monoclonal anti β-Tubulin (E7) antibody (Iowa Hybridoma Bank) 1:5000 and rabbit polyclonal anti-L7a antibody (a gift from A. Ziemiecki) 1:40000.

RNA binding assay

Protein expression and purification were performed as previously described (Bechara et al., 2007). All RNAs were produced using the T7 RNA polymerase (Promega) according to the manufacturer protocol starting from linearized plasmids. The pGEM-T and pTL1 vectors were linearized using PstI, pCR2.1 TOPO with BamH1. *Sod1 5'* region was obtained by digesting pGEM-T *Sod1* full-length with BstXI. Restriction enzymes were purchased at New England Biolabs. RNAs were purified on NucAway spin columns (Ambion) and their quality was verified on an Acrylamide-Urea gel after staining with the Stains-All (Sigma). RNA-Protein interactions were analyzed either by Electro Mobility Shift Assay or by filter binding assay, as previously described (5, 33).

RNA forming structures detection

The presence of a G-quartets structure in the *Sod1* mRNA was tested both by binding assay and by reverse transcription with different primers along the *Sod1* mRNA, as previously described (Schaeffer et al., 2001) in the presence of Na+ or K+ in both experiments. For the primer extension assays, RT was performed as described (Schaeffer et al., 2001) and using the following primers γ-^{32}ATP 3' end labeled.

I 5'CTCTTCAGATTACAGTTT3'; primer II 5'GTACGGCCAATGATGGAATG3'; primer III 5'GGATTAAAATGAGGTCCTGC3'; primer IV 5'CTTCTGCTCGAAGTGGATG3'; primer V 5'CTTCAGCACGCACGC3'.

The *Sod1*-64 RNA boundaries were determined as previously described (Schaeffer et al., 2001).

Chemical and enzymatic probing the *Sod1*-5'UTR region to determine the *Sod1*-64 structure.

5'UTR *Sod1* RNA (5pmoles) was renatured at 40°C for 15 min in the appropriate native buffer (50 mM Hepes buffer pH 7.5 for DMS or Borate buffer pH 8 for CMCT, 5 mM Mg Acetate, 50 mM KOH acetate, 2 mM β-mercaptoethanol). Chemical modifications were performed in 20 µl final volume using either 1µl of DMS diluted 1/2 (v/v) in ethanol or 60 µg of CMCT (Carbodiimide), at 20°C for 5 and 15 min, respectively and in the presence of 2µg of *E. coli* total tRNA. Enzymatic modifications were performed with V1 (0,0002U and 0,001U), T1 (0,05U, and 0,1U) and T2 (0,05U and 0,1U) nucleases, followed by a phenol/chloroform (v/v) extraction. After ethanol precipitation and solubilization in the appropriate buffer, modified RNAs were reverse transcribed using the labeled primer III, sequencing reactions and gel analysis were carried out as previously described (Brunel and Romby, 2000; Schaeffer et al., 2001).

Polyribosomes Purification.

Polyribosomes purification and analysis was performed as previously described (Khandjian et al., 2004). Fifteen fractions of 800 µl each were collected from sucrose gradient. 100 µl of each fraction were ethanol-precipitated, resuspended in 50 µl of Laemmli buffer and analyzed by immunoblot. The other 700 µl of each fraction were treated with Trizol (Invitrogen) to purify RNA. The quality of RNAs was verified on a 1% agarose gel and by O.D. measurement.

Quantitative Real time PCR

The reverse transcription reactions were performed with 2µg of RNA using the ThermoScript RT-PCR system (Invitrogen). The PCR reactions were carried out with the qPCR core Kit for Syber Green I (Eurogentec), in an ABI PRISM 7000 (Applied BioSystems). To amplify the *Sod1* cDNA the following primers were used: F-

5’ACCATCCACTTCGAGCAGAA3’; and R-5’AGTCACATTGCCCAGGTCTC3’. The level of expression of the *Sod1* was normalized to the standard housekeeping gene, *Hprt*, that was amplified with the F-5’GTAATGATCAGTCAACGGGGGAC3’ and the R-5’CCAGCAAGCTTGCAACCTTAACCA3’ primers. Relative changes in mRNA amounts were calculated based on the Δ_{CT} method (Livak and Schmittgen, 2001).

Acknowledgment

We thank Enzo Lalli and Edouard Khandjian for discussion and critical reading of the manuscript, and A. Ziemiecki for kind gift of anti-L7a anti-serum. This study was supported by INSERM, CNRS, Agence Nationale de la Recherche (BB and HM), Fondation Jerôme Lejeune, GIS-Maladies Rares (BB and JLM) and CHU de Nice.
EB is recipient of a fellowship from Fondation pour la Recherche Médicale. MCD is recipient of a fellowship from ARC. LD is a recipient of a fellowship from "Marie Curie Mobility Program"

Competing interests. The authors have declared that no competing interests exist.

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Figures Legends

Figure 1.

FMRP specifically binds *Sod1* mRNA.

FMRP binding to *Sod1* mRNA is not dependent on K^+. Labelled G-quartets RNA (N19) or *Sod1* full length mRNA were incubated with increasing amounts of recombinant His-FMRP in the presence of K^+(a) or Na^+(b). FMRP-*Sod1* binding was not affected by ionic conditions while, as expected, the presence of Na^+ affected FMRP binding to N19.

(c) Gel-shift experiments were performed using a ^{32}P-labeled N19 probe incubated with 0.1 pmole of recombinant His-tagged FMRP, in the presence of increasing amounts of unlabelled competitors, ranging from 10^{-9} to 10^{-7}M [lane 3-5 (N19), lane 6-8 (*Sod1)*, lane 9-11 (N8)]. Lane 1, no protein control, lane 2, no competitor control. Note that both N19 (G-quartets-containing RNA and positive control) and *Sod1* compete equally for binding to FMRP, whereas N8 (negative control) only competes out at high concentration (non-specific binding).

Figure 2.

FMRP binds a 64 base fragment of *Sod1* mRNA via its C terminal region.

a) Schematic representation of *Sod1* mRNA and the fragments subcloned from full-length cDNA and used to map the binding domain of FMRP on *Sod1* mRNA.

b) Filter binding assay using FMRP and ^{32}P-labeled N19. The competition was performed using various regions of unlabeled *Sod1* mRNA: *Sod1*-5' region, *Sod1* mid region, and *Sod1*-3' region. The graph depicts the fraction of bound labelled N19 RNA plotted against unlabelled competitor RNA concentration.

c) Binding specificity of FMRP to *Sod1*-64 fragment. Filter binding assay using FMRP and ^{32}P-labelled *Sod1* mRNA. Competition was performed with different unlabelled mRNA fragments. The *Sod1*-64 RNA fragment shows a competition profile similar to the one of *Sod1* full-length mRNA.

d) Filter binding assays using various recombinant RNA-binding domains of FMRP: KH1, KH2 and the C-terminal domain containing the RGG box and ^{32}P-labeled RNAs reveal that the FMRP C-terminal domain (FCT) displays equal affinity for *Sod1* mRNA or G-quartets containing the N19 fragment, whereas the KH domains are not able to bind *Sod1* mRNA.

e) Filter binding assay using increasing amount of recombinant His-FMRP and ^{32}P-labeled RNA fragments in the presence of K^+ or Na^+. FMRP-*Sod1*-64 RNA binding is not dependent on ionic conditions, excluding the presence of a G-quartets forming structure RNA.

Figure 3.

Secondary structure of the *Sod1*-64 RNA fragment

Enzymatic (a) and chemical (b) probing of in vitro-transcribed *Sod1*-5' region. Cleavage and modification sites were detected by primer extension using the ^{32}P- 5'-end-labelled primer IV. The resulting cDNA was separated on 8% polyacrylamide/8 M urea sequencing gel and analyzed by autoradiography. RNA sequencing reactions were run in parallel. The nature and positions of different loops and stems are indicated at right. Increasing concentrations of RNase V1 (V1), RNase T1 (T1) (left panel) or chemical agents (DMS, CMCT) (right panel) were added before the reverse transcription step (-) indicates the lanes where the untreated RNA was loaded.

c) RNA secondary structure model of the *Sod1*-64 mRNA fragment showing results from enzymatic cleavage and chemical modification experiments. White and black arrows

represent weak, moderate and strong RNase T1 cleavage sites, respectively. White and black triangles represent weak, moderate and strong RNase V1 cleavage sites, respectively. Dashed and white circles represent weak, moderate, and strong modifications by DMS, CMCT, respectively. "x" represents reverse transcriptase pauses.

Figure 4.

FMRP does not affect *Sod1* mRNA stability but rather controls its translation status.

a) Primary cultured hippocampal neurons derived from *Fmr1* knock-out or wild-type mice were incubated with 5μM ActinomycinD for 12 hours. *Sod1* mRNA levels in cells expressing or not FMR1 was monitored at various time points after treatment. The ratio of these levels is shown.

b) Cytoplasmic RNA was extracted from cells and mice tissues expressing or not FMRP. *Sod1* mRNA levels were analysed by qRT-PCR and normalized to *Hprt* in each sample. As shown in the diagram, *Sod1* mRNA levels were not affected by the absence of FMRP.

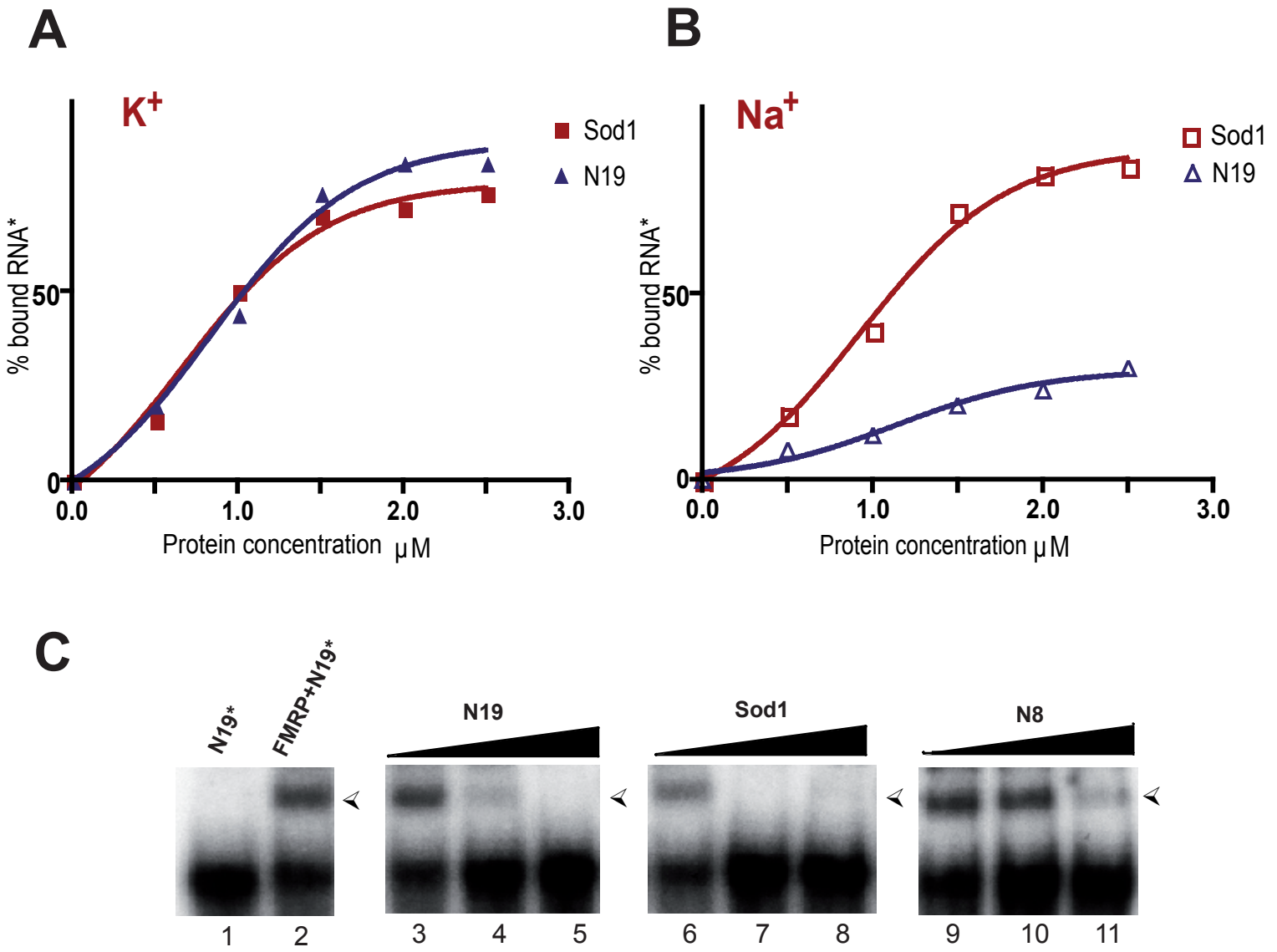
c-d). Polyribosome association of *Sod1* mRNA in STEK cell lines expressing or not FMR1 and in brain obtained from wild type and FMR1 null mice. RNA purified from fractions corresponding to monosomes, light polyribosomes and heavy-sedimenting polyribosomes were pooled and the amount of *Sod1* mRNA in each pool was determined by quantitative RT-PCR, normalized to *Hprt* in each sample. *Sod1* mRNA is less associated to light polyribosomes in the absence of FMRP.

Figure 5.

Decreased level of Sod1 protein in *FMR1* null cells, brain, embryo

a) Western blot analysis of one FMR1+ STEK clone (where *FMR1* was reintroduced; Castets et al., 2005) and one STEK *FMR1* null clone. The result shown on the left panel is

representative of the analyzed clones. (On the right panel) Densitometric analysis showing a significant decrease of Sod1 expression, comparing five wild-type rescued clones and five *FMR1* knock-out clones. Two independent experiments were quantified. Results are the average of *Sod1* levels normalized for beta-tubulin expression (Student'st-test, P= 0,05). The same analysis described in (a) was repeated for mouse total brain (b), mouse hippocampus (c), mouse cerebellum (d) and mouse 10*dpc* embryo extracts (e) obtained from wild-type and *FMR1* null mice.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K+
Sod1
N19
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0.0
1.0
2.0
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Protein concentration µM
B
Na+
Sod1
N19
% bound RNA*
50
0
0.0
1.0
2.0
3.0
Protein concentration µM
C
N19*
FMRP+N19*
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Sod1
N8
1
2
3
4
5
6
7
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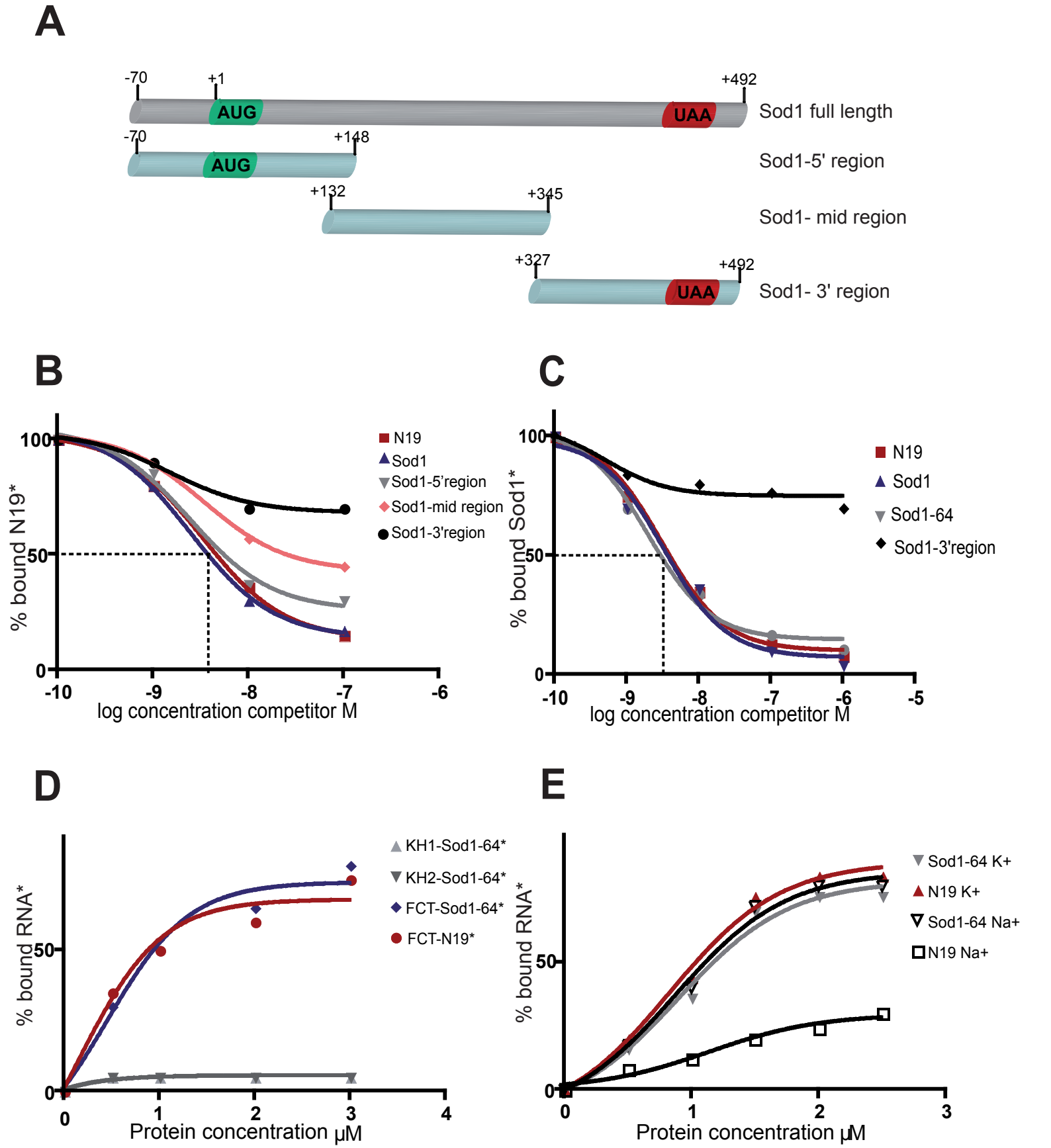
9
10
11

Figure 1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+1
+492
AUG
UAA
Sod1 full length
-70
+148
AUG
Sod1-5' region
+132
+345
Sod1- mid region
+327
+492
UAA
Sod1- 3' region
B
100
50
0
% bound N19*
-10
-9
-8
-7
-6
log concentration competitor M
N19
Sod1
Sod1-5'region
Sod1-mid region
Sod1-3'region
C
100
50
0
% bound Sod1*
-10
-9
-8
-7
-6
-5
log concentration competitor M
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Sod1-64
Sod1-3'region
D
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0
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1
2
3
4
Protein concentration µM
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KH2-Sod1-64*
FCT-Sod1-64*
FCT-N19*
E
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0
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3
Protein concentration µM
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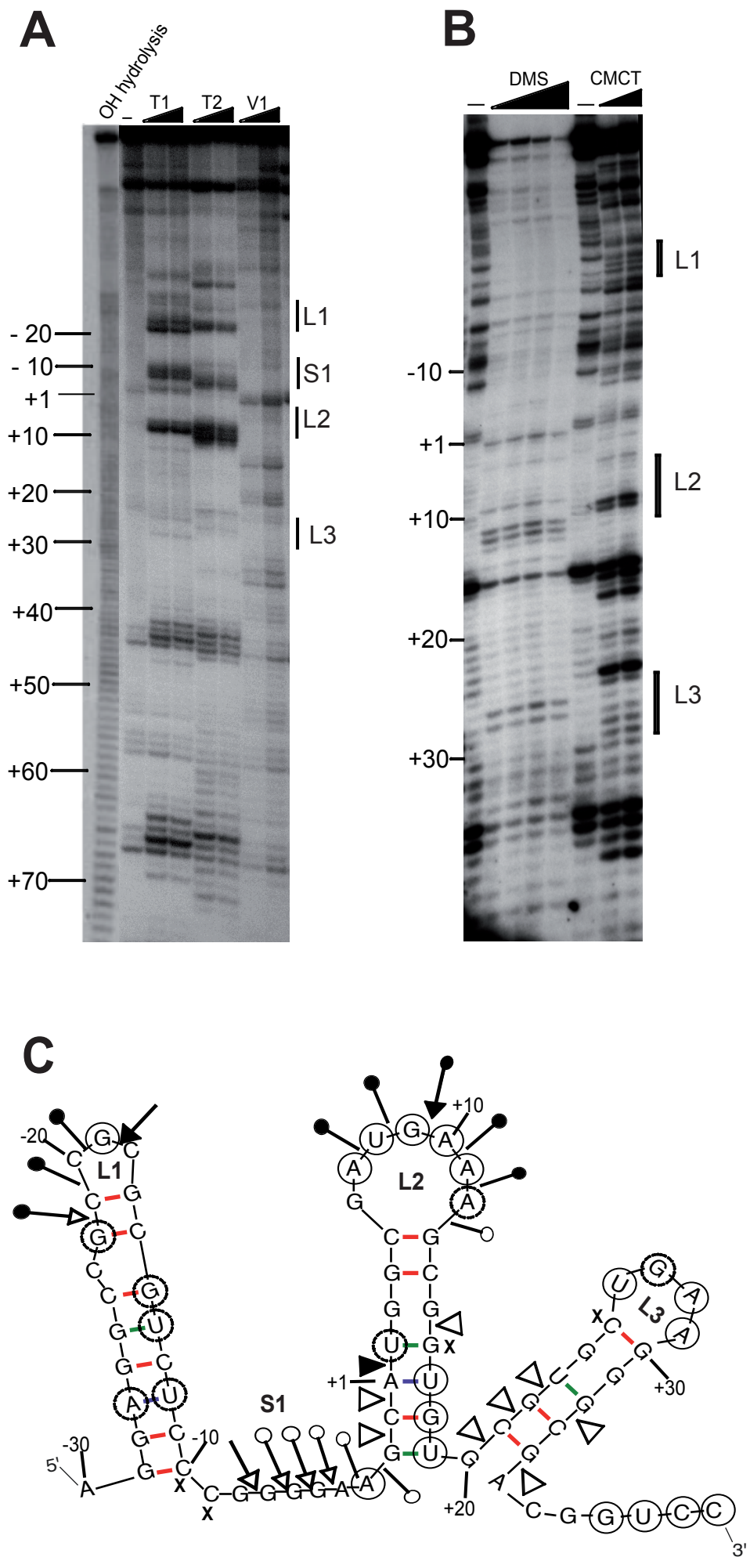
N19 K+
Sod1-64 Na+
N19 Na+

Figure 2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T2
V1
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- 10
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+20
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+40
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S1
L2
L3
B
DMS
CMCT
-10
+1
+10
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L1
L2
L3
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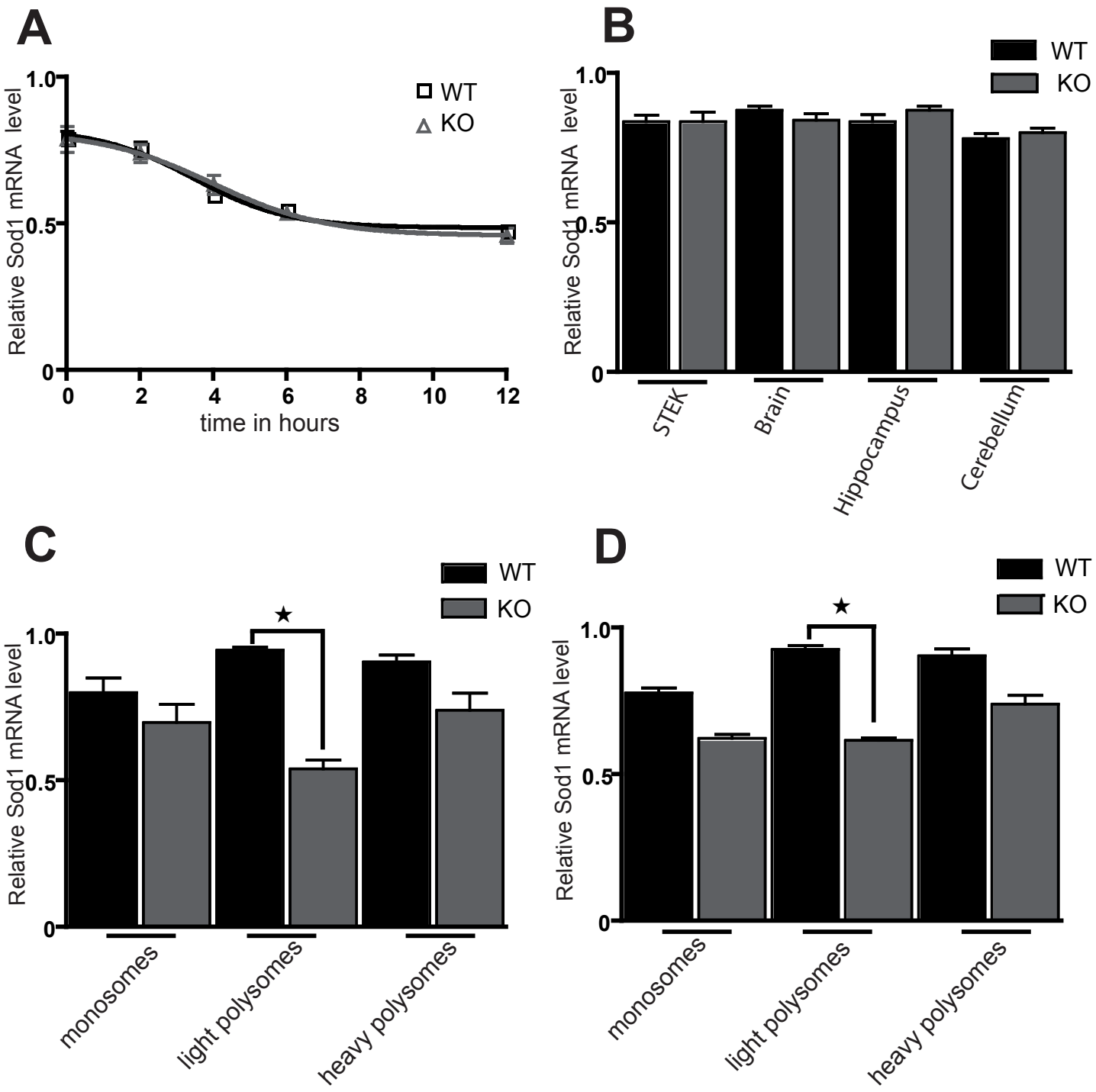
5'
3'
X

Figure 3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WT
KO
0
2
4
6
8
10
12
time in hours
B
Relative Sod1 mRNA level
1.0
0.5
0
WT
KO
STEK
Brain
Hippocampus
Cerebellum
C
Relative Sod1 mRNA level
1.0
0.5
0
WT
KO
★
monosomes
light polysomes
heavy polysomes
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Relative Sod1 mRNA level
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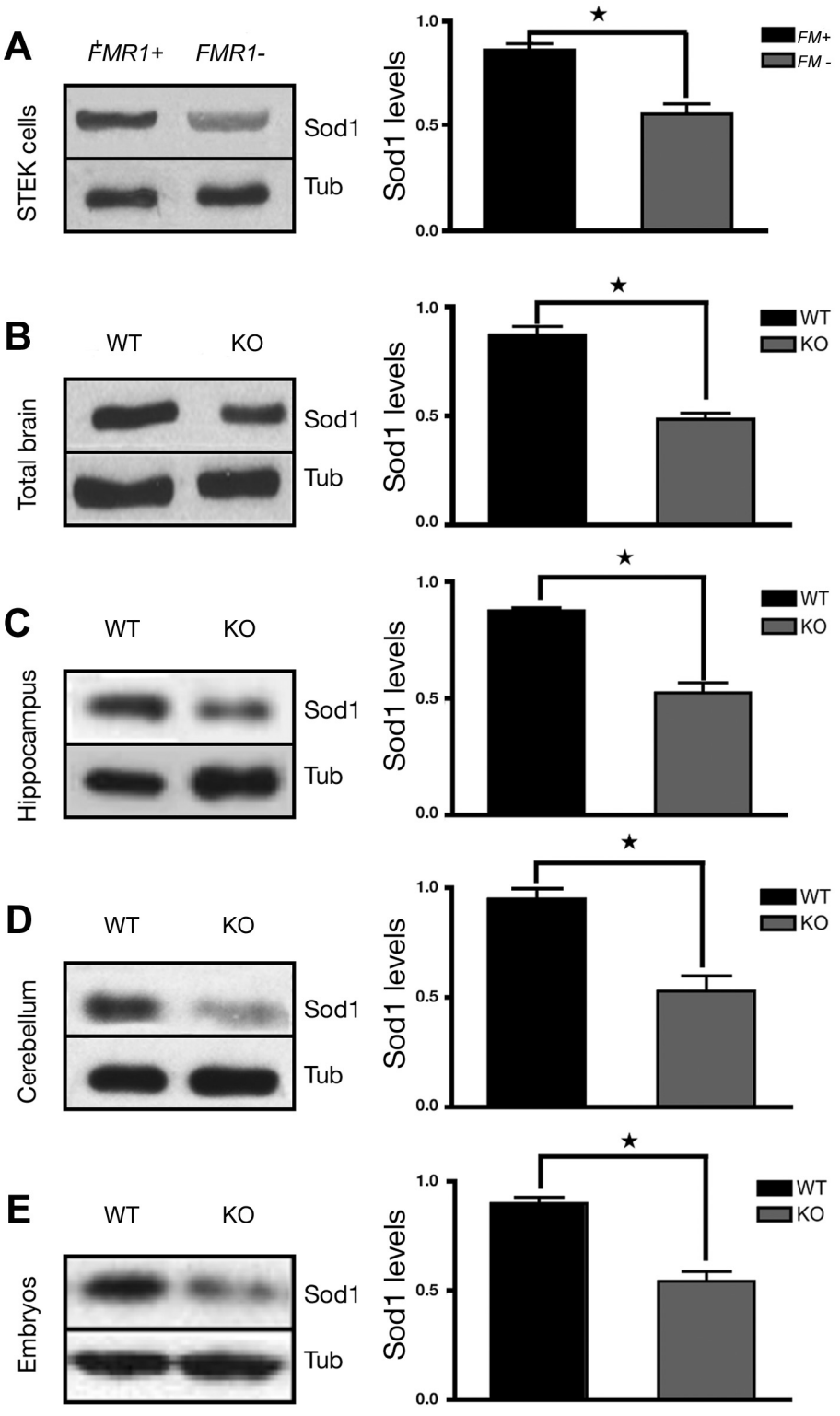
0
WT
KO
★
monosomes
light polysomes
heavy polysomes

Figure 4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FMR1+
FMR1-
STEK cells
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Tub
Sod1 levels
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0.5
0.0
FM+
FM -
B
WT
KO
Total brain
Sod1
Tub
Sod1 levels
WT
KO
C
WT
KO
Hippocampus
Sod1
Tub
Sod1 levels
WT
KO
D
WT
KO
Cerebellum
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Sod1 levels
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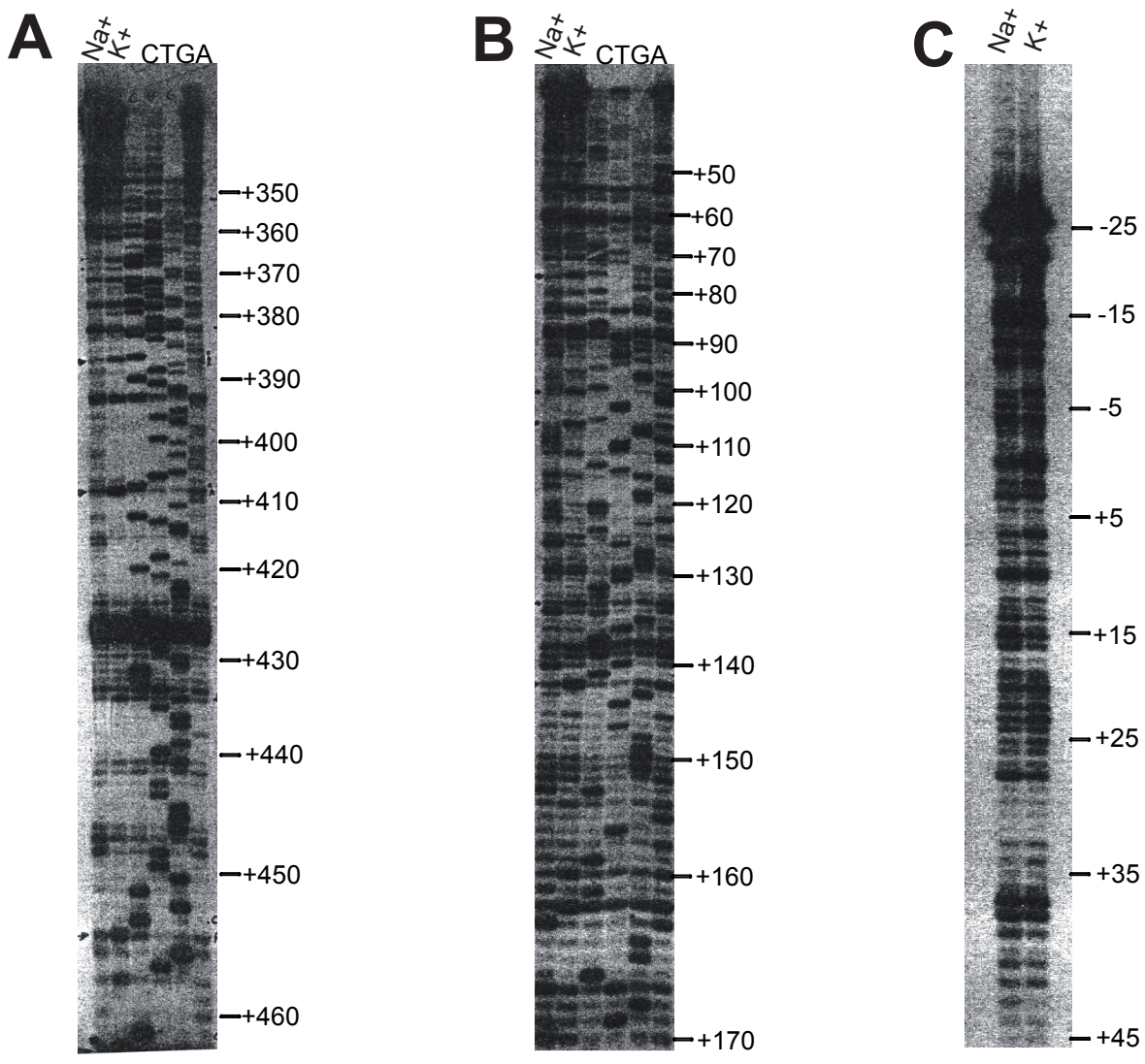
Figure 5



Supplemental figure

Publication 2

FMRP is suggested to be component of granules that are in a state of dynamic flux, in activity- regulated equilibrium with the surrounding translational control granules. Several of FMRP interactors were shown to be RNA binding proteins. FXR1P and FXR2P, two homologues and interactors of FMRP were predicted to have similar functions as those of FMRP simply by homology comparison. Indeed, the three proteins share high homology degree in both their sequence and structural domains, but the fact that FXR1P and FXR2P were never correlated directly to any disease, their molecular functions were poorly investigated. The role of FXR1P in both muscle development and translation regulation started to emerge but still needs a profound analysis to be more understood.

Aim : Does FXR1P show any affinity for FMRP targets ? if so, what would be the influence of FXR1P on the RNA binding properties of FMRP ?

Procedure : To analyze the RNA binding specificity and affinity, we used RNA binding assays in presence of the G-quartet as specific competitor. To study the affinity of the heterodimer FMRP/FXR1P, equal amount of protein was used in the same kind of binding experiments. We therefore dissected the dynamic of FMRP/FXR1P/G-quartet association and dissociation by time lapse RNA binding assays.

Results : Only FXR1P Isoe showed an affinity to G-quartets lower than that of FMRP whereas the two other isoforms FXR1P Isod and Isoa bind unspecifically the G-quartet structure. FMRP/FXR1P heterodimer has a higher dynamic association and dissociation with and from RNA than the homodimers.

Conclusion : we tried to analyze the RNA binding affinities of FMRP in presence of its interactors. We showed that despite the high homology degree between FMRP and FXR1P, both proteins exhibit different affinity for RNA (at least for RNAs harbouring a G-quartet structure) and that FXR1P may have more a “synergic” effect rather than compensatory for the absence of FMRP.

Published online 14 December 2006 *Nucleic Acids Research, 2007, Vol. 35, No. 1* ***299–306***
doi:10.1093/nar/gkl1021

Fragile X related protein 1 isoforms differentially modulate the affinity of fragile X mental retardation protein for G-quartet RNA structure

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Received September 6, 2006; Revised and Accepted November 13, 2006

ABSTRACT

Fragile X syndrome, the most frequent form of inherited mental retardation, is due to the absence of expression of the Fragile X Mental Retardation Protein (FMRP), an RNA binding protein with high specificity for G-quartet RNA structure. FMRP is involved in several steps of mRNA metabolism: nucleocytoplasmic trafficking, translational control and transport along dendrites in neurons. Fragile X Related Protein 1 (FXR1P), a homologue and interactor of FMRP, has been postulated to have a function similar to FMRP, leading to the hypothesis that it can compensate for the absence of FMRP in Fragile X patients. Here we analyze the ability of three isoforms of FXR1P, expressed in different tissues, to bind G-quartet RNA structure specifically. Only the longest FXR1P isoform was found to be able to bind specifically the G-quartet RNA, albeit with a lower affinity as compared to FMRP, whereas the other two isoforms negatively regulate the affinity of FMRP for G-quartet RNA. This result is important to decipher the molecular basis of fragile X syndrome, through the understanding of FMRP action in the context of its multimolecular complex in different tissues. In addition, we show that the action of FXR1P is synergistic rather than compensatory for FMRP function.

INTRODUCTION

Fragile X related genes are members of a small gene family whose founding member is the Fragile X Mental Retardation 1 gene (*FMR1*). Inactivation of *FMR1* causes Fragile X syndrome, the most common cause of inherited mental retardation (1,2). The other members of this family, *FXR1* and *FXR2*, are autosomal and have not been associated so far with any human disease (2–4). Animal models have been generated for *Fmr1* deficiency, recapitulating the phenotype of Fragile X syndrome (5,6). *Fxr2* null mice are viable and show some behavioral phenotypes, such as hyperactivity, similar to those observed in *Fmr1* knockout mice (7). *Fxr1* null mice die shortly after birth most likely because of heart and/or respiratory failure due to alterations in muscle development (8). In *Xenopus*, complete or partial inactivation of *xFxr1* expression has dramatic muscle-specific effects (9). In vertebrates, members of the FXR protein family are structurally very similar and share a high degree of sequence homology in clustered regions corresponding to functional domains (2–4). Like FMRP, FXR1P contains several RNA binding domains: two KH domains and one RGG box. It also contains a nuclear localization signal (NLS), a nuclear export signal (NES) and a protein–protein interaction domain (2,10). They also share the same gene structure, derived from their common ancestor in *Drosophila melanogaster* (11). FXR proteins are able to bind RNA (3,4), but binding specificity has been studied in detail only for FMRP. Indeed, even if a few hundreds of different RNAs have been proposed to be putative targets of FMRP, only two structures are specifically bound by this protein, the G-quartet and the kissing complex (12–14) and one sequence, a poly(U) stretch (15). FXR1P has been reported to bind AU rich element (ARE) and, through the interaction with this element, to regulate the expression of the proinflammatory cytokine tumor necrosis factor (TNFα) in macrophages (16). In the cytoplasm the three FXR proteins are associated with polyribosomes (17), while they share only two interacting proteins, CYFIP2 and MSP58, with FMRP (2,18,19). The *FXR1* primary transcript is alternatively

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spliced, with the possibility to generate upto 15 isoforms (20), see also www.ncbi.nlm.nih.gov/IEB/Research/Acembly/. Of notice, some of these isoforms are differentially expressed in various tissues (21). Up to date, the ability of full-length FXR1P and FXR2P to bind a G-quartet RNA structure in a specific manner has not been reported. We analyzed here the RNA binding properties of the three most abundantly expressed FXR1P isoforms and show that they have different affinities for the G-quartet RNA structure. Since all protein members of the FXR family are able to heterodimerize with FMRP, they are believed to act together (4). In the present study we determined that, when complexed to FMRP, FXR1P isoforms can modulate its affinity for G-quartet RNA and also the dynamics of this complex. Our data demonstrate that FXR1P has a synergistic molecular function with FMRP rather than a redundant role.

MATERIALS AND METHODS

Purification of recombinant proteins

Glutathione *S*-transferase (GST)-FMRP produced in the baculovirus system was purified as described previously (22). pET21a/FMRP (ISO1) vector was described previously (23). To construct pGex-4T-1/FMRP, ISO1 cDNA was excised from pTL1/FMRP ISO1 and subcloned into the EcoRI/NotI sites of pGex-4T-1 (Amersham). To construct pET21a/FXR1P, Isoe, Isod and Isoa isoforms were amplified by PCR using the primers (Eurogentec): EcoRI forward-5′-GGCGAATTCATGGCGGACGTGACGGTG-3′; XhoI reverse-5′-GCCCTCGAGTTATGAAACACCATTCAGGAC -3′, the PCR consisted of 1 cycle at 94°C for 4 min, 30 cycles of three steps each, 94°C for 30 s followed by 60°C for 30 s and 68°C for 2 min using the *Pfx* polymerase (Invitrogen). PCR fragments were purified, digested and cloned into the EcoRI/XhoI sites of pET21a (Novagen). The sequences of the cDNAs corresponding to the different FXR1P isoforms were verified by sequencing. The proteins were produced in bacteria and purified following the manufacturer's protocol. GST-MSP58 was produced and purified as described previously (19).

GST-pull down

GST-pull down assays were performed as described previously (22). Briefly, an increasing amount of recombinant His-FXR1P (1, 2 or 4 μM) was mixed with 4 μM of GST-FMRP. Pull down assays were carried out in the following buffer: [50 mM Tris–HCl (pH 7.4) at 4°C, 1 mM $MgCl_2$, 1 mM EDTA, 150 mM KCl, 1 mM DTT], as described (22). After washing with the same buffer, the proteins bound to the beads and their interactors were eluted using 30 mM glutathione and separated by electrophoresis on 8% SDS–polyacrylamide gels. FMRP was visualized by immunoblot using the 1C3 monoclonal antibody (24), FXR1P was revealed by the 3FX monoclonal antibody (21). The proteins were also visualized on gel by Coomassie staining.

RNA binding assays

The different RNA fragments used in this study, N19 [RNA sequence derived from *FMR1* cDNA and containing a G-quartet forming structure (13)] and N8 [RNA sequence not containing G-quartet structures and corresponding to the 3′-untranslated region (3′-UTR) of *PP2Ac* (25)], were cloned in pTL1 plasmid. For filter binding assay, pTL1 plasmids linearized with PstI were *in vitro* transcripbed with T7 RNA polymerase (Promega) (13). The RNAs were purified using the NucAway Spin columns (Ambion). RNAs were then ethanol precipitated and resuspended in a appropriate buffer. For binding experiments, N19 was labeled co-transcriptionally by incorporation of [α-^{32}P]ATP. Labeled RNAs were purified on a 1% low-melting agarose gel (Ambion). Labeled RNAs (80 000 c.p.m., 5 fmol) were renatured for 10 min at 40°C in 4 μl of binding buffer [50 mM Tris–HCl (pH 7.4) at 4°C, 1 mM $MgCl_2$, 1 mM EDTA, 150 mM KCl, 1 mM DTT] in the presence of 8 U RNasin (Invitrogen), 0, 1 μg of *Escherichia coli* total tRNA and 0.01% BSA. An increasing amount of protein was then added to the RNA. RNA–protein complexes were formed for 10 min on ice. After incubation, binding solutions were passed through MF-membrane filters (0.45 HA, Millipore) and washed with 2 ml binding buffer. Filters were air dried and the amount of radioactivity was measured by Cerenkov counting. Data were plotted as percentage of total RNA bound versus the protein concentration. Competition experiments to determine the relative binding strength of the different proteins to G-quartet RNA were carried out using labeled N19 RNA incubated with 1 pmol of protein in the presence of increasing concentrations of unlabeled competitors. FMRP was used as an internal positive control. For association rate determination, 5 fmol of labeled N19 were incubated with 1 pmol of the appropriate protein in the binding buffer between 10 and 300 min on ice. For dissociation rate determination, 5 fmol of labeled N19 were incubated with 1 pmol of the appropriate protein in the binding buffer for 10 min on ice, 10^{-6} M of competitor RNA (N19 or N8) were then added to the mixture and incubated between 10 and 300 min. Each binding curve is the result of at least three independent experiments performed with three replicates for each binding point. All data obtained for the different experiments of RNA binding, calculating the standard deviation for each binding point, are shown in Supplementary Data. All the values and curves were analyzed using the PRISM Graphpad version 4 Software.

RESULTS

Our first aim was to assess whether FXR1P is able to bind G-quartet RNA structure, which is considered to be a frequent structure recognized by FMRP and present in many of its mRNA targets (12,13,26). Due to extensive alternative splicing of *FXR1* mRNA, at least seven isoforms of FXR1P are differentially expressed in various tissues (20). We decided to study the RNA binding properties of three FXR1P isoforms: Isod and Isoa (Figure 1), the two isoforms most highly expressed in brain (3), and Isoe (Figure 1) that is a FXR1P isoform highly expressed during myogenesis and in adult cardiac and skeletal muscle (21). The FXR1P-Isod and Isoa isoforms both lack exon 12 and 15 and only differ in their C-terminus due to the choice of a different splicing acceptor site in the mRNA of the FXR1P-Isoa isoform, resulting in a frameshift that induces an early stop codon

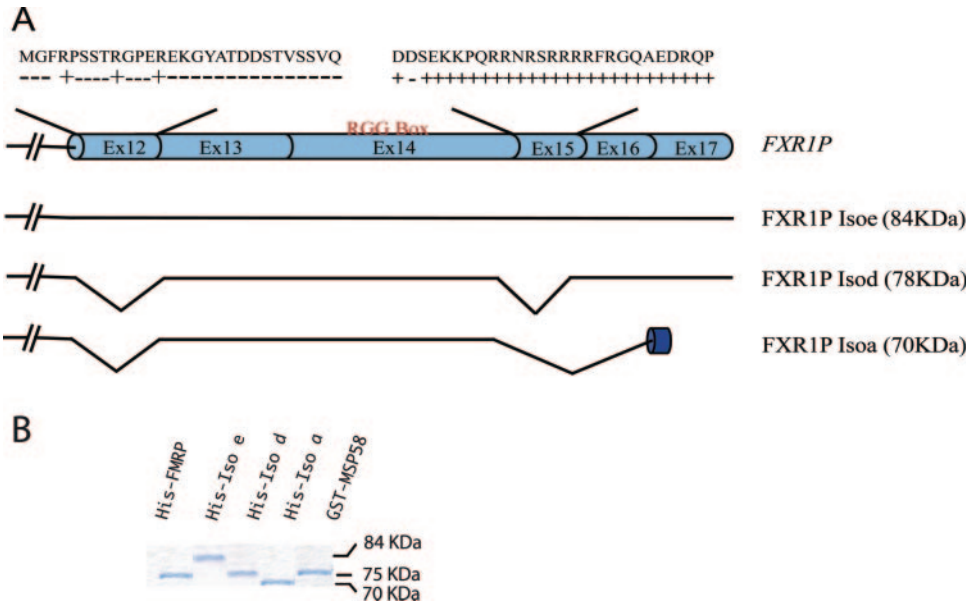

Figure 1. The FXR1P isoforms. (**A**) Schematic representation of the C-terminal region of the three FXR1P isoforms analyzed: Isoe 84 kDa, Isod 78 kDa, Isoa 70 kDa. In the upper part of the figure the alternatively spliced sequences are indicated. A (+) under each amino acid indicates the predicted ability of the sequence to bind RNA accordingly to the algorithm described by Terribilini and coworkers (28). (**B**) Production of recombinant proteins. Equal amounts of Hist-FMRP, His-FXR1P-Isoe, Isod, Isoa and GST-MSP58 were loaded on a 10% SDS–PAGE gel and revealed by Coomassie blue staining.

(Figure 1A). On the other side, it is interesting to underline that the only differences between FXR1P-Isod and Isoe isoforms are the insertion of 28 amino acid encoded by exon 12 and the presence of 27 amino acid encoded by exon 15 (20,21) (Figure 1A). This 27 amino acid stretch is strongly recognized as a putative RNA binding motif by two different predictive programs available online [http://bindr.gdcb.iastate.edu/RNABindR/main.aspx (27) and http://129.130.115.77/cgi-bin/bindn.pl (28)], whereas the sequence of exon 12 does not apparently display such properties. The presence or absence of exon 15 raises then the possibility that the 3 isoforms share different RNA binding abilities.

As the tissue distribution of FXR1P isoforms had not been investigated completely, we performed RT–PCR on various RNA samples extracted from cell lines and tissues. FXR1P containing exon 15 RNA was detected at very low level in brain, and in particular in the cerebellum, cortex and hippocampus, as well as in the neuroblastoma cell line NG108, together with FXR1P isoforms lacking exon 15 (data not shown).

To investigate the G-quartet binding properties of the three FXR1P isoforms, we generated in a bacterial system recombinant FXR1P isoforms: Isoe, Isod and Isoa (Figure 1A) (3,21), FMRP ISO1 (29) and as a control MSP58, a recently described G-quartet binding protein (19), tagged with His or GST (Figure 1B).

In a filter binding assay, recombinant FMRP protein produced in bacteria displays the same affinity for RNA containing a G-quartet structure as recombinant FMRP produced in an insect cell system (data not shown), confirming that the system of production does not change FMRP affinity for G-quartet RNA, in agreement with studies by Darnell and colleagues (30). Also it has been shown that FMRP acts as a nucleic acids chaperone in low-salt binding conditions (31) and is also able to bind RNA non-specifically, raising the possibility of introduction of a bias in the assesment of its binding affinities, as already suggested (13,32). Considering the high level of homology that exists between the FXR proteins (4), we reasoned that FXR1P could also display the same properties of aspecific binding to RNA. As a result, to assess the RNA binding properties of FXR1P isoforms, we used the rigorous and sensitive RNA competition assays, which alleviate the contribution of aspecific binding (13,25). Using the previously described filter binding assay (30), we observed that FXR1P-Isod and Isoa isoforms do not bind specifically G-quartet RNA structure since the amount of bound G-quartet radiolabeled probe is not competed by either the unlabeled G-quartet RNA [N19, corresponding to the portion of the *FMR1* transcript containing the G-quartet structure (13)] or another RNA not containing G-quartet structures and not binding FMRP [N8, corresponding to the 3′-UTR of the PP2Ac transcript (25)] (Figure 2B). Indeed, at the equilibrium state, the dissociation constant (K_d) is around 5 μM for FXR1P-Isod isoform and 0.8 μM for FXR1P-Isoa. Conversely, FXR1P-Isoe binds G-quartet RNA but with a lower affinity compared to FMRP or MSP58 (Figure 2A). As little as 1 nM of competitor RNA is able to displace 50% of FMRP from G-quartet labeled probe, whereas ∼10 nM are necessary for FXR1P-Isoe (Figure 2A). When we used as competitor the N8 probe, that does not bind FMRP (25), no binding was observed for all proteins analyzed here (Figure 2B). To confirm that FXR1P-Isoe interaction with G-quartet RNA is specific for the structure, we performed the binding assay either in the presence of K^+ or in the presence of Na^+. Indeed, FXR1P-Isoe, like FMRP and MSP58 (13,19) is unable to bind G-quartet containing *FMR1* RNA in the presence of Na^+, a cation destabilizing the G-quartet structure (Figure 2C). This finding suggests that the effect observed is not due to the recognition of a specific RNA sequence, but to the G-quartet structure localized in

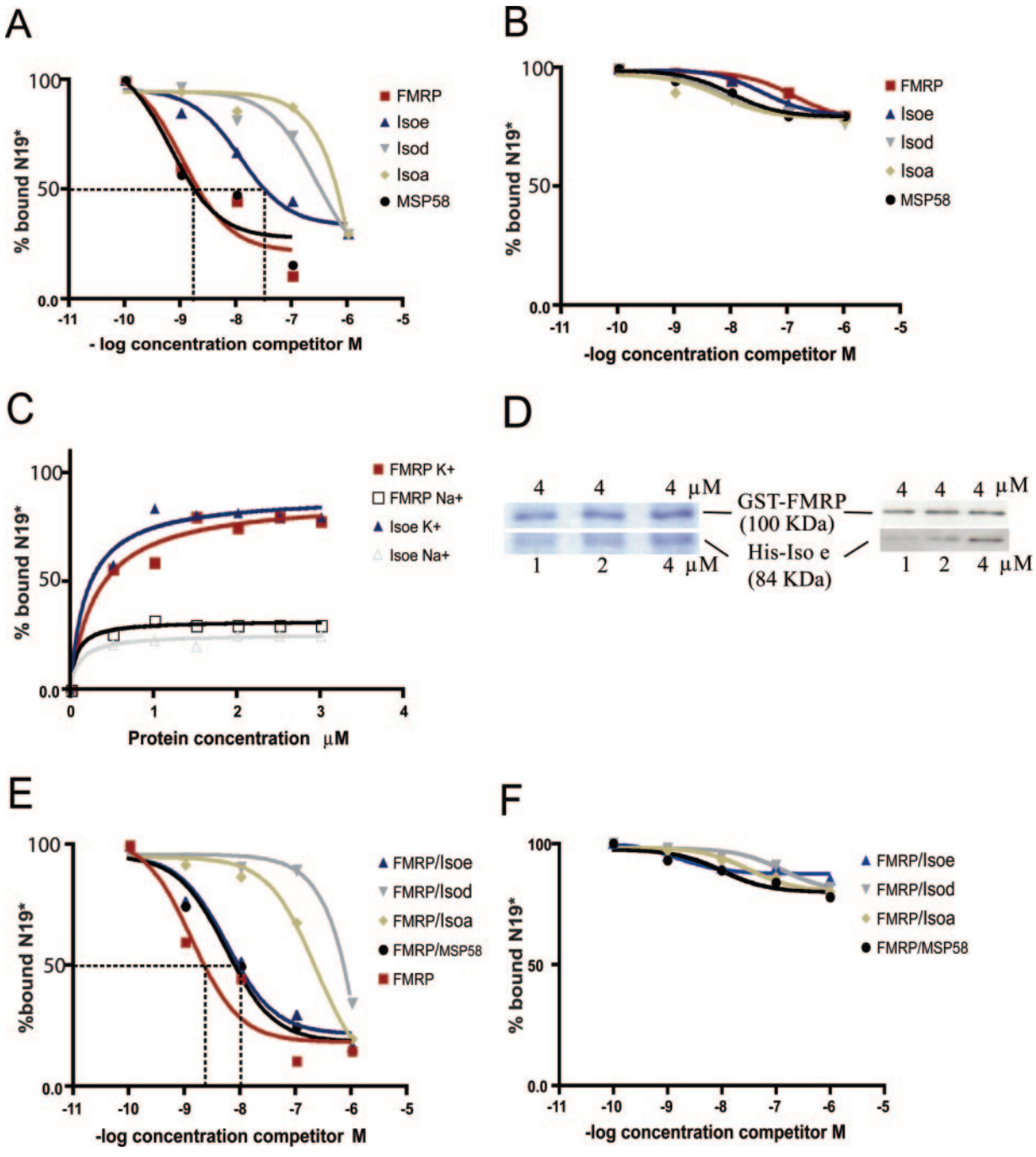

Figure 2. RNA binding properties of FMRP and FXR1P isoforms. (**A**) Filter binding assay using FMRP, FXR1P-Isoe, Isod, Isoa and MSP58. The RNA probe used is ^{32}P-labeled N19 RNA, and competition was performed using the same unlabeled RNA. (**B**) The same experiment was repeated using as competitor the N8 RNA sequence, not containaing any G-quartet forming structure. (**C**) Filter binding assay was repeated with an increasing amount of FMRP and Isoe in the presence of Na^+ or K^+. (**D**) GST-pull down was performed as described in Materials and Methods. On the right part of (D), proteins used in GST-pull down assay were revealed by immunoblot. FMRP was detected by monoclonal 1C3 antibody, FXR1P by the monoclonal 3FX antibody. Lane 1: 4 µM GST-FMRP complexed with 1 µM His-FXR1P, Lane 2: 4 µM GST-FMRP complexed with 2 µM His-FXR1P, Lane 3: 4 µM GST-FMRP complexed with 4 µM His-FXR1P. On the left part of (D), proteins used in GST-pull down experiment were revealed by Coomassie stained gel. (**E**) Competition assay to determine the K_d at the equilibrium state binding FMRP, the heterodimers FMRP/Isoe, FMRP/Isod, FMRP/Isoa and the complex FMRP/MSP58, with the ^{32}P-labeled N19 probe and competed with unlabeled N19. (**F**) The same experiment described in (E) was repeated using the N8 RNA as unlabeled competitor.

the assayed RNAs (13). In addition, we repeated the same analysis by competing the binding of ^{32}P-labeled N19 probe with the G-quartet forming RNA structures obtained from the 5′-UTR of PP2Ac (that contains four G-quartet forming structures) (25) and obtained the same results (data not shown) that are described in Figure 2A using N19 RNA competition.

Since FXR1P isoforms display different G-quartet binding specificity, we asked whether they would affect differently FMRP binding affinity to G-quartet. First, we verified the

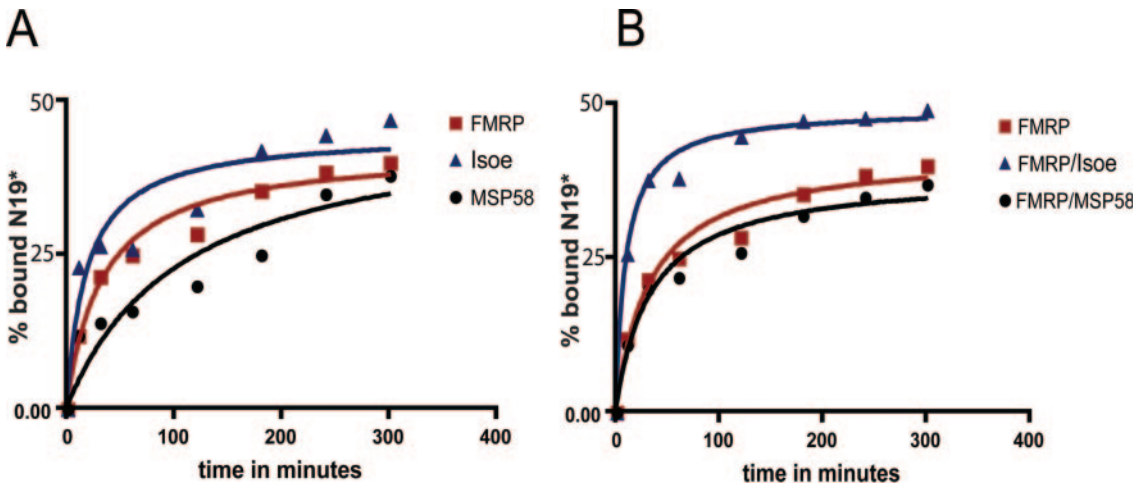

Figure 3. Association rate of FMRP, Isoe and MSP58. (**A**) Each protein was mixed with ^{32}P-labeled N19 RNA probe for a time lapse of 10, 30, 60, 120, 180, 240 and 300 min and then each reaction was filtered and the amount of retained radioactivity evaluated. (**B**) The same experiment described in (A) was repeated with the complex FMRP/MSP58 and FMRP/Isoe as indicated in the figure.

amounts of FXR1P and FMRP that integrate the heterodimer complex. To this purpose, we performed GST-pull down experiments by mixing 4 μM of GST-FMRP with increasing amounts (1, 2, 4 μM) of His-FXR1P-Isoe, Isod or Isoa (In Figure 2D, the results are shown only for interaction between FMRP and FXR1P-Isoe). The beads were then treated with glutathione and the eluted proteins were revealed by immunoblot using the two monoclonal antibodies 1C3 (24) and 3FX (21) recognizing FMRP and FXR1P, respectively. As shown in Figure 2D, the ratio of released FXR1P and FMRP is around 1:1 when mixed in stochiometric amounts. This result shows that when the two proteins are mixed *in vitro* their association is dose-dependent and, on the other side, also shows that our results are not due to an unbalanced ratio of the two interacting proteins in the FXR heterodimers.

Subsequently, we tested the ability of the FXR1P-Isoe/FMRP heterodimer to bind G-quartet RNA. Indeed, the FXR1P-Isoe/FMRP complex binds G-quartet RNA with a comparable affinity as the FMRP homodimer at different concentrations of competitor RNA (Figure 2E). Surprisingly, FXR1P-Isod and Isoa inhibit FMRP binding to G-quartet RNA when these form a heterodimer with the latter protein (Figure 2E). As a control, MSP58 protein, that binds G-quartet RNA in a specific manner (19), was used (Figure 2E), and its binding to FMRP leads to the same results as when FMRP is complexed to FXR1P-Isoe. When we used the N8 probe as a negative control, no displacement of the equilibrium was observed, as shown in Figure 2F.

In view of these results, we decided to better dissect the dynamics of FXR1P/G-quartet RNA and FXR1P/FMRP/G-quartet RNA interactions. We evaluated the velocity of interaction of the two FXR proteins with G-quartet RNA. For this purpose 1 pmol of each protein was mixed with 5 fmol of labeled N19 RNA. At different time points (10, 30, 60, 120, 240 or 300 min), the assay was stopped and the amount of radioactivity bound by the proteins evaluated by the filter binding assay. The time necessary for FMRP/FXR1P-Isoe heterodimer to bind the half amount of total bound RNA ligand was estimated to be 9.93 min, which is lower than the time employed by FMRP or FXR1P homodimers (33.65 and 19.04 min, respectively) to bind the same amount of RNA probe (Figure 3A and B). This result indicates that the Kon for the heterodimer is higher than the Kon for both homodimers. Conversely, the presence of MSP58 complexed with FMRP did not influence its binding to G-quartet RNA.

We then investigated the kinetics of FXR1P-Isoe and FMRP dissociation from the G-quartet RNA structure, when both bind to it as homodimers or as heterodimers. FMRP was mixed with ^{32}P-labeled N19 RNA and the complex was allowed to form for 10 min on ice. Then 1 μM of cold RNA [N19 or N8, as a negative control (25)] was added, the reaction was stopped after 10, 30, 60, 120, 240 or 300 min and the amounts of retained radioactivity evaluated by filter binding assay. In this experiment, it is interesting to observe (Figure 4A) that the FXR1P-Isoe homodimer releases 50% of total bound RNA after only 15 min. As expected from its higher affinity, the FMRP homodimer releases the same amount of bound RNA after a longer lapse of time, around 45 min. The negative controls are shown in Figure 4B. In this case the binding of recombinant proteins to G-quartet containing RNA was competed using the N8 probe. Indeed, only 20–25% of the binding is competed after more than 4 h of incubation.

Finally, the heterodimer FXR1P-Isoe/FMRP and the complex MSP58/FMRP were analyzed. The effect of the heterodimer was dramatic: 50% of the labeled RNA was released after 5 min and 65% of labeled RNA was released after 10 min (Figure 4C), while the interaction with MSP58 does not change significantly the dynamics of the interaction between the G-quartet RNA and FMRP. In Figure 4D the negative controls are shown, confirming the specificity of the action described in Figure 4C. This data shows that formation of the FXR1P-Isoe/FMRP heterodimer increases the dynamics of protein–RNA interaction, favoring the release of bound mRNA.

DISCUSSION

FMRP is a component of multimolecular complexes involved in different steps of mRNA metabolism (2,12). A growing list

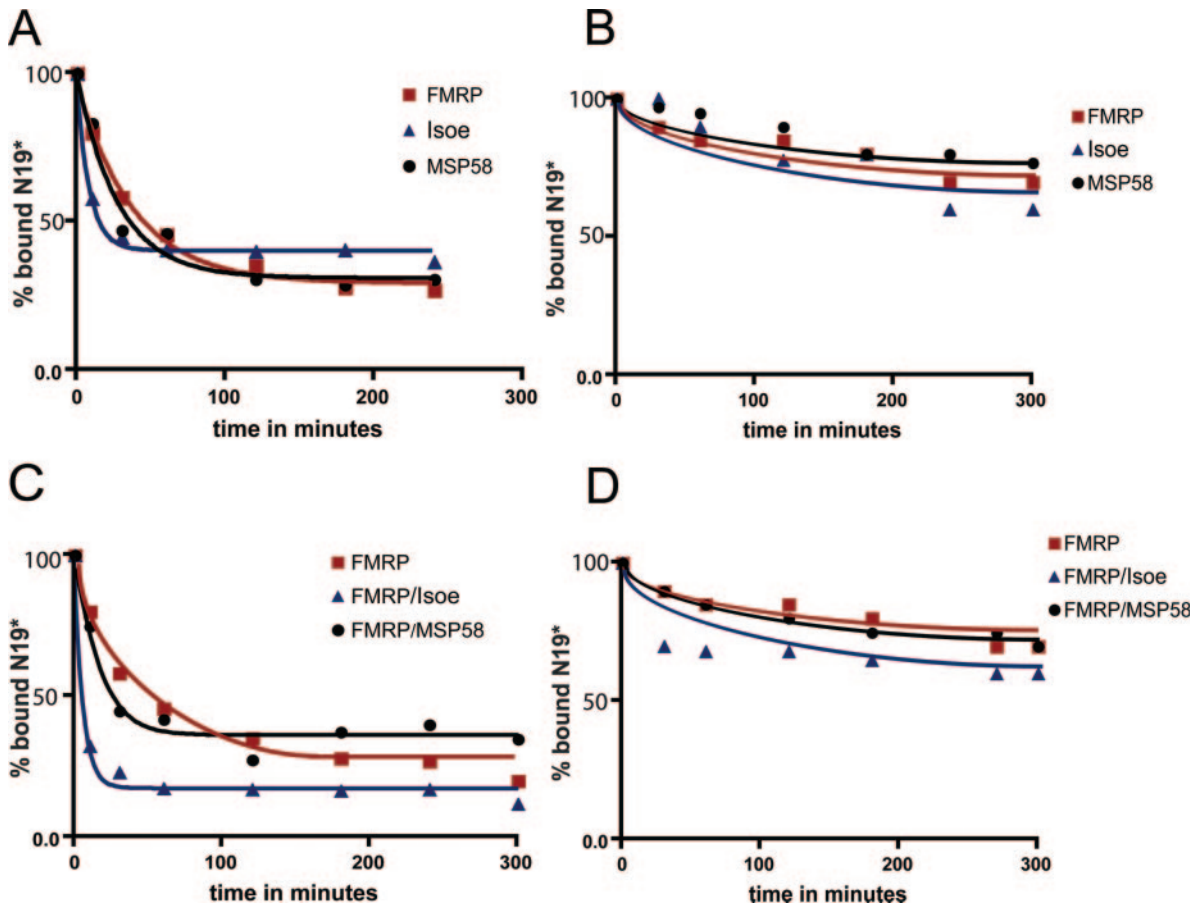

Figure 4. Dissociation rate of FMRP, Isoe, MSP58 and the two complexes FMRP/Isoe and FMRP/MSP58. (**A**) Each protein was mixed with ^{32}P-labeled N19 RNA probe for 10 min on ice and then an equal amount of unlabeled N19 RNA was added as competitor to each reaction, which was then filtered after a precise time lapse of 10, 30, 60, 120, 180, 240 and 300 min. The radioactivity retained on the filter was evaluated. (**B**) The same experiment described in (A) was repeated using unlabeled N8 RNA as competitor. (**C**) The same experiment described in (A) was performed using the protein complexes FMRP/Isoe and FMRP/MSP58. (**D**) The same experiment described in (C) was performed using the cold N8 RNA as competitor.

of proteins interacting with FMRP has been described, most of them being RNA binding proteins (19,33). In addition, several hundreds of mRNAs have been described as putative targets of FMRP (12,26), however the functional significance of most of the multiple interactions established by FMRP is still elusive (12,34). A widely accepted hypothesis proposes that FMRP can transport mRNA in mRNPs shuttling between structures where RNA translation is repressed and polyribosomes (2). In the absence of FMRP, the equilibrium in mRNP normally containing FMRP is perturbed, resulting in the deregulation of the expression and localization of a subset of its target mRNAs (12,26). Based on these considerations, we reasoned that RNA binding proteins belonging to the same mRNP complex, FMRP and its interacting proteins may enter in contact with the same mRNAs and decided to test the ability of FXR1P to bind the same mRNA targets and to influence its affinity for them. Up to date, the functions of FXR1P (or FXR2P) were inferred to be, by homology and analogy, similar to that of FMRP (1). Here we propose that it is not the case, at least for FXR1P.

First, we tested FXR1P affinity for G-quartet forming RNA structures. It is surprising that among the three isoforms analyzed only one, the Isoe is able to bind a G-quartet RNA forming structure, present in a large amount of putative target RNAs of FMRP (26). The three FXR1P isoforms share the same RGG box domain. However, it has been reported that, even though a peptide corresponding to the RGG box of FMRP binds specifically G-quartet forming RNA (30), the corresponding peptide of the RGG box of FXR1P does not (35), strongly suggesting that the RGG box of FXR1P is not sufficient *per se* to bind the G-quartet structure. The only difference between the two isoforms Isoe and Isod are two short sequences of 28 (exon 12) and 27 amino acid (exon 15) (cf. Figure 1A). Only this latter amino acids stretch appears to have putative RNA binding properties and is encountered solely in the Isoe isoform able to bind specifically the G-quartet structure. This 27 amino acid stretch encoded by FXR1P exon 15 being in close proximity to the RGG box of FXR1P encoded by sequences of exon 14 (Figure 1A), it may contribute to the binding to G-quartet mRNA structures together with the RGG box. Alternatively, the presence of this additional sequence in the FXR1P-Isoe, as compared to the other shorter isoforms, may alter the structure of the C-terminal portion of FXR1P, thereby allowing the binding. In a similar way, a different affinity for RNA was also shown for different FMRP isoforms. Indeed, ISO18, a minor isoform of FMRP lacking a small portion of exon 17 (29), is still able to bind G-quartet RNA (36),

like ISO1 and ISO7 (13,30). In addition, ISO 18 is able to bind the 3′-UTR of *FMR1* mRNA (36), that, conversely, is not bound by ISO1 and ISO7 (13,30).

In adult muscle, where both FMRP and FXR2P are absent, the FXR1P isoforms encountered both contain exon 15 sequences and correspond to a doublet of 82 (Isog) and 84 kDa (Isoe) (20,21,37). Their ability to bind G-quartet RNA structure suggests that, in this tissue, specific RNAs might be recognized by FXR1P via the interaction with G-quartet forming structure. Since FXR1P absence has a strong impact during muscle development (8,9), its RNA binding capacities are critical *per se*, independently from FMRP's fonction. Indeed, a recent knock-down analysis for *xFxr1* produced a list of putative FXR1P target RNAs. Interestingly, several of their human homologues harbor a putative G-quartet structure [(9), and our unpublished data]. In addition, very little is known concerning the precise function of FXR1P in muscle, and it is possible that muscle-specific FXR1P interacting proteins might modulate its affinity for RNA.

Our analysis to dissect the binding capacities to G-quartet RNA FXR1P and its heterodimer with FMRP yielded unexpected findings. We observed a dramatic effect of the FMRP/FXR1P heterodimer on the dynamics of complex formation with G-quartet RNA. This effect was not observed when FMRP and its other partner MSP58 were mixed together, suggesting that MSP58 can probably compete for the same binding site as FMRP. In addition, the interaction of FMRP with FXR1P-Isoa or Isod strongly reduced FMRP specificity for G-quartet RNA. These different behaviors of the two FMRP-interacting proteins illustrate the complexity of the functions and interactions that take place in FMRP-containing mRNPs and in different tissues. FXR1P-Isod and Isoa are the FXR1P isoforms with the highest expression in brain, suggesting that in neurons FMRP interacts mostly with these two isoforms that might regulate negatively its action. Since in brain and cerebellum FXR1P-Isoe mRNA is expressed at a low level as revealed by RT–PCR (our unpublished data), probably only a very small portion of FMRP may be regulated by FXR1P-Isoe. Conversely, FMRP and FXR1P-Isoe and Isog isoforms are co-expressed in myoblasts and in myotubes, suggesting a particular regulation of G-quartet containing target mRNAs during muscle differentiation but not in adult muscle, where FMRP is not expressed anymore (21). The present study highlights the functional differences between FXR1P isoforms and therefore emphasizes the importance of the extensive tissue-specific alternative splicing undergone by *FXR1* mRNA. In view of these results, it is clear that in each mRNP the ratio between FMRP and FXR1P different isoforms becomes important to precisely regulate FMRP function. The modulation of the affinity and/or of the dynamics observed for the FXR1P/FMRP heterodimer may reflect a regulation of the exchange of mRNAs between mRNPs or trafficking granules and polyribosomes.

The interaction domain of the two FXR proteins is localized in the N-terminal region of both proteins. This domain mediates the interaction between FMRP and several other proteins (FXR2P, CYFIP1, CYFIP2, NUFIP and 82-FIP) (10,33). On the other hand, despite the high level of homology, the N-terminal region of FXR1P seems to interact only with CYFIP2 and FXR2P (18). CYFIP2, together with CYFIP1, that only interacts with FMRP, belongs to a small family of proteins linking FMRP to the Rac pathway (18,33,38). We have previously proposed that the CYFIP proteins might modulate the ability of the FXR family members to homo and/or heterodimerize (18).

FXR1P and FXR2P are believed to have distincts but overlapping function in conjunction with FMRP, with the possibility to partially compensate for its absence. Our results show here a completely different function for two different FXR1P isoforms, which modulate the action of FMRP. This data reveals how a full understanding of FMRP function may be achieved through the deciphering of the global action of FMRP-containing mRNP complexes.

ACKNOWLEDGEMENTS

The authors are grateful to Alexandra Charlesworth for help. B.B. is supported by CNRS, Fondation J. Lejeune, GIS Maladies Rares, Fondation pour la Recherche Médicale, FRAXA Foundation and 'Contrat d'interface' from INSERM and CHU de Nice. E.B. is recipient of a fellowship from Fondation pour la Recherche Médicale. E.L. is supported by CNRS, Fondation pour la Recherche Médicale and ARC. L.D. is supported by the FRAXA Foundation and by a short term fellowship from the Boeringher Ingelheim Fund and the Conquer Fragile X Foundation. E.W.K. is supported by the NSERC. The authors also thank the support of the French Embassy in Canada for their Travelling Exchange Programm. Funding to pay the Open Access publication charges for this article was provided by CNRS.

Conflict of interest statement. None declared.

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Publication 3

Using a yeast two hybrid system, MSP58 was shown to interact specifically with FMRP. In the cytoplasm, MSP58 is associated with polyribosomes. MSP58 showedan affinity for RNA homopolymers. As an interactor of FMRP that shows to some extent the same localization, my contribution to this study was to analyze the ability of MSP58 to recognize and bind G-quartets as FMRP.

We demonstrated that MSP58 binds specifically the G-quartets structure with an affinity similar to that of FMRP.

We further analyzed the dynamics of FMRP/MSP58 G-quartet association and dissociation and showed that MSP58 acts in a different way than the other interactor, FXR1P (see publication 2)

Human Molecular Genetics, 2006, Vol. 15, No. 9 ***1525–1538***
doi:10.1093/hmg/ddl074
Advance Access published on March 29, 2006

The nuclear MicroSpherule protein 58 is a novel RNA-binding protein that interacts with fragile X mental retardation protein in polyribosomal mRNPs from neurons

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Received February 14, 2006; Revised and Accepted March 19, 2006

The fragile X syndrome, the leading cause of inherited mental retardation, is due to the inactivation of the fragile mental retardation 1 gene (*FMR1*) and the subsequent absence of its gene product FMRP. This RNA-binding protein is thought to control mRNA translation and its absence in fragile X cells leads to alteration in protein synthesis. In neurons, FMRP is thought to repress specific mRNAs during their transport as silent ribonucleoparticles (mRNPs) from the cell body to the distant synapses which are the sites of local synthesis of neuro-specific proteins. The mechanism by which FMRP sorts out its different mRNAs targets might be tuned by the intervention of different proteins. Using a yeast two-hybrid system, we identified MicroSpherule Protein 58 (MSP58) as a novel FMRP-cellular partner. In cell cultures, we found that MSP58 is predominantly present in the nucleus where it interacts with the nuclear isoform of FMRP. However, in neurons but not in glial cells, MSP58 is also present in the cytoplasmic compartment, as well as in neurites, where it co-localizes with FMRP. Biochemical evidence is given that MSP58 is associated with polyribosomal poly(A)+ mRNPs. We also show that MSP58, similar to FMRP, is present on polyribosomes prepared from synaptoneurosomes and that it behaves as an RNA-binding protein with a high affinity to the G-quartet structure. We propose that this novel cellular partner for FMRP escorts FMRP-containing mRNP from the nucleus and nucleolus to the somato-dendritic compartment where it might participate in neuronal translation regulation.

INTRODUCTION

The fragile X syndrome (FXS) is the leading cause of inherited mental retardation, due to the silencing of the fragile X mental retardation 1 gene (*FMR1*) and the subsequent absence of FMRP, its gene product. FXS affects 1/4000 males and 1/7000 females worldwide and is characterized by moderate to severe mental retardation. The phenotype is complex and is also accompanied by physical abnormalities such as facial dysmorphism, postpubertal macro-orchidism in males and mild connective tissue dysplasia (1–4). FMRP is an RNA-binding protein widely expressed in mammalian tissues (5) and particularly abundant in neurons (6) and is an element of messenger ribonucleoprotein (mRNP) complexes associated with brain polyribosomes (7–9). Its presence within the translational apparatus suggests that it is involved in the translational control of certain mRNAs. In addition, FMRP is present in mRNA cargoes that are transported along neurites and dendrites (10,11). Its interaction with the RNA-induced silencing complex in the cytoplasm suggests

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that it can also play a role in the control of the stability of mRNA and/or it acts as translational inhibitor via the interaction with the AGO proteins (12).

Two KH domains and an RGG box modulate the ability of FMRP to bind RNA. The RGG box has high affinity for the RNA G-quartet structure (13,14) present in several of FMRP's mRNA targets (15), whereas the KH2 domain seems to recognize synthetic RNAs aptamers presenting a loop–loop pseudoknot-specific motif or 'kissing complex' (16). In *Fmr1* null mice, a series of mRNA display change, both in localization and abundance, pointing out the critical role that FMRP plays in the targeting of specific mRNAs (17,18). It is proposed that the absence of FMRP would lead to alterations in the local synaptic synthesis of proteins that are essential for synaptic development and maturation. One of the consequences of the absence of FMRP is the presence of abnormal looking immature and surnumerary neuronal dendritic spines in the brains of fragile X patients (19,20), which ultimately lead to mental retardation in FXS patients.

FMRP is able to interact directly through its N-terminal domain with a series of protein partners. Among these, FXR1P and FXR2P are two closely related paralogues that show high levels of homology with FMRP (21,22) and can form heterodimers with FMRP. The FMRP-interacting protein 82-FIP is an RNA-binding protein that is present in polyribosomal mRNPs (23), whereas NUFIP1, also an RNA-binding protein, is a nucleocytoplasmic shuttling protein that is associated with active synaptoneurosomes (24). Two paralogous proteins, CYFIP1 and CYFIP2, that interact with FMRP also provide a link to the Rho GTPase pathway that controls actin cytoskeleton dynamics (25,26). More recently, IMP1, a zip-code protein involved in mRNA transport, has also been found to directly interact with FMRP (27). RanBPM, a protein involved in the microtubule-organizing centre has been reported to bind to the C-terminus of FMRP (28). In addition, other RNA-binding proteins such as nucleolin, YB-1/p50, Pur-α and Staufen have been detected in complexes containing FMRP, but it is not known whether they interact directly or indirectly with FMRP (29–31). Only a few non-RNA-binding proteins have been shown to interact with FMRP, including myosin Va (31) and Lgl in *Drosophila* (32).

In this study, we describe a novel FMRP-interacting protein named MicroSpherule Protein 58 (MSP58). We provide evidence that MSP58 associates with polyribosomal poly(A+) mRNP and is also found as a polyribosome component in synaptoneurosomes. Moreover, we show that MSP58 is a novel nuclear RNA-binding protein able to bind G-quartet RNA.

RESULTS

MSP58 is a novel FMRP-interacting proteins

To identify new FMRP-interacting proteins specific to neurons, we screened a human fetal brain cDNA library using a yeast two-hybrid system, with the major FMRP Iso7 form as a bait. From $\sim 2.8 \times 10^6$ clones screened, 78 positive clones displayed adenine and histidine prototrophy as well as β-galactosidase activity. PCR and restriction analysis of selected colonies showed that among these, three clones were redundant and carried a 1.7 kb insert. This insert corresponded to the full-length cDNA of a human nuclear/nucleolar protein of 58 kDa called MSP58 (33). The predicted open-reading frame encodes a protein of 462 residues. The cDNA obtained from the yeast two-hybrid screening also harboured the 5′ and 3′-untranslated regions which were identical to the previously established sequence (33).

The specificity of the interaction of MSP58 with FMRP was confirmed in yeast by co-transforming the AH109 strain with plasmids encoding MSP58 and FMRP (Table 1). Given the high degree of similarity between all members of the FXR family, we also tested the ability of MSP58 to interact with FXR1P and FXR2P, the two other members of the FXR family. MSP58 was found to interact with FMRP, FXR1P and FXR2P (Table 1), whereas no interaction was detected with control plasmids bearing unrelated proteins cDNA. The strong interaction between MSP58 and the FXR proteins induced us to further investigate FMRP/MSP58 interaction.

MSP58 is a highly evolutionarily conserved nuclear protein

MSP58 was initially shown to interact with the nucleolar protein p120, and its overexpression led to enlargement of nucleoli (33). Also, MSP58 was reported to play a role in modulation of Daxx-dependent transcriptional repression (34). Recently, a study revealed that MSP58 behaves as an oncogene and that its transformation activity can be inhibited by physical interaction with the PTEN tumour suppressor (35). Moreover, it was reported that MSP58 associates to and stabilizes the transcriptional activity of the bHLH trancription factor STRA13 (36). Finally, MSP58 was shown to interact with Mi-2β in the nucleolus and to up-regulate ribosomal gene transcription (37). However, despite of all these data, the cellular role of MSP58 remains unclear because divergent properties/functions have been attributed to the protein.

We searched for sequences displaying similarities with human MSP58 cDNA and found homologues in species as distant as human (hMSP58, also called MCRS1), mouse (mMSP58), *Xenopus laevis* (xMSP58 or xMCRS1), zebrafish (zMSP58 or zMCRS1), quail (qMSP58 or qTOJ3) and finally *Drosophila melanogaster* (dMSP58 or CG1135-PA) (Fig. 1A). Alignment of all sequences revealed that all homologues share a high level of similarity. Indeed, human and mouse MSP58 proteins are 98.7% identical, differing by only six residues over 462, whereas human shares 81% identity with its frog counterpart. As the presence of highly conserved residues stretches should delineate domains essential for MSP58 function(s), we performed computational analysis of these sequences. The N-terminal part contains a highly conserved domain bearing a putative bipartite nuclear-localization signal (amino acids 32–46), as well as a putative nucleolar-localization signal (amino acids 44–56). This stretch of residues 44–56 KRRSSR-IKRKKFDDELVSS is 100% conserved over species, showing slight divergences only in *Drosophila*, suggesting functional relevance of this domain. Further down the sequence, a putative monopartite nuclear-localization signal (amino acids 113–123) is detected, whereas a coil–coil domain is predicted at position 301–350. Despite the high level of conservation of the central part of MSP58, i.e. amino acids 125–294, no similarity to

Table 1. MSP58 interacts with FMRP, FXR1P and FXR2P in yeast

	Plasmid 1	Plasmid 2	Growth on AHLT medium	β-galactosidase activity	Interaction
Interaction test	pGBKT7/FMRP	pACT2/MSP58	++++	++++	+
	pGBKT7/MSP58	pACT2/FMRP	++++	+++	+
	pGBKT7/FXR1P	pACT2/MSP58	++++	+++	+
	pGBKT7/FXR2P	pACT2/MSP58	++++	+++	+
Positive control	pGBKT7/FMRP	pACT2/FXR1P	++++	++++	+
	pGBKT7/p53	pACT2/T antigen	++++	++++	+
Negative control	pGBKT7/MSP58	pACT2	−	N/A	−
	pGBKT7/MSP58	pACT2/T antigen	−	N/A	−
	pGBKT7	pACT2/MSP58	−	N/A	−
	pGBKT7/p53	pACT2/MSP58	−	N/A	−

AH109 yeast strain was co-transformed with the indicated plasmids. Test for interaction was performed by plating the transformants on a medium depleted in adenine and histidine, to check for activation of the reporter genes *ADE2* and *HIS3*. All transformants positive for interaction on medium depleted in adenine and histidine showed a blue colour in the presence of X-Gal, confirming the *Lac Z* activation. Interaction could be detected between FMRP and MSP58, FXR1P and MSP58 as well as FXR2P and MSP58. Growth was comparable to strains transformed with FMRP, FXR1P, FXR2P as well as with p53 and the T antigen which are known to interact, whereas no growth was observed when MSP58 was transformed together with the empty pGBKT7 vector or with pGBKT7-p53 which is an unrelated protein.

known functional domains were found. The C-terminus of all MSP58 homologues contains a highly conserved forkhead-associated (FHA) domain (Fig. 1B). FHA domains are known to interact with phosphorylated residues and indeed, MSP58 FHA domain was shown to specifically bind phosphorylated residues of STRA13 and PTEN (35,36).

MSP58 N-terminal domains interact with the phosphorylation domain of FMRP

To confirm the results obtained in yeast and to verify whether the FMRP/MSP58 interaction was direct and specific, we performed a series of pulldown assays using the FMRP full-length protein, as well as FXR1P (Iso7) and FXR2P. These proteins were produced and labelled with [^{35}S]methionine by *in vitro* transcription–translation using the rabbit reticulocyte lysate, whereas human MSP58 was produced as a fusion protein with glutathione *S*-transferase (GST) in bacteria. The GST–MSP58 fusion protein immobilized on glutathione–Sepharose was incubated with either *in vitro*-translated FMRP, FXR1P, FXR2P or luciferase as a negative control. The three members of the FXR family were shown to bind to the immobilized GST–MSP58, whereas luciferase did not (Fig. 2A). No binding to GST was observed, confirming the specificity of the interaction. To verify that the interaction between FMRP and MSP58 was direct and not RNA-dependent, GST pulldowns between *in vitro*-translated FMRP and GST–MSP58 bound to beads were performed in the presence of RNase A, RNase T1 and DNase I. This treatment did not alter FMRP binding to MSP58 (data not shown), indicating that the interaction occurs at the protein–protein level.

To refine the area of interaction between MSP58 and FMRP, MSP58 fragments were fused to GST and tested for their ability to retain ^{35}S-radiolabelled FMRP. As shown in Figure 2B, MSP58 N-terminus encompassing amino acids 1–124 was sufficient to bind FMRP, and *a fortiori* the stretch of amino acids 1–294 as well as the full-length MSP58, indicating that FMRP-binding domain lies in the N-terminus. In contrast, to determine the region of FMRP that binds MSP58, several FMRP variants and deletion constructs were tested for their ability to bind GST–MSP58. As MSP58 has been previously reported to behave as a nuclear protein, we also tested its ability to bind the nuclear FMRP isoform (Iso6) lacking exon 14 (amino acids 426–490) that encompasses the NES. As shown in Figure 2C, FMRP full-length (Iso7) and Iso6 and all FMRP versions truncated before (amino acids 1–526) and after the RGG box (1–554) were able to bind recombinant GST–MSP58. Because FMRP version truncated at amino acids 426 was not able to bind MSP58, although FMRP Iso6 lacking amino acids 426–490 still interacts with MSP58, we concluded that the binding domain to MSP58 lies between amino acids 490 and 526 (Fig. 2E). Interestingly, it encompasses the phosphorylation domain of FMRP which has been mapped at amino acids 444–526 (38–40). This domain is also present in FXR1P and FXR2P and displays a highly conserved block of residues among the three proteins (Fig. 2D).

We then questioned whether point mutations in the serine 500 residue of FMRP, known to be phosphorylated (38), would affect its binding to MSP58. Substitution of the S500 by the unphosphorylable residue alanine or by the acidic residue aspartate, which mimics a charged phosphorylated serine, did not, at least *in vitro*, affect the binding of MSP58.

Subcellular localization of MSP58

According to the few reports available in the literature, MSP58 is supposed to be either exclusively nucleolar or nucleolar and nucleoplasmic (33,34,36,37) or even cytoplasmic (35). It is worth mentioning that most of these studies utilized cells transiently expressing an HA-tagged MSP58 that was revealed with anti-HA antibodies, whereas the cellular distribution of the *bona fide* endogenous MSP58 has not been fully documented. We first raised an anti-MSP58 antibody in rabbit against the central part of human protein (amino acids 125–294).

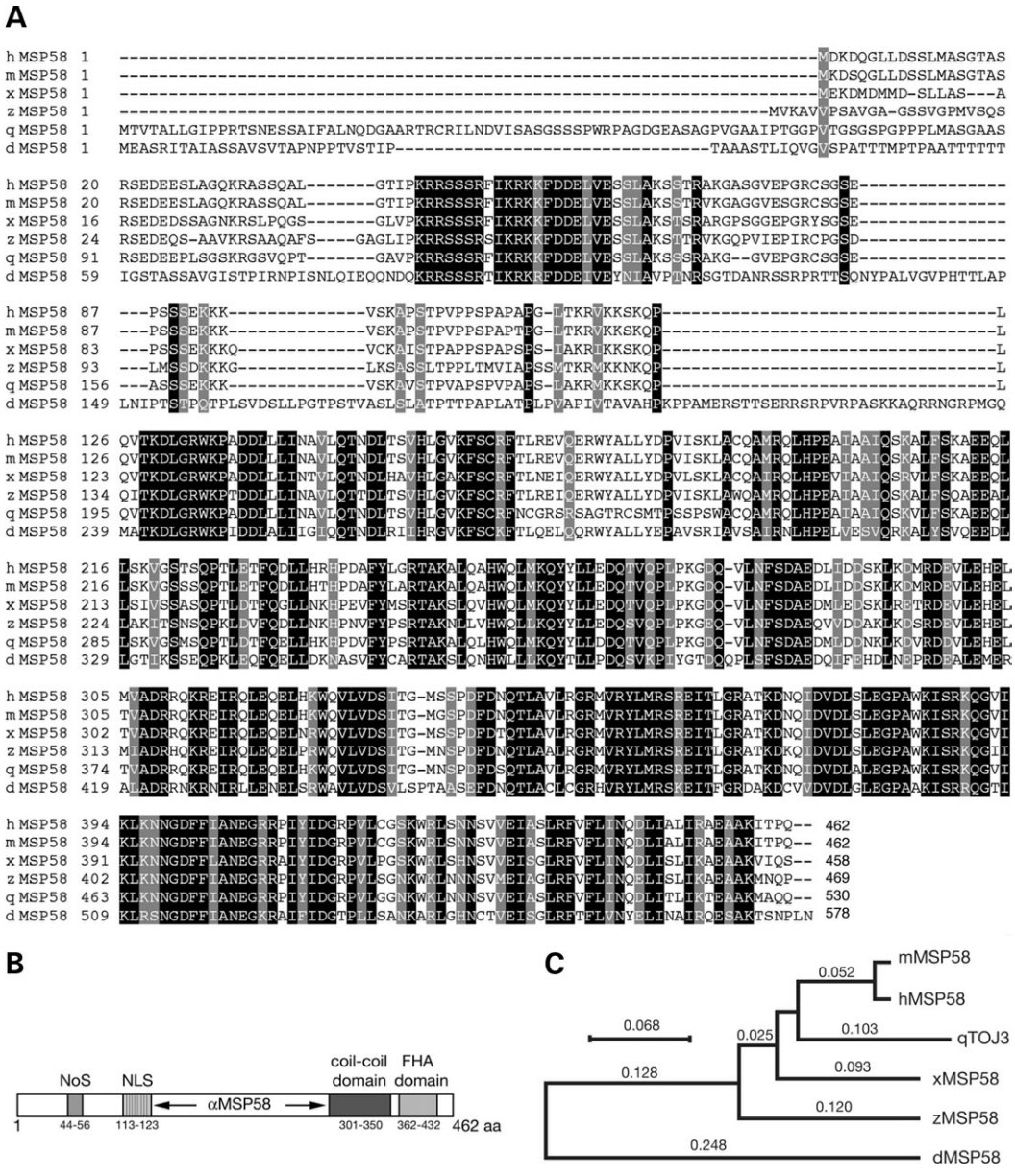

Figure 1. MSP58 is highly evolutionarily conserved. (**A**) MSP58 is widely represented among species as divergent as human (h), mouse (m), *Xenopus* (x), zebrafish (z), quail (q) and *Drosophila* (d). The amino acids highlighted in black are conserved among all species; those highlighted in grey are similar. Note the high level of conservation among the different species, especially in the C-terminal portion of the protein. Clusters of amino acid identity can be detected all along the sequence, particularly in the predicted functional domains. (**B**) Analysis of MSP58 sequences among species reveals conserved features. The N-terminus bears a putative nucleolar-localization signal (NoS) as well as putative monopartite nuclear-localization signal (NLS). In the second half of the protein lies a predicted coiled-coil domain and finally in the C-terminus a highly conserved FHA domain. (**C**) Phylogenetic tree of the MSP58 family. Sequences alignments used to construct the tree were obtained using Clustalx software. The phylogenetic tree was then generated using the program PhyloWIN and the neighbour-joining algorithm, with 500 bootstrap replicates. Respective lengths of the tree branches are indicated and illustrate the ratio of amino acid differences between sequence pairs.

One antiserum was obtained, and after immuno-affinity purification, its specificity towards MSP58 was determined by both immunoblot analyses and immunofluorescence staining using HeLa and Cos-1 cells transfected with an expression vector for *hMSP58*. In addition, we observed that different staining patterns were obtained depending on the procedures used to fix the cells prior to the immunofluorescence stainings, and we assumed that the discrepancies observed in its subcellular localization reported by others were due to the fixation procedures. A classical 10 min 4% PFA fixation followed by a 0.5% Triton permeabilization treatment revealed an exclusively nucleolar staining (Fig. 3A), whereas a 10 min fixation with 4% PFA in the presence of 0.5% Triton showed both nuclear and nucleolar localization (Fig. 3B). In contrast, a combined fixation and permeabilization procedure using formaldehyde in a mixture of acetone and methanol

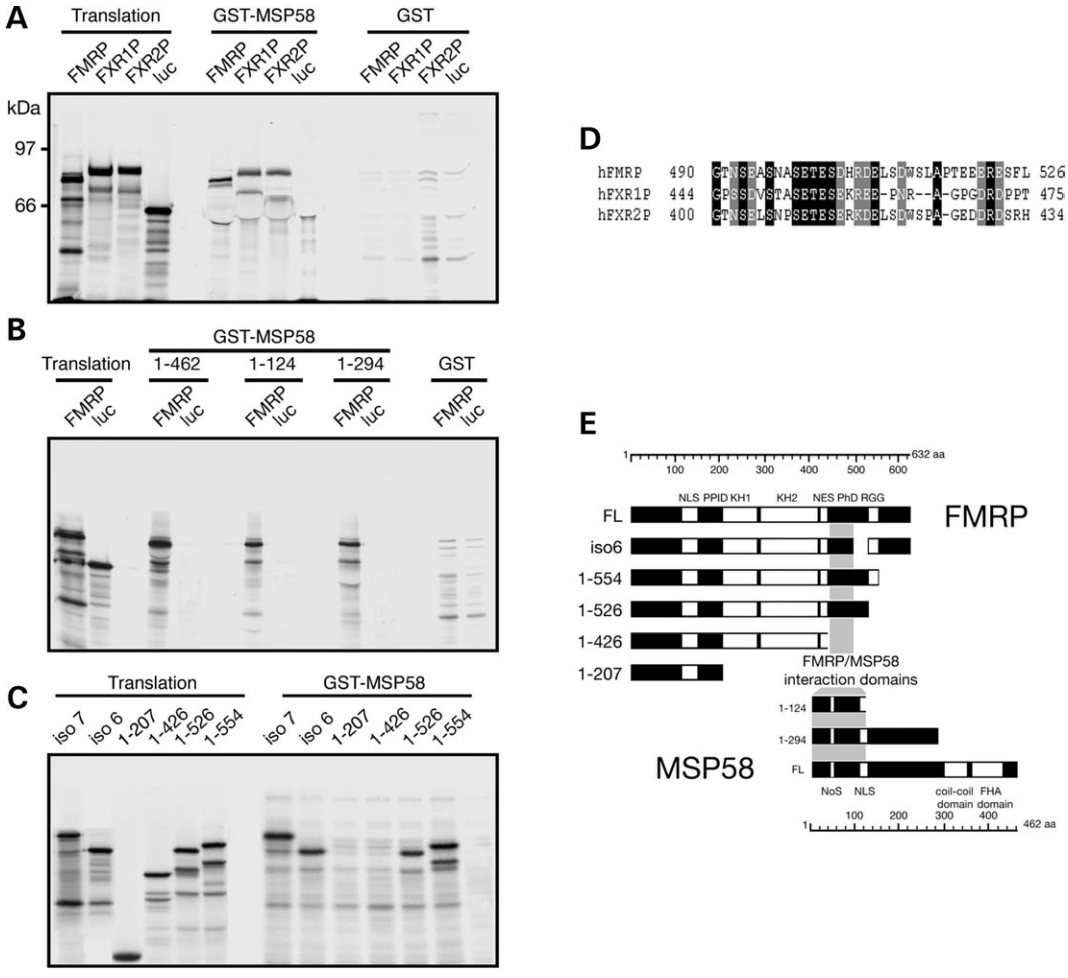

Figure 2. *In vitro* interaction of FMRP with MSP58 in a pulldown assay. (**A**) Pulldown assay using 1 μg of the fusion protein GST–MSP58 and *in vitro*-translated ^{35}S-labelled FMRP, FXR1P and FXR2P. (**B**) *In vitro*-translated FMRP binds to GST–MSP58 as well as to its amino acids 1–124 and 1–294 fragments, indicating that MSP58 N-terminal domain is the minimal binding domain for FMRP. (**C**) *In vitro*-translated FMRP Iso7 and the nuclear Iso6 form as well as the N-terminal truncated FMRP fragments were used to map the domain of interaction between FMRP and MSP58. FMRP Iso7 and Iso6 as well as FMRP versions truncated just before (amino acids 1–526) and after the RGG (amino acids 1–554) were able to bind recombinant GST–MSP58. FMRP fragments truncated more N-terminally: amino acids 1–207 and 1–426 did not bind to GST–MSP58. (**D**) The domain encompassing amino acids 490–526 that binds MSP58 corresponds to the phosphorylation domain of FMRP and is highly conserved in FXR1P and FXR2P. (**E**) Schematic diagram summarizing the data presented in (A–C).

(2/19/19 by vol) allowed to visualize both nucleoplasmic and nucleolar distribution of MSP58 and also a faint cytoplasmic staining. In control analyses, no staining was detected when the antibodies were pre-incubated with recombinant purified GST–MSP58 amino acids 125–294 that was used for immunization (Fig. 3D). On the basis of these results, we concluded that MSP58 lies predominantly in the nucleus, whereas a small fraction is detected in the cytoplasm.

MSP58 recruits FMRP nuclear Iso6 into the nucleolus

To better visualize *in vivo* the subcellular localization of MSP58, we fused its cDNA to the red fluorescent protein (RFP) and transfected Cos-1 cells with this construct. Exogenous RFP–MSP58 strongly accumulates in the nucleolus but is also present in the nucleoplasm (Fig. 4A). We also noticed that upon overexpression of RFP–MSP58 nucleoli became enlarged, in agreement with Ren *et al*. (33). This nucleolar accumulation was also reproducible in HeLa and *Fmr1*−/− STEK cells (data not shown). The specificity of the distribution was assessed by immunostaining of the RFP–MSP58 fusion protein with anti-MSP58 and indeed a co-labelling of RFP–MSP58 and the endogenous MSP58 was observed (not shown). In this series of analyses, we were not able to detect a cytoplasmic staining due to the relative extremely strong nucleolar accumulation of exogenous RFP–MSP58. Alternatively, we speculate that high levels of RFP–MSP58 present in the nucleolus might have deleterious effects on the metabolism of the cell and prevents normal nuclear export of the protein.

To study *in vivo* the interaction between MSP58 and FMRP, we used in transfection experiments the two constructs encoding, respectively, RFP–MSP58 and the nuclear Iso6 FMRP fused to GFP. In single transfected Cos-1 cells, GFP–FMRP Iso6 localizes in the nucleoplasm and is excluded from

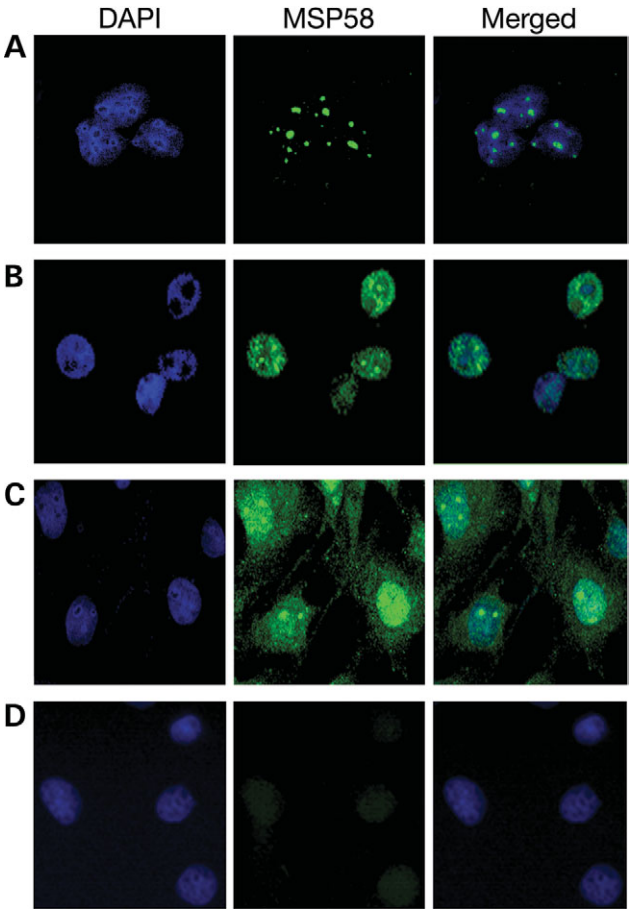

Figure 3. Apparent cellular distribution of MSP58 is conditioned by the fixation procedures prior to immuno-labelling with anti MSP58 antibodies. Cos-1 cells were proceeded for three fixation regimes. (**A**) A 10 min fixation with 4% PFA followed by a 30 min permeabilization step with 0.5% Triton reveals an exclusively nucleolar staining. (**B**) A 10 min fixation with 4% PFA in the presence of 0.5% Triton shows a nuclear and nucleolar localization. (**C**) In contrast, a one-step fixation/permeabilization with formaldehyde/methanol/acetone mixture (2/19/19 by vol) not only displays a nucleoplasmic and nucleolar distribution of MSP58, but allows the detection of cytoplasmic MSP58. Shown in (**D**) is a control experiment performed on cells fixed as described in (C) and incubated with anti-MSP58 antibodies pre-incubated with recombinant GST–MSP58 amino acids 125–294 protein fragment used for immunization. Nuclei were stained with DAPI.

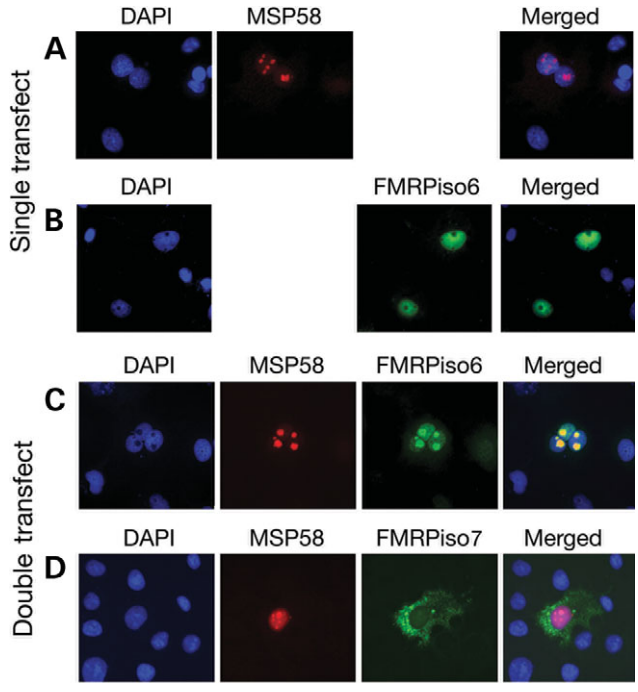

Figure 4. MSP58 recruits FMRP Iso6 into the nucleolus. (**A**) In Cos-1 cells, the majority of transiently expressed RFP–MSP58 is localized in the nucleolus. (**B**) Transiently expressed GFP–FMRP Iso6 is exclusively nucleoplasmic. (**C**) In cells co-expressing nuclear GFP–FMRP Iso6 and RFP–MSP58, both proteins co-localize in the nucleolus. (**D**) In cells double-transfected with cytoplasmic GFP–FMRP Iso7 and RFP–MSP58, MSP58 fails to attract FMRP Iso6 which remains in the cytoplasm.

the nucleolus (Fig. 4B). Interestingly, co-expression of RFP–MSP58 and GFP–FMRP Iso6 drastically altered the nucleoplasmic distribution of the latter. Indeed, in co-transfected cells, GFP–FMRP Iso6 was clearly recruited into the nucleolus (Fig. 4C). To ascertain that this phenomenon was not artefactual owing to the presence of the fusion proteins RFP and GFP, which are known to form homo and heteromultimers *in vivo*, we transfected Cos-1 cells with untagged versions of pTL1-FMRP Iso6 together with pTL1-MSP58 and revealed both proteins by immunofluorescence staining using anti-MSP58 antibody and mAb1C3 to FMRP, and the same phenomenon was observed (data not shown). To assess whether the ability of MSP58 to attract FMRP Iso6 in the nucleolus was not cell-type-specific, RFP–MSP58 and GFP–FMRP Iso6 were also co-expressed in different cell lines, i.e. HeLa and *Fmr1*−/− STEK cells, and similar observations were made suggesting that MSP58 has the ability to drag and retain FMRP Iso6 in the nucleolus.

In a second set of experiments, we co-transfected RFP–MSP58 with the cytoplasmic GFP–FMRP Iso7 and observed that GFP–FMRP Iso7 remained in the cytoplasm, whereas RFP–MSP58 accumulated in the nucleoplasm and the nucleolus (Fig. 4D). These results strongly suggest that MSP58 recruits the nuclear FMRP Iso6 while leaving the FMRP full-length in the cytoplasm. Indeed, MSP58 failed also to translocate the cytoplasmic FXR1P and FXR2P as seen in transfection assays with expression vectors coding for these homologues (data not shown).

Expression of MSP58 in rat tissues

Because *MSP58* expression was previously studied in murine tissues by RT–PCR or northern blot analyses (36), we determined the distribution of the protein by immunoblotting analyses using our new specific anti-MSP58 antibody. In agreement with previous studies at the mRNA level, MSP58 is detected in all tissues tested, albeit at different levels (Fig. 5). The highest levels of MSP58 were detected in spleen and testis, although strong signals were also present in brain. In muscular tissues such as cardiac and skeletal striated muscle, only trace amounts of MSP58 could be detected.

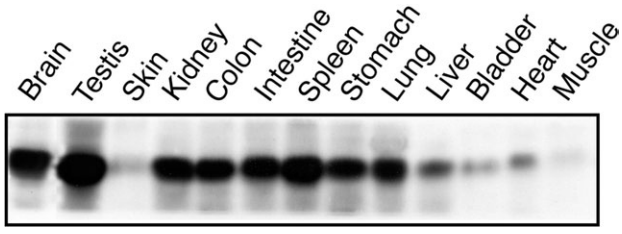

Figure 5. MSP58 is widely expressed in rat tissues. Distribution of FMRP in extracts from different tissues and organs from adult rat. Equal amounts of proteins (~60 μg) were subjected to immunoblot analyses using anti-MSP58 serum.

MSP58 co-localizes with FMRP in the cytoplasm of neurons

Because high levels of MSP58 are detected in brain extract, we determined its distribution in adult rat brain sections by immunohistochemical approaches using the MSP58 antibody. We observed strong staining in all nuclei throughout the brain with a specific nuclear localization in all glial cells (Fig. 6). Surprisingly, strong MSP58 staining was also detected in all cortical pyramidal neurons present in the cortex, and in neurons of the CA3 region of the hippocampus as well as in Purkinje cells in the cerebellum, where it co-localized with FMRP. We failed to detect MSP58 in the nucleolus, most probably due to the difficulties of penetration of the antibody in a fixed tissue. Essentially, the same distributions of MSP58 and FMRP were seen in paraffin-embedded sections of rat brain (data not shown). These observations illustrate that MSP58 is abundant in the cytoplasm of neurons, contrary to other cells, and co-localizes with FMRP and are in favour of an interaction between MSP58 and FMRP.

To further document the cytoplasmic localization of MSP58 in neurons, electron microscopy analyses were performed on immunogold-labelled rat brain sections using the purified MSP58 antibody. In granular cells of the cerebellum, MSP58 was restricted to the nuclei, whereas in Purkinje cells additional cytoplasmic localizations could be observed (Fig. 7A). At higher magnification, MSP58 could be detected associated with the ER, both in Purkinje cells (Fig. 7B) and in CA3 hippocampal neurons (Fig. 7C).

To verify whether MSP58 was also present in neurites, as is the case for FMRP, we used primary cultures of rat hippocampal neurons and double stained them with anti-MSP58 (revealed in red) and anti-FMRP (in green) and analysed both distribution by confocal microscopy. In neurons in primary culture, the majority of MSP58 is detected in the nucleus and nucleolus whereas FMRP was not. In contrast, while FMRP was predominantly detected in the cytoplasm, a substantial amount of MSP58 co-localized with FMRP in the cytoplasm (Fig. 8A). When a high gain that resulted in the saturation of the fluorescent signal in the cell body was used, a granular-like punctuate MSP58 staining was also clearly observed in the dendritic arborizations. At higher magnification, co-localization of FMRP and MSP58 was evident in the majority of granule-like structures in neurites (Fig. 8B).

MSP58 is present in polyribosomal mRNP complexes

It is well established that FMRP is present in mRNP complexes associated with heavy sedimenting polyribosomes prepared from total brain (8,9,41). Because MSP58 is strongly expressed in brain and is also encountered in the cytoplasm of neurons close to the ER (Figs 6 and 7), we asked whether it could be present within the translation machinery. We prepared brain polyribosomes as previously described and analysed by velocity sedimentation through sucrose density gradients (8). In the presence of Mg^{2+}, MSP58 was detected in fractions corresponding to heavy sedimenting polyribosomes and its distribution along the gradient mirrors that of FMRP (Fig. 9A). In the presence of EDTA that dissociates ribosomes into their subunits concomitant with the release of free mRNP complexes, MSP58 and FMRP were detected sedimenting in the same fractions, suggesting that MSP58 is present in mRNP complexes that also carry FMRP (Fig. 9B).

In neurons, polyribosomal aggregates are also present in dendritic spines and synapses and these structures contain FMRP (7,42). We therefore asked whether MSP58 would also escort mRNPs to synaptic polyribosomes. To test this hypothesis, we prepared a polyribosomal fraction from purified synaptoneurosomes and analysed this fraction by velocity sedimentation through sucrose density gradients. Two main results were obtained: first, FMRP is indeed associated with synaptosomal polyribosomes, in contradiction with the results obtained by Zalfa *et al*. (43), and secondly that MSP58 also is present in these structures (Fig. 9C). FMRP as well as MSP58 could be released from synaptosomal polyribosomes after treatment with the chelating EDTA agent (data not shown).

MSP58 is a novel RNA-binding protein associated with poly(A+) mRNP

We questioned the ability of MSP58 to be present in poly(A)+ mRNPs by performing oligo(dT) selection on purified 150–500S rat brain polyribosomes treated with EDTA to dissociate ribosomal subunits and to release their associated mRNPs. We observed that MSP58 is retained onto the oligo(dT) column and is eluted together with FMRP at 0.5 M NaCl. These results suggest that, similar to FMRP, MSP58 is an RNA-binding protein associated with poly(A+) mRNPs pointing out its ability to bind RNA *in vivo* as well as with other interacting proteins present in RNP complexes (Fig. 10A). Because most of the proteins interacting with FMRP present on polyribosomes are known to be RNA-binding proteins (8), we searched for the ability of MSP58 to bind RNA and performed RNA-homopolymers binding assays with purified recombinant GST–MSP58. Indeed, GST–MSP58 could selectively bind to polyG and polyU, but not to polyA or polyC (Fig. 10B), a pattern of binding to RNA homopolymers similar to that observed for FMRP (44). Interestingly, the binding activity to polyG and polyU was retained in a truncated version of MSP58, suggesting that the RNA-binding domain lies within the N-terminal domain of MSP58 encompassing amino acids 1–124. It should be noted that this domain appears also to bind FMRP

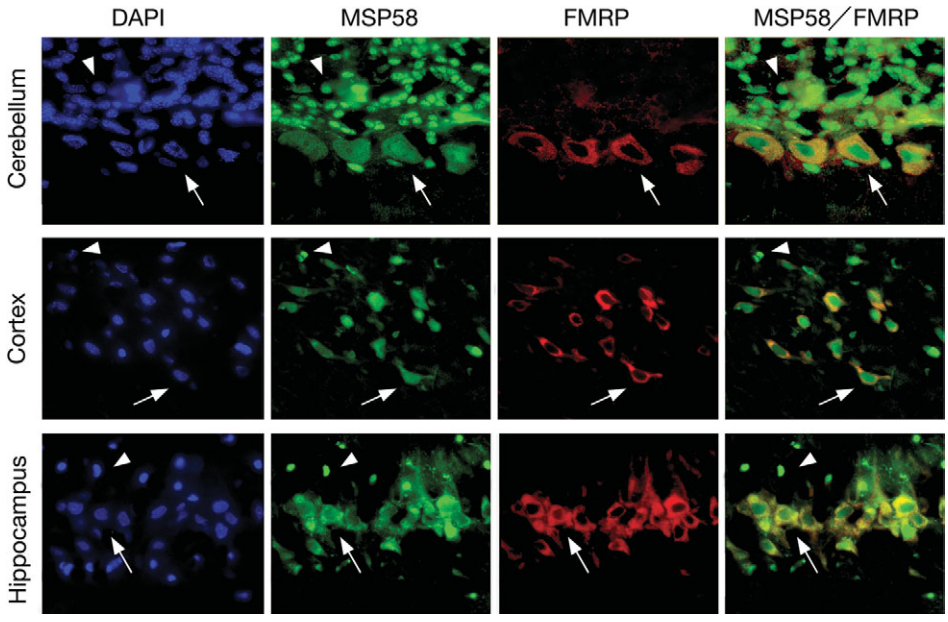

Figure 6. MSP58 and FMRP distribution in rat brain. Immunostaining of rat brain sections were carried out using anti-MSP58 polyclonal serum (green) and anti-FMRP mAb1C3 (red) and couterstained with DAPI. In small glial cells (arrow heads), MSP58 is concentrated in the nucleus, whereas in Purkinje cells of the cerebellum, and in pyramidal neurons in the cortex and in neurons in the CA3 region of the hippocampus, MSP58 is both nuclear and cytoplasmic (arrows) and co-localizes with cytoplasmic FMRP.

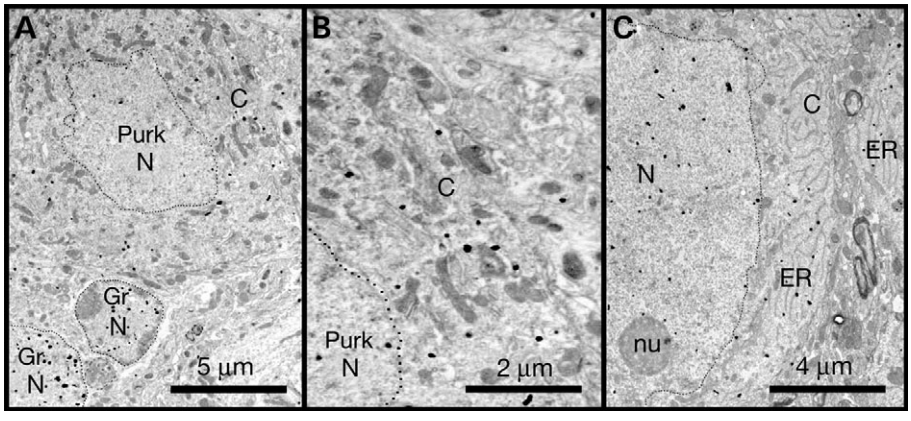

Figure 7. MSP58 is present in the nucleus and in the cytoplasm of neurons. (**A**) Electron micrograph of the soma of labelled Purkinje and granular cells. Immunogold particles are present in both nuclei, whereas only the Purkinje cell displays a cytoplasmic MSP58 distribution. (**B**) Higher magnification of the Purkinje cell shown in (A). In a CA3 neuron (**C**), MSP58 is detected in the nucleus as well as in close association with the endoplasmic reticulum (ER). Purk, Purkinje; Gr, granular; N, nucleus; nu, nucleolus; C, cytoplasm.

(Fig. 2E). Despite our efforts, no consensus RNA-binding sequence could be detected in this domain, suggesting that it should contain a yet uncharacterized sequence.

Because RNA homopolymers correspond to synthetic RNA segments, the affinity of MSP58 to these structures might not reflect its ability to bind biologically relevant mRNA structures encountered *in vivo*. Therefore, we questioned whether it could have affinity to a well-known mRNA structure called G-quartet. This structure is a recognition motif that allows binding with high affinity to FMRP and is present in several FMRP-mRNA targets, and particularly in the coding region of *FMR1* mRNA (13,14). Electrophoretic mobility shift assays were performed with recombinant GST–MSP58 in the presence of the N19 fragment corresponding to *FMR1* mRNA G-quartet minimal binding site (14). The results showed that MSP58 was able to bind N19, whereas a fragment corresponding to amino acids 125–294 was not (Fig. 10C). To ascertain that MSP58 binding to the G-quartet structure was not due to a general affinity for RNA, we performed nitrocellulose filter-binding assays (13,14) which allowed to titrate by competition assays the specificity of the binding of MSP58 to the G-quartet N19 sequence. Indeed, similar to

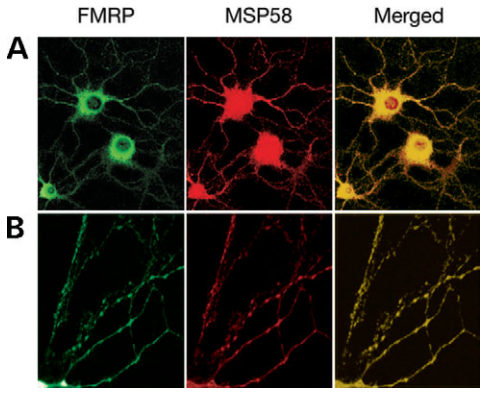

Figure 8. MSP58 co-localizes with FMRP in the cytoplasm and neurites in primary cultured hippocampal neurons. Double-labelling of FMRP (green with antibody 1C3) and MSP58 (red with anti-MSP58) showing co-localization of MSP58 and FMRP in the cytoplasm (**A**) as well as in neurites (**B**) as dot-like punctuations.

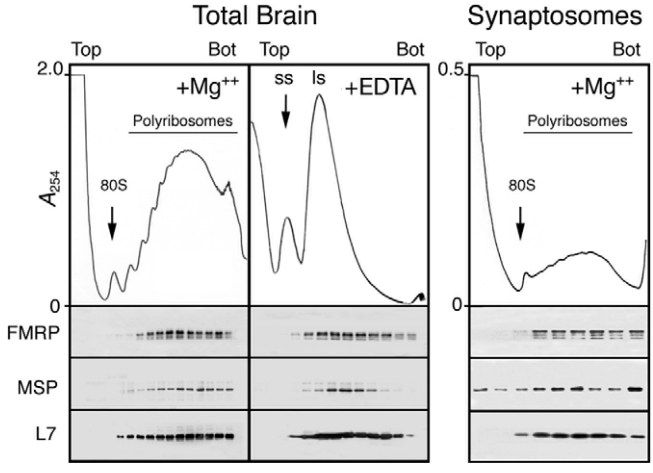

Figure 9. MSP58 co-sediments with FMRP in polyribosomes prepared from total brain and from synaptoneurosomes. Aliquots (containing 10 OD at A260 nm) of total rat brain polyribosomal preparations were analysed by sedimentation through sucrose density gradient in the presence of $MgCl_2$ or after treatment with 25 mM EDTA. Synaptoneurosomal polyribosomes were prepared as described in Materials and Methods and two OD at A260 nm analysed by sedimentation in a smaller sucrose density gradient. Fractions from the sucrose gradients were tested for the presence of MSP58, FMRP and L7A ribosomal protein (L7) using the rabbit anti-MSP58 antibody, mAb1C3 and rabbit anti-L7a serum, respectively. 'ss' and 'ls' indicate small and large ribosomal subunits, whereas 80S indicates the position of monosomes.

FMRP, the binding of MSP58 to the N19 RNA was efficiently competed by the same sequence, whereas an unrelated fragment of *FMR1* mRNA called N8 was unable to displace the binding (Fig. 10D). The binding of MSP58 to the N19 sequence was clearly dependent on the presence of the cation K^+, known to stabilize the G-quartet structure as previously demonstrated (13,14), whereas reduced binding was observed in the presence of Na^+ (Fig. 10E). All these results taken together suggest that MSP58 is a novel RNA-binding protein with high affinity to a G-quartet motif.

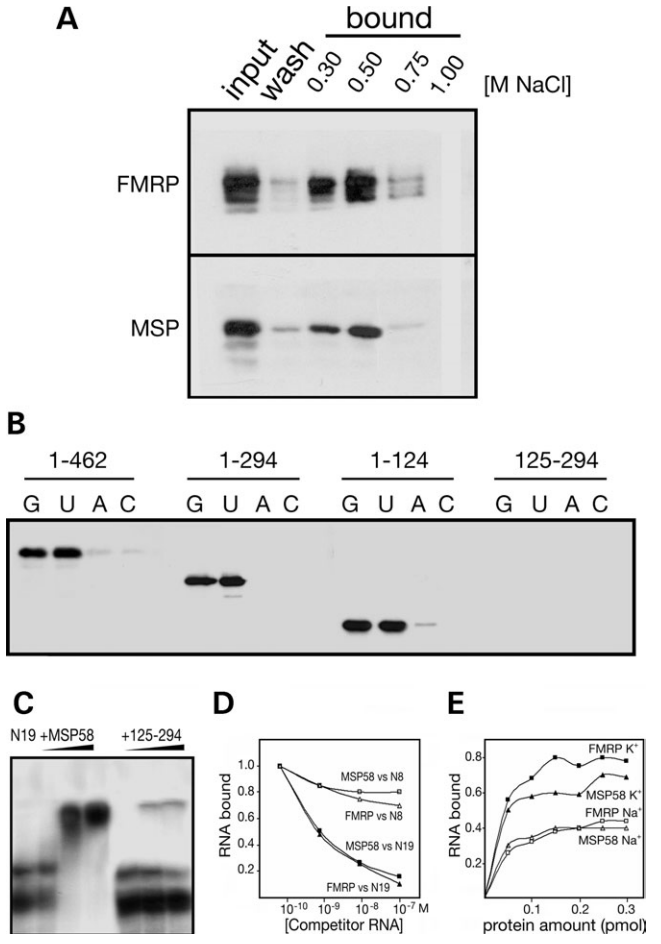

Figure 10. MSP58 is a novel RNA-binding protein. (**A**) MSP58 associates with poly(A)+ mRNP from brain polyribosomes. MSP58 is retained on oligo(dT) cellulose matrix loaded with EDTA-dissociated brain polyribosomes. The column was first washed (W) and then eluted with increasing salt concentrations from 0.3 M to 1 M NaCl and the majority of MSP58 was recovered in the 0.5 M NaCl fraction, together with FMRP, indicating their presence in poly(A)+ mRNPs. (**B**) MSP58 binds RNA homopolymers in an *in vitro* binding assay. Purified recombinant GST–MSP58 selectively binds to poly(G) and poly(U), but not to poly(A) or poly(C). This pattern of binding appears similar to that observed for FMRP. Only MSP58 fragments containing the N-terminal amino acids 1–124 domain were able to bind poly(G) and poly(U), suggesting that the RNA-binding domain of MSP58 lies in this region. No binding on homopolymers was observed for GST alone. (**C**) Electromobility shift analyses. Labelled N19 RNA was incubated with increasing amounts of MSP58 or as controls the MSP58 125–294 fragment. (**D**) Nitrocellulose filter-binding assay. Competition experiments on nitrocellulose filter-binding assay to determine the relative affinity of GST–MSP58 for the N19-G-quartet structure. Fraction of bound N19 (^{32}P-labelled), retained on either FMRP or GST–MSP58, plotted against competitor (either N19 or N8) RNA concentration. Each point reflects the results obtained in three independent experiments. (**E**) MSP58/G-quartet interaction occurs in the presence of the stabilizing cation K^+ and is disrupted in the presence of Na^+.

DISCUSSION

In this study, we have identified a novel partner for FMRP. MSP58 is evolutionarily conserved from fly to man. Its N-terminal part contains putative nucleolar and nuclear localization signals which seem to control its targeting to the nuclear compartment and is necessary to bind FMRP, whereas

FMRP appears to bind MSP58 through its C-terminal domain (amino acids 444–526) that contains a phosphorylation domain. Despite the fact that mutations on the putative phosphorylated serine 500 did not seem to affect the binding to MSP58 *in vitro*, we cannot exclude that *in vivo* phosphorylation might modulate the interaction between MSP58 and FMRP. Additional serines (Ser 494, 497, 504) that are highly evolutionary conserved in the phosphorylation domain of the FXR proteins have not been yet tested for their putative phosphorylation.

Previous studies have suggested that MSP58's functions are related to transcriptional regulation in the nucleus and nucleolus. This assumption is based on the observation that it has been exclusively detected in the nuclear compartment where it was reported to interact with transcription factors (34,36) and to up-regulate ribosomal gene expression (37). Our study reveals an additional important function of MSP58 as an RNA-binding protein presumably involved in translation regulation. We have shown that MSP58 is present in the cytoplasm of neurons in association with poly(A+) mRNP present in heavy sedimenting brain polyribosomes. The case of such a nuclear protein that is also present in association with the translation machinery is not isolated, because several proteins that have been characterized initially as nuclear proteins, such as hnRNP A1, PTB, La, hnRNP, YB-1/p50 and nucleolin, turned out to be present also in the cytoplasm and even implicated in translation control [for discussion and cross-references see (45)]. Of particular interest is YB-1/p50, which has been originally defined as a transcription factor, but which is the major protein of cytoplasmic mRNPs (46). Moreover, several FMRP-interacting proteins such as FXR1P, FXR2P, 82FIP and NUFIP are also nucleocytoplasmic shuttling proteins associated with polyribosomes (23,24). However, to our knowledge, MSP58 is the only FMRP partner which is also present in the nucleolus.

In co-transfection experiments, we observed a clear co-localization between MSP58 and FMRP nuclear Iso6 in the nucleolus. FMRP Iso6 being normally exclusively nucleoplasmic and excluded from the nucleolus (47), we conclude that overexpression of MSP58 results in the translocation of FMRP Iso6 into the nucleolar compartment. This suggests that in overexpression assays a fraction of FMRP can enter the nucleolus, as previously shown by immunoelectron microscopy studies performed on Cos-1 cells (48,49). Even though FMRP lacks the nucleolar targeting sequence present in FXR1P and FXR2P (50), the exceptional detection of FMRP in the nucleolus, if relevant, might result from an alternate mechanism, perhaps by interaction with FXR1P or FXR2P or even with other nuclear proteins. The binding of MSP58 to FMRP could directly participate in the translocation of FMRP-containing mRNP in the nucleolus.

Recent data indicate that the initial steps of RNA recognition and the packaging of mRNP complexes by RNA-binding proteins to be transported in the cytoplasm occur in the nucleus (51–53). We propose that FMRP binds its target mRNA in the nucleoplasm to be assembled in an mRNP with MSP58 and other RNA-binding proteins and RNA. The binding of MSP58 might change the conformation of FMRP and therefore affect the binding of its RNA targets on the RGG box, owing to the close proximity of the phosphorylation MSP58-binding domain and the RGG box. Moreover, the ability of MSP58 to bind with high affinity RNA G-quartet structures suggests that it might, *in vivo*, compete with FMRP for the binding of mRNA harbouring this structure. The protein composition of the FMRP-containing mRNP complexes might indeed modulate the type of mRNA targeted to the nascent mRNP. In addition to its central role in ribosome biogenesis, the nucleolus has been proposed to be the site of the assembly of pre-mRNP complexes (54,55). According to this point of view, the nucleolus would function as a nuclear checkpoint to verify the functional integrity and relevancy of these particles. Following this attractive model, it can be envisioned that the FMRP-containing mRNPs transit in the nucleolus where their potential functionality are verified before they are exported in the cytoplasm. Together with the other known FMRP-interacting proteins, such as FXR1P, FXR2P and 82-FIP, the interaction between MSP58 and FMRP in mRNP at the polyribosomal level might modulate FMRP function in the control of translation.

In neurons, a population of FMRP/MSP58-containing mRNP will be sorted from the neuronal cell body at a hypothetic RNP-triage centre (56,57) to be translocated at very distant locations in the form of mRNP granules. These mobile structures contain a reservoir of mRNAs that are maintained in a repressed state during migration (58) until they reach the synapse where FMRP is thought to play a key role in the control of their local translation. The presence of MSP58 in polyribosomes at the synapse as well as its ability to bind G-quartet containing mRNA raises the possibility that it would play a role, in concert with FMRP and/or other factors, in the control of local *de novo* protein synthesis, an essential phenomenon for synaptic development and maturation.

In summary, we have shown that MSP58 is a nucleocytoplasmic shuttling protein able to bind RNA and that it is associated with the cytoplasmic translation apparatus both in the cytoplasm and at the synapse. We therefore propose that in neurons MSP58 may be part of structures that are transported from the nucleus to translation sites, in a similar way as FMRP. However, the present study does not allow us to conclude whether MSP58 is an active essential partner to translocate mRNPs or whether it is dispensable to the cellular machinery. Also, it will be necessary to study whether MSP58 neuronal distribution is altered in *Fmr1* KO neurons. Throughout this paper, we have presented FMRP as a central key molecule and hypothesized that its affinity to RNA could in theory be modulated by protein interactors. An other diametrically opposite view would be that FMRP modulates the activity of MSP58. As the world of RNA-binding proteins is expanding, understanding the role of MSP58 and of other RNA-binding FMRP interactors, will be essential to unravel the functions altered by the absence of *FMR1* expression in the FXS.

MATERIALS AND METHODS

Yeast two-hybrid screen

Full-length cDNA of human FMRP Iso7 from the pTL1-*FMR1* Iso7 was subcloned in-frame with the DNA-binding domain

of the transcription factor Gal4 (Gal4 DNA-BD) into the *Eco*RI/*Pst*1 sites of the yeast expression vector pGBKT7 (Yeast Two-Hybrid System, Clontech). Human fetal brain cDNA library screening was performed by mating the AH109 Matα expressing the bait with the Y187 strain pre-transformed with the human fetal brain library encoded by the pACT2 vector (Matchmaker pre-transformed library, Clontech). Strains resulting from the mating carried the *ADE3*, *HIS3* and *LacZ* reporters under the control of *Gal4* responsive elements. From $\sim 2.8 \times 10^6$ clones screened, 78 positive colonies showed adenine and histidine prototrophy and β-galactosidase activity. Among these, PCR and restriction analysis showed that three colonies were redundant and carried a 1.7 kb insert. This insert corresponded to the full-length cDNA of human MicroSpherule protein of 58 kDa (MSP58 or MCRS1, GenBank accession no. Q96EZ8).

Purification of recombinant fusion proteins and production of anti-MSP58 antibodies

For expression of recombinant GST–MSP58, the coding sequence of MSP58 or the fragments corresponding to amino acids 1–124, 1–294 and 125–294 were amplified by PCR using the primers (*Eco*RI F: 5′-CGG GAA TTC GAC AAA GAT TCT CAG GGG CT-3′, *Xho*I R: 5′-GGC GAG CTC TCA CTG TGG TGT GAT CTT GG-3′, 125*Eco*RI F: 5′-GCC GAA TTC CCA CTT CAG GTG ACC AAG G-3′, 294*Bam* R: 5′-CGG GGA TCC CTT GAG CTT ACT GTC ATC AAT C-3′ and 124*Bam* R: 5′-CGG GGA TCC CTG TTT ACT CTT CTT CAC ACG C-3′) and subcloned into the *Eco*RI/*Xho*I or *Eco*RI/*Bam*HI sites of pGex-4T-1 (Amersham). Fusion proteins were expressed in BL21(DE3) *Escherichia coli* strain (Stratagene) grown in liquid LB until OD≥1 and induced overnight with 1 mM IPTG. Bacteria were then collected and the expressed fusion proteins purified in non-denaturing conditions on glutathione–Sepharose beads, according to manufacturers's protocole (Amersham Pharmacia Biotech). Fusion protein was eluted from the beads with 10 mM reduced glutathione in 50 mM Tris–HCl (pH 8.0). Protein yields were estimated by Coomassie staining using as standard different concentrations of bovine serum albumin (BSA) ranging from 0.2 to 1 μg/μl. The GST–MSP58 amino acids 125–294 were used to produce antibodies in rabbit using standard protocol, and anti-MSP58 IgG were affinity purified with the same fusion protein used for immunization.

GST-pulldown assays

FMRP and its truncated variants, FXR1P and FXR2P, were produced by *in vitro* transcription–translation in rabbit reticulocyte system in the presence of [^{35}S]methionine (Amersham) according to manufacturer's instruction (Promega, Madison, WI, USA). Five microlitres of *in vitro*-translated proteins were mixed with 1 μg of GST–MSP58 and its variant recombinant proteins bound to beads and incubated 2 h, at room temperature, under constant rotation in 500 μl of pulldown buffer (10 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP-40, Protease Inhibitor Cocktail, Roche). Beads were collected by spinning (3000 *g*, at room temperature for 2 min) and washed four times with the pulldown buffer. Final wash was removed and beads were resuspended in 50 μl of SDS sample buffer. One-third of the sample was loaded on a 7.5% SDS–PAGE. Gel was then dried and exposed for 3 days to a Biomax film (Kodak).

RNA studies

Homopolymer binding assays. Binding assays were performed according to established procedures. Briefly, 0.5 μg of recombinant GST-tagged MSP58 were incubated with immobilized poly(G), poly(U), poly(A) or poly(C) polyacrylhydrazido-agarose beads (Sigma) in 0.5 ml of binding buffer containing 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5% NP-40 and 2.5 mM $MgCl_2$ supplemented with Protease Inhibitor Cocktail (Roche) for 1 h at room temperature. After incubation, the beads were washed four times with binding buffer and bound proteins eluted by addition of SDS sample buffer followed by heat denaturation. Proteins were separated by SDS–PAGE and blotted either with anti-GST antibody (Amersham) used at 1:25 000 or with anti-MSP58 serum. Control analyses were also performed with ^{35}S-labelled MSP58 produced in the rabbit reticulocyte lysate system (data not shown). *In vitro*-translated proteins were detected after exposure of the gel to an X-ray film.

RNA-binding assays. The different RNA fragments N19 and N8 derived from *FMR1* RNA were synthesized by *in vitro* transcription with T7 RNA polymerase from pTL1 (47) derivative plasmids linearized with *Pst*I. The RNAs were purified using the NucAway Spin columns (Ambion). RNAs were then ethanol precipitated and resuspended in the appropriate buffer. For binding experiments, N19 was labelled co-transcriptionally by incorporation of [α-^{32}P]ATP. Labelled RNAs were purified on a 1% low-melting agarose gel (Ambion). Labelled RNAs (80 000 c.p.m., 5 fmol) were renatured for 10 min at 40°C in 40 μl of binding buffer [50 mM Tris–HCl pH 7.4 at 4°C, 1 mM $MgCl_2$, 1 mM EDTA, 150 mM KCl, 1 mM dithiothreitol (DTT)] with 8 U of RNasin (Invitrogen), 10 μg of *E. coli* total tRNA and 0.01% BSA. The RNA was then added to increasing amount of proteins. RNA–protein complexes were formed for 10 min at 0°C. After incubation, binding solutions were passed through MF-membrane filters (0.45 HA, Millipore) and washed with 2 ml binding buffer. Filters were air-dried and the amount of radioactivity was measured by Cerenkov counting. Data were plotted as percentage of total RNA bound versus the protein concentration. Competition experiments to determine the relative binding strength of MSP58 to G-quartets were carried using ^{32}P-labelled N19 RNA incubated with MSP58 (0.1 pmol) in the presence of increasing concentrations of unlabelled competitors. FMRP was used as an internal positive control. Binding of FMRP and MSP58 to the G-quartet were performed in the presence of 150 mM of either K^+ or Na^+.

Cell culture, primary neuron culture and transient transfection assays

HeLa S3, Cos-1 and STEK *Fmr1* KO/TSV40 cells were propagated and maintained in DMEM supplemented with 10%

FBS and antibiotics (100 U/ml penicillin, 50 mg/ml streptomycin). Transfection assays with different vectors were performed in the presence of Effectene according to manufacturer's recommendations (Qiagen). Primary neuron culture was prepared from rat hippocampi as described (59).

Protein studies

Organs were removed from animals, processed according to standard protocols and protein extracts prepared for SDS–PAGE as described (60). Immunoblot analyses were performed using mAb1C3 to FMRP, rabbit polyclonal antisera directed against MSP58 and anti-L7 ribosomal protein. Detection of bound antibodies was performed with HRP-coupled secondary antibodies followed by ECL reaction.

Immunofluorescence studies

Immunocytofluorescence. Cos-1 cells grown on glass cover slips were washed three times with ice-cold phosphate-buffered saline (PBS) then fixed with the fixation and permeabilization protocols described in Results. To detect endogenous MSP58, the anti-MSP58 was used at 1:200 (overnight at 4°C), followed by incubation (90 min at room temperature) with Alexa fluor 488 goat anti-rabbit IgG (Molecular Probes). Control experiments were performed in the presence of antibodies that were pre-incubated with the recombinant purified GST–MSP58 amino acids 125–294 used for immunization. For direct visualization of MSP58 in eukaryotic cells, MSP58 was fused to the RFP by subcloning its cDNA from the pGex-4T-1/MSP58 full-length in the *Eco*RI–*Sal*I sites of the polylinker of a modified version of peRFP graciously provided by Paul De Koninck.

Immunohistofluorescence. Adult rats, deeply anaesthetized with ketamine xylol (40 mg/kg, i.p.) were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was removed, transferred to cryoprotective solution (HistoPrep, Fisher Scientific), frozen and serially cut into longitudinal sections (5 μm) with a Leica CM1900 cryostate. Sections were treated as described (23) and mounted on slides and processed for double immunofluorescence using rabbit anti-MSP58 (at 1:200 dilution) and anti-FMRP mAb1C3 antibody. Double immunofluorescence staining were performed by separate and sequential incubations of each primary antibody diluted in PBS at 4°C overnight, followed by the respective secondary antibody coupled to Alexa594 or Alexa 488 (Molecular Probes) and incubated at room temperature for 3 h. Samples were analysed with a Nikon EclipseE800 microscope equipped with a Hammamatsu CCD camera. Images were then treated with the Adobe PhotoShop software program.

Electron microscopy. Pre-embedding immunogold labelling was performed as described (61). Briefly, free floating fixed rat brain sections were blocked for 30 min in 0.8% BSA, 0.1% cold fish skin gelatin (Amersham, Piscataway, NJ, USA) and 5% normal goat serum in Tris-buffered saline (TBS), followed by incubation with MSP58 antiserum for 48 h at 4°C. Sections were washed and incubated with 0.5 nm gold-conjugated secondary antibody (AuroProbe, Amersham, 1:80 in TBS) for 12 h at 4°C and the gold particles were enhanced with silver solution (IntenSE, Amersham). Sections were treated with 1% OsO_4, dehydrated in graded ethanol, then in propylene oxide and embedded in Durcupan ACM (Fluka). Specimens were sectioned and examined using a Philips Tecnai 12 electron microscope.

Polyribosome preparation and analysis

Total brain polyribosomes were prepared from young Sprague-Dawley rats (7 days old) and purified as described (8). For EDTA–mRNPs studies, the fractions of sucrose gradients corresponding to 150–500S were pooled, diluted with 1 volume of buffer (20 mM Tris–HCl, pH 7.4, 100 mM KCl, 1.25 mM $MgCl_2$) and the particles pelleted by centrifugation in a Sorvall TH-641 rotor for 2 h at 34 000 r.p.m. at 4°C. The polyribosomal pellets were resuspended in 20 mM Tris–HCl, pH 7.4, 100 mM KCl, 1% NP-40 containing 25 mM EDTA and analysed after centrifugation in a linear 5–35% (w/w) sucrose density gradient for 3.5 h at 34 000 r.p.m. at 4°C.

For preparation of synaptosomes, 16 cortices from young animals (7 days old) were homogenized by hand using a glass homogenizer (30 strokes) in a buffer containing 20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 1.25 mM $MgCl_2$, 0.32 M sucrose, 1 mM DTT, 50 μg/ml cycloheximide, 5 U/ml RNasine (Amersham Pharmacia) and protease inhibitors (Mini Complete, Roche Biochemicals). Purification of synaptosomes followed a two-step procedure. After centrifugation at 640*g* for 10 min, the supernatant was layered on Percoll gradient and the synaptosomal fraction obtained as described (62). The second step of purification was performed by floatation in an Optiprep gradient (63). The purified synaptosomal fraction was then resuspended in buffer containing 1% NP-40 and the released polyribosomes were concentrated after ultracentrifugation through a sucrose pad (45% w/w) in a Sorval TH-641 rotor at 34 000 r.p.m. for 3 h. The polyribosomal pellet was resuspended in buffer containing 1% NP-40 and analysed by velocity sedimentation in a 15–45% (w/w) isokinetic sucrose gradient and centrifuged in a 4 ml tube with a Sorvall TST 60.4 rotor at 30 000 r.p.m. for 2 h at 4°C. All gradients were fractionated by upward displacement using an ISCO UA-5 flow-through spectrophotometer set at 254 nm and connected to a gradient collector.

Each collected fraction was precipitated overnight at −20°C after addition of 2 volumes of ethanol. The precipitated material was collected by centrifugation at 12 000 r.p.m. for 20 min and solubilized in SDS sample buffer before immunoblot analyses. FMRP was detected with mAb1C3, MSP58 with its corresponding antiserum and ribosomal L7 protein with rabbit anti-L7 serum. As synaptosomes account for a very minor fraction of the brain, we used a total of 16 cortices per extraction, and performed the sedimentation analyses in small 4 ml tubes instead of the standard 11 ml used to analyse total polyribosomes extracted from three brains. Finally, the sensivity of the UV detector used to follow the polyribosomal profile was tuned to scale 0.5 (optical density at 254 nm) instead of 2.0 (compare the profile of total brain versus synaptosomes in Fig. 8).

ACKNOWLEDGEMENTS

We thank Roumiana Gulemetova and Richard Kinkead for helpful discussions concerning brain fixation and preparation, Paul De Koninck for the neuron primary cultures and for the modified peGFP and peRFP plasmids and Philippe Lemieux and J. Grosgeorge for technical assistance. L.D. was a recipient of a postdoctoral fellowship from the FRAXA Research Foundation (USA) and was supported by traveling grants from the Conquer fragile X Foundation and the Boerhinger Ingelheim Fund. M.G. holds a Jerome Lejeune (France) fellowship. This work was supported by grants from the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council of Canada (to E.W.K.) and from La Fondation de la Recherche Médicale, GIS Maladies Rares and FRAXA Foundation (to B.B.).

Conflict of Interest statement. None declared.

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Discussion

A look at the RNA binding properties of the FMRP and its interactors !

Characterization of FMRP/*Sod1* mRNA interaction

Does FMRP bind specifically to Sod1 mRNA?

FMRP presents a general affinity for RNAs, but it binds specifically to only a small subset of RNA sequence/structure (Schaeffer et al. 2001). The huge amount of data provided about putative FMRP mRNA targets lacks crucial information about the specificity of FMRP/"target" mRNA interaction. Among the hundreds of putative FMRP targets (Brown et al. 2001; Miyashiro et al. 2003), specific interaction was assessed in detail for only two mRNAs, *FMR1* (Schaeffer et al. 2001) and *PP2A* (Castets et al. 2005b) in an appropriate and reliable experimental procedures using both physiological conditions and Protein/RNA binding assays in presence of specific and unspecific RNA competitors to eliminate any kind of bias.

Sod1 mRNA was identified as a FMRP putative target "*in vivo"* by Miyashiro and colleagues. Based on their observations (Miyashiro et al. 2003) we have analyzed FMRP/*Sod1* mRNA interaction. Using both the experimental conditions developed by Schaeffer and colleagues, and the presence of adequate competitors, we have proven that FMRP binds specifically the *Sod1* mRNA with an affinity similar to that observed for the G-quartet (Schaeffer et al. 2001).

How does the recognition FMRP/Sod1 occur ?

When first FMRP was shown to recognize and bind specifically to the G-quartet structure, several bioinformatics programs were developed to search for sequences that may adopt a G-quartet structure. Based on alignment with a "canonical sequence" these programs had been used by many groups while analyzing FMRP targets as putative G-quartet containing mRNA. Relying on such procedure might be distrustful simply because manipulating these programs in an "un-proficient" manner would lead to both false positive and negatives. In another hand, these programs do not take neither the purine quartets into account nor the possibility of a distant G-quartet formation. Thus, the presence of G-quartets needs to be confirmed experimentally. We have combined two assays to search for any G-

quartet structure in the *Sod1* mRNA. First, we performed binding assays in presence of increasing amount of FMRP in buffers containing either Na^+ or K^+ which would respectively destabilize or stabilize a G-quartet structure, altering therefore its binding to FMRP. We observed that FMRP/*Sod1* interaction was not affected in either buffer, leading to a preliminary conclusion that *Sod1* mRNA might not contain a G-quartet. To definitely exclude any bias, the *Sod1* mRNA was reverse transcribed in presence of either Na^+ or K^+. For both reactions, the same pattern of migration was observed on polyacrylamide gel (for experimental procedure, see results and material and method of publication 2) confirming the absence of a G-quartet in the Sod1 mRNA and therefore, classifying the Sod1 mRNA as the second novel *in vivo* validated FMRP sequence/structure target.

Using site boundary determination as described (Schaeffer et al. 2001), we identified a 64 base fragment of the *Sod1* mRNA able to be recognized and bound specifically by FMRP with the same affinity as that of the full length Sod1 mRNA. The border positions of this fragment were at -30 and +34 spanning both sides the *Sod1* AUG start codon. Existence of bioinformatics programs allowing to predict a secondary structure of a RNA such as the "MFOLD" (http://helix.nih.gov/apps/bioinfo/mfold.html) was a first attempt do discover the putative structure of this fragment.

The secondary structure delivered by MFOLD was a single long stem loop interrupted by 2 bulges. If any constraints are subjected to the program, it will function by joining both ends of the fragment and to anneal bases that would pair. This could be a first indication about the putative secondary structure of an RNA that should be confirmed by experimental procedures. Aiming to better define the structure adopted by this 64 base fragment in solution, we used a panel of chemical and enzymatic modifications as described (Brunel and Romby 2000). This technique is based on the reactivity of RNA molecules towards chemicals or enzymes that modify or cleave specific atomic positions in RNA respectively. Results obtained from these experiments were applied to MFOLD program that calculated a best fitting free energy for the structure that resulted as a succession of three independent stem-loop structures separated by short single stranded regions. This structure was named SSLIP (*Sod1* Stem Loops Interacting with FMRP). Binding assays in presence of competitors showed that SSLIP is only bound by the C-terminus region of FMRP. Since the C-terminal domain of FMRP contains the RGG box that binds to the G-quartets RNA structure (Darnell et al. 2001), and more recently an G-rich "undefined sequence/structure" present in the *PSD95* mRNA (Zalfa et al. 2007) (Nevertheless, alignment with the "canonical sequence" predicted the presence of a G-quartet structure (Todd et al. 2003), that was experimentally

proven (H. Moine, personal communication)) and in the APP mRNA (Westmark and Malter 2007), we found that SSLIP is able to compete the G-quartets for binding to FMRP C-terminus region. This observation lead us to hypothesize that FMRP recognizes similar structural features in both RNAs (e.g. specific atoms properly spatially oriented and/or in the correct electrostatic environment) that may involve an induced-fit mechanism for the recognition. As already discussed, the RGG box was shown to be flexible and unstructured (low complexity sequence composition) and that the interaction between the RGG box peptide and various G-quartets showed a large heterogeneity in both the conformation of the RNA targets and their RGG binding mode which could be the basis of recognition specificity (Ramos et al. 2003).

What is the functional significance of FMRP/Sod1 mRNA interaction?

FMRP was widely considered as a translational inhibitor for so called "*in vitro"* and *"in vivo"* models. Nevertheless, taking in consideration the vast repertoire of cell models, the overexpression of FMRP and the simplistic way to enhance or repress translation in rabbit reticulocyte lysate, the results obtained by several groups should be revisited in a more accurate interpretation. FMRP could shuttle not only between the nucleus and the cytoplasm but also between several different RNPs involved in both translational activation and repression suggesting that it might be involved not "exclusively" in the translation inhibition. Recently Zalfa and colleagues revealed a hidden facet of FMRP acting on the stabilization of PSD95 mRNA, although the G-rich sequence/structure recognized by the C-terminus region of FMRP was poorly defined by the authors (Zalfa et al. 2007). Strikingly, another group showed that FMRP recognizes a G-rich sequence in the APP mRNA, but does not act on the stability of this mRNA (Westmark and Malter 2007). Considering that FMRP could have distinct effects on mRNAs, its function would strongly depend on the mRNA sequence/structure that it binds to.

Aiming to understand the significance of FMRP/SSLIP interaction , we first proceeded by the analysis of the *Sod1* mRNA turnover in presence and absence of FMRP in two different cell types and observed that FMRP does not affect the stability of SSLIP containing mRNA. However, when analyzing the distribution of the *Sod1* mRNA on mouse brain polyribosomes, we showed that *Sod1* mRNA is less associated to active translating polyribosomes in the absence of FMRP. This alteration was also observed at the protein level

where the Sod1 protein expression was decreased in the absence of FMRP. These data lead us to conclude that FMRP may act as a translation enhancer on SSLIP containing mRNAs.
Two major traits were observed in this study, first the ability of the C-terminus region to bind specifically to a novel structure most probably by fit mechanism and second the role of FMRP that it might have on its target mRNAs depend mostly on the structure adopted by the mRNA and recognized by FMRP.
The presence of the triple stem loops around the AUG start codon may reflect to some extent the role of these structures profiting from FMRP to drive them to polyribosomes. It will be very interesting to decipher the mechanism by which FMRP could bind a region of mRNA spanning its AUG start codon and activate its translation. A recent work by Marzi and colleagues provided new insights in the regulation of translation initiation by both trapping ribosomes and displacement mechanism in prokaryotes (Marzi et al. 2007). May this mechanism be the same in eukaryotes? If so, could FMRP be involved in such regulation?

SOD , oxidative stress, brain and mental retardation, any link ?

Under normal conditions, a dynamic equilibrium exists between the production of reactive oxygen species (ROS) and the antioxidant capacity of the cell. ROS includes superoxide, hydroxyl, peroxyl, alkoxy, and nitric oxide (NO) free radicals. Superoxide is the first reduction product of molecular oxygen, and it is an important source of hydroperoxides and deleterious free radicals. Hydrogen peroxide (H_2O_2) reacts with reduced transition metals such as iron, via the Fenton reaction, to produce the highly reactive hydroxyl radical. Most toxic effects are due to hydroxyl radical formation, which also initiates lipid peroxidation. Normally, the ROS within the cells are neutralized by antioxidant defense mechanisms. Superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) are the primary enzymes involved in direct elimination of ROS whereas glutathione reductase and glucose-6-phosphate dehydrogenase are secondary antioxidant enzymes, which help in maintaining a steady concentration of glutathione and NADPH necessary for optimal functioning of the primary antioxidant enzymes. Oxidative stress occurs when ROS levels exceed the antioxidant capacity of a cell. These ROS are highly toxic and react with lipids, proteins and nucleic acids, and lead to cell death via apoptosis or necrosis.

Oxidative stress and brain

The brain is highly vulnerable to oxidative stress due to its limited antioxidant capacity, higher energy requirement, and higher amounts of lipids. The brain makes up about 2% of body mass but consumes 20% of metabolic oxygen. The vast majority of energy is used by the neurons. Due to the lack of glutathione-producing capacity by neurons, the brain has a limited capacity to detoxify ROS. Therefore, neurons are the first cells to be affected by the increase in ROS and shortage of antioxidants and, as a result, are most susceptible to oxidative stress. Indeed, *Sod1* mRNA is present in dendrites and in axons meaning that is an axonally synthesized protein essential for axon development and axonal transport (Willis et al. 2005). Gene mutations in *Sod1* can cause familial amyotrophic lateral sclerosis (SLA). Several *Sod1* mutants found in SLA patients perturb fast axonal transport (De Vos et al. 2007) and can cause motor axonopathy in zebrafish (Lemmens et al. 2007). Sod1 is also regulated by cellular stress, suggesting that the axonal localization of its mRNAs may provide a mechanism to locally respond to axonal injury.

Oxidative stress and mental impairment

Fragile X syndrome

Recently, using drosophila model, Zhang and colleagues analyzed by two dimension electrophoresis the misregulation of proteins in the absence of FMRP and interstingly they showed an alteration in the expression of two enzymes (1-cys peroxiredoxin in brain and peroxiredoxin and thioredoxin peroxidase in testis) concluding that oxidative stress occurs in in *FMR1* null flies (Zhang et al. 2005).

Another study, using the mouse model, showed that brains from *Fmr1*-knockout mice, display higher levels of reactive oxygen species, nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activation, lipid peroxidation and protein oxidation than brains from wild-type mice, indicating the presence of markers of oxidative stress in brains lacking FMRP (El Bekay et al. 2007).Modifications of oxidative stress have been linked to anxiety, sleep trouble and autism, all features of FXS patients.

Autism

The brain is highly vulnerable to oxidative stress, particularly during the early part of development that may result in neurodevelopmental disorders such as autism. In fact, recent evidence points towards increased oxidative stress in autism.

Several studies have suggested alterations in the enzymes that play a vital role in the defense mechanism against damage by ROS in autism. For instance, compared to controls, patients with autism showed decreased activity of glutathione peroxidase reduced levels of total glutathione and lower redox ratio of reduced glutathione (GSH) to oxidized glutathione and decreased catalase and Sod activity (Yorbik et al. 2002; Ming et al. 2005).
since the alteration of Sod1 expression may generate the modification of the cellular reduction/oxidation, and may represent one of the molecular defects resulting in the complex phenotype of Fragile X patients, our study sheds new light on the physiopathology of the Fragile X syndrome.

Another aspect of FMRP analyzed during my thesis was the determination of its interactors RNA binding properties and the influence that they might exert on FMRP when present together.

The RNA binding properties of FMRP were mainly assessed regardless its belonging to a macromolecular complex where the majority of its interactors are also RNA binding proteins. The high homology degree in both sequence and functional domains that FMRP shares with its two homologues FXR1P and FXR2P suggests that these proteins may have a similar or redundant function and that they could to some extent compensate the absence of FMRP, therefore explaining the heterogeneity of symptoms in FXS patients. Nevertheless, any experimental indication about their ability to bind mRNA was provided. We have analyzed the ability of two FMRP partners : FXR1P and MSP58 to bind mRNAs and tried to mimic the "*in vivo*" behavior of FMRP in its containing complex, by a "simplistic" *in vitro* procedure where FMRP was in presence of either interactor.

Could FXR1P or MSP58 bind specifically the G-quartet structure ?

The *FXR1* pre-mRNA is highly subjected to alternative splicing giving rise to several FXR1P isoforms that are differentially expressed in tissues, during development and also

during cell differentiation (Khandjian et al. 1998; Dubé et al. 2000). We have chosen to analyze the RNA binding properties of three FXR1P isoforms , Isoe (muscle specific) together with Isod and Isoa that are mainly expressed in brain (Bechara et al. 2007). In parallel we analyzed the MSP58 affinity to mRNAs (Davidovic et al. 2006). MSP58 is a nucleocytoplasmic shuttling RNA binding protein that it is associated with the cytoplasmic translation apparatus both in the cytoplasm and at the synapse. In neurons MSP58 may be part of structures that are transported from the nucleus to translation sites, in a similar way as FMRP (Davidovic et al. 2006).

Filter binding assays in presence of competitors showed that MSP58 binds specifically the G-quartets structure with an affinity similar to that of FMRP, whereas among the three FXR1P isoforms, only Isoe showed a specific binding to G-quartets despite a lower affinity compared to that of FMRP but neither Isod nor Isoa recognized specifically the G-quartet structure. This was the first evidence that despite their high homology degree, FXR1P has at least a different affinity for RNAs than that of FMRP and that it may act differently than FMRP on its targets. It was shown that a peptide corresponding to the RGG box of FXR1P is not able *per se* to recognize the G-quartet structure (Zanotti et al. 2006), which was not the case for the full length protein (Bechara et al. 2007). Indeed, two major observations emerged from our analysis. First, the three FXR1P isoforms do not exhibit the same specificity for G-quartets structure, which could be explained by the presence of 27 amino acids encoded by exon 15 present in Isoe. The sequence analysis of these amino acids showed that they reveal an affinity for RNA; what should remain to be investigated is whether these amino acids influence the RNA binding affinity of Isoe by their direct interaction with RNA or by modulating the structure of the C-terminus region of Isoe rendering it more flexible for specific RNA binding. Second, the fact that the RGG box peptide of FXR1P was not able to recognize a G-quartet structure (Zanotti et al. 2006) could be due either to its uncorrect folding or stability in solution as that observed for the FMRP RGG peptide. Another explanation for its limited ability to interact with RNA in a sequence-specific manner might be due to its recognition sequence that is too short. The presence in the full length protein of multiple domains are therefore tethered together to create a much larger binding interface that specifically recognizes a longer sequence.

How would FMRP react "vis à vis" of G-quartets in presence of its interactors ?

At the equilibrium stage, the formation of the heterodimer FMRP/FXR1P Isoe showed a slight effect on the affinity of FMRP for G-quartets, the same case was observed for FMRP/MSP58 heterodimer. However, both Isod and Isoa reduced both the affinity and specificity of FMRP to G-quartets. The heterodimer FMRP/FXR1P Isoe also showed an increased dynamic in both association and dissociation with G-quartets. This observation could be explained by the fact that protein-protein interactions play crucial role in the RNA-binding specificity. It has been shown that RNA binding domains (RBD) from different proteins can cooperate to recognize RNA through a combination of weak protein–RNA. Moreover, The formation of heterodimers through interactions between an RBD and another protein can increase the specificity of the RNA interaction as well. For example, the binding of the RRM of spliceosomal U2B" to a stem–loop in U2 small nuclear (sn)RNA requires an interaction with the U2A' protein (Price et al. 1998). Another example was elucidated with the recent structures of the archaeal and eukaryotic exosomes have revealed extensive protein–protein interactions between proteins that contain both KH and S1 domains in the core of the protein complex (Buttner et al. 2005). These interactions can position the S1 domains of specific exosome subunits to recognize the RNAs that are targeted for degradation.

These different behaviors of the two FMRP-interacting proteins illustrate the complexity of the functions and interactions that take place in FMRP-containing mRNPs and in different tissues. FXR1P-Isod and Isoa are the FXR1P isoforms with the highest expression in brain, suggesting that in neurons FMRP interacts mostly with these two isoforms that might regulate negatively its action. Our study highlights the functional differences between FXR1P isoforms and therefore emphasizes the importance of the extensive tissue-specific alternative splicing undergone by FXR1 mRNA. In view of these results, it is clear that in each mRNP the ratio between FMRP and FXR1P different isoforms becomes important to precisely regulate FMRP function. The modulation of the affinity and/or of the dynamics observed for the FXR1P/FMRP heterodimer may reflect a regulation of the exchange of mRNAs between mRNPs or trafficking granules and polyribosomes.

Conclusions & Perspectives

FMRP is one of the most analyzed RNA binding proteins involved in a genetic disorder. Its absence results in a dramatic impairment of synaptic connections and plasticity leading to mental retardation. Several groups had deployed consequent efforts to understand the molecular functions of FMRP and to decipher the reigning enigma of how the absence of a single protein can result in such a disease. The ability of FMRP to bind mRNAs, its shuttling between the nucleus and the cytoplasm, its belonging to several mRNPs and its involvement in RNA trafficking and local protein synthesis had mainly participated to place some parts of the puzzle which is still far from being completely assembled.

I deeply believe that both the identification and characterization of FMRP mRNA targets, in an appropriate manner, together with the analysis of the functional significance of FMRP/RNA interaction and the post transcriptional regulation that it exert on its targets, are critical steps in understanding the molecular basis of the Fragile X syndrome.

During my thesis I have analyzed the FMRP/*Sod1* mRNA interaction demonstrating that FMRP is able to differentially regulate its mRNA targets depending most probably on the mRNA structure recognized by FMRP. On another hand I have also demonstrated that the affinity of FMRP for its target mRNAs can be influenced by its interactors and that its function might be regulated by the latter. Further analysis of the FMRP/RNA complex and FMRP/Protein complex would help in deciphering the molecular mechanism(s) by which FMRP act and would enlighten the hidden facet of FMRP.

The diversity and divergence of biological materials and technical procedures used by several laboratories helped to some extent in revealing some functional aspects of FMRP, nevertheless it created a wide range of controversy that escorted FMRP since its birth fouling its reputation and making it poorly understood.

Appendix

Human Molecular Genetics, 2005, Vol. 14, No. 6 ***835–844***
doi:10.1093/hmg/ddi077
Advance Access published on February 9, 2005

FMRP interferes with the Rac1 pathway and controls actin cytoskeleton dynamics in murine fibroblasts

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Received December 13, 2004; Revised and Accepted January 27, 2005

Fragile X syndrome, the most common form of inherited mental retardation, is caused by absence of FMRP, an RNA-binding protein implicated in regulation of mRNA translation and/or transport. We have previously shown that dFMR1, the *Drosophila* ortholog of FMRP, is genetically linked to the dRac1 GTPase, a key player in actin cytoskeleton remodeling. Here, we demonstrate that FMRP and the Rac1 pathway are connected in a model of murine fibroblasts. We show that Rac1 activation induces relocalization of four FMRP partners to actin ring areas. Moreover, Rac1-induced actin remodeling is altered in fibroblasts lacking FMRP or carrying a point-mutation in the KH1 or in the KH2 RNA-binding domain. In absence of wild-type FMRP, we found that phospho-ADF/Cofilin (P-Cofilin) level, a major mediator of Rac1 signaling, is lowered, whereas the level of protein phosphatase 2A catalytic subunit (PP2Ac), a P-Cofilin phosphatase, is increased. We show that FMRP binds with high affinity to the 5′-UTR of *pp2acβ* mRNA and is thus a likely negative regulator of its translation. The molecular mechanism unraveled here points to a role for FMRP in modulation of actin dynamics, which is a key process in morphogenesis of dendritic spines, synaptic structures abnormally developed in Fragile X syndrome patient's brain.

INTRODUCTION

Fragile X syndrome, the most common cause of inherited mental retardation, is due to mutations in the *FMR1* gene, resulting in the absence of functional FMRP (fragile X mental retardation protein) (1). In almost all cases, mutations consist in an expansion of CGG trinucleotides repeats. Apart from mental retardation, several features characterize Fragile X phenotype including facial dysmorphism, post-pubertal macro-orchidism and connective tissue dysplasia (2). The shape and density of dendritic spines, which are actin-rich synaptic structures, are altered in patients and in FMRP deficient mice brain. These observations suggest a defect in maturation and/or function of synapses that is thought to be at the basis of mental retardation (3,4). FMRP contains at least three RNA-binding domains, two KH domains and one RGG box. The latter binds with high affinity to RNA G-quartet structures formed by intrastrand annealing of four guanine-rich tracts (5,6). FMRP is associated with polyribosomes (7) and is most likely involved in translational control (8–11), perhaps through interaction with the RNAi machinery (12,13). A point-mutation (I304N) in the KH2 domain has been reported in a patient with an unusually severe phenotype (14) and it has been shown that the KH2-I304N mutant FMRP fails to associate with elongating polyribosomes (15). Several approaches have led to the identification of few hundreds of putative mRNA targets (5,11,16,17), but the specificity of interaction between FMRPs and most of these mRNAs remains to be confirmed. Moreover, consequences of FMRP absence for expression and/or subcellular localization of

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proteins encoded by these mRNAs, as well as correlations with phenotypic features, have been studied only in a few cases.

FMRP is part of large mRNP complex (7,11,18). Several FMRP interacting proteins have been described including its two close paralogs, FXR1P and FXR2P (Fragile X Related Protein 1/2) (19), NUFIP1 (Nuclear FMRP Interacting Protein 1) (20,21), 82-FIP (82 kDa-FMRP Interacting Protein) (22) and the two closely related proteins CYFIP1 and CYFIP2 (Cytoplasmic FMRP Interacting Protein 1/2) (23). Interestingly, CYFIP proteins interact physically with Rac1 and are genetically linked with this small Rho GTPase in *Drosophila* (24–26). Rac1 plays a key role in actin cytoskeleton remodeling (27,28) and notably controls formation, maturation and maintenance of dendritic spines (29–31). Moreover, mutations affecting several components of Rho GTPases pathways have been identified in mentally retarded patients (32,33) and are associated with dendritic spine defects in the corresponding mouse models (34).

In this study, we designed a cellular model consisting of murine fibroblasts which express either no or mutant FMRP and compared them to FMRP positive cells. Using this model, we have identified a novel molecular link between FMRP and the Rac1 pathway: indeed, Rac1 activation leads to relocalization of four FMRP main interactors (CYFIP1, FXR1P, NUFIP and 82-FIP) to actin-containing domains called actin rings. Reciprocally, Rac1-induced actin reorganization is modified in FMRP deficient cells and in cells expressing FMRP mutated in KH1 or in KH2 domain. In these cells, the level of phospho-ADF/Cofilin (P-Cofilin), a major mediator of Rac1-dependent actin remodeling, is reduced, whereas the level of the catalytic subunit of protein phosphatase 2A (PP2Ac), which controls P-Cofilin dephosphorylation (35–37), is increased. We demonstrate that FMRP can bind the 5′-UTR of *pp2acβ* mRNA with high affinity via well-conserved G-quartet structures, suggesting a direct mechanism of translational repression. Thus, our findings implicate FMRP in the control of actin cytoskeleton remodeling through the modulation of PP2Ac expression.

RESULTS

FMRP interacting proteins relocalize to actin ring areas in PDGF-stimulated fibroblasts

To characterize the interaction between FMRP and Rac1 pathway, we have used a set of immortalized fibroblast cell lines derived from a *Fmr1* knock-out mouse cell line: these cells express either wild-type *FMR1* (FMR1+), *FMR1* alleles with a point-mutation in the KH1 domain (the analogous I241N mutation to the I304N patient mutation in KH2 domain, $FMR1^{KH1}$) or in the KH2 domain (I304N, $FMR1^{KH2}$) or no *FMR1* (FMR1−) (Supplementary Material, Fig. S1). Using immunofluorescence co-staining, we first analyzed the intracellular distribution of Rac1, FMRP and four of its interacting proteins relatively to actin staining. Cells were serum starved and then treated with PDGF for 20 min. PDGF is a growth factor which induces a signaling cascade leading to Rac1 activation and to transient formation of specific actin structures, called actin rings (reviewed in 38). Activated Rac1 was previously reported to relocalize in dorsal ruffles associated with these actin rings (39). P21-activated kinase 1 (PAK1), a direct downstream target of Rac1, is also recruited to these dynamic actin structures after PDGF treatment (40).

We indeed observed that Rac1 moves to actin ring areas after PDGF treatment (Fig. 1B). In this context, we expected that CYFIP1 subcellular localization would be of particular interest, because this protein was shown to interact with activated Rac1 (24,26). While CYFIP1 was found homogeneously distributed in cytoplasm of non-induced cells (Fig. 1A), as previously reported (23), PDGF treatment led to CYFIP1 relocalization in actin ring areas (Fig. 1B). We then analyzed FMRP distribution and observed that it is not detectably modified after PDGF induction (Fig. 1B). However, not only FXR1P, but also 82-FIP and NUFIP1 (the latter two proteins being mostly nuclear in serum-starved cells) did relocalize to these regions upon PDGF activation (Fig. 1B). We checked whether FXR1P relocalization also occurs in NIH-3T3 fibroblasts and indeed, we observed its recruitment close to actin ring areas (Supplementary Materials, Fig. S2). These relocalizations occurred in both FMR1+ and FMR1− cells, demonstrating that FMRP is not required for recruitment of its partners to actin polymerization sites (data not shown). These observations support the existence of a connection between Rac1 and FMRP interacting proteins.

PDGF-induced actin cytoskeleton reorganization is enhanced in *FMR1* mutant fibroblasts

Dendritic spine morphology and function, that appear affected in fragile X syndrome patients brain, depend on a dynamic and precise organization of the actin cytoskeleton network controlled by Rho GTPases (41). We thus analyzed Rac1-induced actin cytoskeleton remodeling in the absence of FMRP. We compared actin cytoskeleton reorganization in FMR1+ and FMR1− cells at several time points after PDGF induction, using phalloidin-FITC staining. Before stimulation, both FMR1+ and FMR1− cells display stress fibers (Fig. 2A). As expected, actin rings characteristic of PDGF stimulation were visible at 10 min after treatment in both cell types (Fig. 2B). Quantitative analysis of cells with rings revealed that 14% of FMR1+ fibroblasts displayed this type of structures at 10 min, whereas this percentage was much higher in FMR1− cells, reaching 47% (Fig. 2C). Proportion of cells with actin rings remained higher in FMR1− cells than in FMR1+ cells also 30 min after PDGF treatment (Fig. 2C). Consistently, the percentage of $FMR1^{KH1}$ and $FMR1^{KH2}$ mutant cells exhibiting rings 20 min after PDGF treatment was 2-fold higher than in FMR1+ cells (Fig. 2D). Macropinocytosis has previously been reported to occur under Rac1 activation and has been connected to circular ruffles (42). We did not observe major changes in this process in FMR1−, $FMR1^{KH1}$ and $FMR1^{KH2}$ cells (data not shown).

Thus, Rac1-induced actin remodeling is enhanced in FMR1−, $FMR1^{KH1}$ and $FMR1^{KH2}$ mutant cells, further emphasizing an involvement of FMRP in Rac1-induced actin cytoskeleton reorganization events.

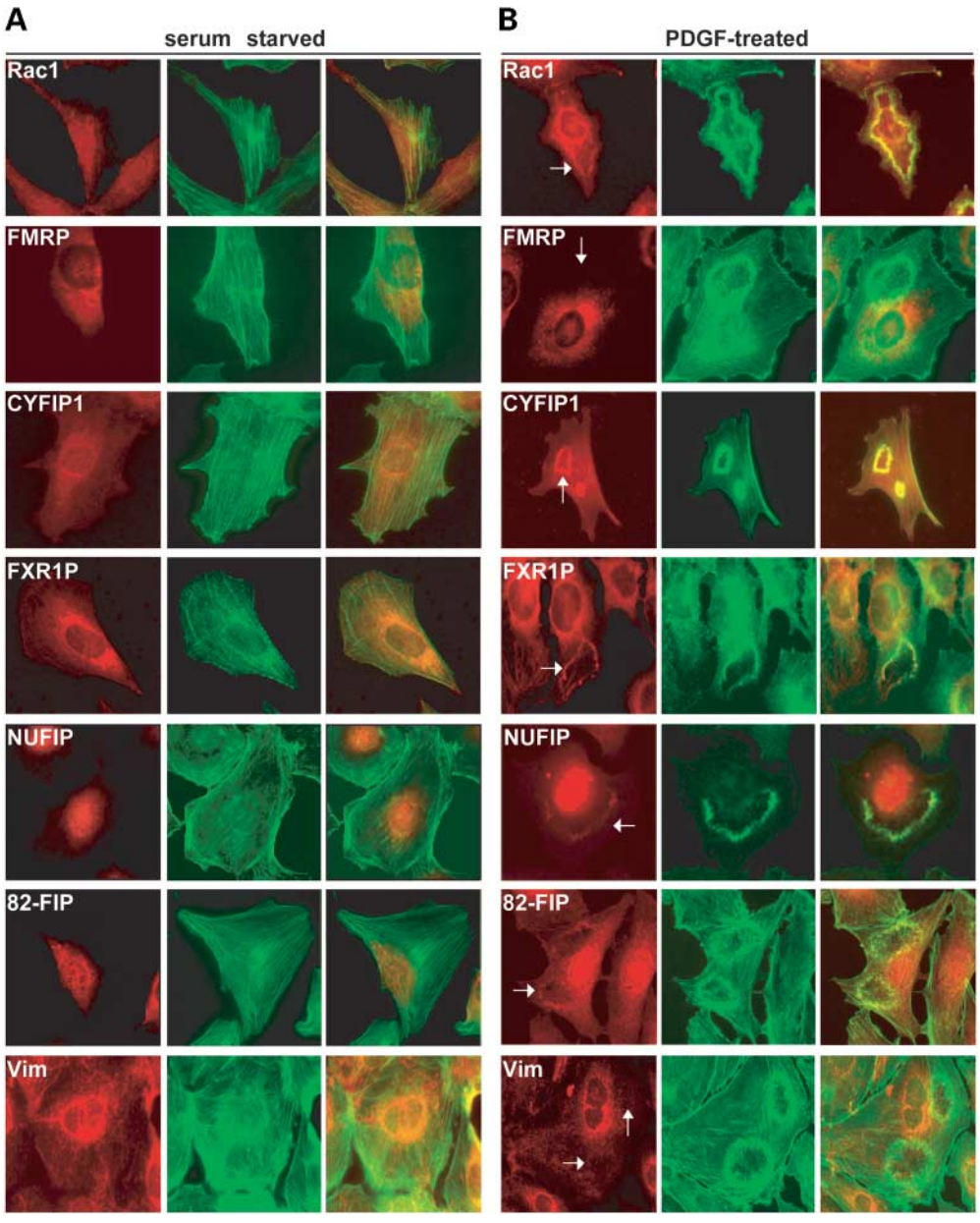

Figure 1. Relocalization of four FMRP partners to actin ring areas. Localization of FMRP and some of its interactors in serum-starved fibroblasts (**A**) and 20 min after PDGF induction (**B**). Rac1, Vimentin, FMRP and its interactors are labeled in red (left column). Actin is labeled in green (phalloidin-FITC, middle column). Merge (right column) corresponds to superposition of indicated protein and phalloidin-FITC labelings. Arrows in (B) indicate actin ring areas. Similar results were obtained in control experiments performed without phalloidin-FITC staining. Vimentin is used as a negative control.

Level of the catalytic subunit of protein phosphatase 2A, a phospho-Cofilin phosphatase, is increased in FMR1−growing cells

Because FMRP is involved in translational regulation, we set out to identify proteins that are misexpressed in FMR1− cells and that could account for the altered PDGF-induced actin phenotype in FMR1− fibroblasts. For this purpose, we compared the proteomes of FMR1+ and FMR1− cells using 2-D gel electrophoresis. Differentially expressed proteins were identified by mass spectrometry (our unpublished data). One of the major proteins found is the beta isoform of the PP2Ac. This enzyme can dephosphorylate P-Cofilin (35–37), two small homologous proteins acting at the end of Rac1 pathway to enhance actin depolymerization (reviewed in 43,44).

We confirmed this quantitative difference by comparing PP2Ac expression level in several FMR1+ and FMR1− clones. As Rho GTPases are involved in G1-phase regulation in fibroblasts (45) and PP2Ac is known to be particularly abundant in this phase (46), we synchronized cells in G1 before protein extraction. PP2Ac level was indeed significantly

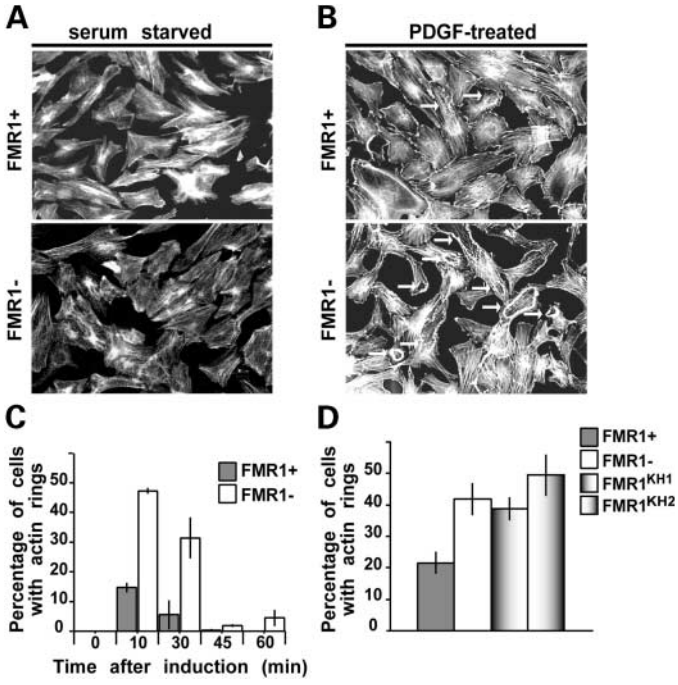

Figure 2. Enhanced actin remodeling response of *FMR1* null (FMR1−) and KH-mutant ($FMR1^{KH1}$ and $FMR1^{KH2}$) fibroblasts upon PDGF treatment. Actin cytoskeleton labeling with phalloidin-FITC (**A**) in serum-starved cells and (**B**) 20 min after PDGF treatment. As expected, PDGF treatment leads to formation of actin ring structures (arrows in B). (**C**) Quantification of cells exhibiting actin rings in two FMR1+ clones and in two FMR1− fibroblasts clones, at different time points after PDGF addition. One representative experiment is shown. Five hundred cells per clone were analyzed. For each cell type, mean and standard deviation between both clones were calculated. (**D**) Quantitative analysis of FMR1-, $FMR1^{KH1}$, $FMR1^{KH2}$ and FMR1+ cells with actin rings 20 min after PDGF treatment.

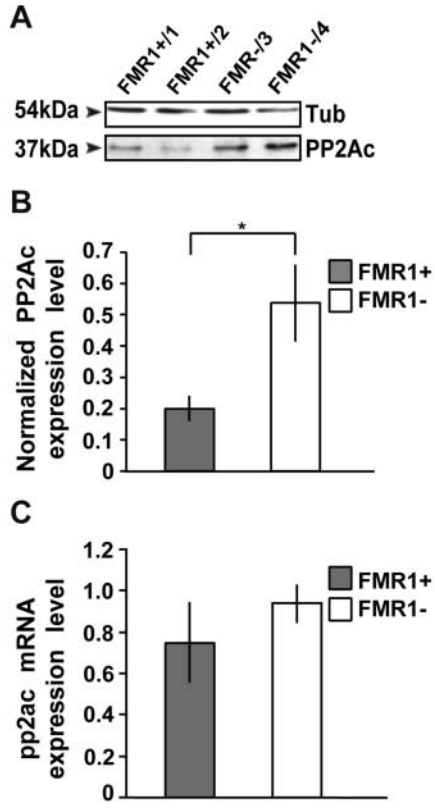

Figure 3. Increased level of protein phosphatase 2A catalytic subunit (PP2Ac) in *FMR1* null cells (FMR1−). (**A**) Western blot analysis of two FMR1+ (+/1, +/2) and two FMR1− (−/3, −/4) clones. (**B**) Densitometer analysis showing significant increase of PP2Ac amount in FMR1− clones (−/3, −/4) compared with FMR1+ clones (+/1, +/2). Two independent experiments were quantified. Results are means of PP2Ac amounts normalized to Tubulin (Student's *t*-test, $P < 0.05$). (**C**) No significant difference was observed at mRNA level, as determined by LightCycler real-time PCR.

higher (2-fold) in FMR1− cells compared with FMR1+ cells (Fig. 3A and B). No significant difference was observed at mRNA level (Fig. 3C), in agreement with previous data demonstrating that PP2Ac expression is regulated at the post-transcriptional level (46).

Phospho-Cofilin level is reduced in FMR1−, $FMR1^{KH1}$ and $FMR1^{KH2}$ mutant fibroblasts

Rac1-induced reorganization of actin cytoskeleton is mediated by a signaling transduction cascade, resulting in the activation of LIMK1, which phosphorylates, and thus inactivates, Cofilin (43). As we identified an increased level of P-Cofilin phosphatase PP2Ac in FMR1− fibroblasts, we analyzed whether P-Cofilin amount is changed in FMR1− cells compared with FMR1+ cells. Indeed, using western blot analysis, we found that P-Cofilin level was significantly decreased (by 50%) in FMR1− cells (Fig. 4B). Conversely, no quantitative difference in global amount of Rac1, LIMK1 and total Cofilin was observed between FMR1+ and FMR1− cells (Fig. 4A). The decreased P-Cofilin and the increased PP2Ac level are also observed in cells expressing mutant FMRP, this phenotype being especially strong in $FMR1^{KH2}$ mutant cells (Fig. 4C).

The reduced level of the inactive form of Cofilin may account for the FMRP-dependent difference in actin reorganization that was observed after PDGF treatment.

pp2acß mRNA specifically interacts with FMRP

Several *in vitro* and *in vivo* data support the role of FMRP as a translational repressor (8–10). Therefore, we asked whether the beta isoform of *pp2ac* (*pp2acß*) mRNA is a direct target of FMRP. The ability of FMRP to bind to *pp2acß* mRNA was tested as previously described: we determined the FMRP affinity for this mRNA by measuring its ability to disrupt binding of ^{32}P-labeled N19 RNA by GST–FMRP in gel shift experiments (6). N19 is a short fragment of *FMR1* mRNA (nucleotides 1470–1896) that contains a G-quartet structure and binds with high affinity to FMRP. Subfragments of *pp2acß* mRNA (full length, 5′-UTR, 3′-UTR) were tested and we found that its 5′-UTR did show an affinity for FMRP similar to that observed for N19 itself (Fig. 5A and B).

G-quartet forming regions can be detected by comparing reverse transcriptase elongation on RNA templates in the presence of either K+ or Na+: stabilization of G-quartet structures by K+, but not by Na+, results in cation-dependent pauses

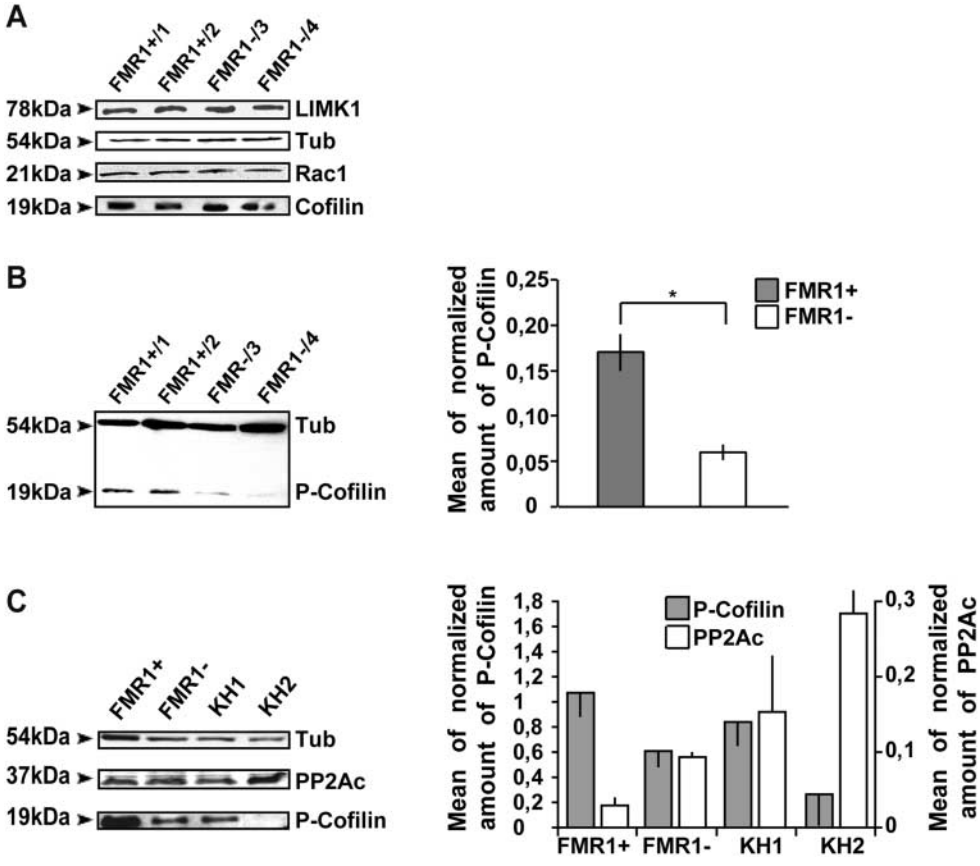

Figure 4. Decreased level of phospho-Cofilin (P-Cofilin) in *FMR1* null (FMR1-) and KH-mutant ($FMR1^{KH1}$ and $FMR1^{KH2}$) fibroblasts. (**A**) Western blot using anti-Rac1, anti-LIMK1 and anti-Cofilin antibodies on total protein extracts of two FMR1+ clones (+/1, +/2) and two FMR1- clones (-/3, -/4) reveal no significant difference in total amount of these proteins. (**B**) Amount of P-Cofilin was determined in same conditions using a specific antibody. Densitometer analysis indicates a 2-fold reduction of P-Cofilin amount in FMR1− clones (−/3, −/4) compared with FMR1+ clones (+/1, +/2) (normalization to Tubulin). Means and standard deviations were calculated from two independent experiments (Student's *t*-test, $P < 0.002$). (**C**) Western blot analysis and its quantification reveal both a decreased P-Cofilin and an increased PP2Ac level in $FMR1^{KH1}$ (KH1) and in $FMR1^{KH2}$ (KH2) cells compared with FMR1+ cells.

visible on a gel (6). This allowed us to identify two strong and two weak G-quartet pauses in the 5′-UTR of *pp2acß* mRNA (Fig. 5C). One is localized only 18 nucleotides before the ATG of the messenger: FMRP binding on this G-quartet is thus likely to produce translational repression of the mRNA, as previously shown for the *FMR1* G-quartet itself (6). Alignment of sequences corresponding to G-rich regions of *pp2acß* 5′-UTR in mammals are shown in Figure 5D. High conservation of these non-coding sequences argues in favor of their functional importance. Altogether, these results show that FMRP is able to bind *pp2acß* mRNA with high affinity and specificity, most likely via G-quartet structures.

DISCUSSION

Functional properties of FMRP have been extensively studied, but its precise mechanism of action and the pathways leading to mental retardation in its absence are still poorly understood. The goal of this work is to characterize connection(s) existing between FMRP and Rac1 pathway, given the importance of this Rho GTPase in nervous system development and in control of dendritic spine formation (31,47). The first indication for existence of such a connection was provided by demonstrating that the CYFIP1/2 proteins are interactors of both Rac1 and FMRP and that the three orthologous genes show genetic interaction in *Drosophila* (23–26). Furthermore, *dRac1* mRNA has been reported to be associated with dFMR1–mRNP complex (48).

We have studied the effect of FMRP function on Rac1-induced actin cytoskeleton dynamics in murine fibroblasts. We compared cells that express wild-type FMRP to cells lacking FMRP or expressing the well-known KH2 mutant (I304N) or its equivalent in the KH1 domain (I241N). Fibroblasts are commonly used to study actin remodeling mechanisms that are also implicated in growth cone extension in neurons (27,28,49,50), since mechanisms of cytoskeletal actin reorganization leading to membrane protrusions are believed to be similar in all cells (44,50). Moreover, neurons are not the only cells affected in fragile X syndrome, because clinical features also include facial dysmorphism and joints hyperextensivity (2). Finally, this model allows us to study the effect of KH1 or KH2 point-mutation, the latter identified in a severely affected patient. Both mutant proteins are associated with mRNP particles but not with actively translating polyribosomes (15) (data not shown for the KH1 mutant).

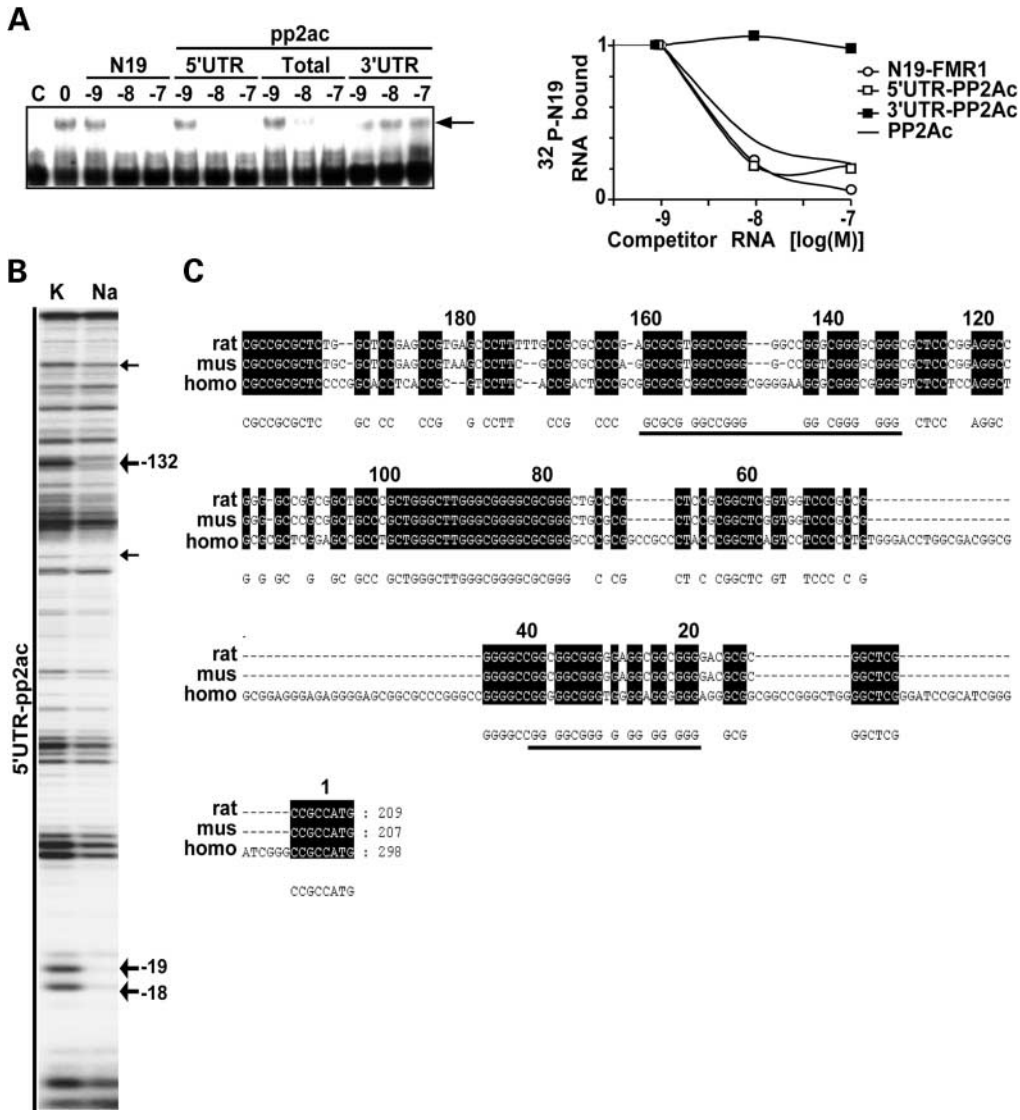

Figure 5. FMRP binding on *pp2Acβ* mRNA via G-quartets. (**A** and **B**) Determination of the binding strength of various subfragments of *pp2Acβ* mRNA, using gel retardation experiments. ^{32}P-labeled N19 subfragment of *FMR1* containing G-quartet was incubated with 0.1 pM GST–FMRP, in the presence of increasing amount of unlabelled competitors. Lane C: control without protein; Lane 0: control without competitor; numbers are logs of competitors concentrations (N19-FMR1, complete *pp2Acβ* mRNA (total), 5′-UTR of *pp2Acβ* mRNA and 3′-UTR of *pp2Acβ* mRNA). The graph depicts the fraction of ^{32}P-N19 bound RNA, plotted against competitors RNA concentrations determined by densitometer analysis. (**C**) Cation-dependent termination of reverse transcription in the 5′-UTR of *pp2Acβ* mRNA. Strong and weak pauses of reverse transcriptase are, respectively, indicated by large and thin arrows. Numbers correspond to positions of strong pauses, +1 being A of the ATG codon. (**D**) Localization and conservation of the two stable G-quartet structures among mammals.

We show in this study that Rac1 activation leads to relocalization of four FMRP-interacting proteins (CYFIP1, FXR1P, NUFIP and 82-FIP) to actin-containing domains involved in processes protrusions. Relocalization of these proteins is not FMRP-dependent, as lack of FMRP does not abolish their recruitment. However, this finding suggests that the assembly and composition of some FMRP-containing complex are modulated by Rac1. We also observed an enhanced Rac1-induced actin remodeling in FMR1–, FMR1^{KH1} and FMR1^{KH2} mutant cells. This correlates with a decreased P-Cofilin level and an increased PP2Ac level in these cells. We, furthermore, showed that *pp2acβ* mRNA is bound by FMRP with high affinity and contains in its 5′-UTR domains able to form G-quartet structures.

PP2A is a phospho-serine/threonine phosphatase ubiquitously expressed in eukaryotic cells. The core enzyme consists of one of two closely related isoforms (α and β) of the catalytic subunit, associated with one of the two isoforms of the structural subunit PR65/A. PP2A is involved in many processes such as regulation the of cell cycle events, translational control and cytoskeleton reorganization (51). Moreover, PP2A was shown to interact physically with Cofilin and to dephosphorylate it (35–37). Cofilin, a downstream component of Rac1 cascade, is a small actin-binding protein, which plays a key role in actin cytoskeleton dynamics, enhancing actin depolymerization and causing actin filaments branching and severing (44,47). Thus, the difference in Rac1-induced actin

remodeling that we observed in FMR1−, $FMR1^{KH1}$ and $FMR1^{KH2}$ mutant fibroblasts may be accounted for by decreased phosphorylation of Cofilin via increased PP2Ac.

The pool of active Cofilin is likely to be higher in FMR1−, $FMR1^{KH1}$ and $FMR1^{KH2}$ cells compared with FMR1+ cells. Indeed, we found a decrease in P-Cofilin level without change in global amount of the protein. This may, at a first glance, appear contradictory with the observation of an enhanced response to Rac1 signaling in the absence of functional FMRP, because Rac1 is known to act through the inhibition of Cofilin. It has, however, been shown that both a decrease in P-Cofilin level and Cofilin over-expression induce the same changes as observed after expression of constitutively active Rac1 (36,47). Indeed, a global and/or local increase of the ratio of Cofilin to P-Cofilin leads to an increase in actin turnover. This creates free barbed ends and maintains a pool of actin monomers, thereby increasing the rate of actin polymerization. On the other hand, the inactivation of Cofilin through Rac1 signaling pathway allows local actin polymerization, which is also required for the extension of their processes (44). Thus, a global and/or local balance between kinase(s) and phosphatases activities is crucial to precisely control the cycling of phosphate on Cofilin. As Cofilin action on spine actin dynamics is implicated in the regulation of synaptic plasticity (52), an alteration of Cofilin phosphorylation may play a role in the alteration of dendritic spines observed in fragile X patients and in *Fmr1* null mice brain.

We propose that the effect of FMRP on Rac1 signaling depends at least in part on translational repression of *pp2acβ* mRNA. We found that $FMR1^{KH1}$ and $FMR1^{KH2}$ mutant cells display the same phenotype than those which lack FMRP. Thus, the association of FMRP with polyribosomes is required for its interference with Rac1 signaling. Moreover, *pp2acβ* mRNA is a likely target of FMRP, because we showed that FMRP binds specifically and with high affinity to its 5′-UTR. This fits with previous observation that PP2Ac expression is regulated at the translational level (46).

We identified four G-quartet structures in *pp2acβ* 5′-UTR. Similar repetitions of RNA motifs have previously been described for iron response elements (53), differentiation control elements (DICE) (54) and for the UCAU sequence bound by Nova1, a protein containing three KH domains (55). Relations between the number of RNA motifs and the functional significance of RNA–protein interaction have been established in some cases. For example, translational inhibition by hnRNP E1 is only observed when at least two DICE elements are repeated in a reporter mRNA (54). Thus, FMRP binding on multiple G-quartet sites could cause translational repression by a similar mechanism. Alpha and beta isoforms of PP2Ac are very homologous, and alpha isoform may also be a target of FMRP, as we have noticed the presence of potential G-quartet forming sequences in its 5′-UTR.

Electrophysiological analysis in hippocampal slices of *Fmr1* knock-out mice has revealed an alteration of synaptic plasticity, manifested by enhanced metabotropic glutamate receptors-dependent long term depression (LTD) (56). It is worth to note that PP2A has also been implicated in the modulation of LTD (57), in metabotropic glutamate receptors signaling transduction (58,59) and in other alterations of synaptic plasticity (such as depotentiation induced by high theta-burst stimulation) (60).

In conclusion, we have shown that FMRP alters Rac1 signaling in mammalian fibroblasts and modulates P-Cofilin and PP2Ac levels. Further investigations are now required to determine whether these alterations also take place in neurons and whether they could participate in the synaptic structure and plasticity defects that are considered to be at the basis of the mental impairment in fragile X syndrome.

MATERIALS AND METHODS

Establishment of stably transfected murine fibroblasts lines

The fibroblastic 3T-6A STEK cell line, which shares the same origin but does not correspond to the one previously described by Mazroui *et al.* (10), was established from mouse *Fmr1* null C57Bl/6J embryos (mouse strain gR2700 available from The Jackson Laboratory), according to the procedure of Todaro and Green (61). Subcultures were propagated as uncloned mass cultures for a period of 6 months before being considered as stable. Cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 units/ml penicillin, 50 mg/ml streptomycin). These cells were transfected using Effectene^{TM} (Qiagen), either with pTL10 vector containing *FMR1* isoform 1 fused to FLAG epitope (62,63), by the same vector containing *FMR1* isoform 1 with a point-mutation in KH1 or in KH2 domain or with an empty pTL10 vector. The pIREShyg3 plasmid (Clontech-BD Biosciences) was co-transfected with pTL10 vectors. Hygromycin (150 μg/ml) was added 48 h after transfection and resistant clones were isolated and amplified. Expression of FMRP was controlled in each clone by immunoblot with 1C3 antibody (Supplementary Material, Fig. S1). Thirteen hygromycin resistant clones (five in which *FMR1* is expressed, one in which *FMR1* mutated in KH1 domain is expressed, two in which *FMR1* mutated in KH2 domain is expressed and five *FMR1* null, referred to, respectively, as FMR1+, $FMR1^{KH1}$, $FMR1^{KH2}$ and FMR1−) were selected. All experiments were performed on several randomly chosen clones: data are presented for some but results were always consistent for the others. Morphology of FMR1+ and FMR1− cells are similar in normal growth conditions (Supplementary Material, Fig. S1).

Site directed mutagenesis of FMRP KH1 or KH2 domain

We performed site directed mutagenesis to introduce the I304N point-mutation in KH2 domain or an equivalent one (I241N) in KH1 domain using the 'QuickChange Site-Directed Mutagenesis Kit' (Stratagene) according to manufacturer instructions and using the following oligonucleotides for KH2 and KH1 corresponding sequences, respectively:

- GTACTCATGGTGCTAATA**A**TCAGCAAGCTA GAAAAGTACCTG/CAGGTACTTTTCTAGCTTGCT GA**T**TA TTAGCACCATGAGTAGTAC
- GAAAGCTGA**A**TCAGGAGATTGTGGACAAGTCAG/ CTGACTTGTCCACAATCTCCTGA**T**TCAGCTTTCC.

Cell culture

Stably transfected cell lines were cultured in DMEM supplemented with 10% FCS and hygromycin (150 μg/ml) until they reach 80% confluence. NIH-3T3 fibroblasts were cultured in DMEM supplemented with 10% newborn calf serum.

To induce Rac1 activation, PDGF (platelet-derived growth factor BB, R&D Systems) was added to a final concentration of 5 or 10 ng/ml to serum-starved cells (16 h in DMEM+ 0.1% serum). For synchronization in G1-phase, cells were serum-starved (20 h in DMEM+ 0.1% serum) and then cultured 6 h in DMEM+ 10% FCS.

Immunofluorescence, immunoblot and antibodies

Cells were fixed for immunofluorescence experiments as previously described (63). Fixed cells were rinsed with PBS and incubated with specific antibodies for Rac1 (1/500, Upstate Biotechnology), CYFIP1 (1/500), FMRP (1C3, 1/1000) (64), FXR1P (830, 1/500) (65), NUFIP1 (1541, 1/250) (21), 82-FIP (1666, 1/250) (22) or in PBS. After PBS rinses, goat anti-mouse/rabbit-Alexa594 and/or AlexaFluor^{TM}488 Phalloidin (Molecular Probes) were then added. Cells were then rinsed and mounted in Kaiser's glycerol gelatin (Merck). Immunofluorescence was analyzed using a Leica DB microscope.

Immunoblot analysis was performed as previously described (63). Membranes were probed overnight at 4°C with 1C3 antibody (1/2000), anti-P-Cofilin (1/1000), anti-Cofilin (1/500, Ozyme), anti-LIMK1 (1/500, Santa Cruz Biotechnology), anti-Rac1 (1/500, Upstate Biotechnology) or anti-PP2Ac (1/500, Upstate Cell Signaling) and with anti-Tubulin (1/5000) (Chemicon), and then incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies (1/5000). Immunoreactive bands were visualized with the Supersignal West Pico Chemiluminescent Substrate (Pierce).

CYFIP1 mouse monoclonal antibody was raised and affinity purified against the synthetic peptide DEIITILDKYLKSGDGEGTPC (CYFIP1 amino acids 1217–1236). Western blot and immunofluorescence analyses on CYFIP1 transfected and mock transfected COS cells as well as on fibroblasts have shown that it specifically recognizes a 140 kDa band corresponding to CYFIP1 (data available on request). Macropinocytosis was assessed by measuring uptake of 10 kDa dextran as previously described (66).

Two-dimensional electrophoresis

Cells were harvested by centrifugation and resuspended in 10 mM Tris, 1 mM EDTA, and 250 mM sucrose. Lysis was performed in four volumes of 2.5 M thiourea, 8.75 M urea, 5% CHAPS, 50 mM DTT and 25 mM spermine. DNA was eliminated by 30 min ultracentrifugation at 90 000 rpm. A total of 150 mg of proteins were diluted in 400 μl of rehydratation buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.4% ampholytes, 20 mM DTT), which were used to rehydrate home-made pH4–8 immobilized pH gradient strips. Isoelectric focusing was conducted for 60 000 V/h at a maximum of 3000 V using the MultiphorII system (Amersham-Pharmacia, Sweden). Strips were then equilibrated for 20 min by rocking first in a solution of 0.15 M bisTris/0.1 M HCl, 6 M urea, 2.5% SDS, 30% glycerol, 0.5 M DTT and then in 0.15 M bisTris/0.1 M HCl, 6 M urea, 2.5% SDS, 30% glycerol, 0.3 M iodoacetamide. They were then embedded onto a 12% SDS/PAGE gel in 800 μl of 1% agarose. The gels buffer consisted of 0.18 M Tris/0.1 M HCl, the cathode buffer contained 0.2 M taurine/25 mM Tris, 0.1% SDS and the anode buffer was 0.384 M glycine/50 mM Tris, 0.1% SDS. Gels were run 25 V for one hour then 400 V/500 mA/12.5 W/gel for 5 h. Fixation was performed 1 h in 30% ethanol, 10% acetic acid and overnight in 30% ethanol, 0.5 M potassium acetate and 1 mM potassium tetrathionate. Staining of gels was done 20 min in 0.2 M potassium carbonate, 0.01% formaldehyde, and 1.25×10^{-3}% sodium thiosulfate and blocked in 0.3 M Tris, acetic acid 2%. Gels were scanned and protein differences between FMR1+ and FMR1− fibroblasts were analyzed. Corresponding spots of interest were excised from the gel and analyzed by Maldi-TOF as previously described (67).

LightCycler real-time PCR

RNA extraction from FMR1+ and FMR1− fibroblasts synchronized in G1-phase was performed using RNASolv^{R}Reagent (Omega Bio-Tek) and 1 μg of RNA was retro-transcribed using AMV Reverse transcriptase (Roche), according to manufacturer instructions. *pp2acβ* and *hprt* cDNA, used as a control, were amplified by real-time PCR, as previously described (6), using, respectively, the following oligonucleotides:

- GCCATGGACGACAAGGCG/TTTACAGGAAG TAGTCTGGGG
- AGAGGTCCTTTTCACCAGCAAG/ATTATGGACAG GACTGAAAGAC.

Gel shift and identification of mRNA G-quartet structures

GST–FMRP protein production and purification, gel shift assay as well as identification and characterization of mRNA G-quartets were performed as previously described (6). We used *pp2acβ* cDNA clone from rat (NM_017040) (68). Subcloning of 3′-UTR was performed by PCR, using following oligonucleotides: CCTATAAATTCCTCCCCAG and CTCTCTAAATTGGG AAGTTT. The 5′-UTR was obtained by digesting the full-length cDNA by *Nco*I at the ATG position.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank Professor James Bamburg for anti-P-Cofilin antibody and Dr Hitoshi Nakagama for PP2Ac cDNA. We are grateful to Solange Pannetier, Fabrice Klein, Sandra Tremblay, Isabelle Kolb-Cheynel and Eric Flatter for help with experiments. We also warmly thank Didier Devys, Dominique Helmlinger, Hervé Seznec and Yvon Trottier for discussions. B.B. is indebted to Enzo Lalli and Astrid Lunkes for critical reading of the

manuscript. This study was supported by funds from 'Human Frontier Science Program' (RGP0052/2001), NIH (R01 HD40612-01), INSERM, CNRS and 'Fondation Jérôme Lejeune'. M.C. is recipient of an 'Allocation de Recherche de l'Ecole Normale Supérieure (Paris)' and E.W.K. is supported by 'Fragile X Research Foundation of Canada' and by the CIHR.

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Le syndrome de l'X fragile est la cause la plus fréquente de retard mental héréditaire. Ce syndrome est du à l'absence de la protéine FMRP (Fragile X Mental Retardation Protein). FMRPest exprimée dans de nombreux tissus, et surtout dans les neurones et dans les spermatogonies. Elle possède un signal de localisation nucléaire (NLS), et un signal d'export nucléaire (NES), des motifs de liaison à l'ARN (deux domaines KH et une boîte RGG). Bien que la sublocalisation et le rôle de FMRP dans le noyau ne soient pas encore connus, dans le cytoplasme FMRP est associée aux polyribosomes faisant partie d'un complexe ribonucléoprotéique où elle interagit avec ses deux homologues FXR1P et FXR2P. Deux structures de liaison pour FMRP ont été identifiées et caractérisées: le "purine-quartet" (cette structure est aussi présente dans l'ARNm de *FMR1*) et le « kissing complex». L'ARNm de *FMR1* est associé aux complexes polyribosomiques synaptiques ainsi que sa traduction sont modulées par l'activité synaptique. Donc, la présence d'un NLS et d'un NES suggère que FMRP fasse la navette entre le noyau et le cytoplasme pour le transport de l'ARNm. Plusieurs ARN ont été identifiés comme cibles potentielles de FMRP. Ces ARN sont derégulés chez les souris Fmr1 nulles, mais la signification fonctionnelle de l'interaction FMRP/ARN reste toujours partiellement connue.

L'objectif principal de ma thèse étant la compréhension du mécanisme d'action de FMRP, ce projet a été abordé en deux points principaux :

-Recherche de l'influence des protéines qui interagissent avec FMRP sur sa capacité (affinité) à se lier à l'ARN

-Recherche de nouvelles séquences/structures cibles de FMRP et analyse du rôle de l'interaction FMRP/ARN.

Nous avons pu montrer une interaction spécifique uniquement entre l'isoforme musculaire de FXR1P avec la structure de G-quartet. Cela nous a permis d'établir un rôle synergique et non compensatoire de FXR1P sur FMRP.

D'un autre côté, nous avons démontré l'interaction spécifique de FMRP avec une nouvelle structure présente dans l'ARNm de la Sod1 que nous avons appelé SSLIP (Sod1 Stem Loops Interacting with FMRP). La distribution de SSLIP sur les polyribosomes est altérée en absence de FMRP ce qui conduit à une faible expression de la protéine Sod1. En utilisant un système de gène rapporteur, nous avons montré que l'interaction FMRP/SSLIP favorise la traduction de la Sod1 ce qui nous a permis d'établir un nouveau mécanisme d'action de la protéine FMRP sur ces cibles ARN.