UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CENTRO DE BIOTECNOLOGIA
DEPARTAMENTO DE BIOFÍSICA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Importância de motivos conservados do fator de início de tradução 2β (eIF2β) na síntese de proteínas e na distribuição subcelular desse fator em células humanas

TESE DE DOUTORADO

GABRIELLE DIAS SALTON

Porto Alegre, 2011.

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Gabrielle Dias Salton

Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS como requisito parcial para a obtenção do grau de Doutor.

Orientador: Dr. João Antonio Pêgas Henriques
Co-orientador: Dr. Guido Lenz

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LISTA DE ABREVIATURAS E UNIDADES

aa – aminoácido (*aminoacid*)

AMP – adenosina monofosfato (*adenosine monophospahate*)

ANOVA – análise da variância (analysis of variance)

ATP - adenosina trifosfato (*adenosine triphospahate*)

BSA – albumina sérica bovina (*bovine serum albumin*)

CBC – complexo de ligação a *cap* (*cap binding complex*)

cDNA - ácido desoxiribonucléico complementar (*deoxyribonucleic acid complementary*)

CK2 – caseína-cinase 2 (*casein kinase 2*)

CRM1 – região 1 de manutenção cromossomal ou exportina 1 (*chromosomal region maintenance 1 or exprotin 1*)

Cy3 – cianina 3 (*cyanine 3*)

DAPI - 4'-6-diamidino-2-fenilindol (*4',6-diamidino-2-phenylindole*)

DNA - ácido desoxiribonucléico (*deoxyribonucleic acid)*

DNA-PK – proteína-cinase dependente de DNA *(DNA-dependent protein kinase)*

EGFP - variante da proteína verde fluorescente melhorada (*enhanced green fluorescent protein*)

eEF – fator de alongamento da tradução (*eukaryotic elogation factor*)

eFR - fator de término da tradução (*eukaryotic release factor*)

eIF - fator eucariótico de início de tradução (*eukaryotic initiation factor*)

EJC – complexo de junção de exons (*exon-junction complex*)

FITC – fluorosceina-isotiocianato (*fluorescein isothiocyanate*)

GDP –guanosina-difosfato (*guanosine diphosphate*)

GTP –guanosina-trifosfato (*guanosine triphosphate*)

HSP – proteínas de choque térmico(*heat shock proteins*)

IRES – sítio interno de entrada ribossomal (*internal ribosome entry site*)

kDa – quiloDalton (*kilodalton*)

LMB – leptomicina B (*leptomycin B*)

μCi-microcurie (*microcurie*)

MDa – megadalton (*megadalton*)

m^7GTP - 7-metil-guanosina (*7-methyl-guanosine*)

moAb – anticorpo monoclonal (*monoclonal antibody*)

Metionil-tRNAi^{Met} – tRNA iniciador carregado com metionina (*initiator tRNA*)

mRNA - ácido ribonucléico mensageiro (*messenger RNA*)

mTOR - cinase proteína alvo de rapamicina de mamíferos (*mammalian target of rapamycin)*

NB – corpos nucleares (*nuclear bodies*)

NES – sinal de exclusão nuclear (*nuclear exclusion signal*)

NLS – sinal de localização nuclear (*nuclear localization signal*)

NMD – *nonsense-mediated mRNA decay*

NPC – complexo de poros nucleares (*nuclear pore complex*)

NTS - sinal de translocação nuclear (*nuclear translocation signal*)

PABP – proteína de ligação a poly-A (*poly-A binding protein*)

PAGE – eletroforese em gel de poliacrilamida (*polyacrylamide gel electrophoresis*)

PBS – tampão salina fosfato (*phosphate buffered saline*)

PCR - reação em cadeia da polimerase (*polymerase chain reaction*)

PD – dobro populacional (*population doubling*)

PKA – proteína-cinase dependente de AMP cíclico (*cAMP-dependent protein kinase*)

PKC – proteína-cinase C (*protein kinase C*)

PKR – proteína-cinase ativada por RNA dupla fita (*protein kinase activated by double-stranded RNA*)

PML – proteína de leucemia promielocítica (*promyelocytic leukemia protein*)

PP1 – proteína fosfatase 1 (*protein phophatase-1*)

PTC – códon de terminação prematuro (*premature termination códon*)

RNA - ácido ribonucléico (*ribonucleic acid*)

RNase – ribonuclease (*ribonuclease*)

RNAi - ácido ribonucleico de interferência (*interference RNA*)

rRNA - ácido ribonucléico ribossomal (*ribosomal RNA*)

4E-BP – proteína de ligação a eIF4E (*eIF4E binding protein*)

SDS - dodecil sulfato de sódio (*sodium dodecyl sulfate*)

siRNA – pequeno RNA de interferência (*small interference RNA*)

snRNPs – pequenas ribonucleoproteínas nucleares (*small nuclear ribonucleoproteins*)

TC - complexo ternário (*ternary complex*)

TetR – repressor de tetraciclina (*tetracycline repressor*)

Tris - Tris-hidroximetil aminometano (*tris(hydroxymethyl)aminomethane*)

tRNA - ácido ribonucléico transportador (*transfer RNA*)

UPF - proteínas *up-frameshift*

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RESUMO

Um dos principais reguladores da síntese proteica é o fator 2 do início da tradução de eucariotos (eIF2), formado por três subunidades não idênticas: α, β, e γ. As funções citoplasmáticas da subunidade β, essenciais à integridade do processo, estão relacionadas à presença de dois domínios conservados: um deles composto por três blocos de seis a oito resíduos de lisinas e localizado na região amino terminal; e o outro constituído por quatro cisteínas que formam um motivo dedo de zinco localizado na região carboxiterminal. Em *Saccharomyces cerevisiae*, a expressão de eIF2β desprovido dos blocos de lisinas foi capaz de inibir o crescimento celular. Entretanto, até o momento não são relatados dados em células de mamíferos. Além de sua fundamental função na regulação do processo de síntese proteica no citoplasma, alguns estudos têm demonstrado que as três subunidades de eIF2 apresentam localização nuclear, mas essa também é uma questão ainda pouco explorada. Os resultados apresentados nessa tese mostram que a expressão de eIF2β desprovido dos blocos de lisinas causa uma redução acentuada na síntese proteica e, consequentemente, uma significativa diminuição da proliferação e viabilidade de células humanas. As análises da distribuição subcelular demonstram que eIF2β selvagem superexpresso está localizado no citoplasma, mas é capaz de translocar para o núcleo e acumular no nucléolo de maneira dependedente de ligação a RNA. Tanto os blocos de lisinas, quanto os resíduos de cisteínas mostram-se essenciais à retenção nucleolar de eIF2β. Além disso, sua exportação nuclear é mediada pela exportina CRM1 e é dependente de sua região amino terminal. Considerando os dados obtidos nesse trabalho, pode-se concluir que a ausência dos blocos de lisinas em eIF2β apresenta um efeito antiproliferativo em célula humanas e que a proteína eIF2β deve desempenhar funções no nucléolo relacionadas a algum processo que envolva ligação a RNA. Nesse contexto, pode-se observar também que, tal como o eIF2β, vários outros eIFs apresentam localização nuclear e participam em diferentes processos que ocorrem no núcleo, e que podem estar relacionados a um mecanismo de checagem da qualidade de mRNAs recém-sintetizados. Assim, os diferentes eIFs atuam em processos vitais à célula no núcleo e, nesse compartimento, também são importantes à regulação da expressão gênica.

ABSTRACT

The eukaryotic initiation factor 2 (eIF2) is one of the main regulatory molecules in the protein synthesis. It is composed by three non identical subunits γ, β, and α: The cytoplasmic functions of the β subunit, that are essential to the integrity of the process, are related to the presence of two conserved domains: one of them composed of three stretches of six to eight lysine residues and located in the amino-terminal region, and the other consisting of four cysteines that form a zinc finger motif located in the carboxyl-terminal portion. In *Saccharomyces cerevisiae*, the expression of recombinant eIF2β without of the polylysine stretches was able to inhibit cell proliferation, however no data are reported in mammalian cells so far. Additionaly to its fundamental role in regulating the process of protein synthesis in the cytoplasm, some studies have shown that eIF2 may act in the nucleus, but this issue is also still underexplored. Our results indicate a strong reduction in protein synthesis and consequently a significant decrease in cell proliferation and viability when the eIF2β without polylysine stretches is expressed in human cells. Analysis of cellular distribution of eIF2β indicates that it is located in the cytoplasm, but can translocate to the nucleus and accumulate in the nucleolus in a RNA-dependent manner. Both polylysine stretches and cysteine residues are essential for nucleolar retention of this factor. The eIF2β nuclear exclusion is mediated by exportin CRM1 and is dependent of its amino-terminal region. In conclusion, the results presented here show that the absence of polylysine stretches in human eIF2β has an antiproliferative effect in human cells and that the eIF2β protein should play a role related to some processes involving RNA in the nucleolus. In this context, we can also observe that, like eIF2β, others eIFs also present nuclear localization and participate in different processes in the nucleus which can de related to proofreading mechanism of newly synthesized mRNAs. In this way, several eIFs play a role in vital cellular processes in the nucleus and, in this compartment they also are important to regulation of gene expression.

ESTRUTURA DA TESE

Esta tese está dividida da seguinte forma: uma introdução geral, os objetivos (gerais e específicos), um capítulo escrito na forma de artigo científico e outro capítulo apresentando resultados complementares, uma discussão geral, as conclusões, as perspectivas e os anexos.

Na introdução, são descritos o processo de síntese proteica e os principais componentes da maquinaria de início da tradução. Os fatores eucarióticos de início da tradução (eIFs) são caracterizados e uma análise mais detalhada do fator, eIF2, especificamente sua subunidade beta, é apresentada. Em sequência, é introduzida a abordagem de tradução nuclear, bem como a descrição da localização e das funções conhecidas dos eIFs no núcleo. Após, é apresentada a relação dos diferentes eIFs com situações patológicas, especialmente com a tumorigênese.

O Capítulo 1 apresenta um estudo que abordou o efeito da superexpressão de uma forma mutante específica de eIF2β em células humanas. Também foi analisada a distribuição subcelular da forma selvagem e de formas mutantes de eIF2β. Esses dados originaram um manuscrito que está em fase de submissão na revista RNA Biology.

O Capítulo 2 mostra um resultado complementar relacionado ao Capítulo 1, que caracteriza a diminuição da síntese proteica global causada pela superexpressão de uma forma mutante de eIF2β em comparação a uma droga clássica inibidora do processo de tradução.

Em seguida, é apresentada uma discussão geral que aborda os resultados desses dois capítulos, e a sua importância para a contribuição científica desse estudo, uma conclusão final e as perspectivas do trabalho.

Os anexos são constituídos de um manuscrito submetido à revista RNA Biology que descreve uma completa revisão sobre a localização e funções nucleares dos diferentes eIFs, sua associação com o processo de tradução nuclear, com a regulação da expressão gênica, bem com o processo de tumorigênese; de um artigo científico sobre terapia celular com células tronco mesenquimais em modelo murino para o diabetes mellitus publicado na revista Stem Cells and Development; e do *curriculum vitae*.

1. INTRODUÇÃO

1.1 CONSIDERAÇÕES INICIAIS

A expressão gênica em eucariotos é regulada em muitos níveis incluindo transcrição, poliadenilação e *splicing* do mRNA, exportação de mRNAs do núcleo para o citoplasma, degradação do mRNA, além da tradução de mRNAs (Clemens & Bommer, 1999).

A tradução do mRNA em proteínas representa um passo final no processo de expressão gênica que leva à formação do proteoma a partir das informações contidas no genoma das células. A regulação da tradução é um mecanismo que é utilizado para modular a expressão gênica em diferentes situações biológicas, desde o desenvolvimento embrionário até a diferenciação, crescimento e metabolismo celulares. As células utilizam o processo de tradução para ajustar os níveis protéicos de acordo com as condições em que a célula se encontra (Clemens & Bommer, 1999; Kuersten & Goodwin, 2003). Diferentes vias de sinalização de sinais da célula podem atuar sobre o processo de tradução e regulá-lo em suas diferentes fases. Há vantagens para a célula em ser capaz de controlar a síntese de proteínas (tanto globalmente, quanto individualmente) sem a necessidade de sintetizar ou degradar os mRNAs produzidos. Essas vantagens incluem a capacidade de responder rapidamente a mudanças do ambiente extracelular e a habilidade de produzir quantidades abundantes de proteínas específicas de um conjunto de mRNAs estocados (Clemens & Bommer, 1999).

Os níveis globais de síntese proteica têm um papel chave no controle da proliferação celular, de modo que a desregulação desse processo pode resultar em expressão gênica anormal e proliferação celular descontrolado, encontrado em doenças humanas, como o câncer. Estudos focando os mecanismos moleculares de regulação da síntese proteica tornam-se importantes para o entendimento desse processo de controle da

expressão gênica em células normais e patológicas, além de poder identificar novos alvos terapêuticos.

1.2 A SÍNTESE PROTEICA

A síntese proteica é um dos mecanismos biossintéticos celulares mais complexos, e seu entendimento é um dos maiores desafios na história da bioquímica. Os processos de ligação de RNAs transportadores (tRNAs), formação da cadeia peptídica e translocação ribossômica são, do ponto de vista mecânico, praticamente idênticos em todos os organismos conhecidos (Kozak, 1983, Hershey & Merrick, 2000;Hernández, 2009). Este é um processo vital para qualquer célula e envolve centenas de macromoléculas, na sua grande maioria evolutivamente conservadas. Na célula eucariótica, a síntese proteica requer a participação de mais de setenta diferentes proteínas ribossomais, vinte ou mais enzimas para ativar os precursores dos aminoácidos, uma dúzia ou mais de enzimas auxiliares e outros fatores protéicos específicos para a iniciação, alongamento e término da síntese de polipeptídeos. Existem talvez cerca de cem enzimas adicionais para o processamento final dos diferentes tipos de proteínas existentes e quarenta ou mais tipos de tRNAs e RNAs ribossomais (rRNAs). Dessa forma, aproximadamente trezentas diferentes macromoléculas devem cooperar para que a síntese polipeptídica ocorra de forma adequada. Muitas dessas macromoléculas estão organizadas em estruturas tridimensionais complexas, que interagem com outras moléculas de classe bioquimicamente distinta das suas. É relevante ressaltar que a tradução é um dos processos que consome a maior parte da energia produzida na célula, além de os fatores protéicos envolvidos nesse processo representarem mais de 50% da quantidade total de proteínas celulares (Kozak, 1983;

Merrick, 1992, Hershey and Merrick, 2000). Didaticamente, o processo de tradução no citoplasma pode ser dividido em três etapas, descritas a seguir.

1.2.1- Etapa de início da síntese proteica

Nessa fase, ocorre a ligação do tRNA iniciador carregado com a metionina inicial (Metionil-tRNAi^{Met}) à subunidade ribossomal 40S e posterior ligação desse complexo ao RNA mensageiro (mRNA). É essencial ressaltar que diferentes fatores de início da tradução (eIFs) medeiam todo esse processo (Kozak, 1999; Preiz & Hentz, 2003; Sonemberg & Hinnebusch, 2009; Jackson et al., 2010) (processo detalhado nas páginas 30-33).

1.2.2- Etapa de alongamento da cadeia polipeptídica

Nessa fase, após a formação do ribossomo 80S completo no códon de iniciação, os aminoacil-tRNAs são adicionados ao sítio aceptor (A) ribossomal e o peptídeo no sítio P. O fator eucariótico de alongamento (eEF) eEF1A ligado a GTP associa-se ao aminoacil-tRNAs e apresenta-o no sítio A ribossomal. O correto pareamento das bases dos códon-anticodon leva a hidrólise de GTP pelo ribossomo. Ocorre a formação da ligação peptídica realizada pela atividade enzimática ribossomal, peptidil-transferase. Isso resulta em um tRNA vazio no sítio P e um peptidil-tRNA no sítio A. É realizado então um movimento de translocação no qual o fator eEF2, por hidrólise de GTP, auxilia o movimento do mRNA, fazendo com que o tRNA vazio alcance o sítio de saída ribossomal (E), o peptidil-tRNA alcance o sítio P e um novo aminoacil-tRNA contendo o anticódon correspondente localize-se no sítio A. Esse processo é repetido de forma cíclica, dando continuidade a síntese da cadeia polipeptídica, até que um códon de terminação seja encontrado (Kapp & Lorsch, 2004).

1.2.3- Etapa de término da síntese proteica

O término da tradução ocorre em resposta à presença de um códon de terminação no sítio A ribossomal. O resultado final deste processo é a liberação do polipeptídeo após a hidrólise da ligação peptídica no peptidil-tRNA no sítio P. A atividade enzimática de peptidil-transferase do ribossomo é responsável por catalisar essa reação de hidrólise em resposta à atividade dos fatores de liberação (eRF) de classe 1 (eRF1) e de classe 2 (eRF3). O fator eRF1 reconhece codons de terminação apresentados no site A, enquanto o eRF3 é uma GTPase que estimula a terminação da tradução de forma dependente de GTP (Zhouravleva et al., 1995; Frolova et al., 1996; Frolova et al., 2000). Desse modo, assim que um códon de terminação é encontrado, um complexo ternário composto por um eRF1, eRF3 e GTP promove a hidrólise da ligação peptidil-tRNA pelo ribossomo, o que resulta na liberação da proteína recém-sintetizada (Kapp & Lorsch, 2004).

1.2.4 Características básicas da maquinaria de início da tradução eucariótica

1.2.4.1 Os ribossomos

Nos eucariotos, o número de proteínas ribossomais está em torno de 82 (49 proteínas associadas à subunidade 60S e 33 proteínas associadas à 40S) (Lewin, 2001; Nazar, 2004; Henras et al., 2008). Em eucariotos, a subunidade 60S contém três rRNAs distintos (28S, 5,8S e 5S), e a subunidade 40S, apenas um tipo de rRNA (18S) (Leary & Huang, 2001; Faticca & Tollervey, 2002; Granneman & Baserga, 2004; Nazar, 2004; Henras et al., 2008).

1.2.4.2 O RNA transportador

Uma característica no processo de síntese proteica é a utilização do tRNA como ponte de ligação entre os aminoácidos e o mRNA. No sistema eucariótico, a metionina inicial é transportada por um tRNA diferente do utilizado no transporte das demais metioninas da cadeia polipeptídica, denominado Metionil-tRNAi^{Met}, que é aminoacilado por uma aminoacil-sintetase específica. Normalmente, a metionina inicial é removida ao término da síntese da cadeia polipeptídica. A utilização dessas formas de tRNAs especiais para a iniciação se faz necessária para o reconhecimento do códon de iniciação da síntese proteica e para fatores que atuam nesse processo (Kozak, 1983; Hinnebush, 2000; Kolitz & Lorsch, 2010).

1.2.4.3 O RNA mensageiro

Em eucariotos, o mRNA apresenta algumas características particulares, se comparado ao mRNA procariótico, a citar:

A - O pré-mRNA recém-transcrito frequentemente sofre modificações pós-transcricionais para se tornar um mRNA maduro, passível de ser transportado ao citoplasma e ser traduzido (Cramer et al., 2001; Proudfoot et al., 2002; Zorio & Bentley, 2004). As principais modificações bioquímicas para a obtenção de um mRNA maduro são: a) adição de 7-metil-guanosina (m^{7}GTP) à extremidade 5' – denominada de estrutura *cap* –, conectada por uma ligação trifosfato 5'-5' feita pela enzima guanilil-transferase. *Cap* é fundamental para a estabilidade e a eficiência da tradução da mensagem, pois sua presença parece induzir a ligação da subunidade ribossomal 40S ao mRNA; b) a adição de um segmento de poliadenosina de 50 a 150 nucleotídeos, denominado cauda poli (A), às extremidades 3' da maioria dos mRNAs é realizada pós-transcricionalmente pela enzima

poli(A)-polimerase. A cauda de poli (A) está relacionada com estabilidade da mensagem e sua eficiente tradução; c) o *splicing* do mRNA, que consiste na excisão de introns específicos e junção dos exons. A retirada dos introns ocorre após duas reações de transesterificação para a união dos exons e liberação do intron. Esse processo é mediado por um complexo denominado spliceossomo.

B - Os mRNAs eucarióticos utilizam em sua grande maioria o primeiro códon AUG da mensagem para início de síntese proteica. Através de um levantamento de mais de 200 sequências-líder de mRNAs eucarióticos, Kozak (1984) identificou nucleotídeos adjacentes ao códon AUG (A/GXXAUGG). Estes formariam um ambiente favorável para o início da síntese proteica em eucariotos, sem, no entanto, caracterizar uma interação rRNA-mRNA análoga à de procariotos. Nesse contexto, as posições -3 e +4 (sendo +1 o A do AUG) são as mais conservadas, contendo purinas. A alteração de uma dessas posições para pirimidinas reduz significativamente a eficiência de utilização desse códon AUG como sítio de início de tradução. Em levedura, no entanto, essas posições não são críticas (Dasso et al., 1990). O tamanho da região 5' não traduzida do mRNA parece não influenciar na tradução da mensagem, mas a presença de uma extensão mínima entre a extremidade 5' da mensagem e o AUG é necessária para que este seja reconhecido corretamente como ponto inicial da tradução em eucariotos (Kozak, 1991). Também, em eucariotos, com raras exceções, as mensagens são sempre monocistrônicas, codificando, portanto, uma única proteína (Kozak, 1983). Existe também a possibilidade de o ribosomo reconhecer um AUG interno em mRNAs contendo grampos do tipo IRES (*internal ribosome entry site*) e de a tradução ocorrer de maneira independente de *cap* em diferentes circunstâncias fisiológicas (Hellen & Sarnow, 2001; Kozak, 2001; Kozak, 2005).

1.2.4.4 Os fatores de iniciação da tradução em eucariotos

No processo de início da síntese proteica ocorre a associação transitória de fatores proteicos, que medeiam o processo de iniciação da síntese proteica com a subunidade ribossomal 40S, o mRNA e o Metionil-tRNAi^{Met} (Preiz & Hentz, 2003; Gebauer & Hentze, 2004; Kapp & Lorsch, 2004; Sonemberg & Hinnebusch, 2009; Jackson et al., 2010). Tais fatores normalmente são desligados da subunidade ribossomal menor com a chegada da subunidade maior e com o início da etapa de alongamento da tradução. Em eucariotos, até o momento, já foram isolados cerca de treze diferentes fatores de iniciação os quais, em sua maioria, são compostos por várias subunidades e ainda podem apresentar diferentes isoformas (Kapp & Lorsch, 2004; Jackson et al., 2010).

A maioria dos eIFs está envolvida na formação de complexos com os vários componentes da maquinaria traducional, associando-se a estes de maneira transitória. As principais funções dos eIFs no processo de síntese proteica estão descritas na Tabela 1.

Tabela 1. Funções dos diferentes eIFs no processo de síntese proteica.

eIF	Funções na tradução do mRNA	Referências
eIF1	Apresenta papel chave no reconhecimento do códon de início AUG por atuar como um regulador negativo da liberação de fosfato de eIF2 no complexo de pré-iniciação 43S (formado pela subunidade ribossomal 40S, complexo ternário, eIF3, eIF1, eIF1A e eIF5). Promove a montagem e estabilização do complexo de pré-iniciação 43S.	Pestova et al., 1998; Algire et al., 2005; Maag et al., 2005; Cheung et al., 2007; Passmore et al., 2007; Nanda et al., 2009; Maduzia et al., 2010
eIF1A	Modula a dissociação de eIF1 do complexo de pré-iniciação 43S. É importante no processo de varredura do mRNA pelo ribossomo e na determinação do códon de início AUG.	Pestova et al., 1998; Maag & Lorsch, 2003; Cheung et al., 2007; Fekete et al., 2007; Passmore et al., 2007; Mitchell & Lorsch, 2008
eIF2	É formado por três subunidades, denominadas eIF2α, eIF2β e eIF2γ, que permanecem associadas durante todo o ciclo. Este fator forma o CT de inicio de síntese proteica juntamente com Metionil-tRNAi^{Met} e uma molécula de GTP (eIF2/GTP/ Metionil-tRNAi^{Met}), e tem a função de promover a ligação de Metionil-tRNAi^{Met} à partícula 40S do ribossomo. Para haver a ligação da subunidade ribossomal 40S ao mRNA, é necessário que esta se encontre associada ao complexo ternário.	Kozak, 1989; Kimball, 1999; Sonemberg & Dever, 2003; Gebauer & Hentze, 2004; Sonemberg & Hinnebusch, 2009; Jackson et al., 2010
eIF2B	É responsável pela troca de GDP por GTP ligado a eIF2 após cada ciclo de iniciação. A afinidade de eIF2 por GDP é 100 vezes maior que por GTP e a troca de GDP por GTP não ocorre espontaneamente, tornando necessária a atividade desse fator para a reciclagem de	Merrick, 1992; Yang & Hinnebusch, 1996; Kimball et al., 1998; Pavitt et al., 1998; Asano et al., 1999;

eIF	Funções na tradução do mRNA	Referências
	eIF2. Composto por cinco proteínas, o fator elF2B é formado por subunidades regulatórias alfa, beta e sigma e pelas subunidades catalíticas lambda e epslon. As subunidades sigma e epslon ligam-se à subunidade beta de eIF2.	Sonemberg & Dever, 2003; Gebauer & Hentze, 2004; Jackson et al., 2010
eIF3	É o mais complexo dos fatores, pois contém diferentes subunidades e participa de múltiplas etapas do início da tradução. Liga-se à subunidade 40S do ribossomo, auxiliando a dissociação das sbunidades ribossomais. Estimula a ligação do complexo ternário eIF2/GTP/Met-tRNAi à subunidade 40S. A ligação do mRNA ao complexo de pré-iniciação 43S também é estimulada por eIF3. O fator eIF3 humano contém treze subunidades nomeadas de eIF3a até eIF3m. Cinco destas, denominadas eIF3a (p170), eIF3b (p116), eIF3c (p110), eiF3g (p44), eIF3i (p36), são ortólogas às cinco subunidades de levedura TIF32, N1P1, PRT1, TIF34 e TIF35, respectivamente.	Asano et al., 1997; Asano et al., 1998; Phan et al., 1998; Hershey & Merrick, 2000; Hinnebuch, 2000; Browning et al., 2001; Phan et al., 2001; Preiss & Hentze, 2003; Unbehaun et al., 2004; Hinnebusch, 2006; Damoc et al., 2007; Zhou et al., 2008; Sonemberg & Hinnebusch, 2009; Jackson et al., 2010
eIF4A	Tem como função desfazer as estruturas secundárias presentes no mRNA, funcionando possivelmente como uma RNA helicase. A caracterização bioquímica desse fator demonstrou atividade de desenrolar RNA dupla fita na presença de ATP. Possui sítio de ligação a ATP, um motivo rico em arginina para ligação a RNA e contém o motivo D-E-A-D característico de DNA helicases. Há três isoformas em mamíferos, eIF4AI, eIF4AII e eIF4AIII, sendo que eIF4AII de humanos é altamente homóloga a eIF4AI (89% de identidade), sendo funcionalmente equivalente, mas sua expressão é tecido-específica.	Trachsel, 1988; Yoder-Hill et al., 1993; Pause et al., 1994; Nielsen & Gingras et al., 1999; Li et al., 1999; Li et al., 2001; Preiss & Hentze, 2003; Gebauer & Hentze, 2004; Sonemberg & Hinnebusch, 2009; Jackson et al., 2010

eIF	Funções na tradução do mRNA	Referências
	O eIF4AIII interage com eIF4G e requer eIF4B para sua eficiente atividade de desdobramento do mRNA.	
eIF4B	É um estimulador da atividade de eIF4A. A proteína humana contém um motivo de reconhecimento de RNA (RRM) na extremidade amino, uma região central rica em asparagina, arginina, tirosina e glicina, e uma sequência adjacente rica em arginina, importante para a ligação ao RNA e para a atividade de estimular eIF4A. O RRM pode estar relacionado com a interação aos ribossomos.	Methot et al., 1994; Methot et al., 1996; Preiss & Hentze, 2003; Sonemberg & Hinnebusch, 2009; Jackson et al., 2010
eIF4E	A principal função desse fator é ligar-se à estrutura *cap* no mRNA. A interação está relacionada com resíduos de triptofano conservados. O eIF4E é um alvo regulatório no processo de início da tradução e atua na proliferação e sobrevivência celular, além de transformação maligna. A atividade de eIF4E é regulada por uma família de supressores traducionais denominados de proteínas de ligação a 4E (4E-BPs) que em mamíferos consistem de três membros: 4E-BP1, 4E-BP2 e 4E-BP3. O estado de fosforilação de 4E-BPs controla a ligação de 4E-BPs a eIF4E de modo que, formas hiperfosforiladas de 4E-BPs ligam-se a eIF4E e previnem a interação desse com eIF4G, inibindo a tradução dependente de *cap*.	Altmann & Trachsel, 1989; Pause et al., 1994; Matsuo et al., 1997; Gingras et al., 1998; Gingras et al., 1999a; Gingras et al., 1999b; Preiss & Hentze, 2003; Preiss & Hentze, 2003; Gebauer & Hentze, 2004; Mamane et al., 2004; Graff et al., 2007; Sonemberg & Hinnebusch, 2009; Jackson et al., 2010
eIF4G	Em mamíferos, apresenta duas isoformas, eIF4GI e eIF4GII, que possuem 46% similaridade. Esse fator possui sítios de ligação a eIF4E, eIF4A, eIF3 e a proteína de ligação a poli (A) (PABP). Evidências revelam que eIF4G tem como função trazer os fatores necessários para desenrolar a estrutura secundária do	Lamphear et al., 1995; Gradi et al., 1998; Wells et al., 1998; Gingras et al., 1999; Preiss & Hentze, 2003;

eIF	Funções na tradução do mRNA	Referências
	mRNA junto ao *cap* na orientação correta. Sua porção aminoterminal contém sítio de ligação a eIF4E e sua porção carboxiterminal liga-se a ribossomos, provavelmente por interação com eIF3 e também apresenta afinidade por eIF4A. Funciona como uma plataforma proteica que tem como principais objetivos o posicionamento dos demais fatores e a circularização do mRNA.	Gebauer & Hentze, 2004; Sonemberg & Hinnebusch, 2009; Jackson et al., 2010
eIF4F	É formado por eIF4A, eIF4B, eIF4E e eIF4G e interage com o *cap* e com o eIF3 associado ao ribossomo através de eIF4G. Desse modo, esse complexo executa a função pivô, intermediando a ligação entre ribossomo e o mRNA.	Gingras et al., 1999; Preiss & Hentze, 2003; Gebauer & Hentze, 2004; Sonemberg & Hinnebusch, 2009; Jackson et al., 2010
eIF5	É uma proteína com atividade GTPásica ribossomo-dependente e tem como função hidrolisar GTP ligado a eIF2, permitindo a junção das subunidades ribossomais no códon de início AUG. A ligação de eIF5 ao eIF2 ocorre entre a região carboxiterminal de eIF5 e a subunidade beta de eIF2.	Chakravarti et al., 1993, Chevesich et al., 1993; Das et al., 1997; Das & Maitra, 2000; Das & Maitra, 2001; Sonemberg & Dever, 2003; Singh et al., 2004; Jackson et al., 2010
eIF5A	É a única proteína na natureza que contém um resíduo de hipusina, um aminoácido incomum modificado pós-traducionalmente pela ação das enzimas deoxihipusina-sintetase e deoxihipusina-hidroxilase. eIF5A interage fisicamente com os componentes da maquinaria de tradução, como componentes estruturais dos	Moldave, 1985; Hershey et al., 1990; Park et al., 1993; Park et al., 1997; Chen & Liu, 1997; Jao & Chen, 2006;

eIF	Funções na tradução do mRNA	Referências
	ribossomos e fatores de alongamento, sugerindo que eIF5A se liga especificamente a ribossomos ativos. Além disso, essa ligação a ribossomos ativamente traduzindo parece ser dependente de hipusina. Sugere-se que eIF5A estimule a formação da primeira ligação peptídica, sendo considerado uma proteína que faz a conecção entre a fase de início da tradução e a de alongamento da cadeia polipeptídica.	Park, 2006; Zanelli et al., 2006; Zanelli & Valentini, 2007
eIF5B	É conservado em eucariotos e é um homológo de IF2 de eubactérias. Apresenta sua região aminoterminal variável e suas porções central (de ligação a GTP) e carboxiterminal conservadas. A homologia funcional também ocorre entre eIF5B e IF2 uma vez que ambos empregam mecanismos similares para promover a iniciação da tradução do mRNA e apresentam uma atividade GTPase ribossomo-dependente. eIF5B tem como função realizar a hidrólise de GTP livre, proporcionando a dissociação subsequente de eIF1, eIF1A e eIF3 da subunidade ribossomal 40S para permitir a junção das subunidades ribossomais.	Kolakofsky et al., 1968; Lee et al., 1999; Pestova et al., 2000; Pestova & Hellen, 2000; Dever et al., 2001; Roll-Mecak et al., 2001; Lee et al., 2002; Antoun et al., 2003; Unbehaun et al., 2004; Preiss & Hentze, 2003; Pisarev et al., 2006; Sonemberg & Hinnebusch, 2009; Jackson et al., 2010
eIF6	Sua sequência primária codifica uma proteína de 245 aminoácidos e não apresenta homólogos em eubactérias. A proteína eIF6 é extremamente conservada e é quase idêntica de espécies de arqueobactérias até seres humanos. Sua sequência apresenta identidade de 77% entre fungos e humanos. O	Russell & Spremulli, 1979; Valenzuela et al., 1982; Si et al., 1997; Sanvito et al., 1999; Si & Maitra, 1999; Miluzio et al., 2009; Jackson et al., 2010

eIF	Funções na tradução do mRNA	Referências
	eIF6 regula os processos de biogênese ribossomal e início da tradução. No processo de início da tradução, possui função antiassociativa, de modo que se liga à subunidade 60S e previne a sua associação com a subunidade 40S.	

1.2.5 O processo de início de síntese proteica em eucariotos

O início da síntese proteica, em eucariotos, é um processo complexo que envolve a ligação de Metionil-tRNAi^{Met} à subunidade 40S do ribossomo e a subsequente associação ao mRNA. Esse processo é auxiliado por uma série de fatores proteicos e é a fase reguladora do mecanismo global de síntese proteica. O início da tradução pode ser dividido em quatro etapas: formação do complexo ternário; formação do complexo de pré-iniciação 43S; associação ao mRNA; e formação do complexo de iniciação 48S. Maiores detalhes poderão ser encontrados nas seguintes revisões: Merrick, 1990; Hershey, 1991; Merrick, 1992; Altmann & Trachsel, 1993; Merrick, 1994; Gray & Wickens, 1998; Preiss & Hentze, 2003; Gebauer & Hentze, 2004; Kapp & Lorsch, 2004; Sonenberg & Hinnebusch, 2009; Jackson et al., 2010).

Conforme ilustrado na Figura 1, o processo de síntese proteica inicia com a formação do complexo ternário, onde ocorre a ligação de eIF2 ao Metionil-tRNAi^{Met} na presença de GTP (quadro I). O complexo ternário liga-se à partícula 40S associada ao fator eIF3 (Gebauer & Hentze, 2004). Assim é formado o complexo de pré-iniciação 43S, que é composto, então, pelo complexo ternário ligado à partícula ribossomal 40S associada aos fatores eIF3, eIF1, eIF1A e eIF5. O complexo 43S reconhece o mRNA ligado ao complexo

eIF4F (eIF4A, eIF4B, eIF4E e eIF4G) através da ligação de eIF3 com eIF4G. Dessa forma, eIF4G atua como uma plataforma proteica que permite a interação entre eIF4E, eIF4A, eIF3 e proteína de ligação à poli (A) (PABP), causando a circularização do mRNA (Wells et al., 1998). O eIF4E liga-se diretamente à estrutura *cap* 5'e o eIF4A promove o desdobramento de estruturas secundárias no mRNA por sua atividade de helicase. Esta ação de eIF4A é assistida pela proteína de ligação a RNA, eIF4B (quadro II). Com isto, a extremidade 5' do mRNA adquire a conformação ideal e eIF4F, em conjunto com eIF3 e PABP ligado à cauda poli (A), posiciona o mRNA no complexo 43S, formando o complexo 48S. O complexo 43S migra sobre o mRNA no sentido 5' → 3' em um processo ativo que requer hidrólise de ATP, até encontrar o códon de iniciação AUG em um contexto favorável definido por Kozak (1989) (quadro III).

Os eIF1 e eIF1A desempenham um papel neste processo de varredura e, como eIF2, atuam também na detecção do correto códon de início (Nanda et al., 2009). Ao atingir o códon AUG, uma pequena pausa é necessária para a ligação da subunidade 60S do ribossomo. Nesse intervalo, o fator eIF5 promove a hidrólise de GTP ligado ao eIF2 e a liberação de eIF2-GDP, bem como dos fatores eIF1, eIF3 e eIF5, deixando o Metionil-tRNAi^{Met} no sítio P da subunidade ribossomal 40S. A união da subunidade ribossomal 60S ao complexo 40S-Metionil-tRNAi^{Met}-mRNA é facilitado pela dissociação desses outros fatores (Gebauer & Hentze, 2004; Kapp & Lorsch, 2004; Sonenberg & Hinnebusch, 2009; Jackson et al., 2010). Este evento dispara uma segunda etapa de hidrólise do GTP em eIF5B estimulada pelo ribossomo 60S. Como consequência, eIF5B ligado a GDP (eIF5B-GDP) dissocia-se do complexo ribossomal propiciando o acoplamento perfeito da subunidade 60S, formando assim o complexo ribossomal 80S (Pestova et al., 2000) (quadro IV).

O complexo eIF2-GDP, que resulta da finalização de cada ciclo do início da tradução, deve ser reciclado para sua forma ativa ligada a GTP, eIF2-GTP, permitindo que eIF2 participe de um novo ciclo de iniciação. Esta reação de troca de GDP por GTP em eIF2 é catalisada pelo fator eIF2B (Hinnebuch, 2000; Sonenberg & Hinnebusch, 2009; Jackson et al., 2010).

O processo de tradução (quadro V) continua com a fase de alongamento da cadeia polipeptídica. A inserção do aminoacil-tRNA no sítio A ribossomal ocorre pela atuação do fator eEF1-GTP. Após a entrada do complexo aminoacil-tRNA/eEF1-GTP no síto A do ribossomo, a hidrólise do GTP em eEF1 causa sua liberação. A ligação peptídica ocorre por estímulo de eIF5A e atuação da enzima peptidil-transferase. O processo de alongamento é então prosseguido (Preiss & Hentze, 2003; Kapp & Lorsch, 2004).

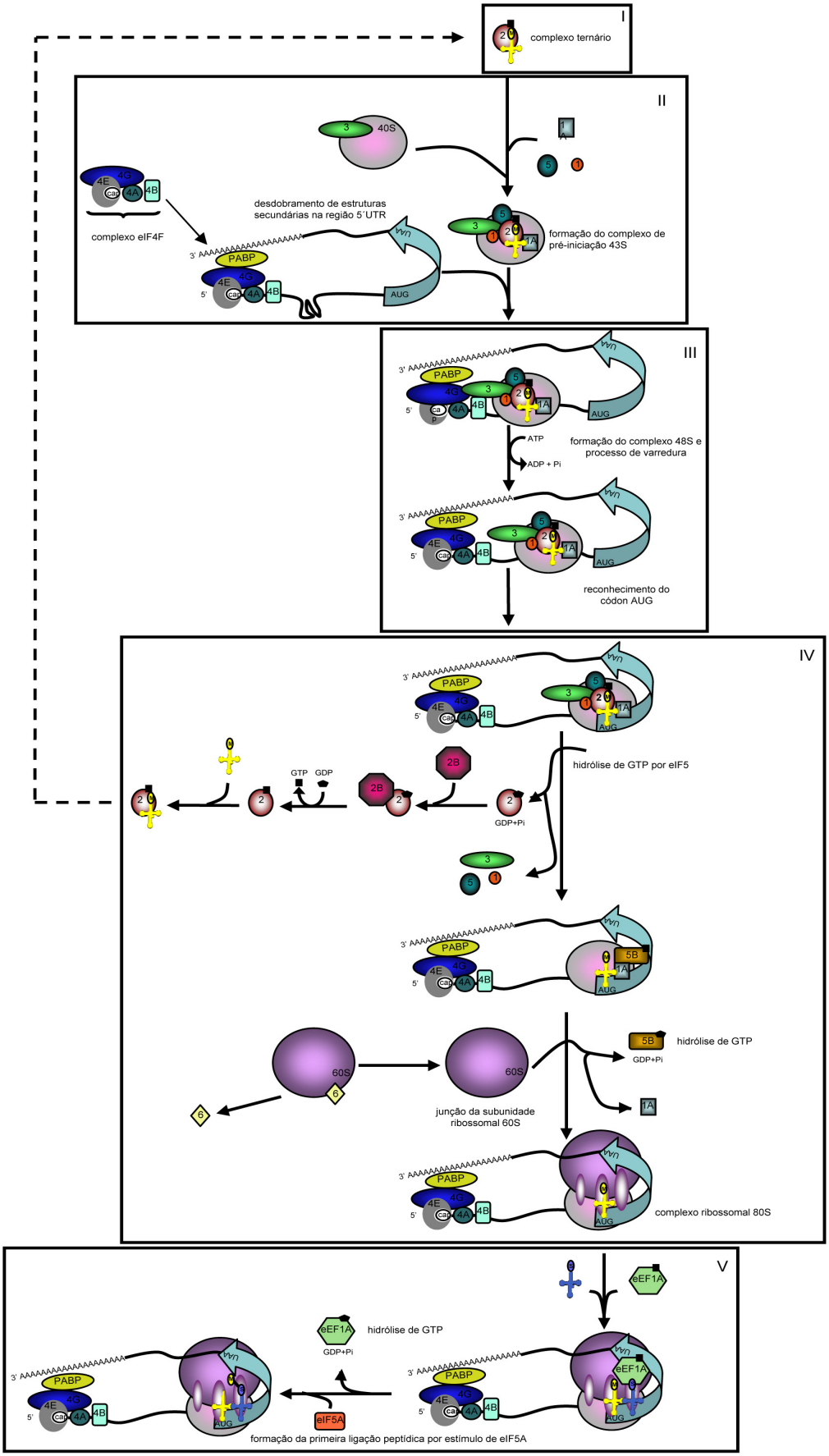

I
complexo ternário
II
40S
desdobramento de estruturas secundárias na região 5´UTR
complexo eIF4F
formação do complexo de pré-iniciação 43S
PABP
AUG
III
formação do complexo 48S e processo de varredura
ATP
ADP + Pi
reconhecimento do códon AUG
IV
hidrólise de GTP por eIF5
GTP
GDP
GDP+Pi
60S
junção da subunidade ribossomal 60S
hidrólise de GTP
complexo ribossomal 80S
V
eEF1A
hidrólise de GTP
eIF5A
formação da primeira ligação peptídica por estímulo de eIF5A

Figura 1. Processo de início de tradução. Os diferentes passos do processo de início de tradução estão representados nas cinco divisões da figura. I - formação do complexo ternário. II – formação do complexo de pré-iniciação 43S. III – formação do complexo 48S, processo de varredura do mRNA e reconhecimento do códon de início AUG. IV – hidrólise de GTP por eIF5 e eIF5B; formação do complexo ribossomal 80S. V – formação da primeira ligação peptídica. Fatores de início de tradução (eIFs), subunidades ribossomais e tRNA são representados como formas coloridas. eIFs são retratados por sua porção numérica seguida pela letra da sua subunidade, quando necessário. As subunidades ribossomais são retratadas como 40S e 60S. tRNAs são ilustrados como formas de cruz. A estrutura *cap* é nomeada cap. Adaptado de Salton et al. (Anexo 1)

1.2.6 A regulação global da síntese proteica em eucariotos

A forma mais eficaz de controle global da síntese de proteínas, em eucariotos, encontra-se na regulação da atividade ou na quantidade dos complexos envolvidos no processo de início de tradução. A atividade desses complexos é modulada, principalmente, pela fosforilação e desfosforilação de vários sítios-alvo presentes em muitos dos fatores de iniciação (Merrick, 1992).

A disponibilidade de eIF2-GTP e, portanto, de complexo ternário é determinada pela atividade de eIF2B. Quando eIF2 está fosforilado na serina 51 da subunidade alfa, há um aumento da afinidade entre eIF2-GDP e eIF2B. Isso causa o sequestro de eIF2B e com isso a troca de GDP por GTP não ocorre (Rowlands et al., 1988), o que acarreta uma redução de complexos ternários e, consequentemente, uma queda global na frequência de iniciação (Dever, 2002; Sonenberg & Dever, 2003; Gebauer & Hentze, 2004; Hershey, 2010; Kimball & Jefferson, 2010).

Outra forma de regulação global da síntese proteica reside na disponibilidade de eIF4E. Esse fator precisa associar-se à proteína eIF4G, para, assim, possibilitar a ligação do complexo de pré-iniciação 43S ao mRNA. Contudo, há uma família de proteínas, as 4E-

BPs, que podem competir com eIF4G pela ligação a eIF4E. Nesse caso, é o estado de fosforilação dessas proteínas que vai determinar a sua afinidade por eIF4E. Quando hipofosforiladas, as 4E-BPs sequestram eIF4E, mas no estado fosforilado perdem afinidade pelo mesmo, que fica livre para associar-se com eIF4G (Sonenberg & Dever, 2003; Gebauer & Hentze, 2004). A fosforilação das proteínas 4E-BPs pode ser regulada pela atividade da cinase proteína alvo de rapamicina de mamíferos (*mammalian target of rapamycin*) (mTOR), de modo que ao fosforilar 4E-BPs, a mTOR estimula a síntese proteica e, conseqüentemente, o crescimento celular (Gingras et al., 2001; Dever, 2002; Gingras et al., 2004; Kimball & Jefferson, 2010).

Existem também outras formas de controle traducional para regular a expressão de diferentes genes. A descrição desses mecanismos pode ser encontrada nas seguintes revisões (Gebauer & Hentze, 2004; Stoneley & Willis, 2004; Kozak, 2005; Valencia-Sanchez et al., 2006; Sonenberg & Hinnebusch, 2009; Fabian et al., 2010; Hershey, 2010).

1.3 TRADUÇÃO NUCLEAR

1.3.1 Modelo de tradução nuclear

A tradução nuclear permanece uma questão controversa, sendo relatados, no entanto, vários indícios de sua ocorrência. Muitos estudos sobre o assunto demonstram que um ou poucos ciclos de tradução conhecidos como *pionner round* (rodada inicial) da tradução do mRNA devem ocorrer (Ishigaki et al., 2001; Chiu et al., 2004). Esse processo deve ocorrer em mRNAs recém-sintetizados, provavelmente como um mecanismo de checagem da qualidade desses RNA quanto a presença de codons de terminação prematuros (PTCs). Esse mecanismo de rodada inicial da tradução pode, se necessário,

induzir a maquinaria de degradação de mRNAs contendo PTCs através do processo denominado *nonsense mRNA decay* (NMD) (Fortes et al., 2000; Ishigaki et al., 2001; Baker & Parker, 2004; Maquat, 2004; Chang et al., 2007). Os PTCs podem ser originados por erros de transcrição ou *splicing*, bem como por mutações sem sentido (Maquat & Carmichael, 2001). A tradução destes mRNAs pode produzir proteínas truncadas com atividades dominante-negativas, ou ainda, que possam apresentar ganho de função deletéria. O NMD pode reduzir os níveis de mRNAs carregando PTCs em 5% a 30%, tanto nas frações associadas ao núcleo, quanto nas frações citoplasmáticas de células eucarióticas (Cheng & Maquat, 1993; Maquat, 1995; Frischmeyer & Dietz, 1999; Dahlberg et al., 2003). Desta forma, a rodada inicial da tradução e o NMD devem estar relacionados como um mecanismo de vigilância contra mRNAs contendo PTCs. Diferentes modelos de localização subcelular para a ocorrência do processo de rodada inicial da tradução e NMD indicam sua ocorrência no citoplasma (Moriarty et al., 1998; Dreumont et al., 2004; Maquat, 2004; Sato et al., 2008; Sato & Maquat, 2009, Maquat et al., 2010; Muhlemann & Lykke-Andersen, 2010) e associado ao núcleo (Frischmeyer & Dietz, 1999; Ishigaki et al., 2001; Maquat, 2002; Wagner & Lykke-Andersen, 2002; Wilkinson & Shyu, 2002; Dahlberg & Lund, 2004; Maquat, 2004; Sato et al., 2008).

Um modelo geral para a rodada inicial da tradução e NMD nucleares tem sido proposto. Nesse modelo, no núcleo, a região 5' UTR do mRNA é ligado ao complexo de ligação a *Cap* (CBC) composto pelas subunidades CBP80 e CBP20, que é adicionado durante a transcrição (Lewis & Izaurralde, 1997; Ishigaki et al., 2001). Os fatores eIF3e e eIF4G ligam-se ao mRNA (Chang et al., 2007; Maquat et al., 2010). O ribossomo realiza a varredura do mRNA ligado a CBC e remodela os Complexos de Junção de Exons (EJC) composto por diferentes proteínas (Dostie & Dreyfuss, 2002; Lejeune et al., 2002) e

incluindo o fator eIF4AIII (Chang et al., 2007; Maquat et al., 2010). Durante a tradução de mRNAs sem PTCs, o EJC é remodelado, CBC é trocado por eIF4E e o mRNA é exportado ao citoplasma para o início da síntese proteica propriamente dita. No entanto, se o ribossomo encontra um PTC no mRNA que ainda tem um EJC ligado, ele faz uma pausa para que proteínas *up-frameshit* (UPF) sejam recrutadas para o mRNA, iniciando a destruição do mRNA pelo complexo NMD (Page et al., 1999; Kim et al., 2001; Lykke-Andersen et al., 2001; Wagner & Lykke-Andersen, 2002; Wilkinson & Shyu, 2002; Dahlberg & Lund, 2004; Maquat, 2004; Sato et al., 2008) no qual também participam os fatores eIF4AIII atuando na formação do complexo EJC (Ferraiuolo et al., 2004; Shibuya et al., 2006; Chang et al., 2007; Maquat et al., 2010) e eIF4G atuando como uma proteína *scaffold* (Chang et al., 2007; Maquat et al., 2010). e participando do processamento do mRNA por sua ligação a componentes do spliceossomo (Figura 2).

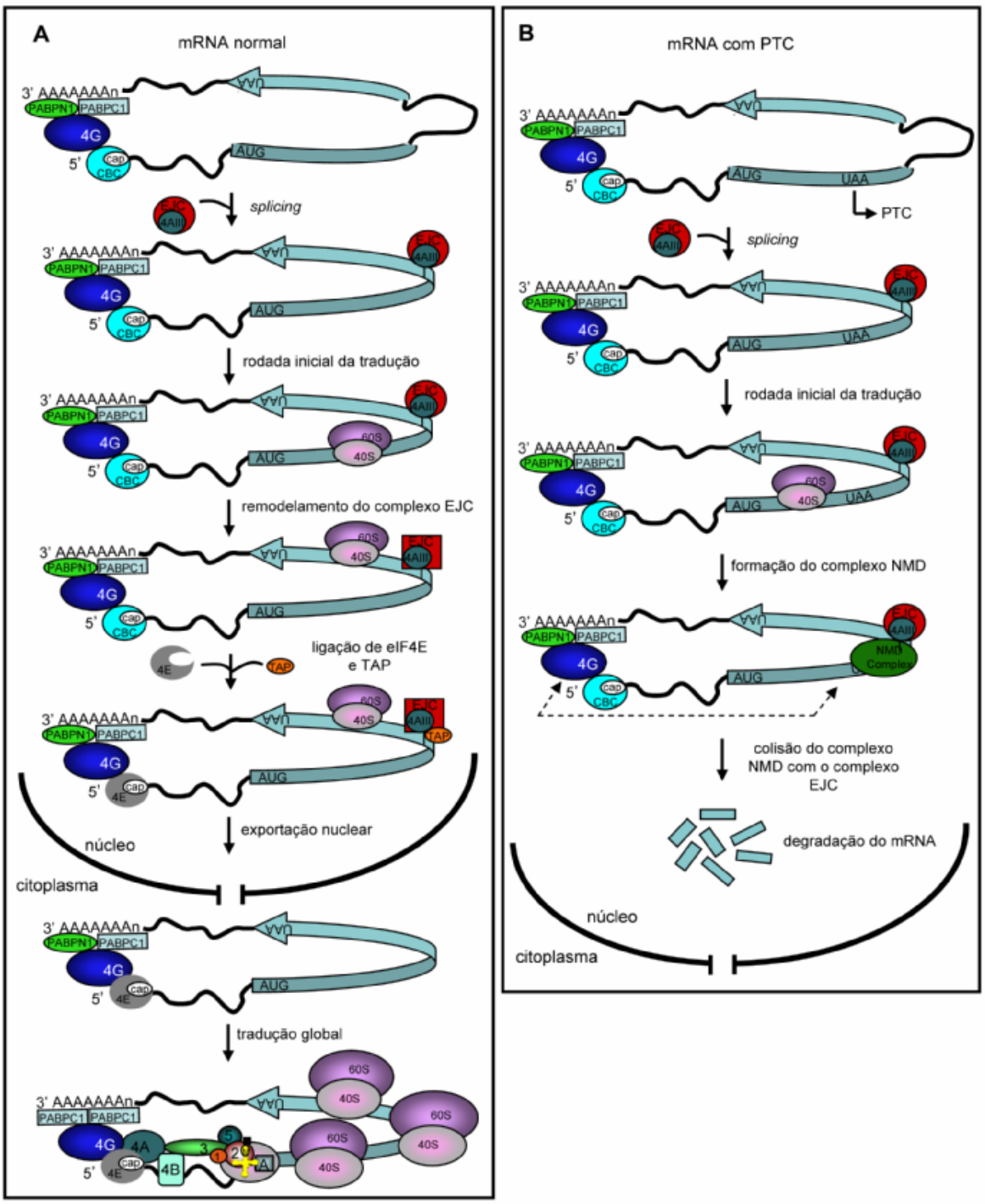


Figura 2. Modelo geral de rodada inicial nuclear da tradução/*nonsense-mediated mRNA decay*. O complexo de ligação a *cap* (CBC) liga-se à região terminal 5`do mRNA durante a transcrição. O fator eIF4G liga-se ao mRNA e eIF4AIII compõe o complexo de junções de exons (EJC). (**A**) O códon de terminação normal está geralmente no exon 3' do mRNA e codons de terminação prematuros (PTC) em exons internos. O ribossomo varre o mRNA ligado a CBC e remove complexos EJC. Durante a tradução do mRNA sem PTC, o EJC é remodelado e CBC é trocado por eIF4E. O mRNA é então exportado ao citoplasma com o auxílio de TAP. (**B**) Se o ribossomo encontra um PTC no mRNA que ainda está ligado a EJC, ele para e diversas proteínas adicionais são recrutadas para o mRNA e

iniciam a degradação do mRNA pelo processo de NMD (*nonsense-mediated mRNA decay*). Os fatores eIF4G and eIF4AIII parecem participar do processo de NMD. 60S – subunidade ribossomal 60S, 40S – subunidade ribossomal 40S, PABPN1 - proteína nuclear 1 de ligação a poli (A), PABPC1 - proteína citoplasmática 1 de ligação a poli (A), 4E – eIF4E, 4G –eIF4G, 4AIII - eIF4AIII, 4A – eIF4A, 4B – eIF4B, 2 – eIF2, 3 – eIF3, 1 – eIF1, 1A – eIF1A, 5 – eIF5, cap – estrutura cap 5', TAP – mediador da exportação nuclear do mRNA. Adaptado de Salton et al. (Anexo 1).

1.3.2 Localização e funções de eIFs no núcleo

Vários estudos têm demonstrado que alguns eIFs localizam-se no núcleo de células eucarióticas, incluindo leveduras, plantas e mamíferos. Além disso, alguns trabalhos também relatam a localização nucleolar de eIFs. A maioria das funções já evidenciadas de eIFs no núcleo estão relacionadas a mecanismos que envolvem o processamento, a exportação de mRNA e rRNA e o processo de NMD. Desse modo, o papel nuclear de eIFs poderia estar associado ao modelo de rodada inicial da tradução/NMD, sugerindo a participação desses fatores em um processo de tradução nuclear. A localização e possíveis funções desses fatores nos compartimentos nuclear e nucleolar estão resumidas na Tabela 2.

Tabela 2. Localização e funções nucleares de eIFs.

eIF	Localização nuclear	Funções nucleares	Metodologia	Referências
eIF1	- nucleoplasmático	- ND	- IF	Bohnsack et al., 2002
eIF1A	- nucleoplasmático - nucleolar	- ND	- IF	Mingot et al., 2004
eIF2α	- associado com a membrana nuclear - nucleoplasmático - nucleolar - em torno do nucléolo - associado a fitas de heterocromatina - ao longo de filamentos nucleares	- apoptose - possível função em reparo de DNA	- ICC - IHC -EM -IF -FC/WB	DeGracia et al., 1997; Lobo et al., 1997; Ting et al., 1998; Goldstein et al., 1999; Lobo et al., 2000; Iborra et al., 2001; Andersen et al., 2002; Leung et al., 2006; Tejada et al., 2009
eIF2β	- nuclear - nucleolar	- possível função em reparo de DNA	- ICC - FC/WB - IF - EM	Ting et al., 1998; Andersen et al., 2002; Bohnsack et al., 2002; Mingot et al., 2004; Leung et al., 2006; Llorens et al., 2006
eIF2γ	- nucleolar	- possível função em reparo de DNA	- EM	Ting et al., 1998; Andersen et al., 2002; Leung et al., 2006
eIF3a	- nuclear	- ND	- IF - FC/WB	Chudinova et al., 2004
eIF3b	- nuclear	- ND	- FC/WB	Morris et al., 2007; Shi et al., 2009

eIF	Localização nuclear	Funções nucleares	Metodologia	Referências
eIF3c	- nuclear	- ND	- FC/WB	Shi et al., 2009
eIF3d	- nuclear	- ND	- FC/WB	Shi et al., 2009
eIF3e	- corpos nucleares - colocalização com corpos nucleares PML	- degradação de proteínas - tráfego nuclear	- IF	Desbois et al., 1996; Morris-Desbois et al., 1999; Guo & Sen, 2000; Yen et al., 2003; Watkins & Norbury, 2004; Zhang et al., 2007; Sha et al., 2009
eIF3f	- nuclear	- degradação ribossomal - apoptose	- IF - FC/WB	Shi et al., 2003; Zhang et al., 2007; Shi et al., 2009
eIF3i	- lado interno do envelope nuclear	- resposta a choque térmico	- IF	Dunand-Sauthier et al., 2002
eIF3k	- nuclear	- apoptose	- IF	Shen et al., 2004; Zhang et al., 2007
eIF3l	- nucléolo	- cofator da RNA polimerase I	- FC/WB	Seither et al., 2001; Yuan et al., 2002
eIF4AIII	- nucleoplasmático - nucleolar - regiões de excisão de introns	- complexo EJC - *splicing* de mRNA - exportação nuclear de mRNA - NMD - resposta a diversas condições de estresse	- EM - IF - ICC	Holzmann et al., 2000; Andersen et al., 2002; Jurica et al., 2002; Jurica & Moore, 2003; Chan et al., 2004; Ferraiuolo et al., 2004;

eIF	Localização nuclear	Funções nucleares	Metodologia	Referências
				Pendle et al., 2005; Leung et al., 2006; Koroleva et al., 2009a; Koroleva et al., 2009b
eIF4E	- regiões de excisão de introns - corpos nucleares PML	- exportação nuclear de mRNA - s*plicing* de mRNA - resposta a privação de soro	- IF - FC/WB - IHC	Lejbkowicz et al., 1992; Lang et al., 1994; Rousseau et al., 1996; Dostie et al., 2000b; Lai & Borden, 2000; Cohen et al., 2001; Iborra et al., 2001; Strudwick & Borden, 2002; Rong et al., 2008; Tejada et al., 2009
eIF4G	- nucleoplasmático - focos nucleares	- processamento de mRNA - NMD - resposta a choque térmico	- FC/WB - IF	Iborra et al., 2001; McKendrick et al., 2001; Coldwell et al., 2004; Kafasla et al., 2009; Ma et al., 2009
eIF5A	- nucleoplasmático - nucleolar - associados a NPC	- transporte nucleo-citoplasmático de mRNA - apoptose	- EM - IF	Rosorius et al., 1999; Lipowsky et al., 2000; Andersen et al., 2002; Jao & Yu Chen, 2002; Jin et al., 2003; Li et al., 2004; Leung et al., 2006; Parreiras et al., 2007; Taylor et al., 2007; Lebska et al., 2009

eIF	Localização nuclear	Funções nucleares	Metodologia	Referências
eIF5B	- colocaliza com a RNA polimerase II - nucelolar	- associado com sítios ativos de tanscrição	- IF	Brogna et al., 2002
eIF6	- nucleolar	- biogênese ribossomal - processamento de rRNA	- EM - IF - FC/WB	Andersen et al., 2002; Basu et al., 2003; Lam et al., 2005; Leung et al., 2006; Gandin et al., 2008; Henras et al., 2008; Biswas et al., 2010

IF - imunofluorescência, ICC - imunocitoquímica, IHC - imunohistoquímica, WB - *western blot*, FC - fracionamento celular, EM - espectrometria de massa, NPC - complexo de poros nucleares, PML – Proteína de Leucemia Promielocítica, ND – não determinado (falta de indícios de possíveis funções nucleares).

1.3.3 Transporte núcleo-citoplasmático

A divisão espacial da replicação e transcrição do DNA no núcleo e a síntese proteica no citoplasma de células eucarióticas tornam necessário o transporte seletivo e regulado de RNAs e proteínas entre esses dois compartimentos (Moroianu, 1999; Komeili & O'Shea, 2001). Tal como outras macromoléculas, a maior parte dos eIFs são translocados ao núcleo e exportados do mesmo por um processo de transporte ativo caracterizado a seguir.

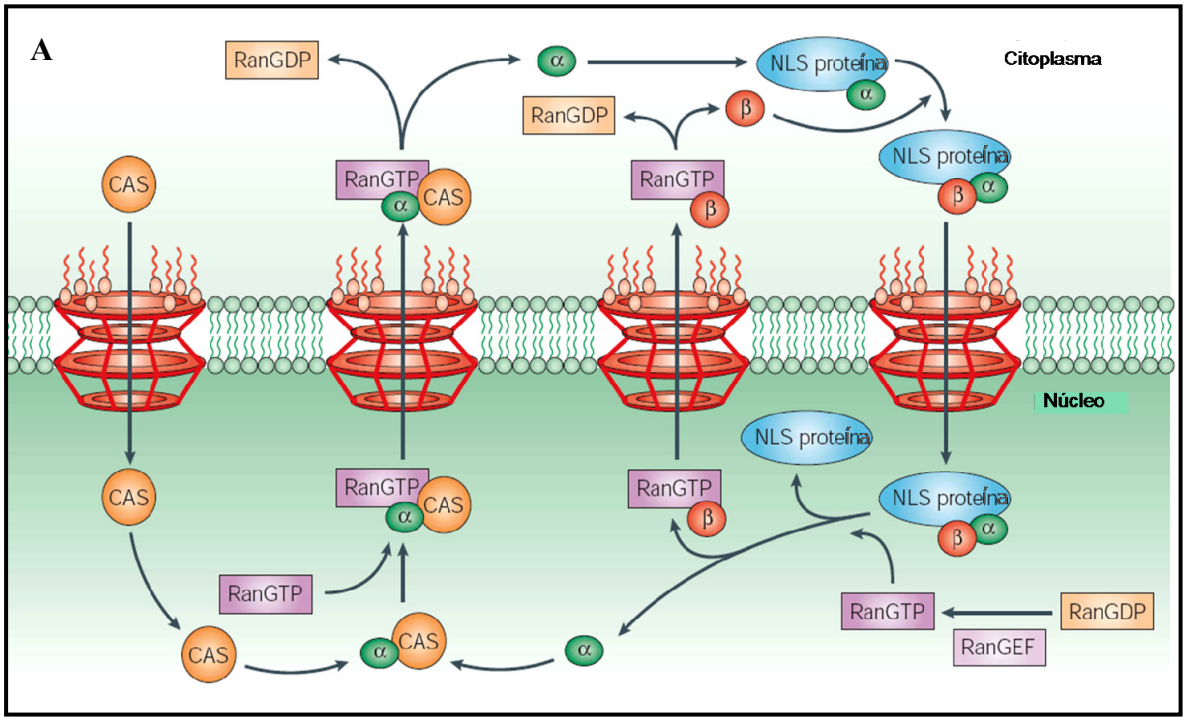
O transporte de moléculas entre o citoplasma e o núcleo ocorre através do envelope nuclear pelos complexos de poros nucleares (NPC), uma estrutura proteica grande de aproximadamente 125 megadaltons (MDa), que capacita a passagem de uma variedade de complexos de até 120 kDa, como é o caso da subunidade maior ribossomal (Reichelt et al.,

1990; Kau & Silver, 2003). Moléculas de até 60 kDa podem difundir-se passivamente pelos NPC, entretanto a maioria das macromoléculas trafega de maneira controlada e sofre um transporte ativo de importação e exportação do núcleo (Ohno et al., 1998). As macromoléculas a serem importadas ao ou exportadas do núcleo contém ou um sinal de localização nuclear (NLS) ou de exclusão nuclear (NES), respectivamente, os quais são reconhecidos por um receptor solúvel e transportados pelo NPC. Os receptores que reconhecem e ligam-se aos NLS e carregam as proteínas do citoplasma ao núcleo através do NPC são denominados de importinas e os que realizam o transporte do núcleo ao citoplasma por ligação ao NES são chamados de exportinas, sendo, entretanto, ambos conhecidos coletivamente como carioferinas (Macara, 2001; Fried & Kutay, 2003; Pemberton & Paschal, 2005; Sorokin et al., 2007).

A interação do receptor com a proteína a ser carregada é regulada por uma pequena RanGTPase que controla o transporte direcional de maneira dependente do estado de seu nucleotídeo (GTP ou GDP). Dessa forma, RanGTP concentra-se no núcleo como consequência da localização do fator de troca de RanGTP (RanGEF), enquanto RanGDP é predominantemente citoplasmática devido à ação da proteína ativadora de RanGTPase (RanGAP) (Kau & Silver, 2003; Kau et al., 2004). A distribuição assimétrica dessas diferentes formas de Ran dirige o transporte núcleo-citoplasmático por catalisar o carregamento e a liberação das proteínas transportadas no compartimento correto (Izaurralde et al., 1997; Gorlich & Kutay, 1999; Moroianu, 1999). Assim, o processo de transporte de importação é realizado de forma que as importinas ligam-se a seus substratos no citoplasma, os transportam através dos NPC e, no núcleo, devido a sua ligação a RanGTP, os liberam. No processo de exportação nuclear, ao contrário, RanGTP promove a ligação das exportinas a seus substratos formando um complexo estável no núcleo. Após a

translocação desse complexo ao citoplama, RanGAP promove a hidrólise de RanGTP para RanGDP, causando a liberação da proteína transportada. Uma vez que a proteína transportada é liberada no compartimento correto, o receptor é reciclado para outra rodada de transporte (Fried & Kutay, 2003, Kau & Silver, 2003; Sorokin et al., 2007) (Figura 3).

Nesse contexto, a maioria dos eIFs possui essas sequências específicas NLS e NES que são reconhecidas pelas maquinarias de importação e exportação, possibilitando, assim, seu transporte núcleo-citoplasmático (Ernst et al., 1987; Qu & Cavener, 1994; Dostie et al., 2000a; Dostie et al., 2000b; Guo & Sen, 2000; Chudinova et al., 2004; Mingot et al., 2004; Parreiras et al., 2007; Sha et al., 2009; Biswas et al., 2010).



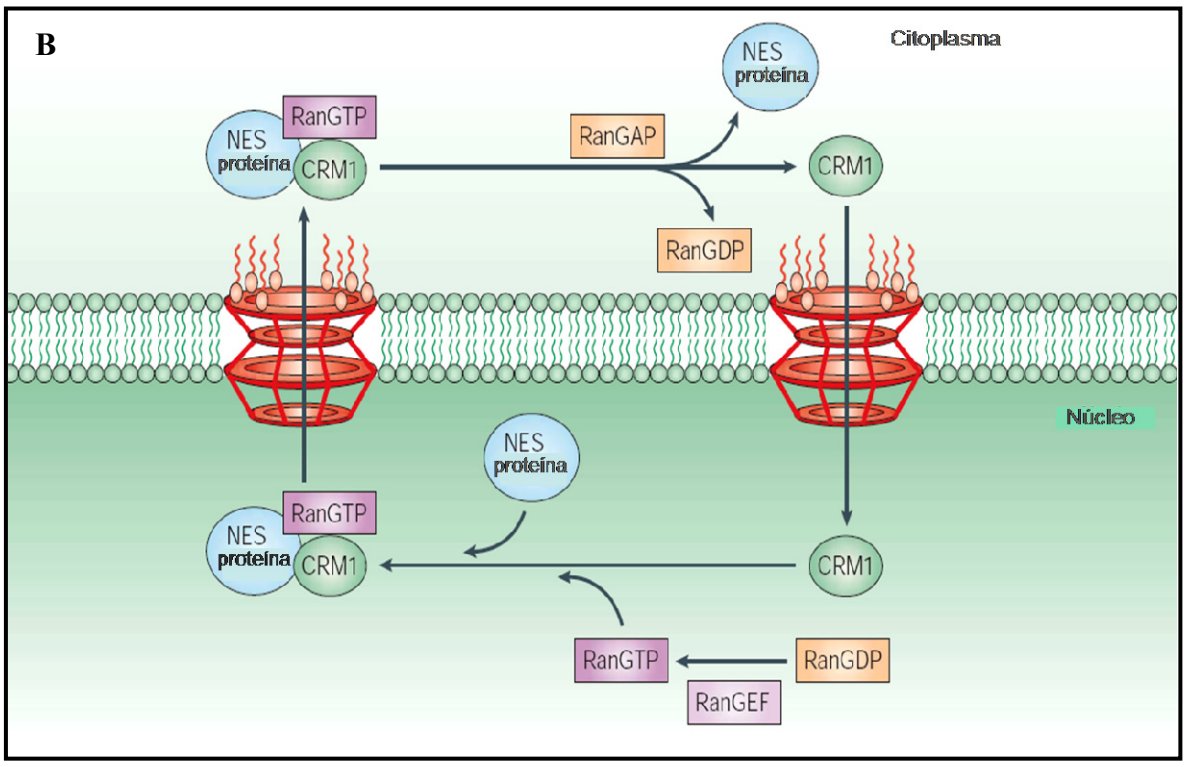


Figura 3. Transporte núcleo-citoplasmático de proteínas. (**A)** No citoplasma, a importina α (α) reconhece proteínas que contêm um sinal de localização nuclear (NLS proteína) e forma um complexo. Esse complexo liga-se à importina β (β) e é translocado através do poro nuclear (estrutura vermelha) ao núcleo. Após a entrada no núcleo, a proteína contendo o NLS é dissociada das importinas por RanGTP. Uma alta concentração de RanGTP é mantida no núcleo por RanGEF, que converte RanGDP a RanGTP. A importina β e RanGTP são reciclados de volta ao citoplasma através do poro nuclear, onde RanGTP é hidrolisado a RanGDP. A importina α é retirada do núcleo pelo receptor de exportação nuclear CAS. Essa exportação envolve a formação de um complexo com RanGTP e, então, CAS é transportado de volta ao núcleo através do poro nuclear. (**B**) Proteínas contendo um sinal de exportação nuclear (NES proteína) ligam-se a CRM1 e RanGTP antes de serem exportados do núcleo. No citoplasma, a hidrólise de RanGTP a RanGDP por RanGAP promove a dissociação do complexo. A CRM1 é então transportada de volta ao núcleo onde pode reassociar-se com outra proteína contendo NES e RanGTP para reiniciar o processo. Adaptado de Kau et al., 2004.

1.4 A SÍNTESE PROTEICA E CONDIÇÕES PATOLÓGICAS

A tradução tem um papel estabelecido na multiplicação celular, de modo que um aumento da síntese proteica ocorre como consequência de mitogênese (Meric & Hunt, 2002) e o seu bloqueio advém de diversos tipos de situações de estresse celular e de ativação ou repressão de vias de sinalização (Gebauer & Hentze, 2004).

Em diferentes doenças, incluindo a transformação e progressão tumoral, as células podem apresentar alterações nos seus níveis de tradução. Algumas doenças são causadas pela diminuição da síntese proteica global, entre as quais pode-se citar: a leucoencefalopatia com perda de substância branca, também conhecida como ataxia infantil com hipomielinização do sistema nervoso central, que decorre de mutações no gene de um eIF específico, o eIF2B (Leegwater et al., 2001; Li et al., 2004); e a miopatia congênita não progressiva, causada pela diminuição da atividade ribossomal (Poche & Kattner, 1987).

Particularmente, células cancerosas parecem necessitar de um estado traducional ativado de forma anormal para sobreviver, de modo que, durante a progressão tumoral, diferentes alterações no controle da tradução ocorrem: 1) variações na sequência de mRNA que aumentam ou diminuem a eficiência traducional, 2) mudanças na expressão ou disponibilidade de componentes da maquinaria traducional, e 3) ativação da tradução através de vias de transdução de sinais anormalmente ativadas. A primeira alteração afeta a tradução de um único mRNA, o qual pode ter um papel primordial na carcinogênese. As segunda e terceira alterações podem levar a mudanças mais globais, como um aumento na taxa global de síntese proteica e ativação traducional de mRNAs envolvidos no crescimento e proliferação celular (Meric & Hunt, 2002).

A fase de início da tradução pode ser regulada por alterações na expressão ou estado de fosforilação dos vários fatores envolvidos (Sonenberg and Dever, 2003; Gebauer and Hentze, 2004; Hershey, 2010). Alterações na expressão dos componentes da maquinaria de início da tradução, incluindo os eIFs, estão presentes em diferentes tipos de tumores (Silvera et al., 2010; Yin et al., 2011), como pode ser verificado na Tabela 3. Evidências têm sugerido os eIFs como um novo grupo de proto-oncogenes ou genes supressores tumorais (De Benedetti & Graff, 2004; Dong & Zhang, 2006). Entretanto, o papel que a modificação na expressão desses eIFs apresentam no desenvolvimento e progressão tumoral ainda não é bem conhecido.

Tem-se evidenciado, também, que a regulação da localização subcelular de diferentes proteínas em compartimentos celulares específicos representam uma forma de regulação da expressão gênica e de resposta a diferentes sinais como liberação de hormônios, de citocinas, de fatores de crescimento, sinais de ciclo celular e do desenvolvimento, resposta imune e condições de estresse (Poon & Jans, 2005). O direcionamento de proteínas supressoras de tumor, fatores de transcrição ou oncoproteínas a seus sítios intracelulares de ação é regulado em diferentes pontos. A alteração em algum desses processos pode resultar em localização subcelular incorreta, o que pode acarretar crescimento celular descontrolado (Kau et al., 2004). Nesse contexto, o melhor entendimento dos mecanismos envolvidos na expressão e disponibilidade dos eIFs, tanto em células normais, quanto tumorais torna-se necessário. Estudar a distribuição subcelular desses componentes da maquinaria de tradução e a regulação de sua disponibilidade celular poderia esclarecer muitas questões relacionadas ao seu papel na tumorigênese. Desse modo, os eIFs e as vias de sinalização envolvidas na ativação do início da tradução representam bons alvos, tanto para o desenvolvimento de drogas, quanto para a terapia

gênica, direcionados ao tratamento do câncer, bem como de outros distúrbios decorrentes de proliferação celular anormal.

Tabela 3. Diferenças dos níveis de expressão de eIFs em cânceres humanos.

eIF	Alterações	Associação com câncer	Referências
eIF2α	Aumento na expressão	Melanomas, cânceres de cólon, estômago e reto, câncer bronquioalveolar de pulmão, carcinoma hepatocelular, tumores cerebrais agressivos; progressão de neoplasmas da tireóide; linfomas agressivos de Hodgkin's e não Hodgkin's	Wang et al., 1999; Lobo et al., 2000; Rosenwald et al., 2001; Wang et al., 2001; Rosenwald et al., 2003; Rosenwald et al., 2008; Tejada et al., 2009; Silvera et al., 2010; Yin et al., 2011
eIF3a	Aumento na expressão	Cânceres de mama, cervical, de pulmão, oesofágico e de estômago	Bachmann et al., 1997; Dellas et al., 1998; Chen & Burger, 1999; Pincheira et al., 2001; Chen & Burger, 2004; Silvera et al., 2010; Yin et al., 2011
eIF3c	Aumento na expressão	Seminomas testiculares, meningiomas e melanomas	Rothe et al., 2000; Baldi et al., 2003; Scoles et al., 2006; Silvera et al., 2010; Yin et al., 2011
eIF3e	Diminuição na expressão	Cânceres de mama, pulmão e cólon	Miyazaki et al., 1997; Marchetti et al., 2001; Buttitta et al., 2005; Traicoff et al., 2007; Silvera et al., 2010; Yin et al., 2011
eIF3f	Diminuição na expressão	Cânceres de pâncreas, vulva, mama e intestino delgado; perda gênica em melanomas	Doldan et al., 2008; Doldan et al., 2008; Shi et al., 2006; Silvera et al., 2010; Yin et al., 2011

eIF	Alterações	Associação com câncer	Referências
eIF3h	Aumento na expressão	Cânceres de mama, próstata, fígado e pulmão	Nupponen et al., 1999; Saramaki et al., 2001; Okamoto et al., 2003; Savinainen et al., 2004; Saramaki & Visakorpi, 2007; Cappuzzo et al., 2009; Silvera et al., 2010; Yin et al., 2011
eIF3i	Aumento na expressão	Cânceres de cabeça e pescoço, fígado e induzidos por cádmio	Joseph et al., 2002; Huang et al., 2004; Joseph et al., 2004; Rauch et al., 2004; Silvera et al., 2010; Yin et al., 2011
eIF4AI	Aumento na expressão	Melanoma e carcinoma hepatocelular	Eberle et al., 1997; Shuda et al., 2000; Silvera et al., 2010; Yin et al., 2011
eIF4E	Aumento na expressão	Leucemias, cânceres de mama, cabeça, pescoço, pulmão, próstata, bexiga, pele, cérvice, esôfago, coloretal, linfomas; correlacionado com o aumento da malignidade em meningiomas, glioblastomas e astrocitomas; associado com a diminuição da sobrevivência em cânceres de próstata avançados	DeFatta et al., 1999; Franklin et al., 1999; Sorrells et al., 1999; Lobo et al., 2000; Berkel et al., 2001; Rosenwald et al., 2001; Nathan et al., 2002; Seki et al., 2002; Van Trappen et al., 2002; Liang et al., 2003; Yang et al., 2003; Chen & Burger, 2004; De Benedetti & Graff, 2004; Lee et al., 2005; Salehi & Mashayekhi, 2006; Salehi et al., 2007; Thumma & Kratzke, 2007; Rosenwald et al., 2008; Assouline et al., 2009; Coleman et al., 2009; Graff et al., 2009; Tejada et al., 2009; Wang et al., 2009; Silvera et al., 2010; Yin et al., 2011

eIF	Alterações	Associação com câncer	Referências
Fosfo-eIF4E	Aumento na expressão	Cânceres de próstata, pulmão, coloretal, cabeça e pescoço; correlacionado com expressão gênica antiapoptótica em linfomas de células B de grande difusão e linfoma de Burkitt`s; associado com tipos histológicos serosos; melhor sobrevivência em tumores ovarianos; associado com estágios precoces da doença em câncer coloretal, gástrico e de pulmão	Lobo et al., 2000; Wendel et al., 2007; Noske et al., 2008; Fan et al., 2009; Graff et al., 2009; Tejada et al., 2009; Silvera et al., 2010; Yin et al., 2011
eIF4GI	Aumento na expressão	Cânceres de pulmão, próstata, mama, cabeça, pescoço e cervical; associado com diminuição da progressão metastática em câncer de mama localmente avançado; associado com câncer de mama inflamatório; associado com o aumento da tradução de ciclina D1 em carcinoma escamoso de pulmão	Brass et al., 1996; Bockmuhl et al., 2000; Sattler et al., 2000; Bauer et al., 2001; Rao et al., 2004; Silvera et al., 2009; Silvera et al., 2010; Yin et al., 2011
eIF4GII	Diminuição na expressão	Câncer de bexiga	Buim et al., 2005; Silvera et al., 2010; Yin et al., 2011
eIF5A	Aumento na expressão	Amplificado em câncer de ovário avançado; associado com progressão metastática em carcinomas coleretal; associado com reduzida sobrevivência livre de progressão e de recorrência em câncer de bexiga	Guan et al., 2001; Guan et al., 2004; Xie et al., 2008; Chen et al., 2009; Luo et al., 2009; Silvera et al., 2010; Yin et al., 2011
eIF6	Aumento na expressão	Cânceres de colon, ovário, cabeça e pescoço; leucemia	Sanvito et al., 2000; Harris et al., 2004; Rosso et al., 2004; Flavin et al., 2008; Silvera et al., 2010; Yin et al., 2011

1.5 O FATOR eIF2

Na década de 1970, foi isolado de células de mamíferos um complexo proteico com massa molecular entre 150 kDa e 160 kDa capaz de se ligar a Metionil-tRNAi^{Met}, o qual foi denominado fator eucariótico 2 de início da tradução (eIF2) (Barrieux & Rosenfeld, 1977; Kaempfer et al., 1978). Esse fator está envolvido no primeiro passo regulatório da biossíntese proteica (Moldave, 1985; Pain, 1986), promovendo a ligação do Metionil-tRNAi^{Met} à subunidade ribossomal 40S em um processo dependente de GTP, e fazendo a seleção do códon AUG para início de tradução em eucariotos (Donahue et al., 1988; Hershey & Merrick, 2000; Hinnebuch, 2000).

1.5.1 Caracterização bioquímica e molecular do fator eIF2

Em seres humanos, o fator eIF2 é formado pelas subunidades não idênticas alfa (36 kDa), beta (38 kDa) e gama (52 kDa) que permanecem associadas durante todo o processo de início da tradução (Barrieux & Rosenfeld, 1977). Essas três subunidades são requeridas para a utilização catalítica de eIF2 durante o início da síntese proteica no citoplasma (Duncan & Hershey, 1983; Hershey, 1991). Nenhuma delas parece existir como um monômero funcional fora do heterotrímero eIF2.

A subunidade alfa de eIF2 é um polipeptídeo cujo estado de fosforilação regula a atividade do heterotrímero eIF2/GTP/Metionil-tRNAi^{Met} (Hershey, 1989). Quando fosforilada em sua serina 51, essa proteína leva a uma inibição da troca de GDP por GTP realizada por eIF2B (Pathak et al., 1988). Isso acarreta uma redução de complexos ternários ativos, por meio da inibição competitiva de eIF2B (Sonenberg & Dever, 2003), ocasionando um bloqueio da síntese proteica global (Dever et al., 1992; Cigan et al., 1993; Hinnebusch, 1994; Dever et al., 1995). A estrutura tridimensional, realizada por difração

de raio X, de dois terços da região aminoterminal de eIF2α humano revelaram dois motivos: um domínio de dobra OB (dobra de ligação a oligonucleotídeo/oligossacarídeo) e um compacto domínio de alfa-hélice (Nonato et al., 2002). A fosforilação de eIF2α é um ponto principal de controle da síntese proteica global nas células eucarióticas e regula a expressão gênica sobre diversas condições como: infecção viral (Clemens & Elia, 1997), apoptose (Srivastava et al., 1998; Saelens et al., 2001), carência de aminoácidos, choque térmico (Hinnebusch, 1997; Kozak, 1999) e estresse do reticulo endoplasmático (Harding et al., 1999). Em mamíferos, essa subunidade pode ser fosforilada por quatro diferentes cinases: (I) inibidor regulado por heme (HRI), (II) proteína cinase ativada por RNA dupla fita (PKR), (III) proteína cinase regulada por nutrientes (GCN2) e (IV) proteína cinase do retículo endoplasmático PKR-like (PERK) (Proud, 2005).

A subunidade gama de eIF2 apresenta três motivos consensuais conservados para ligação a GTP em sua porção aminoterminal (Merrick, 1992) e está implicada em várias funções de eIF2 como ligação a GDP/GTP, a Metionil-tRNAi^{Met} e hidrólise de GTP (Nika et al., 2001). Tem uma similaridade evidente com o fator de elongação procariótico (EF-Tu), principalmente em relação à região de ligação ao tRNA (Hannig et al., 1993; Schmitt et al., 2002). A estrutura do complexo eIF2γ-GDP inativo assimila-se à estrutura do complexo EF-Tu-GTP ativo (Schmitt et al., 2002).

1.5.2 Caracterização da subunidade beta de eIF2

A subunidade beta do eIF2 é uma proteína moderadamente abundante em células de mamíferos. Em seres humanos, apresenta um mRNA de 1416 pares de base e uma sequência polipeptídica de 333 aminoácidos (Pathak et al., 1988). O eIF2β possui duas regiões altamente conservadas que representam domínios de interação com ácidos

nucléicos, conforme ilustrado na Figura S1 do Capítulo 1. Na porção aminoterminal da molécula, existe um domínio composto por três blocos de seis a oito resíduos de lisinas (Pathak et al., 1988) que são as únicas regiões altamente conservadas evolutivamente nessa porção da molécula (Laurino et al., 1999). Esses resíduos conferem uma carga positiva acentuada à essa região, mediando interações com mRNA (Laurino et al., 1999) e com outros eIFs (Das et al., 1997; Asano et al., 1999; Das & Maitra, 2000).

A porção central da proteína apresenta os sítios de ligação a eIF2γ (Hashimoto et al., 2002). A análise da sequência de eIF2β humana indicou a presença de dois motivos consensuais de ligação a GTP (Pathak et al., 1988; Bommer et al., 1991). No entanto, esses motivos estão ausentes em levedura. Dados de experimentos de ligação cruzada com análogos de GTP indicaram uma possível participação de β e γ na ligação a GTP (Merrick, 1992), o que sugere uma forte interação funcional entre as subunidades γ e β de eIF2 durante a formação do complexo ternário (Bommer et al., 1991; Gaspar et al., 1994).

Na porção carboxi terminal, há uma sequência contendo quatro cisteínas (C_2-C_2) que parecem formar uma estrutura de "dedo-de-zinco" (Evans & Hollenberg, 1988; Pathak et al., 1988). Uma análise de mutações realizadas *in vitro*, em *Saccharomyces cerevisiae*, as quais alteravam uma ou mais cisteínas, o espaçamento entre essas cisteínas ou removiam a região C_2-C_2, indicou que estes resíduos são indispensáveis para a função de eIF2β (Donahue et al., 1988; Castilho-Valavicius et al., 1992).

Ensaios *in vitro* revelaram que eIF2β liga-se ao mRNA, enquanto eIF2α e γ não são capazes dessa associação (Laurino et al., 1999). O emprego de uma série de formas truncadas de eIF2β revelou que a capacidade de ligação ao RNA está relacionada com as regiões aminoterminal e carboxi terminal, sugerindo que os blocos de lisina sejam responsáveis pela aproximação inicial não específica e de alta afinidade ao mRNA, e a

região C_2-C_2 seja responsável pela ligação mais específica (Donahue et al., 1988; Laurino et al., 1999). Essa ligação da porção carboxiterminal deve estar relacionada com a seleção do códon de início AUG, visto que análise de mutantes para a região carboxi terminal, em *S. serevisae*, demonstrou que essas mutações permitiam o início da síntese proteica, tendo como códon iniciador o triplete UUG (Donahue et al., 1988).

A subunidade β de eIF2 está envolvida em diversas interações com outros fatores como eIF1 (Hashimoto et al., 2002), eIF3 (Valasek et al., 2002), eIF2B (Kimball et al., 1998; Pavitt et al., 1998; Asano et al., 1999) e eIF5 (Das et al., 1997; Singh et al., 2004). Os três blocos de lisinas presentes na região aminoterminal dessa molécula mostraram ser responsáveis pela ligação dessa subunidade aos fatores de iniciação eIF5 (Das et al., 1997; Das & Maitra, 2000; Singh et al., 2004) e eIF2B (Asano et al., 1999) em leveduras e seres humanos. Assim, eIF2β parece ser um dos principais mediadores de contatos com diversas outras moléculas que participam do início da tradução. O eIF2β está envolvido também nas interações com outras proteínas, como proteína-cinase dependente de DNA (DNA-PK) eIF2β (Ting et al., 1998), caseína-cinase 2 (CK-2) (Llorens et al., 2003) e proteína adaptora Nck-1 (Kebache et al., 2002). Essa ligação com Nck-1 previne a fosforilação de eIF2α e a subsequente parada da tradução em resposta a estresse do retículo endoplasmático (Kebache et al., 2004).

Análises *in vitro* mostram que são também encontrados, na subunidade β de eIF2, importantes sítios de fosforilação para diferentes cinases como: (I) proteína cinase C (PKC), (II) proteína-cinase dependente de AMP cíclico (PKA), (III) CK-2 (Welsh et al., 1994; Suragani et al., 2006) e (IV) DNA-PK (Ting et al., 1998). Os estudos da fosforilação de eIF2β em células de mamíferos têm mostrado que ela ocorre associada a diferentes

condições como choque térmico (Duncan & Hershey, 1984), privação de soro (Duncan & Hershey, 1985), diabetes (Garcia et al., 1996) e nascimento (Luis et al., 1993).

Laurino *et al.* (1999) demonstraram, em *S. cerevisia*e, que eIF2β desprovido dos blocos de lisina presentes na porção aminoterminal da molécula não é capaz de realizar a ligação ao mRNA, embora a associação às demais subunidades do heterotrímero eIF2 continuasse ocorrendo de forma normal. A partir daí, surgiu a hipótese de que uma forma de eIF2β apresentando a deleção dos blocos de lisinas poderia funcionar *in vivo* como um inibidor competitivo da forma selvagem da proteína. Essa hipótese foi corroborada em ensaio de curva de crescimento de *S. cerevisiae*, no qual, sob controle do mesmo promotor, competiam os produtos protéicos de eIF2β selvagens e desprovido dos blocos de lisinas. Observou-se que este último foi capaz de induzir parada do crescimento celular nesse microrganismo (dados não publicados, Laurino, JP) (Figura 4). Além disso, Llorens *et al*. (2006) mostraram que uma forma truncada de eIF2β, consistindo apenas da porção aminoterminal da proteína, é capaz de diminuir 40% da viabilidade de células HeLa.

Desse modo, células humanas expressando um vetor contendo o gene de eIF2β codificando a proteína com a deleção dos blocos de lisinas poderiam apresentar parada de proliferação devido à diminuição acentuada da síntese proteica. Seria possível que essa forma truncada da proteína, competindo com a forma selvagem da célula, pudesse apresentar um efeito antiproliferativo que poderia ser utilizado para terapia gênica antiproliferação celular direcionada para células tumorais.

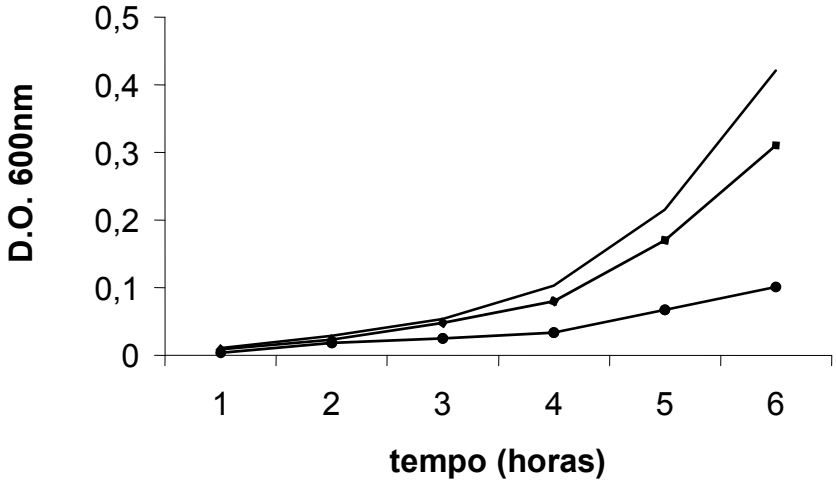


Figura 4. Curva de crescimento de *S. cerevisiae* em meio líquido. Células 167-3c transformadas com o plasmídeo pLeu2 Cen4 (plasmídeo de baixo número de cópias), expressando eIF2β selvagem ou desprovido dos blocos de lisina sob controle de seu próprio promotor (J. P.Laurino, dados não publicados). A linha simples representa a curva de crescimento de células transformadas com o plasmídeo pLeu2 Cen4 vazio; A linha com losangos representa a curva de crescimento de células transformadas com o plasmídeo pLeu2 Cen4 contendo o gene selvagem de eIF2β de *S. cerevisiae*; A linha com círculos representa a curva de crescimento de células transformadas com o plasmídeo pLeu2 Cen4 contendo o gene de eIF2β de *S. cerevisiae* desprovido dos blocos de lisinas.

O eIF2β, como outros eIFs, parece também apresentar modificações no seu padrão de distribuição celular em determinadas condições da célula. Por análise de fracionamento celular, um estudo mostrou que células HeLa, em condições normais ou de privação de soro, apresentam uma fração nuclear de eIF2β endógeno (Llorens et al., 2006). Ao contrário, Bohnsack *et al*. (2002) e Mingot *et al*. (2004) reportaram que eIF2β endógeno não apresenta localização nuclear em condições normais. No entanto, essa proteína modifica sua localização de exclusivamente citoplasmática para predominantemente nuclear após tratamento com o inibidor da exportina 1 (CRM1), leptomicina B (LMB), por 30 min ou 1 h (Bohnsack et al., 2002; Mingot et al., 2004). Além disso, a presença nucleolar de eIF2β também foi identificada em estudos de proteômica do nucléolo de

células HeLa (Andersen et al., 2002; Leung et al., 2006). Assim, o fato de que, até o momento, pouco se sabe sobre a localização e função de eIF2β no núcleo, torna-se importante estudar sua disribuição subcelular e as características moleculares estruturais e funcionais que estão relacionadas, bem como as situações que regulam sua disponibilidade nos diferentes compartimentos celulares. Neste sentido, esta tese de doutorado tenta contribuir com a caracterização de motivos de aminoácidos de eIF2β responsáveis por seu transporte ao núcleo e ao nucléolo, e também sua exclusão desses compartimentos. Isso poderá colaborar com o esclarecimento dos processos nucleares nos quais os diferentes eIFs atuam e, além disso, entender o mecanismo de tradução nuclear, ainda pouco conhecido.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a importância de motivos conservados do eIF2β na síntese de proteínas e na distribuição subcelular desse fator em células humanas

2.2 OBJETIVOS ESPECÍFICOS

- Analisar os efeitos da superexpressão das proteínas eIF2β selvagem e eIF2β com a deleção dos três blocos de lisinas nos processos de síntese proteica, proliferação e viabilidade celulares em células Hek293tetR;
- Analisar a distribuição subcelular de eIF2β em diferentes linhagens de células humanas;
- Identificar motivos de aminoácidos específicos relacionados com a distribuição subcelular de eIF2β.

3. CAPÍTULO I

Proliferation of human cells relies on the evolutionarily conserved amino-terminal region of eukaryotic initiation factor 2β

3.1 APRESENTAÇÃO

Esse capítulo apresenta um trabalho desenvolvido nessa tese que originou um manuscrito o qual foi submetido à revista RNA Biology intitulado "Proliferation of human cells relies on the evolutionarily conserved amino-terminal region of eukaryotic initiation factor 2β". As contribuições de cada autor desse trabalho estão descritas no corpo do manuscrito na página 95 dessa tese.

O interesse de estudar a importância dos resíduos de lisinas presentes na região aminoterminal de eIF2β para a síntese proteica em células humanas decorreu de informações publicadas sobre o seu papel no processo de início de tradução e o efeito de sua deleção em células de *S. cerevisiase*. Um estudo realizado por Das et al. (1997) evidenciou que eIF2β liga-se a eIF5 através de seus blocos de lisinas. Também, Laurino et al. (1999) mostraram que esses resíduos medeiam a interação de eIF2β com o mRNA e Asano et al. (1999) demonstraram que a interação de eIF2β com eIF2B é via blocos de lisinas. Esses dois últimos trabalhos evidenciaram que a deleção desses resíduos diminui o crescimento de *S. cerevisiae*. Um estudo de Bonsack et al. (2002) mostrou que eIF2β pode apresentar localização nuclear e análises de bioinformática realizadas neste estudo mostraram os blocos de lisinas como possíveis NLS da molécula. Além disso, outro estudo de proteômica nucleolar demonstrou que eIF2β está presente no nucléolo de células humanas. Assim, considerando que, até o momento, nada se sabia sobre o efeito da deleção desses resíduos de lisinas em células humanas, nesse estudo foi analisada a importância desses aminoácidos para a manutenção dos processos de síntese proteica, proliferação e viabilidade celulares e seu papel na localização nuclear de eIF2β.

A molécula eIF2β apresenta também, em sua região carboxiterminal, resíduos conservados caracterizados por cisteínas que formam uma estrutura de dedos de zinco na

proteína. Esse tipo de estrutura é responsável pela localização nucleolar de diferentes proteínas. Desse modo, no presente trabalho, também foi analisado o papel desses resíduos na localização nucleolar de eIF2β.

Os resultados obtidos nesse estudo permitiram evidenciar que, tanto quanto em *S. cerevisiae*, os blocos de lisinas de eIF2β são essenciais ao processo de síntese proteica e viabilidade de células humanas. Foi mostrada a presença nuclear e nucleolar de eIF2β e os blocos de lisinas e o motivo de dedo de zinco da proteína eIF2β foram identificados como responsáveis por sua localização nucleolar. Também se pôde evidenciar que a proteína é excluída do núcleo via exportina CRM1 e que sua região aminoterminal medeia essa exportação. Além disso, pôde-se sugerir outros possíveis resíduos relacionados a distribuição subcelular da proteína e inferir possíveis funções nucleares/nucleolares de eIF2β.

3.2 ARTIGO 1 – manuscrito submetido à RNA Biology

TITLE: Proliferation of human cells relies on the evolutionarily conserved amino-terminal region of eukaryotic initiation factor 2β

RUNNING TITLE: eIF2β role in cell survival and its cellular location

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KEYWORDS : eIF2β, protein synthesis, cell proliferation, nucleolar localization, polylysine stretches, zinc finger motif

ABBREVIATIONS

dEGFP, destabilized variant of EGFP; eIFs, eukaryotic initiation factors; eIF2, eukaryotic initiation factor 2; eIF2β, β subunit of eukaryotic initiation factor 2; H-DMEM, High glucose Dulbecco's modified Eagle's medium; LMB, leptomycin B; NES, Nuclear Exclusion Signal; NTS, Nuclear Translocation Signals; ROI, fluorescence in the region of interest

ABSTRACT

Translational initiation factor 2 in eukaryotes (eIF2) plays a crucial role in the initiation and regulation of protein synthesis. The β subunit (eIF2β), which mediates the interactions of eIF2 with mRNA, contains evolutionarily conserved polylysine stretches in the amino-terminal region and a zinc finger motif in the carboxy-terminal. We here report that these polylysine stretches are essential for a sustained cell proliferation and that deletion of these stretches negatively affects both protein synthesis and cell viability of human cell. Moreover, our data also demonstrates that eIF2β translocates from the cytoplasm to the nucleus, accumulating in the nucleolus and that the Nuclear Exclusion Signal (NES) sequence in the amino-terminal region of eIF2β, promotes nuclear exclusion of eIF2β mediated by CRM1 exportin. Finally, we report that the polylysine stretches and the C_2-C_2 zinc finger motif are essential for eIF2β nucleolar localization, involving an RNA binding-dependent mechanism. This study suggests that besides eIF2β being involved in the protein synthesis process, it should act in nuclear processes. Furthermore polylysine stretches of

eIF2β are important for cell proliferation and viability as well as are essential for eIF2β nucleolar localization.

INTRODUCTION

The initiation of protein synthesis in eukaryotes requires the participation of multiple protein components, collectively known as eukaryotic initiation factors (eIFs) [1, 2], which form transient and specific complexes following a precise sequence [3, 4]. eIF2 is a multifunctional heterotrimeric protein that drives the Met-tRNAi to the P site of the small ribosomal subunit in a GTP-dependent manner, a crucial step in the initiation of protein synthesis [5, 6]. In addition, eIF2 is involved in mRNA binding, and start site recognition [7, 8]. The activity of eIF2 can be reduced by a wide range of stress conditions, thus affecting negatively protein synthesis [9]. These effects, which are conserved throughout eukaryotes, from budding yeast to mammals, act mainly through phosphorylation of the α subunit of eIF2 [6].

Previous studies based on genetic approaches with yeast or recombinant mammalian proteins have shown that the β subunit of eIF2 (eIF2β) plays a major role in the binding of eIF2 to other factors: eIF2β binds to eIF2Bε [10, 11], to eIF5 [11, 12], to mRNA [8] and to eIF3 [13]. eIF2β has also been implicated in the interactions of eIF2 with other proteins such as DNA-dependent protein kinase (DNA-PK) [14] and casein kinase 2 (CK-2) [15]. Moreover, eIF2β binds to the adaptor protein Nck-1 [16], preventing the phosphorylation of eIF2α in response to endoplasmic reticulum stress [17].

The carboxyl-terminal region eIF2β contains a zinc-finger motif that contributes to mRNA binding and start-site selection during the scanning process [18]. The amino-terminal region of eIF2β, which is conserved from yeast to humans, contains three polylysine

stretches [19] and phosphorylation sites for CK2 and protein kinase C (PKC) [20]. The polylysine stretches participate in the binding of eIF2β to eIF5, eIF2Bε and mRNA [8, 10, 11, 21]. In yeast, deletion of the polylysine stretches compromises cell growth, which points to an important role for this structural feature [8, 11]. So far, these effects on cell growth have not yet been explored in mammalian cells.

Moreover, studies have demonstrated that the α [22-24] and β [25, 26] subunits of eIF2 are located in the cytoplasm and can also be located in the nucleus of different cell types, but nuclear functions have not yet been described for these proteins. Some other eIFs such as eIF1A, eIF3, eIF4AIII, eIF4E, eIF4GI, eIF5A and eIF6 have been detected in the nucleus or nucleolus of mammalian cells. The role of eIFs within the nucleus is unclear, but several reports indicate that they participate in nucleocytoplasmic mRNA transport, mRNA splicing, ribosomal biogenesis, cytokine regulation, and nonsense-mediated mRNA decay [27-36].

In this work, we studied the role of eIF2β polylysine stretches on protein synthesis, cell growth and viability in human cells as well as the subcellular localization of eIF2β.

MATERIALS AND METHODS

Antibodies and reagents

Mouse anti-IF2β monoclonal antibodies (eIF2β moAbs, catalog number: sc-133209 and sc-9978) were obtained from Santa Cruz Biotechnology. Rabbit anti-TetR polyclonal antibody was from Abcam (catalog number: ab-33249). The human serum used as positive control for nucleolar specific antigen (anti-nucleolus) of ANA Test System was purchased from Immuno Concepts (catalog number: 2023). Anti-rabbit or anti-mouse peroxidase-conjugated and anti-mouse Cy3 or FITC-conjugated antibodies were obtained from Sigma

Chemical. Tetracycline was from Acros Organics (catalog number: 64-75-5) and solubilized in absolute ethanol at 1 mg/mL. Leptomycin B was purchased from Sigma Chemical (catalog number: L2913) RNAse A (20 mg/mL) was purchased from Invitrogen (catalog number: 12091021).

Plasmid constructions

The cDNA encoding full-length human eIF2β (eIF2βWT) was generated by PCR using a HeLa cDNA library (Matchmaker cDNA library; Clontech, Palo Alto, CA, U.S.A.). Deletion of the polylysine stretches was achieved by site-directed mutagenesis with the *Site Mutagenesis System* from Promega using specific primers.

The tetracycline-regulated plasmids were constructed from a pcDNA4/TO plasmid (Invitrogen), where the dEGFP cDNA from the pd1EGFP-N1 vector (Clontech) was subcloned at position 1052 (named pJL). The cDNAs of eIF2βWT and eIF2βΔ3K (eIF2β without polylysine stretches) were cloned into pJL and named pJL:eIF2βWT and pJL:eIF2βΔ3K, respectively. eIF2βWT and eIF2βΔ3K are thus under tetracycline-regulated expression control, whereas dEGFP is constitutively expressed.

Various constructs of eIF2β were generated for the analysis of its subcellular localization. The coding sequences of eIF2βNT (amino-terminal region of eIF2β - amino acids 1 to 157) and eIF2βCT (carboxyl-terminal region of eIF2β - amino acids 158 to 333) were generated by PCR. The fragments of eIF2βWT, eIF2βΔ3K, eIF2βNT or eIF2βCT were cloned into the pcDNA4/TO vector and named pGDS:eIF2βWT, pGDS:eIF2βΔ3K, pGDS:eIF2βNT and pGDS:eIF2βCT, respectively. Deletion of the last two cysteines of the zinc finger motif was achieved by deleting the DNA corresponding to last 29 amino acids in eIF2βWT or eIF2βCT using the restriction enzyme *Ps*TI (named pGDS:eIF2βΔC_2 or

pGDS:eIF2βCTΔC$_2$). To generate an eIF2βNT form without polylysine stretches, the amino-terminal region of eIF2βΔ3K was digested with *Apa*I and *Eco*RV, followed by a blunt end reaction and named pGDS:eIF2βNTΔ3K. In all cases, the resulting sequences were confirmed by DNA sequencing.

Transfection, cell culture and treatments

Hek293TetR (R710-07, Invitrogen, CA, USA) cells were seeded at a density of 1.5×10^5 cells/ml in H-DMEM (High glucose Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum (H-DMEM complete medium) and transfected the following day with pJL, pJL:eIF2βWT or pJL:eIF2βΔ3K using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 6 h, the complete medium was changed and after 24 h, cells were selected with 150 μg/mL of zeocin (Invitrogen, catalog number: R25001).

To study eIF2β subcellular localization, $1.5x10^5$ Hek293 (ATCC CRL-1573), $1x10^5$ MCF7 (ATCC HTB-22) or $0.8x10^5$ HeLa (ATCC CCL-2) cells were plated on poly-D-lysine coated coverslips (Ø10mm) in H-DMEM complete medium and transfected with the plasmids of interest as described, then analyzed using immunocytochemistry.

Cell viability assay

Selected Hek293TetR cells expressing pJL, pJL:eIF2βWT or pJL:eIF2βΔ3K were plated in 96-well plates at a density of 6×10^3 cells per well in H-DMEM complete medium. After 24 h, tetracycline was added (1 μg/ml) and untreated cells were used as controls. After 24, 48, 72 and 96 h of treatment, cell viability was assayed using the tetrazolium salt XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-

carboxanilide) method following the manufacturers (Roche Applied Science, catalog number: 11465015001) recommendations.

Cell Proliferation assay

Cell sorting on a BD FACS Aria (BD Biosciences, San Jose, CA, USA) was used to select populations of Hek293TeTR cells containing pJL, pJL:eIF2βWT or pJL:eIF2βΔ3K and expressing similar and homogeneous intensity levels of dEGFP. The selected cells were plated in 96-well plates at a density of 100 cells per well in complete H-DMEM complete medium at a final volume of 200 µl. Cells were treated with tetracycline (1 µg/ml) for 24, 48, 72, 96 h and a half tetracycline dose was added after each 48 h. After 96 h of culture, the treated and untreated cells were incubated with 1 µCi/well of (methyl-^{3}H)-thymidine triphosphate for 24 h, then the cells were transferred to a Cell Harvesters FilterMate^{TM} Universal Harvester (PerkinElmer, MA, USA) scintillation filter. The samples were counted in a Wallac 1450 Microbeta (PerkinElmer, MA, USA) beta scintillation counter.

Determination of population doubling

Hek293TetR cells expressing pJL, pJL:eIF2βWT or pJL:eIF2βΔ3K were plated at each passage in 24-well plates at a density of 50,000 cells/well and treated with 1 µg/mL tetracycline with renewal of medium and treatments every 48 h. After each passage, cells were counted and population doubling (PD) was determined according to the formula PD = (log N(t)-logN(to))/log 2, where N(t) is the number of cells per well at time of passage, and N(to) is the number of cells seeded at the previous passage. The sum of PDs was then plotted in relation to the time of culture.

Protein synthesis assay

A destabilized variant of EGFP characterized by a rapid protein turnover and a half-life of 1h[54] was used for the protein synthesis assay. Briefly, Hek293TetR cells expressing pJL, pJL:eIF2βWT or pJL:eIF2βΔ3K were plated in 6-well plates at 1.8 x 10^5 cells per well. After 24 h, cells were treated with tetracycline (1 µg/ml) for 24, 48, 72 and 96 h. Treated and untreated cells were harvested, trypsinized and distributed in 96-well for cytometry analysis. Ten thousand events were recorded for the dEGFP expression evaluation in the flow cytometer (Guava Easy Cyte Plus System, Guava Technologies, Millipore, MA, USA). To improve this assay, Hek293TetR cells expressing pJL, pJL:eIF2βWT or pJL:eIF2βΔ3K were sorted for the homogeneous dEGFP expression range by BD FACS Aria II (BD Biosciences, San Jose, CA, USA) and the same procedure described above for protein synthesis measurement was performed.

Western blotting

0.6x10^6 cells were washed once with cold PBS, lysed in 200 µL Laemmli lysis buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol and 0.002% bromophenol blue) and boiled for 5 min. The protein extract from 10^5 cells was loaded per lane, resolved in a gradient SDS-PAGE gel (10-20%) and transferred to nitrocellulose membranes. eIF2β moAb (1:500) or anti-TetR (1:500) was used. The peroxidase-conjugated anti-mouse (1:2,000) or anti-rabbit (1:2,000) was used as a secondary antibody.

Immunocytochemistry and fluorescence analysis

Twenty-four hours after transfection, Hek293, HeLa and MCF7 cells were fixed with 4% formaldehyde/PBS for 20 min, permeabilized with 0.5% Triton X-100/PBS for 10 min, and then incubated with 3% BSA/PBS for 1 h. Cells were incubated overnight at 4ºC with eIF2β moAb (1:100), then incubated with anti-mouse Cy3-conjugated Ig (1:100) for 1 h. The cells were then incubated with anti-nucleolus for 2 h, followed by anti-human FITC-conjugated Ig (1:100) for 1 h, and finally labeled with DAPI 1 μg/mL (Invitrogen) for 10 min. Images were acquired from numerous fields using a 4X or 60X objective (oil immersion) on a confocal microscope (FluoView FV1000, Olympus, Japan). eIF2β gene expression in the cytoplasm, nucleus and nucleolus was analyzed by measuring the Cy3 fluorescence levels in 24-bit mode by confocal microscopy. The fluorescence in the region of interest (ROI) from the biggest nucleolus was measured using the color histogram tool on *Image J v.1.42q* (NIH, USA). Gene expression in the selected ROI was defined as the mean value of red spectra readings from an RGB scale. Identical ROIs were used following the same procedure to analyze the localization of eIF2β in the cytoplasm and nucleus. Readings taken in the cytoplasm, nucleus and nucleolus were normalized with background correction and adjusted to the average cellular volume of the nucleolus (25% of nucleus volume), nucleus (14.5% of total volume) and cytoplasm (85.5% of total volume) in HeLa cells [55, 56]. Data gathered in the nucleolus, nucleus and cytoplasm were plotted as percentage values in relation to the mean total red fluorescence, where the mean total red fluorescence of each eIF2β form is the sum of the red fluorescence from the nucleolus, nucleus and cytoplasm. One hundred cells were analyzed and the number of cells (*n*) quantified from each construct is indicated in the figure legends. To block nuclear

export, transfected HeLa cells were treated with 5 ng/mL leptomycin B for 1 h prior to fixation.

RNA binding assay

Eighty thousand HeLa cells were grown on cover glass and transfected with pGDS:eIF2βWT on the next day. Twenty-four hours after transfection, cells were fixed with methanol for 5 min at -20°C followed by acetone for 2 min at -20°C and permeabilized with 0.1% Triton X-100 for 3 min on ice. The RNAse reaction was performed using 0.4 mg/ml RNAse A/PBS for 60 min at 37°C. The samples were processed for immunocytochemistry using eIF2β moAb and anti-nucleolus.

Statistical Analysis

ANOVA followed by Tukey post-hoc test was used for statistical analysis of multiple groups, with $P < 0.05$ considered significant.

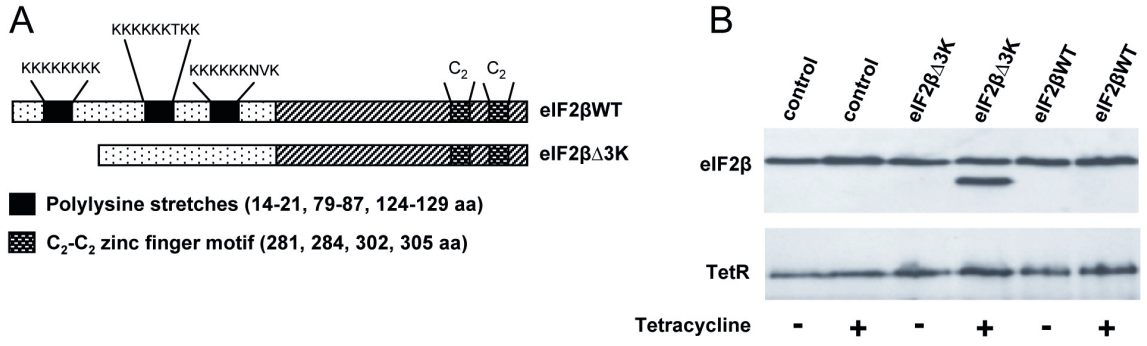
RESULTS

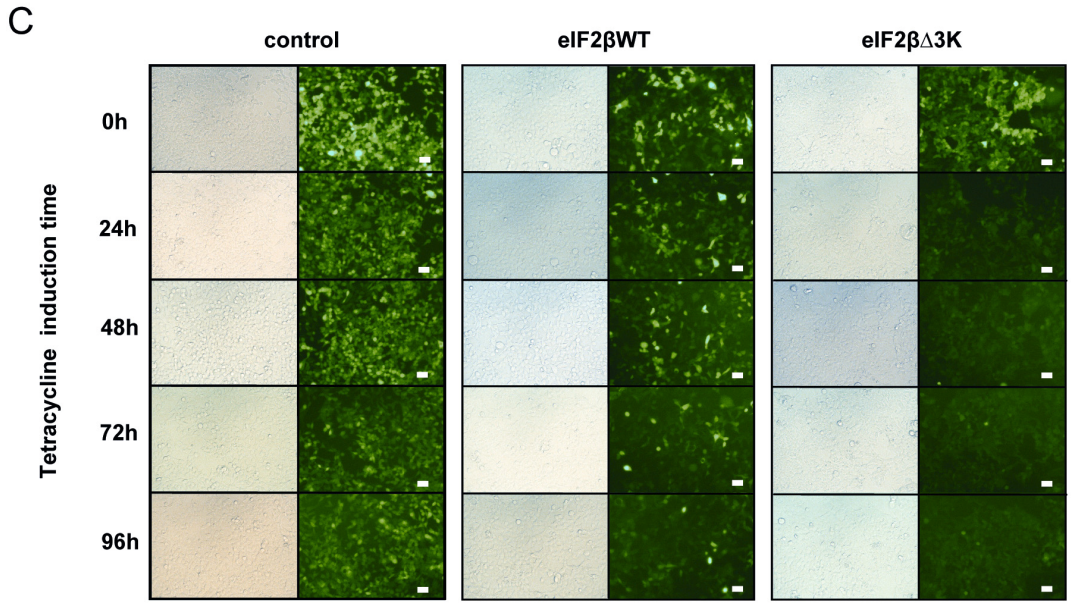
Overexpression of eIF2βΔ3K decreases the protein synthesis

Previous studies in yeast showed that deletion of the polylysine stretches in eIF2β compromised yeast growth [8, 11]. Since no data is currently available in mammalian cells, we performed site directed mutagenesis on the human eIF2β, deleting the polylysine stretches (Fig. 1A) in the amino-terminal region of eIF2β. Wild type eIF2β (eIF2βWT) and mutant eIF2β (eIF2βΔ3K) where then cloned under control of a tetracycline-regulated promoter in a plasmid expressing constitutively a destabilized variant of EGFP (dEGFP)

with rapid turnover but whose expression is dependent on the efficiency of the expressed form of eIF2β (see materials and methods).

Hek293TetR cells were transfected with either the empty vector, the eIF2βWT construct or the eIF2βΔ3K construct and selected with zeocine. Expression of eIF2β before and after tetracycline treatment was analyzed by western-blot in control cells transfected with the empty vector (pJL), with eIF2βWT or eIF2βΔ3K As expected, tetracycline induced the expression of the truncated form of eIF2β in the eIF2βΔ3K transfectants (Fig. 1B). In order to assess the effects of eIF2βΔ3K overexpression on protein synthesis, we analyzed the expression level of dEGFP as a marker of protein synthesis by microscopy and flow cytometry. eIF2βΔ3K expression led to a significant decrease in dEGFP expression after 24h whereas exogenous expression of eIF2βWT did not affect dEGFP expression (Fig. 1C). This was quantitatively confirmed by flow cytometry as dEGFP expression levels decreased drastically. The stable transfectants were first cell sorted based on their dEGFP expression level to obtain a homogeneous dEGFP expression range of each one. Cells were then treated with tetracycline for up to 96 h. Protein synthesis rate as reflected by dEGFP expression decreased approximately 80% at each of the time points for the eIF2βΔ3K plasmid but not for the eIF2βWT plasmid (Fig. 1D) based on the mean fluorescence intensity of each sample. A representative histogram at 96 h of tetracycline treatment is shown in Fig. 1E.


A
KKKKKKKKK
KKKKKKTKK
KKKKKKNVK
C2
C2
eIF2βWT
eIF2βΔ3K
Polylysine stretches (14-21, 79-87, 124-129 aa)
C2-C2 zinc finger motif (281, 284, 302, 305 aa)
B
control
control
eIF2βΔ3K
eIF2βΔ3K
eIF2βWT
eIF2βWT
eIF2β
TetR
Tetracycline
- + - + - +


C
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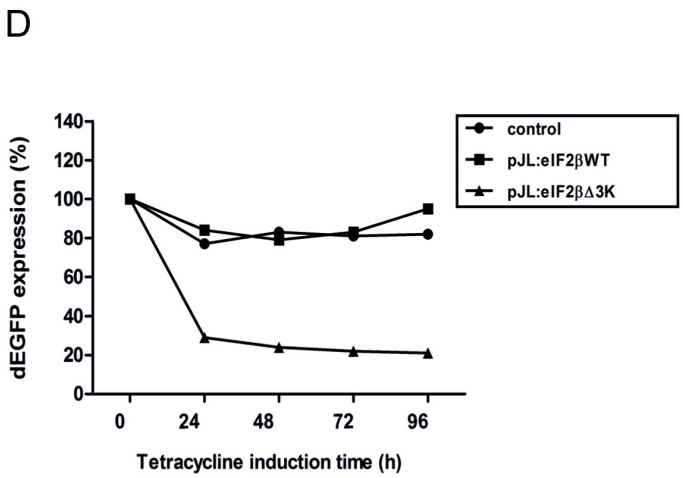
eIF2βWT
eIF2βΔ3K
Tetracycline induction time
0h
24h
48h
72h
96h


D
dEGFP expression (%)
140
120
100
80
60
40
20
0
0
24
48
72
96
Tetracycline induction time (h)
control
pJL:eIF2βWT
pJL:eIF2βΔ3K

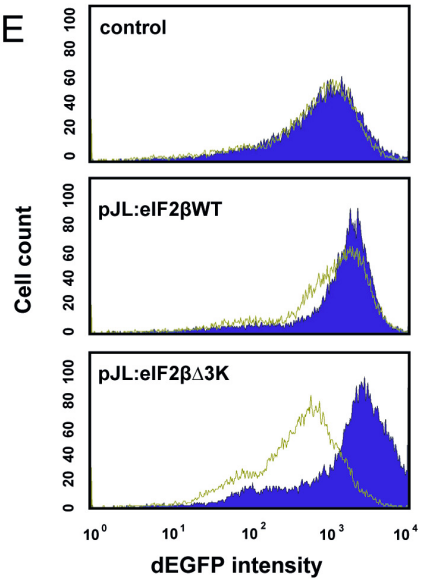
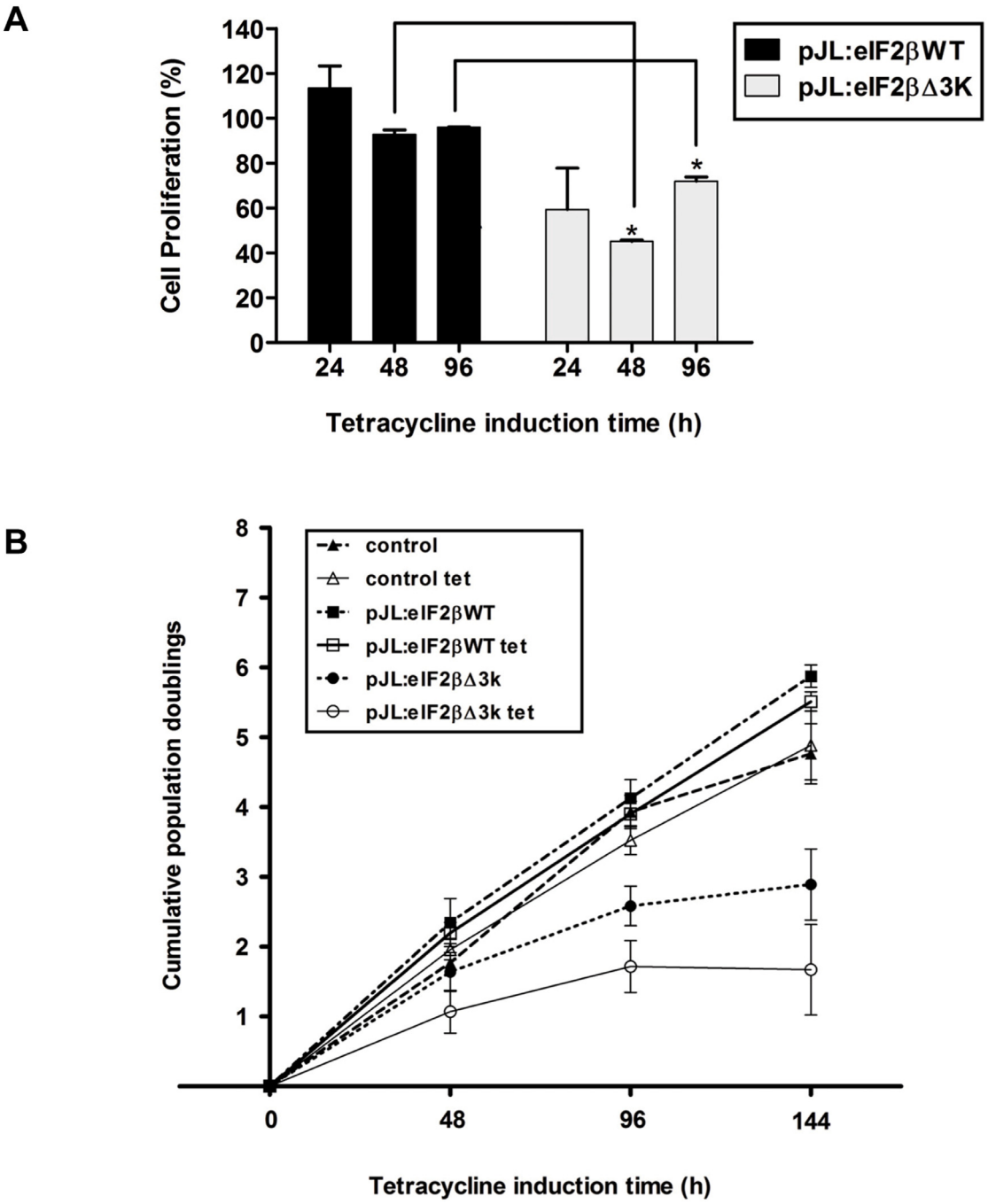

E
control
pJL:eIF2βWT
pJL:eIF2βΔ3K
Cell count
0 20 40 60 80 100
10^0
10^1
10^2
10^3
10^4
dEGFP intensity

Figure 1. eIF2βΔ3K expression decreases protein synthesis. (A) Domain structure of eIF2β contains conserved sequences. Black boxes indicate a polylysine stretches and dotted boxes indicate a cysteine zinc finger domain. (B) eIF2β expression after tetracycline induction. The whole protein cell extract of 10^5 Hek293TetR cells containing pJL (empty vector), pJL:eIF2βWT or pJL:eIF2βΔ3K. were loaded onto an SDS-PAGE gel and subjected to western blot analysis with anti-eIF2β (sc-133209) and anti-TetR. The second line of each construction shows cells that were induced with 1 μg/ml tetracycline for 72 h. The immuno-blot was probed with antibody against protein tetracycline repressor (TetR) to control for protein loading and to show the constitutive TetR expression in the Hek293 cell line. (C) eIF2β expression was induced by tetracycline (1 μg/mL) for 24 to 96 h in Hek293TetR cells containing pJL, pJL:eIF2βWT or pJL:eIF2βΔ3K. Protein synthesis qualitative analysis using dEGFP expression was performed by fluorescence microscopy. (D) After cell sorting, protein synthesis was quantitated by measuring dEGFP expression using flow cytometry in Hek293TetR cells expressing pJL, pJL:eIF2βWT or pJL:eIF2βΔ3K for 24, 48, 72 and 96 h. (E) Flow cytometry representative graph of 96 h tetracycline induction showing the dEGFP expression shift as a consequence of eIF2βΔ3K overexpression. The dEGFP expression results are expressed as percentage (mean) with respect to the time zero (no tetracycline induction) value of the corresponding constructs. pJL= control. The scale bars represent 100 μm.

Deletion of the polylysine stretches in eIF2β decreases cell proliferation and viability

Since overexpression of eIF2βΔ3K reduces protein synthesis, we further analyzed the effect of this truncated form on cell proliferation and viability. Hek293TetR cells containing pJL, pJL:eIF2βWT or pJL:eIF2βΔ3K were sorted as previously described. Cell proliferation was then measured by (methyl-^{3}H)thymidine incorporation. eIF2βΔ3K overexpression led to a decrease of 40%, 55% and 20% in (methyl-^{3}H)thymidine incorporation after 24, 48 and 96 h of tetracycline treatment, respectively (Fig. 2A). This indicates that eIF2βΔ3K overexpression leads to a 40% mean decrease in cell proliferation. Population doubling analysis showed that eIF2βΔ3K expression impairs cell growth in comparison to both empty vector and eIF2βWT expressing cells (Fig. 2B). Deletion of the polylysine stretches in eIF2β also impairs cell viability significantly (Fig. 2C), as

eIF2βΔ3K overexpression led to a 30% decrease in cell viability after 96 h of tetracycline treatment.



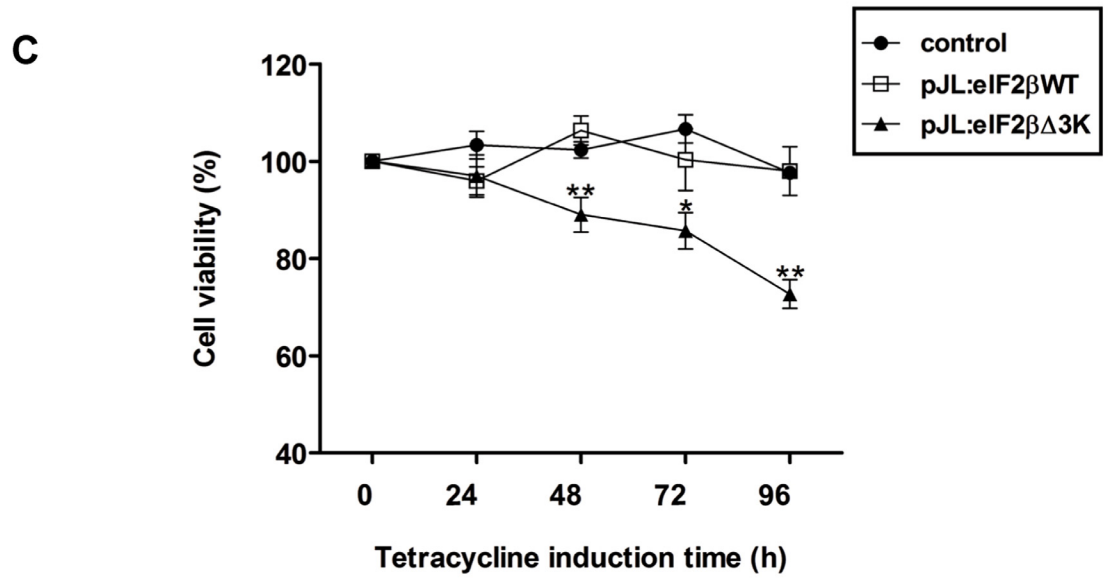


Figure 2. Deletion of the polylysine stretches affects cell viability and proliferation. Gene expression was induced by tetracycline (1 μg/mL) in Hek293TetR cells containing pJL (empty plasmid), pJL:eIF2βWT or pJL:eIF2βΔ3K. (A) Cells were selected by cell sorting to obtain similar dEGFP expression range. Proliferation of the selected cells was analyzed by (methyl-3H)thymidine incorporation after 24, 48 and 96 h of tetracycline induction. The proliferation results are expressed as the percentage (mean ± SEM) with respect to empty plasmid at each time point. (B) Cumulative population doublings of cells treated or untreated with tetracycline. (C) Cell viability was analyzed using XTT method after 24 to 96 h of tetracycline induction. The viability results are expressed as the percentage (mean ± SEM) with respect to the time zero value of the corresponding constructs. ANOVA test was performed on three independent experiments: * indicates $P < 0.05$ and ** indicates $P < 0.01$. pJL = control. Tet = tetracycline treatment.

eIF2β is present in the nucleolus of human cell lines

In silico analysis using MultiLOC [37] and PSORTII [38] subcellular prediction algorithms indicated that eIF2β might be present in the nucleus. We therefore characterized the subcellular localization of eIF2β in three distinct cell lines. Although eIF2β is predominantly cytoplasmic, nucleolar localization of eIF2βWT was clearly observed in HEK293 (Fig. 3A), MCF-7 (Fig. 3B) and HeLa cells (Fig. 4A). Moreover, upon

overexpression of eIF2βΔ3K, nucleolar localization was abrogated in all three cell lines (Fig. 3A, 3B and 4A).

Since the four cysteines residues in the carboxyl-terminal region of eIF2β forming a zinc finger domain (C_2-C_2 motif) (Fig. 1A) are evolutionarily conserved, we investigated whether they play a role in nucleolar localization. For this purpose, further deletion mutants of eIF2β were generated (Fig. 4A, see materials and methods). We first observed that deletion of the last two cysteines of the zinc finger of eIF2βWT (eIF2βΔC_2) strongly decreased nucleolar localization of eIF2β (Fig. 4A and B). This was confirmed with two comparable constructs with full N-terminal deletions (eIF2βCT and eIF2βCTΔC_2). Here again, the presence of the zinc-finger motif was sufficient for eIF2βCT localization to remain totally nuclear with a clear nucleolar recruitment, whereas the mutant form lacking this motif (eIF2βCTΔC_2) abolishes nucleolar but not nuclear localization (Fig. 4A and B). The N-terminal region of eIF2β alone in its native form (eIF2βNT) localized exclusively to the cytoplasm, whereas the N-terminal region of eIF2β with its polylysine stretches deleted (eIF2βNTΔ3K) displayed a strong cytoplasmic localization as well as a discrete nuclear distribution (Fig. 4A and B). Taken together, these results revealed that the polylysine motifs could control the nuclear and nucleolar localization whereas the zinc-finger motif could regulate only the nucleolar localization of eIF2β.

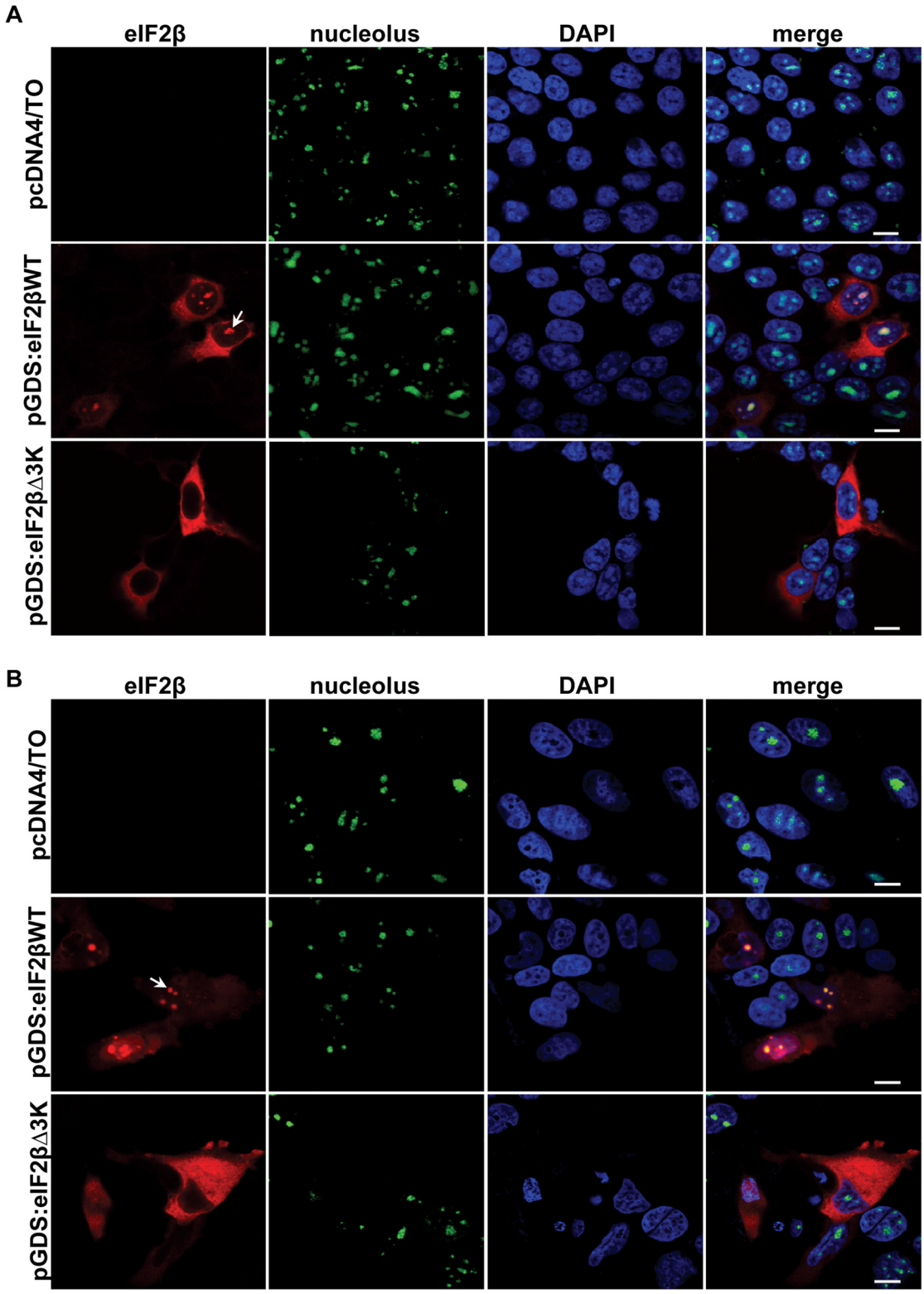
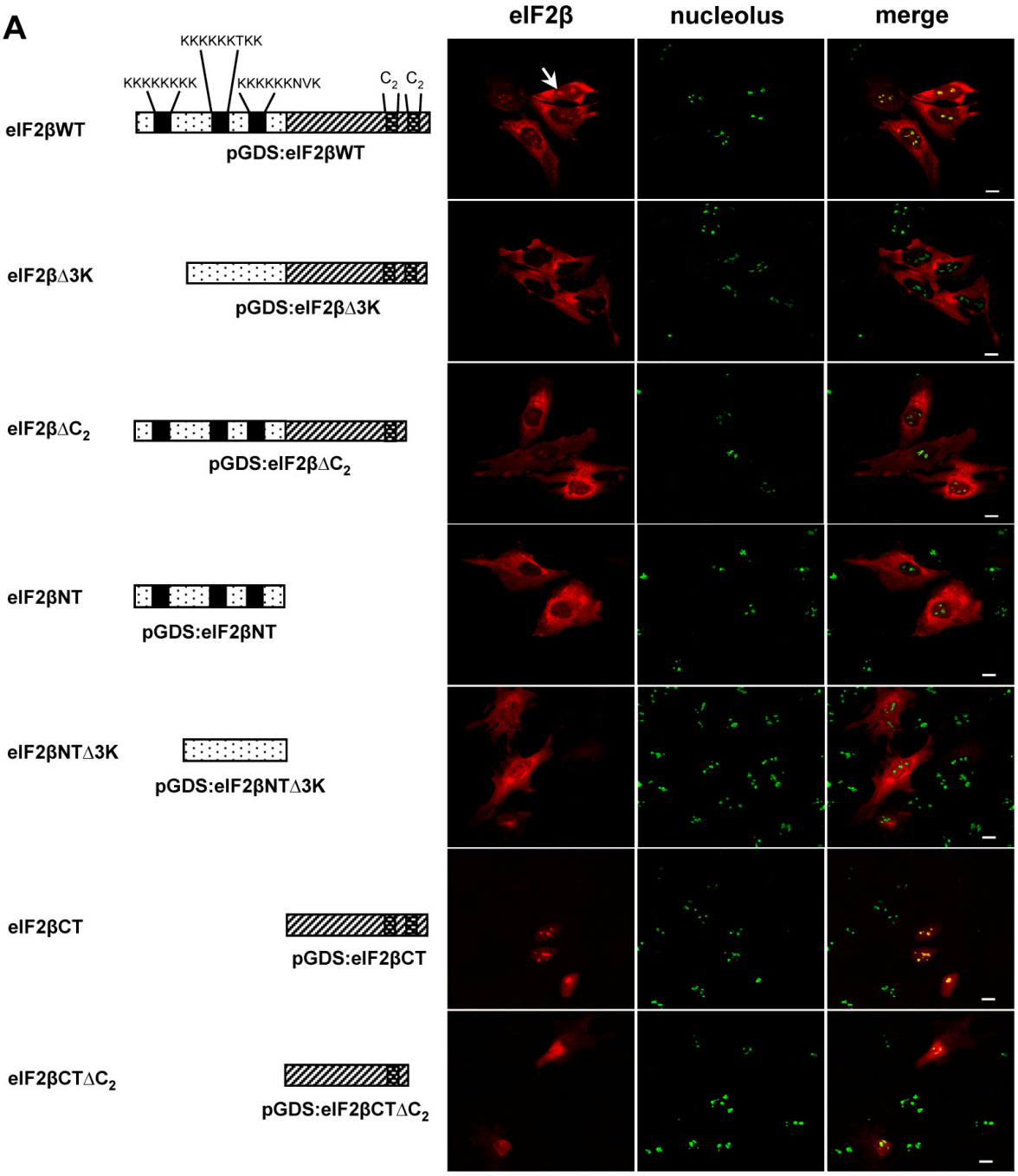


Figure 3. eIF2βWT also exhibits nuclear and remarkable nucleolar localization in human cell lines. (A) Hek293 cells were transfected with empty vector pcDNA4/TO and plasmids expressing the eIF2β forms pGDS:eIF2βWT (eIF2βWT) and pGDS:eIF2βΔ3K (eIF2βΔ3K). Twenty-four hours after transfection, cells were fixed in 4% formaldehyde, permeabilized in 0.5% Triton X-100 and submitted to immunocytochemistry using an anti-

eIF2β monoclonal antibody (moAb) sc-133209 and anti-nucleolus human serum. DAPI was used to stain the nucleus. The subcellular localization was analyzed by confocal microscopy. (B) MCF7 cells were transfected, fixed and analyzed as described above. Arrow shows eIF2β nucleolar staining. The scale bars represent 10 μm.



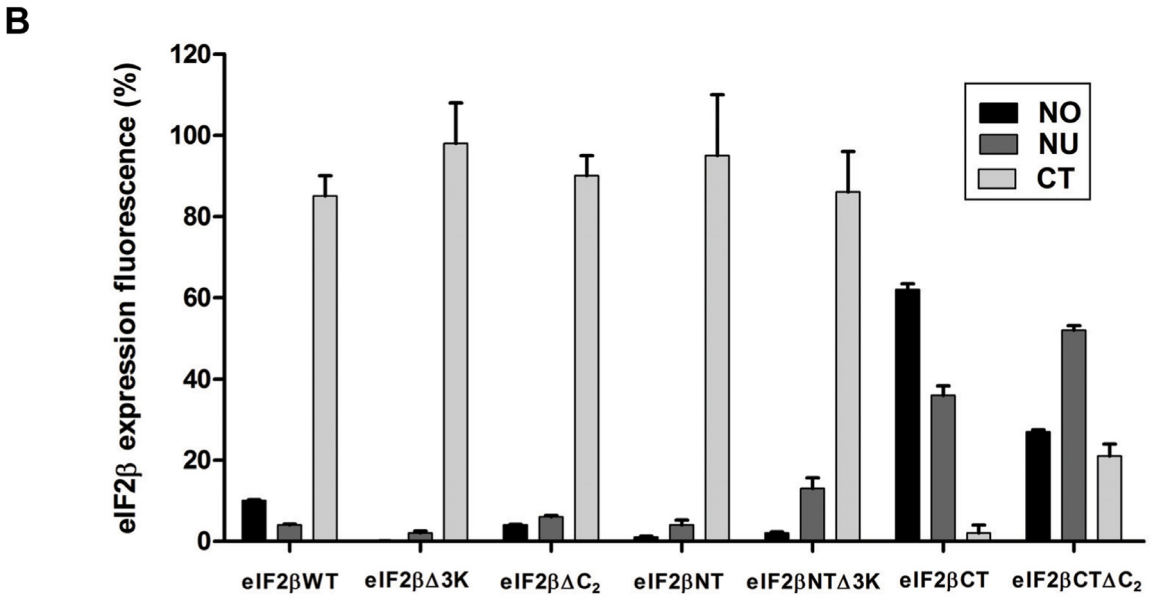


Figure 4. Qualitative and quantitative analysis of the subcellular localization of different forms of eIF2β. (A) To explore the structural features associated with the cytoplasmic, nuclear and nucleolar location of eIF2β protein, different constructs of eIF2β were created and named eIF2βWT, eIF2βΔ3K eIF2βΔC_2, eIF2βNT, eIF2βNTΔ3K, eIF2βCT, eIF2βCTΔC_2. HeLa cells were transfected with plasmids containing the different forms of eIF2β. Twenty-four hours after transfection, cells were fixed in 4% formaldehyde, permeabilized with 0.5% Triton X-100 and submitted to immunocytochemistry using anti-nucleolus human serum and moAb anti-eIF2β sc-133209 to stain all constructed with exception the eIF2βNT and eIF2βNTΔ3K for each it was used the moAb anti-eIF2β SC-9978. Fluorescence analysis was performed by confocal microscopy. This experiment was repeated three times with similar results (*n*= 85, 17, 61, 13, 9, 73, 75). (B) The histogram shows quantitative analysis of the subcellular localization of these eIF2β constructs. The error bars represent the percentage values of red fluorescence mean signal of eIF2β detection ± SEM, normalized by background and adjusted by cellular volume of nucleolus (NO), nucleus (NU) and cytoplasm (CT). The mean total fluorescence (sum of nucleolus, nucleus and cytoplasm red fluorescence values) was taken as 100%. Arrow shows eIF2β nucleolar staining. The scale bars represent 10 μm.

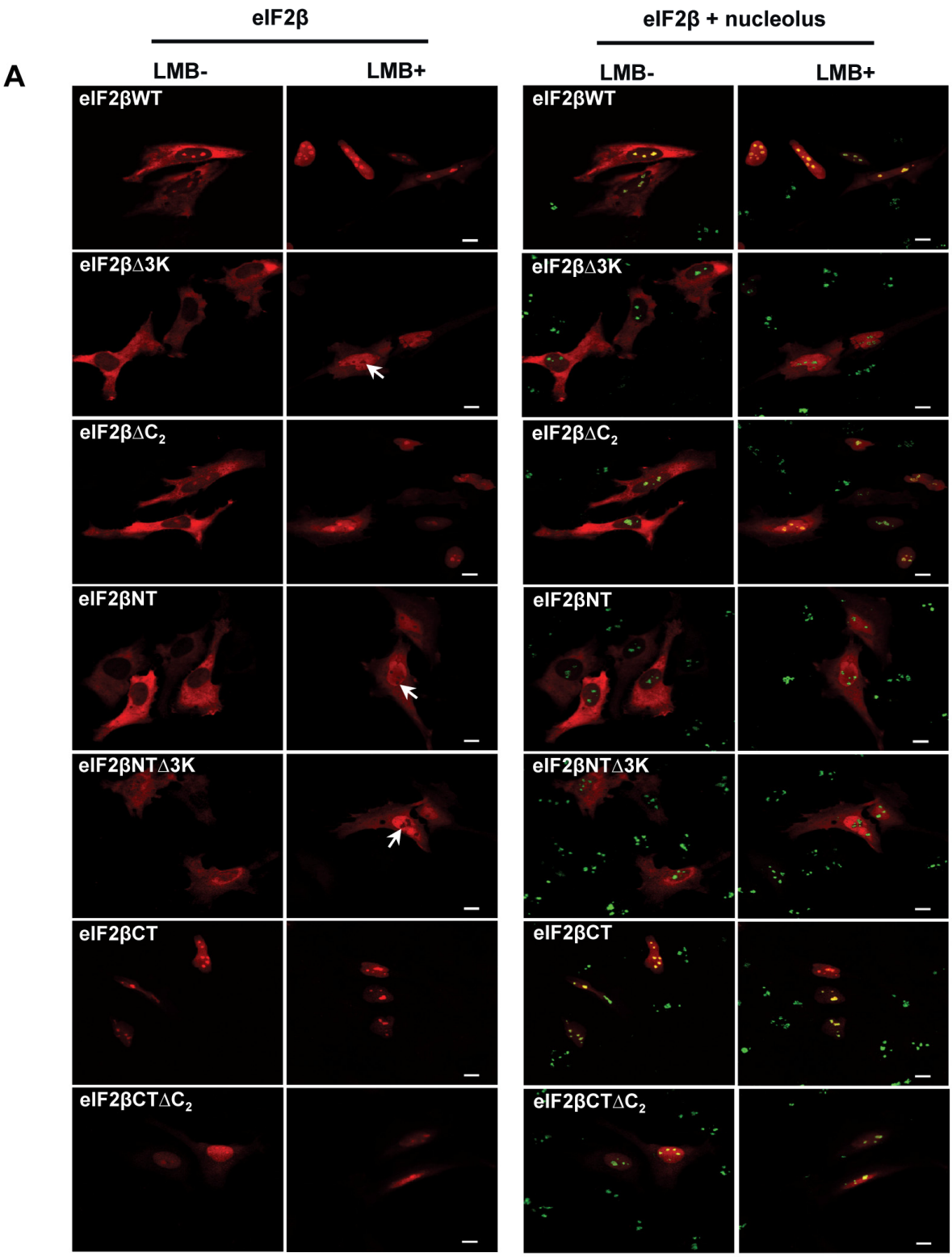
The amino-terminal region of eIF2β is essential for its nuclear export by CRM1

The nuclear export receptor CRM1 (also known as exportin-1) interacts with leucine-rich NES found in a large variety of proteins [39]. Upon treatment with leptomycin B (LMB), a specific inhibitor of CRM1, the localization of endogenous eIF2β shifted from a strictly cytoplasmic to a predominantly nuclear distribution [26]. LMB-treatment of the

eIF2βWT-transfected cells changed the cytoplasmic distribution to an exclusively nuclear and nucleolar localization (Fig. 5A), indicating that CRM1 does indeed affects export of eIF2β from the nucleus. In contrast, the eIF2βCT form lacking the polylysine stretches is spontaneously localized to the nucleus and nucleolus, independently of the LMB treatment. Moreover, the eIF2βNT form, which displays a cytoplasmic distribution in untreated cells, behaved similarly to the WT-form as it became nuclear (but not nucleolar) upon LMB-treatment (Fig. 5A). Together, these results indicate that the amino-terminal region of eIF2β is necessary for its nuclear export by CRM1.

Both the polylysine stretches and the C_2-C_2 motif are necessary for eIF2β nucleolar localization

After LMB treatment, eIF2βWT was detected exclusively in the nucleus and nucleolus whereas the nuclear eIF2βΔC_2-localization was less nucleolar under the same conditions, with a residual cytoplasmic distribution. For the eIF2βΔ3K construct, LMB treatment, led to a predominantly nuclear but not nucleolar localization (Fig. 5A, arrow). The eIF2βCT form remained detectable only in the nucleus and nucleolus, whereas the eIF2βCTΔC_2 form was found predominantly in the nucleus, but was extremely reduced in the nucleolus after LMB treatment (Fig. 5A). After LMB treatment, eIF2βNT and eIF2βNTΔ3K were detected in the cytoplasm as well as in nucleus, but they were absent from the nucleolus (Fig. 5A, arrows). Together these results indicate that the polylysine stretches and C_2-C_2 motif are participating synergistically in the nucleolar localization of eIF2β, as can be concluded from Fig. 5B which summarizes the observed changes in subcellular distribution of the eIF2β constructs upon LMB treatment.


A
eIF2β
eIF2β + nucleolus
LMB-
LMB+
LMB-
LMB+
eIF2βWT
eIF2βΔ3K
eIF2βΔC2
eIF2βNT
eIF2βNTΔ3K
eIF2βCT
eIF2βCTΔC2
eIF2βWT
eIF2βΔ3K
eIF2βΔC2
eIF2βNT
eIF2βNTΔ3K
eIF2βCT
eIF2βCTΔC2

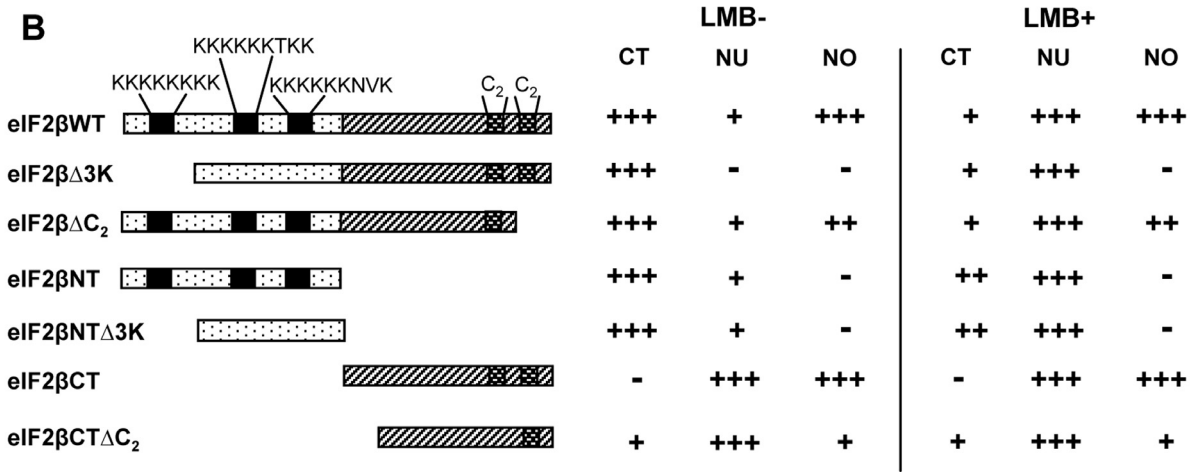


Figure 5. LMB affects the subcellular localization of eIF2β constructs. (A) HeLa cells were transfected with plasmids containing different forms of eIF2β. Twenty-four hours after transfection, cells were treated with LMB 5 ng/mL for 1 h at 37° C and 5% CO_2 and were fixed in 4% formaldehyde, permeabilized in 0.5% Triton X-100 and submitted to immunocytochemistry with moAb anti-eIF2β sc-133209 to stain all constructed with exception the eIF2βNT and eIF2βNTΔ3K for each it was used the moAb anti-eIF2β SC-9978 and anti-nucleolus human serum. The left panel shows eIF2β stained by moAb anti-eIF2β. The right panel shows eIF2β and nucleolar stain by anti-eIF2β and anti-nucleolus human serum respectively. Fluorescence images were collected by confocal microscopy. Arrow shows eIF2β nucleolar negative staining. (B) Summary of the subcellular localization of eIF2β constructs without (LMB-) and with LMB treatment (LMB+) is shown. +, low level of localization; ++, moderate level of localization; +++, high level of localization; -, without localization. Cytoplasmic localization (CT), nuclear localization (NU) and nucleolar localizaton (NO). The scale bars represent 10 μm.

RNA is required for nucleolar localization of eIF2β

eIF2β's main functions in the initiation translation process in the cytoplasm is to bind mRNA and facilitate AUG initial codon recognition. We are focusing on the lysine stretches and the zinc finger motifs that are known to be nucleic acid-binding motifs [8, 18]. Since the nucleolus is the major site of transcription of rRNA and of the processing of

rRNA into pre-ribosomal particles [40, 41], we examined the possible binding role of eIF2β to RNA in the nucleolus.

Using confocal microscopy, we analyzed the effect of RNAse digestion on eIF2βWT localization in HeLa cells. RNAse A treatment of permeabilized transfected HeLa cells extinguished the nucleolar distribution of eIF2β, but not its cytoplasmic localization (Fig. 6). Control experiments demonstrated that RNAse A digestion also extinguished the nucleolar labeling by anti-nucleolus specific antibody without affecting neither DNA nor the nuclear structure as seen with the DAPI counter-stain (Fig. 6). Together, these data indicate that the nucleolar localization of eIF2β is stabilized by its interaction with RNA.

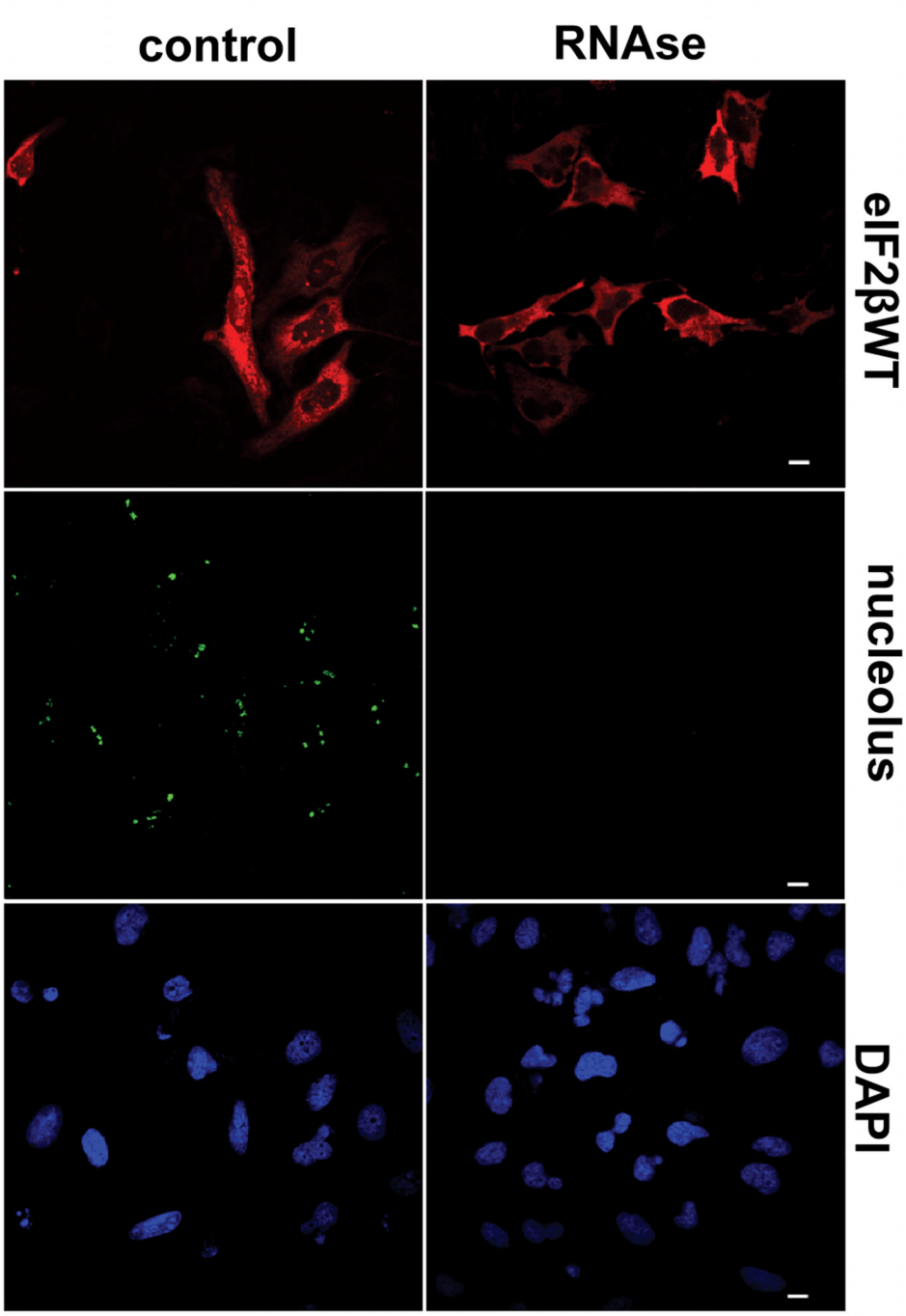


Figure 6. RNA is required for nucleolar localization of eIF2β. HeLa cells were grown on microscopic slides and transfected with pGDS:eIF2βWT. Twenty-four hours after transfection, cells were fixed with methanol (5 min, -20°C) followed by acetone (2 min, -20°C) and permeabilized with 0.1% Triton X-100 for 3 min on ice. The digestion with 0.4 mg/ml RNAse A was performed in PBS for 60 min at 37°C. The cells were washed in PBS and processed for immunocytochemistry using moAb anti-eIF2β sc-133209. Control = Buffer without enzyme. The anti-nucleolus human serum was used to monitor the efficiency of RNAse A digestion. DAPI was used to stain DNA. The scale bars represent 10 μm.

DISCUSSION

The eIF2β protein contains two evolutionarily conserved regions consisting of three stretches of polylysine residues and a C_2-C_2 zinc finger motif. Since the polylysine stretches are implicated in the binding of eIF2β to eIF5, eIF2Bε and mRNA [8, 10, 11, 21], we hypothesized that their deletion may affect the multifactor complex formation [21], and thus the global translation processes. Expression of human eIF2β in which the polylysine stretches were deleted causes a decrease in the rate of protein synthesis which is comparable the classic protein synthesis inhibitor, anisomycin (data not shown). This is in agreement with data from yeast in which the polylysine stretches of eIF2β were deleted [8, 11]. In addition, our data confirms with extended accuracy previous data in humans, where full deletion of the amino-terminal region of eIF2β had been reported to decrease translation rate [25]. Finally the reduction in cell proliferation and cell viability upon expression of eIF2βΔ3K indicates that this truncated protein functionally acts as a dominant negative inhibitor of protein synthesis, competing with the endogenous form of eIF2β, and thereby affecting cell fate. This is particularly relevant since heterozygous deletion of eIF2β has a reported anti-tumoral effect and homozygous deletion of eIF2β leads to embryonic death [42].

Since translation is a process taking place in the cytoplasm, eIF2β is not classically associated with a nuclear localization. However, *in silico* analysis revealed that eIF2β displays features of a nuclear protein and that the polylysine stretches could thus act as nuclear localization signal (NLS). Moreover, eIF2β, together with the other two subunits of eIF2, and other eukaryotic initiation factors such as eIF4A, eIF5A and eIF6 have been identified as nucleolar in HeLa cells by proteomic approaches [43, 44] (Nucleolus Proteome Database -http://www.lamondlab.com/NOPdb/).

Experimentally, we observe that eIF2βWT-overexpression leads to a translocation of the protein to the nucleus, with a strong accumulation in the nucleolus, and find that this nuclear localization of eIF2β is abrogated upon deletion of the polylysine stretches. Surprisingly, when we block the nuclear export with LMB, eIF2βΔ3K, the eIF2β construct lacking polylysine stretches, accumulate in the nucleus, although with slower kinetics than the WT counterpart. This indicates that eIF2β nuclear translocation is strongly but not exclusively regulated by the polylysine stretches which act as an NLS, and further, that the residual nuclear translocation of eIF2β could rely on mechanisms involving other domains of the protein. Such candidate-domains could be one of eIF2βs' four putative Nuclear Translocation Signals (NTS) (Fig. S1 in the supplemental material). The NTS has been recently described as composed of Ser/Thr-Pro-Ser/Thr amino acid sequence in which at least one of the Ser/Thr has to be phosphorylated or replaced by an acidic residue (Asp or Glu) [45]. Amongst the four putative NTSs of eIF2βs, both the amino-terminal motif 09-Asp-Pro-Thr-11, and the carboxyl-terminal motif, 286-Ser-Pro-Asp-288, are highly conserved. This carboxyl-terminal motif could explain the residual nuclear localization of the eIF2β up on LMB-treatment observed after deletion of the polylysine stretches or of the full N-terminal sequence.

Overall, the nuclear localization of eIF2βWT in cells treated with LMB indicates that it actively shuttles through the nucleus in a CRM1-dependent manner. Indeed, LMB-treatment led to a clear nuclear distribution of eIF2β for each of the eIF2β constructs we present in this study, except for eIF2βCT, suggesting that the amino-terminal motif could also harbors the Nuclear Export Signal (NES). Indeed, eIF2β has two quite conserved motifs in Chordata that resemble a NES (98-**V**KD**L**K**I** -103 and 117-**L**DM**I**M**L**-122) [46] (Fig. S1 in the supplemental material).

Furthermore, our data indicates that the nucleolar localization of eIF2β is strongly dependent on the last two conserved cysteines. Indeed, the carboxyl-terminal region eIF2β contains a conserved C_2-C_2 zinc finger motif, these domains having been described to possess nucleolar targeting abilities [47, 48]. The deletion of the two terminal cysteines of eIF2β leads to drastic reduction in its nucleolar distribution, indicating that this zinc finger domain is also implicated in the nucleolar localization of eIF2β. However, deletion of the polylysine stretches also leads to exclusion of eIF2β from the nucleolus in the course of its accumulation into the nucleus upon LMB treatment. The polylysine stretches are thus implicated both in nuclear and nucleolar localization. This finding is supported by data reporting that a lysine rich region in NF-κB inducing kinase (NIK) acts as both as nucleolar and nuclear signal for that molecule [49]. In this way, both polylysine stretches, as C_2-C_2 zinc finger motif are required for nucleolar localization of eIF2β.

The nucleolus is a sub-nuclear compartment where rRNA synthesis and maturation as well as assembly of rRNA into ribosomal particles occur. Our data indicates that eIF2β nucleolar localization is dependent upon its RNA binding. Nucleolar integrity is thus required for eIF2β accumulation at the nucleolus, indicating that it might have nucleolar function involving its binding to RNA. This is consistent with reports showing that nucleolus site is implicated in gene silencing, cell cycle regulation, viral replication, signal recognition particle assembly, senescence, modification of small RNPs, nuclear export pathways, tRNA processing, p53 regulation and telomerase function [40, 41, 50]. Our data are also consistent with the proposed model where eIF2β, in conjunction with other initiation factors, may perform the pioneer round of translation in the nucleus as a proof-reading mechanism of newly synthesized mRNA, followed by a nonsense-mediated mRNA decay to degrade mRNAs containing premature termination codons [34, 36, 51].

Taken together, our data report extensively that eIF2β expression actively controls protein synthesis, and consequently interfere in the cell division and survival. Our main finding are that eIF2β's polylysine stretches are essential in this process and that eIF2β shuttles through the nucleolus. It has been reported that increased eIF2 activity induces neoplastic transformation and is associated with several cancers *in vivo* [52], whereas some potential anti-cancer drugs have been suggested to act through eIF2 regulation thus reducing protein translation rate and cell cycle progression [53]. This is consistent with our data where expression of the truncated form of eIF2β decreased the availability of active eIF2 complexes and reduced the rate of translation initiation, cell proliferation and viability, in addition to modifying subcellular eIF2β localization. Despite this nuclear shuttling, the alterations of eIF2β cytoplasmic functions on the translation process should still be considered as the major factor affecting cell proliferation and viability. We therefore propose that the polylysine stretch domains of eIF2β should be considered as promising targets for therapeutical strategies where an efficient antiproliferative effect is necessary.

SUPPLEMENTARY FIGURES

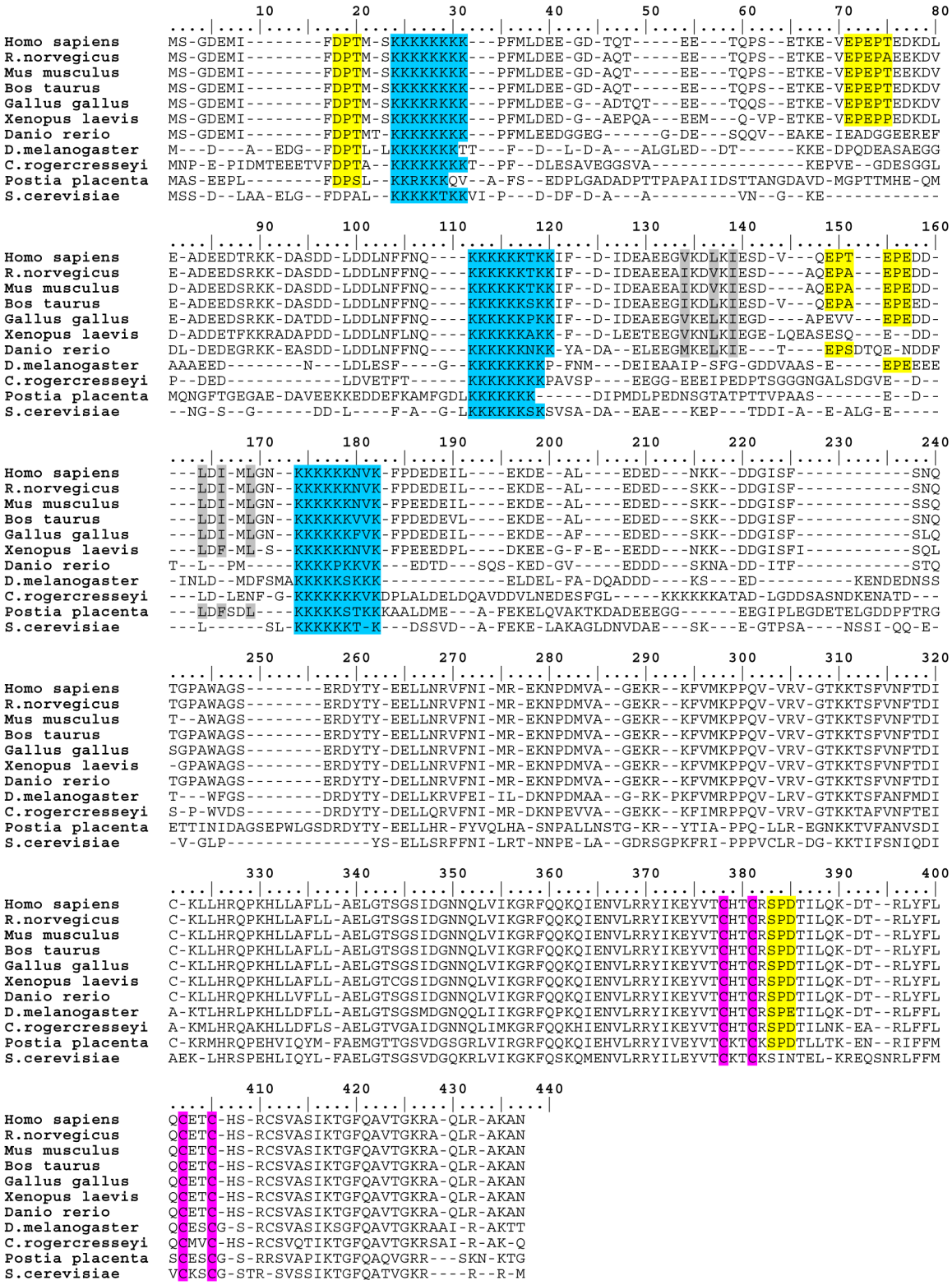


Figure S1. Alignment of eIF2β protein sequences. Sequences of different eukaryotic species were aligned: *Homo Sapiens*; *Rattus novergicus* (*R. novergicus*) ; *Mus musculus*; *Bos Taurus*; *Gallus gallus*; *Xenopus laevis*; *Danio rerio*; *Drosophila melanogaster* (*D. melanogaster*); *Caligus rogercresseyi* (*C. rogercresseyi*); *Postia placenta*; *Saccharomyces cerevisiae* (*S. cerevisiae*). Polylysine stretches are highlighted in blue and C_2-C_2 zinc finger motif in pink. Sequences of potential NTSs (S/T-P-S/T similar motifs) are highlighted in yellow and potential NESs in grey.

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AUTHOR CONTRIBUTIONS

G.D.S. planned all experimental designs, performed all experiments, analyzed and interpreted all data, and wrote the paper. C.C.L. and J.P.L. planned the experimental design, performed some experiments, analyzed and interpreted all data, and wrote the paper. G.L. planned some experiments, interpreted the data and wrote the paper. N.O.M. developed and analyzed fluorescence quantification and statistical data. N.S. and M.C planned and performed some flow cytometry experiments. R.M.X. and E.C.L. planned some experiments. J.A.P.H. planned the experimental design, interpreted the data and wrote the paper.

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4. CAPÍTULO II

4.1 APRESENTAÇÃO

Esse capítulo apresenta resultados gerados nessa tese que não foram inseridos no Capítulo 1, mas que são importantes para a dissussão do tema desenvolvido nesse trabalho. Esse resultado mostra a comparação entre a diminuição da síntese proteica global em células humanas causada pelo inibidor clássico, anisomicina, com aquela causada pela superexpressão da proteína eIF2β desprovida dos blocos de lisinas. São descritas nessa seção a metodologia utilizada, bem como a descrição do resultado.

4.2 Materiais e Métodos

Células Hek293TetR transfectadas com os plasmídeos pJL (plasmídeo vazio), ou pJL:eIF2βWT (plasmídeo expressando eIF2β selvagem) ou pJL:eIF2βΔ3K (plasmídeo expressando eIF2β desprovido dos blocos de lisinas), os quais expressavam também a proteína dEGFP, foram selecionadas com 150 μg/mL de zeocina. Colônias de células expressando dEGFP foram isoladas. Após a seleção e isolamento de populações de células expressando cada plasmídeo, essas foram cultivadas em placas de 6 poços em uma densidade de 1,8 x 10^5 células por poço durante 24h. Após esse período, essas células foram tratadas com 1 μg/ml de tetraciclina por 24, 48, 72 e 96 h para induzir a expressão das formas de eIF2β. Em paralelo, células não tratadas foram cultivadas na mesma densidade e no mesmo intervalo de tempo e utilizadas como controle de indução de expressão. Células tratadas e não tratadas foram tripsinizadas, distribuídas em plascas de 96 poços e analisadas por citometria de fluxo. Dez mil eventos foram adquiridos em citômetro de fluxo Guava Easy Cyte Plus System, (Guava Technologies, Millipore, MA, USA) e a expressão de dEGFP foi analisada.

4.3 Resultados

Para avaliar o efeito da superexpressão de eIF2βΔ3K nos níveis de síntese proteica, a expressão de dEGFP foi utilizada como marcador de síntese proteica e analisada por citometria de fluxo. A figura 5 mostra que a superexpressão de eIF2βΔ3K causa uma diminuição de 65% nos níveis de expressão de dEGFP em todos os tempos testados. Essa diminuição é quase tão acentuada quanto à diminuição de 80% causada pelo inibidor clássico do processo de tradução, anisomicina.

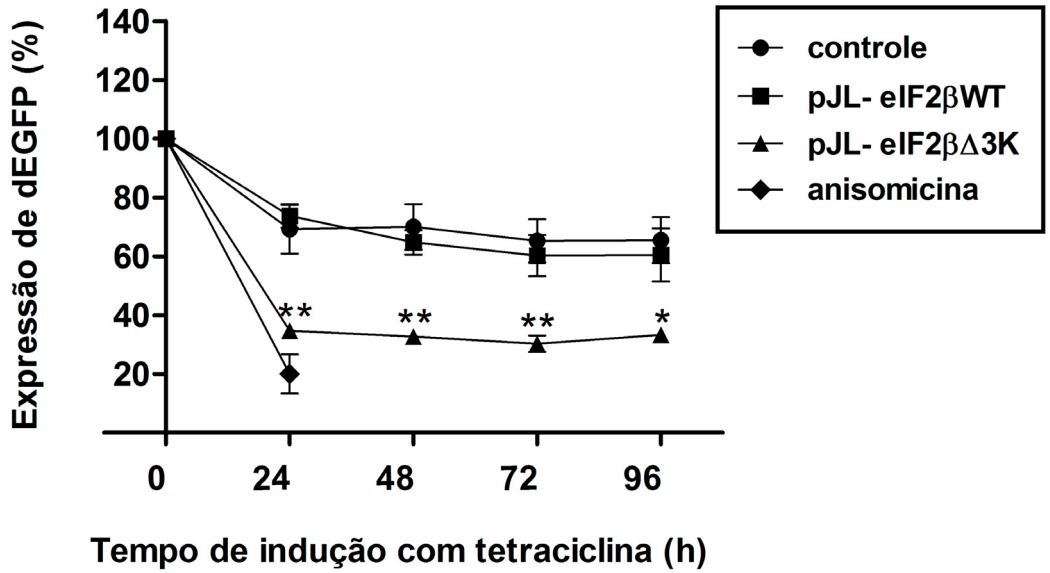

Figura 5. Expressão de eIF2βΔ3K reduz a taxa de síntese proteica. A expressão de eIF2β foi induzida por tetraciclina (1 μg/mL) por 24 a 96h em células Hek293TetR expressando ou o controle (plasmídeo vazio), ou o plasmídeo contendo o cDNA selvagem de eIF2β (pJL:eIF2βWT), ou o plasmídeo contendo o cDNA de eIF2β desprovido dos blocos de lisinas (pJL:eIF2βΔ3K). A síntese proteica foi quantificada baseada nos níveis de expressão de dEGFP por citometria de fluxo. Células Hek293TetR foram tratadas com 5 μg/mL de anisomicina por 24h e foram usadas como controle positivo de inibição da síntese proteica. Os resultados são expressos como percentual (média ± DP) relativo ao valor do tempo zero (sem indução com tetraciclina) de cada construção. O teste de ANOVA foi realizado considerando três experimentos independentes. * indica $P < 0.05$ e ** indica $P < 0.01$.

5. DISCUSSÃO

DISCUSSÃO

5.1 Efeito citostático e citotóxico da superexpressão de eIF2β desprovido do blocos de lisinas

A proteína eIF2β apresenta resíduos extremamente conservados na sua porção aminoterminal caracterizados por três blocos de lisinas. Em *S. cerevisiae*, um efeito letal da deleção desses resíduos foi demonstrado por Asano *et al*. (1999) e Laurino *et al*. (1999). Em células humanas, apesar de Llorens *et al*. (2006) terem demonstrado que a deleção da região aminoterminal de eIF2β diminui a viabilidade celular, o efeito isolado da deleção dos blocos de lisinas para essas células ainda não havia sido explorado até o momento. Assim, esse trabalho, pela primeira vez, evidenciou que a superexpressão de eIF2β desprovido desses três blocos de lisinas (eIF2βΔ3K) é capaz de diminuir a síntese proteica em células humanas (Capítulo 1, Figura 1C e 1D), de tal forma que causa uma acentuada queda na proliferação (Capítulo 1, Figura 2A) e viabilidade dessas células (Capítulo 1, Figura 2C). A diminuição da síntese proteica causada pela superexpressão de eIF2βΔ3K é comparável àquela causada pelo inibidor clássico de síntese proteica, anisomicina, como pode ser observado na Figura 5 (Capítulo 2).

Em relação ao mecanismo de início de tradução, as principais funções dos blocos de lisinas de eIF2β são permitir a ligação do complexo eIF2 ao mRNA, ao eIF5 e ao eIF2B (Kimball et al., 1998; Asano et al., 1999; Laurino et al., 1999). Nossos resultados mostraram que a deleção dessas lisinas é capaz de diminuir a síntese de proteínas em 75%, sugerindo então que as interações de eIF2, indispensáveis à formação do complexo de início de tradução (Singh et al., 2004), devem ser prejudicadas pela ausência dessas lisinas neste fator. Desse modo, diferentes passos fundamentais para o processo de início de tradução podem ser afetados, entre os quais: ligação a eIF5, que causa a hidrólise de GTP e

consequente liberação dos fatores eIF2, eIF3 e eIF1; eficiente ligação do complexo 43S ao mRNA; e reciclagem de eIF2/GTP por ação de eIF2B.

Além de seu papel no mecanismo do processo de início de tradução, mais recentemente tem-se mostrado que eIF2β, tal como a subunidade alfa, tem um papel regulatório no processo de início de síntese proteica. Sua função regulatória parece estar intimamente relacionada a alterações do estado de fosforilação de eIF2α e, consequentemente, ao bloqueio da síntese proteica em diferentes condições de estresse celular. Essa capacidade de regular o estado de fosforilação de eIF2α advém de sua capacidade de ligar-se a proteínas fosfatases. A ligação de eIF2β à proteína adaptadora NcK-1 (Kebache et al., 2002) impede a fosforilação da eIF2α e a consequente parada da tradução global em resposta a estresse do retículo endoplasmático de eIF2β (Kebache et al., 2004). Também, estudos *in vitro* mostraram que eIF2β interage com a proteína ser/thr-fosfatase-1 (PP1) através de um motivo RVxF adjacente ao seu terceiro bloco de lisinas e de um segundo sítio de ligação em sua região carboxiterminal (Wakula et al., 2006). Ao associar-se à fosfatase PP1, quando encontra-se fosforilado pelas enzimas CK2 (Ser^{2} e Ser^{67}) e PKA (Ser^{218}), o eIF2β torna-se um ativador de sua própria defosforilação (Wakula et al., 2006). A propriedade de ligação de eIF2β à PP1 também leva à inibição da defosforilação da serina 51 da subunidade alfa de eIF2. Essa interação não é um fator limitante da taxa de síntese proteica global, entretanto não pode ser excluída a ideia de exercer um controle traducional de um grupo restrito de mRNAs em condições específicas (Wakula et al., 2006).

Nesse contexto, além de ser fosforilado pelas cinases CK2 e PKA, eIF2β também apresenta sítios importantes de fosforilação *in vitro* para as cinases PKC (Welsh et al., 1994; Suragani et al., 2006) e DNA-PK (Ting et al., 1998). Em mamíferos, a fosforilação

desse fator ocorre em diferentes situações de estresse celular como choque térmico (Duncan & Hershey, 1984), privação de soro (Duncan & Hershey, 1985; Llorens et al., 2006), diabetes (Garcia et al., 1996) e durante o nascimento (Luis et al., 1993). Assim, a fosforilação de eIF2β deve ser um processo dinâmico e seu o estado de fosforilação deve ser também um ponto de controle do processo de início da tradução. Essa ideia é comprovada pelo estudo de Llorens et al. (2006), que demonstraram que a inibição da fosforilação das serinas 2 e 67 por CK2 diminui as taxas de síntese proteica. É relevante ressaltar que essa diminuição não é decorrente da falha na formação do trímero eIF2 ou na ligação a eIF5 e assim, deve advir de algum evento posterior à ação de eIF5 (Llorens et al., 2006). Esse conjunto de informações sugere, então, que eIF2β não é apenas uma proteína importante por sua propriedade de interação proteica, mas também por apresentar capacidade de regulação do processo de início de tradução.

Um estudo recente de Heaney et al. (2009) pontuou o papel essencial de eIF2β na sobrevivência de células de mamíferos. Esses autores relataram que a deficiência em homozigose (completa) do gene de eIF2β (EIF2S2) causa letalidade embrionária em camundongos e que a deleção em heterozigoze (parcial) reduz a proliferação de células germinativas de tumor testicular de camundongos. Esses resultados mostram a sensibilidade de células tumorais à disponibilidade de eIF2β (Heaney et al., 2009).

No Capítulo 1, foi evidenciado que a deleção de apenas poucos resíduos específicos da proteína é capaz de diminuir fortemente a proliferação e a viabilidade de células humanas (Capítulo 1, Figura 2A e 2C), mostrando a importância desses resíduos para a sobrevivência celular (Capítulo 1, Figura 2B) e que uma proteína eIF2β truncada pode atuar como dominante negativa em células humanas. Considerando que nesse estudo, mostrou-se que a viabilidade de células humanas é afetada pela disponibilidade de eIF2β

funcional, a modulação dessa molécula ativa torna-se um alvo potencial de terapias antiproliferação celular. Além disso, o papel essencial dos blocos de lisinas de eIF2β pode sugerir esses resíduos como possíveis alvos para o desenvolvimento de drogas que modulem a funcionalidade dessa molécula.

5.2 Distribuição subcelular de eIF2β

Para caracterizar a distribuição subcelular de eIF2β, primeiramente foram realizadas análises de bioinformática da predição da localização subcelular, as quais demonstraram que essa subunidade de eIF2 apresenta predição nuclear e apontou os blocos de lisinas como NLS da proteína (dados não mostrados). No Capítulo 1, foi demonstrado que eIF2β selvagem (eIF2βWT) superexpresso está localizado no citoplasma, mas é capaz de ser translocado para o núcleo e acumular no nucléolo (Figura 3A e 3B). Essa localização nucleolar é abolida quando os blocos de lisinas estão ausentes, bem como quando as cisteínas que formam as estruturas de dedos de zinco são deletadas (Capítulo 1, Figura 4A e 4B). Além disso, a localização nucleolar de eIF2β mostrou-se dependente de RNA, visto que o tratamento com RNAse A foi capaz de extinguir a presença de eIF2β no nucléolo (Capítulo 1, Figura 6). Também demontrou-se que eIF2β é excluído do núcleo pela exportina CRM1, uma vez que com o tratamento com o inibidor de CRM1, leptomicina B, o eIF2β torna-se exclusivamente nuclear e nucleolar (Capítulo 1, Figura 5A e 5B). Sua exclusão do núcleo deve ser mediada por um sinal de exclusão nuclear (NES) presente na região amino terminal, visto que a construção contendo apenas essa região da molécula foi capaz de confinar o eIF2β no citoplasma e a construção contendo apenas a região carboxiterminal apresenta exclusivamente localização nuclear e nucleolar, mesmo após o tratamento com leptomicina B (Capítulo 1, Figura 5A e 5B).

Além de sua importância para a síntese proteica e proliferação de células humanas, nosso trabalho identificou que os blocos de lisinas apresentam participação na translocação nuclear de eIF2β e são necessários para sua localização nucleolar. Os blocos de lisinas, provavelmente não são os únicos NLS de eIF2β, uma vez que, após a inibição da exportação nuclear da forma eIF2βΔ3K, essa fica retida no núcleo, demonstrando que mesmo com a ausência das lisinas, a proteína pode ser translocada ao núcleo. Outros possíveis NLS, denominados sinais de translocação nuclear (NTS) (Chuderland et al., 2008), são encontrados na proteína por comparação de similaridade de sequências (Capítulo 1, Figura S1). Esses resíduos de lisinas de eIF2β devem funcionar como um NLS, mas funcionam principalmente como um sinal de localização nucleolar. Essa idéia é suportada pelos resultados demonstrados por Birbach et al. (2004), nos quais regiões carregadas positivamente constituídas por lisinas atuam tanto como um NLS, quanto como um sinal de retenção nucleolar da proteína cinase induzida por NF-κB (NIK).

Muitas proteínas que apresentam transporte núcleo-citoplasmático contêm tanto NLS, quanto NES. Várias possibilidades parecem significativas para regular o equilíbrio entre os mecanismos de localização e exclusão nucleares. Primeiramente, um desses sinais de transporte núcleo-citoplasmático pode ser estruturalmente exposto e ficar mais acessível para as maquinarias de transporte. Também, modificações como fosforilação podem controlar as localizações subcelulares ou subnucleares de diferentes proteínas (Birbach et al., 2004; Niu et al., 2006). Desse modo, os blocos de lisinas de eIF2β são motivos de aminoácidos que podem ser expostos na superfície da molécula, sendo assim reconhecidos pela maquinaria de importação nuclear ou localização nucleolar. Sua exposição, então, poderia ser modificada para regular a distribuição subcelular ou subnuclear de eIF2β, por processos como fosforilação/defosforilação de resíduos adjacentes a eles. De fato, os sítios

de fosforilação para as cinases CK2 e PKC são adjacentes aos blocos de lisinas 1 e 2, respectivamente (bloco 1: M$\underline{S}^{2}$GDEMIFDPTM$\underline{S}^{13}$**KKKKKKKK**; bloco 2: $\underline{S}^{67}$DDLDDLNFFNQ**KKKKKKTKK**). Esses sítios compõem-se das serinas 2, 13 e 67 para a enzima CK2 e 67 para PKC. É interessante notar que as serinas 2 e 67 de eIF2β são fosforiladas constitutivamente por CK2 (Llorens et al., 2006). Além disso, a defosforilação de eIF2β fosforilado por CK2 é promovida pela proteína fosfotase-2A (Welsh et al., 1994) e pela PP1 (Wakula et al., 2006). Assim, considerando que eIF2β é fosforilado tanto constitutivamente quanto em situacões celulares específicas, pode-se considerar a possibilidade de que a fosforilação ou defosforilação de sítios adjacentes aos blocos de lisinas poderiam ser reguladas e, assim, poderiam expor ou ocultar esses resíduos na superfície da molécula, possibilitando ou não a importação da proteína ao núcleo.

Diversos estudos têm demonstrado que motivos de dedo de zinco possuem a capacidade de direcionar proteínas ao nucleolo, funcionando como sinais de localização nucleolar (Galcheva-Gargova et al., 1998; Yang et al., 1999; Yano et al., 2000; Urrutia, 2003). No Capítulo 1, mostra-se claramente que, além dos blocos de lisinas, há outra região da molécula eIF2β essencial para a localização nucleolar da proteína, a qual corresponde às quatro cisteínas (C_2-C_2) presentes na região carboxiterminal que formam uma estrutura de dedo de zinco (Figura 4A e 4B). Esse motivo, juntamente com os blocos de lisinas, é essencial para localização nucleolar de eIF2β, mas separadamente não é suficiente para desempenhar essa translocação subnuclear (ver Figura 4A e 4B).

Foi demonstrado que uma forma de regulação da localização nucleolar da proteína nucleostemina, uma proteína nucleolar de ligação a GTP que regula a proliferação celular, envolve sua ligação a GTP e a hidrólise de GTP (Tsai & McKay, 2005). Para muitas proteínas de ligação a GTP, a interação com a molécula GTP conduz a uma modificação

conformacional da proteína que afeta sua função e sua atividade, podendo expor motivos de retenção ou de dissociação que levam a mudança de sua afinidade de ligação ao nucléolo. Isso poderia, dessa forma, promover uma forma de regular a função da proteína (Swaminathan, 2005). Estudos têm demonstrado que as subunidades β e γ de eIF2 estão envolvidas em ligação a GTP (Bommer & Kurzchalia, 1989; Bommer et al., 1991) e a ligação do trímero eIF2 a GTP regula sua função no processo de tradução. Assim, a localização nucleolar de eIF2β poderia ser também regulada por essa interação.

Nesse contexto, é relevante ressaltar também que nosso trabalho mostrou que a exclusão nuclear de eIF2β é promovida por sua porção aminoterminal, a qual apresenta sequências semelhantes a NES rico em leucina (Kutay & Guttinger, 2005) (Capítulo 1, Figura S1). Essa exportação nuclear é mediada pela exportina CRM1, que reconhece NES ricos em leucina, corroborando a existência de um NES na região aminoterminal de eIF2β. Como demonstrado na Figura S1 do Capítulo 1, ressalta-se que sequências similares a NES estão presentes na região aminoterminal de eIF2β. Assim, pode-se inferir que a regulação da fosforilação e defosforilação de eIF2β poderia controlar também a exposição de um NES na superfície da molécula. Bonsack et al. (2002) mostraram que eIF2β endógeno apresenta apenas localização citoplasmática, sendo possível então que sítios constitutivos de fosforilação estejam relacionados à exclusão da proteína do núcleo em condições normais.

O nucleolo é conhecido como a fábrica dos ribossomos. É um subcompartimento nuclear que contém aglomerados de rDNA que codificam proteínas ribossomais, rRNA e proteínas. É responsável pela transcrição do rDNA, processamento e modificações do rRNA e formação das partículas ribossomais. Mais recentemente, tem-se mostrado que o nucleolo é também um sensor de estresse da célula, participando de diversos eventos

celulares, como silenciamento gênico, regulação do ciclo celular, replicação viral, formação de partículas de reconhecimento sinal, senescência, modificação de snRNPs, exportação nuclear, processamento de tRNAs, regulação de p53 e função telomerase (Olson et al., 2002; Mayer & Grummt, 2005; Sirri et al., 2008). Os resultados obtidos mostrando que a localização nucleolar de eIF2β é dependente de ligação a RNA (Capítulo 1, figura 6) e que é mediada por motivos de ligação a esse ácido nucléico (blocos de lisinas e resíduos de cisteínas) indicam fortemente que a função nucleolar está relacionada a processos que envolvam RNA. Nesse contexto, mRNAs aberrantes e proteínas UPF estão localizadas no nucléolo de plantas, sugerindo que esse subcompartimento possa estar envolvido no processo de NMD (Koroleva et al., 2009a). Desse modo, pode-se considerar que eIF2β tenha função no processo de NMD no nucléolo das células. Além disso, é plausível inferir que a função de eIF2β no nucléolo possa estar associada não apenas ao rRNA e mRNA, mas também a tRNAs presentes no nucléolo.

Assim, a regulação da translocação de eIF2β ao núcleo e ao nucléolo pode estar relacionada à resposta das células a condições específicas de estresse e desse modo sugerimos hipóteses de possíveis funções nucleares/nucleolares desse fator. Evidentemente mais estudos são necessários para investigar essas funções e descobrir em que tipo de condições celulares elas ocorrem.

Considerando a importância dos blocos de lisinas para a localização nucleolar de eIF2β, é possível que os efeitos de diminuição observados na proliferação de células humanas possa ser devido também ao bloqueio das funções nucleares da proteína. No entanto, a redução da síntese proteica parece ser a principal causa desses efeitos, uma vez que a magnitude da diminuição da taxa de tradução foi similar àquela causada pela anisomicina (Capítulo 2, Figura 5).

5.3 Hipóteses de possíveis funções de eIF2β no núcleo/nucleolo

Considerando todos os dados de distribuição celular gerados nesse trabalho pode-se inferir que eIF2β seja translocado ao núcleo para desempenhar funções no nucleolo. Essas funções parecem estar relacionadas a processos que envolvam RNA. Desse modo, é possível sugerir as seguintes hipóteses de funções:

5.3.1 Reconhecimento do Metionil-tRNAi^{Met} no nucleolo

O eIF2β reconheceria o Metionil-tRNAi^{Met} processado no nucleolo e exportaria o mesmo para o núcleo e para o citoplasma. Nesse sentido, a regulação da fosforilação exporia um NLS de eIF2β em sua superfície, o que permitiria sua ligação a maquinaria de importação nuclear. eIF2β, juntamente com eIF2α, seriam responsáveis pela translocação do complexo eIF2 ao núcleo, e eIF2β e γ ligados a GTP seriam responsáveis por direcionar o complexo eIF2 ao nucléolo. No nucléolo, eIF2β e eIF2γ seriam responsáveis por reconhecer e ligar eIF2 ao Metionil-tRNAi^{Met}, exportando o complexo ternário formado no nucléolo ao citoplasma para o início da síntese proteica.

5.3.2 Regulação da biogênese ribossomal

O eIF2β seria um responsável pela regulação da biogênese ribossomal em situações de bloqueio global da síntese proteica por fosforilação da subunidade α de eIF2 (Kimbal, 1999; Sonemberg & Dever, 2003; Kimbal & Jeferson, 2010). eIF2β seria um sensor de parada da síntese proteica para diminuição da biogênese ribossomal, de modo que, ao sinal de parada caracterizado por um excesso de eIF2 ligado a GDP (eIF2/GDP), os eIF2 ligados a GTP (eIF2/GTP) restantes seriam translocados ao núcleo, direcionados ao nucléolo e

poderiam, por sua capacidade de ligação a RNA, sinalizar a diminuição da biogênese ribossomal.

5.3.3 Função do reparo de DNA por recombinação não-homóloga

No núcleo e no nucléolo, eIF2β estaria envolvido no reparo de DNA induzido por quebras duplas de DNA via recombinação não-homóloga. O eIF2β ligaria-se ao DNA e estabilizaria o complexo proteico da cinase dependente de DNA (DNA-PK), o qual é formado pela subunidade catalíca (DNA-PKcs) e pelo complexo Ku que contém as subunidades que se ligam ao DNA (Ku70 e Ku80) (Khanna & Jackson, 2001; Jackson, 2002; Burma & Chen, 2004). Esse complexo possui uma função essencial na reparação de quebras duplas de DNA e no direcionamento à apoptose frente a danos severos no DNA (Burma & Chen, 2004). Essa ideia é suportada pelo fato de que eIF2β se liga à DNA-PK (Ting et al., 1998) e é fosforilado por ela (Ting et al., 1998; Suragani et al., 2006), ou seja, pode ser funcionalmente regulado por fosforilação por essa cinase. Também, alguns estudos de perfil de expressão gênica corroboram a ideia de uma possível função de eIF2β no mecanismo de reparo de DNA. Lanza et al. (2005) analisaram o perfil de expressão gênica em células endoteliais de veia humana (HUVEC) frente à exposição de doses de raios-X. Esse trabalho demonstrou a modulação de genes relacionados ao processo de reparo de DNA por recombinação não-homóloga: XRCC5, que codifica para a proteína Ku80, sendo esta uma das subunidades do complexo Ku; e BRCA1, o qual é um gene chave na sensibilidade celular a danos de DNA e essencial para a reparação; bem como a superexpressão de dois genes que codificam fatores de início de tradução: o da subunidade β de eIF2 (EIF2S2) e o de eIF3b (EIF3S2). Em outro estudo, Otomo et al. (2004) analisaram o perfil de expressão gênica temporal a radiação ionizante em duas linhagens

de glioblastomas com diferentes radiossensibilidades. Os autores mostraram que os genes relacionados à reparação de DNA estão superexpressos na linhagem mais radiorresistente (U87MG) em relação a mais radiossensível (A172). Os genes de reparo encontrados superexpressos incluem: XRCC5; G22P1, que codifica para a proteína Ku70; e o gene que codifica para a proteína ATR (Proteína relacionada a ATM e Rad3) (Durocher & Jackson, 2001). Esse estudo também evidenciou a superexpressão dos genes EIF2S2 e EIF3S2, os quais também estão relacionados a radiossensibilidade nas linhagens de glioblastomas estudas.

5.4 eIFs no núcleo e tradução nuclear

A ideia de que o processo de tradução do mRNA em proteínas ocorre apenas no citoplasma de células eucarióticas vem a algum tempo sendo questionada (Iborra et al., 2004). Atualmente, a ideia da existência de um processo relacionado à tradução nuclear tem sido amplamente difundida. Esse processo é denominado de *pionner round* (rodada inicial) de tradução nuclear, ou seja, um primeiro ciclo de tradução nuclear, similar à tradução, no qual o mRNA recém-sintetizado é, em seguida, traduzido para ser checado quanto a presença de codon de terminação prematuro (PTC). No momento em que um PTC é encontrado, esse mRNA é degradado pela maquinaria de NMD, evitando sua exportação ao citoplasma e a consequente síntese de proteínas truncadas, que acarreta um gasto de energia desnecessário e pode ser deletério à célula (Fortes et al., 2000; Ishigaki et al., 2001; Wilkinson & Shyu, 2002). Esse processo similar à tradução não deve se tratar de um mecanismo de produção de proteínas no núcleo, mas de um mecanismo de controle da qualidade dos mRNAs recém-sintetizados e, consequentemente, de mais uma maneira de regulação da expressão gênica.

Nesse contexto, diversos trabalhos mostram que, além de seu papel essencial no processo de síntese proteica, os diferentes eIFs apresentam também importantes funções em processos nucelares e/ou nucleolares de células de leveduras, plantas e mamíferos (Tabela 2). Esses processos incluem processamento do mRNA, NMD, biogênese ribossomal, transporte núcleo-citoplasmático de mRNAs, degradação de proteínas e apoptose. Desse modo, o papel de alguns eIFs no núcleo parece estar relacionado ao processo de controle da presença de PTC em mRNAs recém-sintetizados, sugerindo a participação desses eIFs nesse primeiro ciclo de tradução nuclear, mas atuando de maneira bem diferente do que em um processo de síntese proteica propriamente dita. Assim, a questão de que se nesse ciclo de tradução nuclear uma proteína é realmente sintetizada ainda permanece sem esclarecimento. De fato, no núcleo, ainda não foram evidenciadas as funções clássicas dos eIFs vinculadas à síntese de proteínas.

No núcleo, tal como no citoplasma, os eIFs participam de processos-chave na regulação da expressão gênica. As funções nucleares dos eIFs também estão sujeitas a regulação, de modo que diferentes condições fisiológicas das células podem modificar a distribuição subcelular dos eIFs, interferindo assim em suas funções nucleares (Dunand-Sauthier et al., 2002; Shen et al., 2004; Taylor et al., 2007; Zhang et al., 2007; Rong et al., 2008; Koroleva et al., 2009a; Koroleva et al., 2009b; Ma et al., 2009).

O direcionamento de diferentes proteínas até seus sítios intracelulares de ação é regulado em diferentes pontos e a alteração em algum desses processos pode resultar em localização subcelular incorreta, o que pode acarretar crescimento celular descontrolado (Kau & Silver, 2003; Kau et al., 2004). De fato, esse tipo de regulação mostra-se bem caracterizado no processo de tumorigênese, uma vez que em diversos tipos de tumores humanos a distribuição subcelular dos diferentes eIFs apresenta-se alterada (Lobo et al.,

1997; Sanvito et al., 2000; Yen & Chang, 2000; Rosenwald et al., 2001; Rosso et al., 2004; Topisirovic et al., 2004; Traicoff et al., 2007; Flavin et al., 2008; Tejada et al., 2009). Desse modo, a distribuição subcelular de eIFs torna-se um alvo para a regulação da proliferação, sobrevivência e morte das células. Essa propriedade mostra a importância dos eIFs em regular a expressão gênica e estimula a ideia de que esses fatores são considerados fortes alvos terapêuticos para o tratamento do câncer.

6. CONCLUSÕES

6.1 CONCLUSÃO GERAL

Os resultados apresentados nessa tese nos permitem concluir que *in vitro* a expressão de uma forma truncada de eIF2β, apresentando a deleção dos blocos de lisinas, é capaz de diminuir a síntese proteica global de células humanas, interferindo na proliferação e na viabilidade celular. Esses resultados nos permitem concluir também que além de ser uma proteína citoplasmática, eIF2β está localizado no nucléolo de células humanas e sua distribuição subcelular parece ser regulada pelos sistemas de importação e exportação celular mediados por NLS e NES na molécula.

6.2 CONCLUSÕES ESPECÍFICAS

1) eIF2β desprovido dos três blocos de lisinas pode atuar como dominante negativo quando superexpresso em células humanas, inibindo a síntese proteica e consequentemente diminuindo a proliferação e viabilidade celulares;

2) além da localização citoplasmática, eIF2β apresenta também distribuição nuclear e nucleolar em células humanas;

3) a translocação de eIF2β do citoplasma ao núcleo pode ser mediada pelos blocos de lisinas, mas também por outros NTS presentes na molécula;

4) os blocos de lisinas, bem como as cisteínas que formam a estrutura de dedos de zinco, são essenciais para a localização nucleolar de eIF2β e parecem atuar sinergicamente;

5) a localização nucleolar de eIF2β é dependente de RNA;

6) a exclusão nuclear de eIF2β é realizada pela exportina CRM1 e deve ser mediada por um NES presente na região amino terminal;

7) eIF2β deve desempenhar alguma função dependente de ligação a RNA no nucléolo, e assim estar envolvido em eventos que ocorrem no núcleo células eucarióticas.

7. PERSPECTIVAS

Os resultados e as conclusões obtidos nessa tese instigam estudos que explorem os efeitos antiproliferativos da expressão de eIF2β desprovido dos blocos de lisinas, bem como que analisem a funcionalidade de eIF2β no núcleo e nucléolo, um aspecto ainda não explorado até o momento. Assim, sugere-se:

- utilizar a técnica de RNA de interferência contra eIF2β endógeno juntamente com a expressão de eIF2β selvagem ou de eIF2β desprovido dos blocos de lisinas recombinantes, com a finalidade de analisar se os efeitos da deleção desses resíduos são letais a células de mamíferos;

- analisar as interações proteicas citoplasmáticas de eIF2β dependentes dos blocos de lisinas para explorar o mecanismo de ação dessa proteína truncada relacionado à diminuição da proliferação celular; analisar suas interações nucleares/nucleolares como ferramenta para investigar sua função nesses compartimentos celulares, bem como para a regulação de sua translocação ao núcleo e nucléolo; identificar em quais condições celulares isso ocorre, buscando a relevância fisiológica para essa localização. Esse estudo investigaria também o papel do estado de fosforilação de eIF2β na sua distribuição subcelular e a relação do processo de fosforilação/defosforilação com a exposição dos domínios de lisinas e de cisteínas na superfície da molécula;

- analisar a expressão diferencial de eIF2β em células normais e tumorais humanas, bem como a resposta de células tumorais à superexpressão de eIF2β desprovido dos blocos de lisinas nos processos celulares de síntese proteica, proliferação e viabilidade, apoptose, autofagia e senescência; analisar a localização nuclear/nucleolar de eIF2β humano em linhagens de células não tumorais e tumorais, investigando possíveis diferenças no padrão

de distribuição subcelular, bem como na resposta a diferentes situações celulares (normais e/ou de estresse).

- analisar a expressão de um plasmídeo contendo eIF2β desprovido dos blocos de lisinas em modelos tumorais *in vivo*, vislumbrando seu potencial antiproliferativo para o desenvolvimento de uma ferramenta de terapia gênica direcionada;

- investigar *in vitro* NLS, NTS e NES na molécula eIF2β para melhor entender a conformação desta molécula, bem como a regulação de sua distribuição subcelular;

- investigar se a localização nuclear/nucleolar de eIF2β estaria relacionada ao reparo de quebras duplas de DNA via recombinação não-homóloga, uma vez que esse fator foi mostrado interagir com e ser fosforilado pela proteína DNA-PK. Nesse sentido, a forma selvagem e diferentes formas truncadas de eIF2β humano seriam superexpressas em linhagens celulares humanas proficientes e deficientes nas proteínas DNA-PKcs, Ku70 e Ku80. Após a exposição a agentes genotóxicos indutores de quebras duplas, seriam analisadas a eficiência do reparo de danos no DNA e a sobrevivência celular;

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9. ANEXO I

Localization of eukaryotic initiation factors in the nucleus: what are they doing there?

9.1 APRESENTAÇÃO

Essa seção apresenta um manuscrito de revisão submetido à revista RNA Biology intitulado “Localization of eukaryotic initiation factors in the nucleus: what are they doing there?”

Atualmente, tem-se sugerido que o processo de síntese proteica não se restringe ao citoplasma, mas pode ocorrer no núcleo de células eucarióticas acoplada. No entanto, essa ideia ainda permanece controversa na literatura científica. Os principais trabalhos sugerem um processo de tradução nuclear associado ao mecanismo de degradação de mRNAs contendo códons de parada prematuros (PTCs): *nonsense mRNA decay* (NMD). Esse processo parece ser um mecanismo de controle de qualidade dos mRNAs recém-sintetizados. Desse modo, vários componentes da maquinaria de tradução, incluindo os eIFs, têm sido encontrados no núcleo e parecem atuar nesse mecanismo de controle da expressão gênica. Além disso, tem-se mostrado que a regulação da distribuição subcelular dos diferentes eIFs está alterada em doenças como o câncer.

Esse trabalho compila, portanto, os dados que abordam a localização e as funções nucleares dos eIFs de diferentes espécies eucarióticas descritos na literatura científica até o momento. O modelo atual de tradução nuclear vinculada ao processo de NMD é descrito e a relação entre a distribuição subcelular dos diferentes eIFs com alguns tipos de tumores são apresentados, sugerindo a importância desses fatores na regulação da expressão gênica e no processo de tumorigênese.

Todos os autores do trabalho participaram ativamente na proposta e organização da ideia abordada, bem como na elaboração, no desenvolvimento e na revisão final do texto.

9.2 ARTIGO 2

TITLE: Localization of eukaryotic initiation factors in the nucleus: what are they doing there?

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Running title: Nuclear functions of eIFs

Key words: eIF, translation, nuclear localization, nuclear function

Abstract

The eukaryotic translation process is an important step in the regulation of gene expression. Translation initiation is the main regulatory step of the protein synthesis process, and it involves several eIFs. The regulation of the subcellular localization of different proteins in specific cellular compartments represents a form of gene expression regulation. Several eIFs have been found in the nucleus of eukaryotic cells including yeast, plants and mammals. Altered expression of many eIFs is associated with malignant transformation and cancer prognosis, and there is evidence that some eIFs present altered cell distribution and impair different cellular process in several tumor types. Here, we perform an extensive and critical review of the evidence for nuclear localization of eIFs and their nuclear functions. We aimed to provide an overview to understand the significance of eIFs' nuclear functions in nuclear translation, gene expression regulation and pathologic processes, such as tumorigenesis.

Background

Protein synthesis is one of the most complex cellular biosynthetic mechanisms, and understanding it has been a great challenge for the scientific community. This essential cellular process is performed by hundreds of macromolecules, including the transfer RNAs (tRNAs), ribosomal RNA (rRNA) and various ribosomal proteins with several auxiliary

enzymes and other specific protein factors for the initiation, elongation and termination of polypeptide synthesis.

In eukaryotes, the initiation of protein synthesis is the main regulatory step of the entire process and is aided by a series of protein factors known as eukaryotic initiation factors (eIFs), which are involved in the formation of transient complexes that possess various components of the translational machinery.[1, 2]

The steady-state translation steps

The initiation of protein synthesis occur in the cytoplasm eukaryotic cells and it is a complex process that involves the binding of methionyl initiator tRNA (Met-tRNAi) to the 40S subunit of the ribosome and the subsequent association with messenger RNA (mRNA). This process is dependent on several eIFs that are involved in the formation of a transient complex with several components of the translational machinery. The translation process can be divided into four stages: formation of the ternary complex, formation of the pre-initiation complex, association with mRNA, and formation of the initiation complex.

As has been extensively reviewed elsewhere,[2-4] this process occurs as follows:

As described in Fig. 1, the first step is the assembly of the ternary complex (box I), comprising eIF2 bound to GTP and Met-tRNAi (eIF2-GTP-Met-tRNAi). After its formation, the ternary complex binds to the 40S ribosomal subunit. The resulting complex, comprising the ternary complex that is bound to the 40S ribosomal particle and associated with others factors eIF3, eIF1, eIF1A and eIF5, is called the 43S pre-initiation complex (box II).[2] Next, the 43S pre-initiation complex binds to mRNA, and this process is dependent upon the eIF4F complex, which includes the factors eIF4E, eIF4G, eIF4A, eIF4B and poly(A)-binding protein (PABP). This complex causes the circularization of

mRNA; in conjunction with eIF3 and the PABP bound to the 3'-poly(A) tail, this complex loads the mRNA onto the 43S pre-initiation complex, forming the 48S complex. The 43S pre-initiation complex then migrates in the 5'→ 3' direction on the mRNA to search for the initiation codon (box III). When the 43S complex reaches an initiation codon AUG in a favorable sequence context (Kozak sequence), which was defined by,[5] codon-anticodon base pairing takes place between the initiation codon and the Met-tRNAi in the ternary complex. At this point, the hydrolysis of GTP that is bound to eIF2 is triggered and promoted by the GTPase-activating protein (GAP) eIF5. After GTP hydrolysis, eIF2 that is bound to GDP (eIF2G-DP) and the other factors (eIF1, eIF3 and eIF5) are released. The joining of the 60S ribosomal subunit to the 40S-Met-tRNAi mRNA complex is facilitated because the other factors have dissociated. This event triggers a second step of eIF5B-mediated GTP hydrolysis, allowing 60S subunit coupling to form the 80S ribosome complex.[6] GDP-bound eIF5B (eIF5-GDP) dissociates from the complex (box IV). The translation process continues in the elongation stage with the insertion of the next tRNA that is bound to eukaryotic elongation factor 1 (eEF1)-GTP. After this complex enters the ribosome E site, GTP hydrolysis of eEF1 causes its release, forming the first peptide bond under eIF5A stimulation (box V).

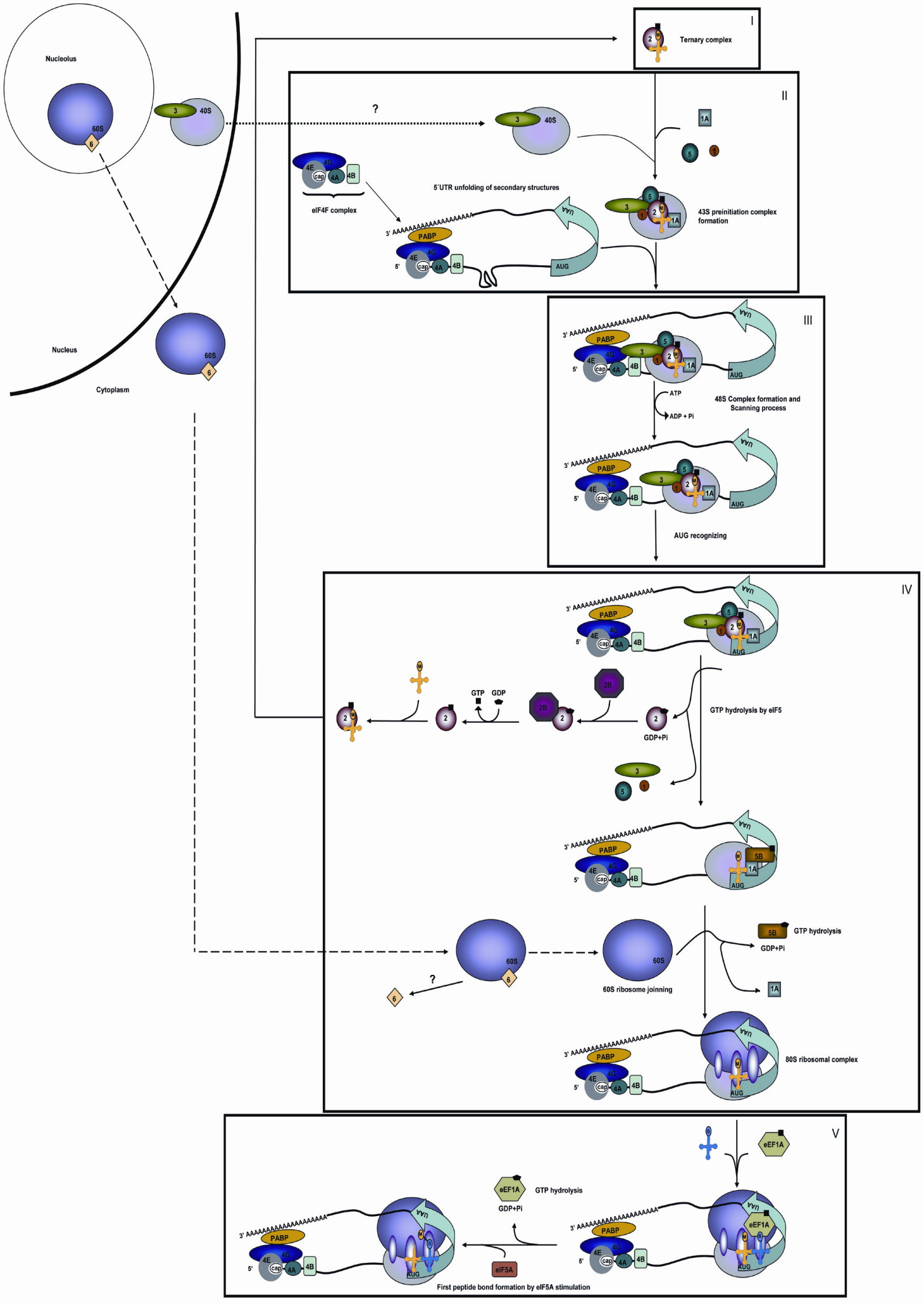
Nucleolus
60S
6
40S
3
Nucleus
60S
6
Cytoplasm
I
Ternary complex
2
II
?
40S
3
1A
5
1
eIF4F complex
4E
4G
cap
4A
4B
5'UTR unfolding of secondary structures
3' AAAAAAAAAAAAAAAAAA
UAA
PABP
5'
AUG
43S preinitiation complex formation
III
ATP
ADP + Pi
48S Complex formation and Scanning process
AUG recognizing
IV
GTP
GDP
2B
GTP hydrolysis by eIF5
GDP+Pi
5B
60S ribosome joinning
GTP hydrolysis
80S ribosomal complex
V
eEF1A
GTP hydrolysis
GDP+Pi
eIF5A
First peptide bond formation by eIF5A stimulation

Figure 1. The steady-state translation initiation process. In the nucleus eIF6 binds to 60S ribosome and eIF3 binds to 40 ribosome allowing ribosomal subunits dissociation. These complexes are exported to cytoplasm. The different steps of eukaryotic initiation translation process are divided in the boxes. I - ternary complex formation. II - 43S complex formation. III - 48S complex formation and scanning of mRNA/initial codon recognition. IV - GTP hydrolysis by eIF5 and eIF5B and 80S ribosomal complex formation. V - first peptide bond formation. The eIFs, ribosomal subunits and tRNA are depicted as colored shapes identified in the figure. The eIFs are labeled only with the numerical portion of their respective names followed by letter of their subunit when necessary. The ribosomal subunits are presented as 40S and 60S. tRNAs are illustrated as cross-shaped. The 5'cap structure is labeled "cap".

Currently, thirteen eIFs are known to participate in the initiation of the protein synthesis process. Most of these are composed of different subunits, and some have several isoforms. A majority of the eIFs are involved in the formation of complexes that contain several components of the translation machinery that associate transiently with the initiation complex. The main characteristics and functions of all of the eIFs in cytoplasmic steady-state translation are described in Table 1.

Table 1. Functions on protein synthesis of eIFs in mammals.

eIF	Subunits/ Isoforms	Molecular weight (kDa)	Characteristics and functions on translation process	References
eIF1		13.5	- promotes a stable complex with 40S ribosome subunit positioned at the correct AUG codon. - acts on the dissociation of ribosomal subunits by binding to the 40S complex and stabilizing the pre-initiation complex	[1, 2]

eIF	Subunits/ Isoforms	Molecular weight (kDa)	Characteristics and functions on translation process	References
eIF1A		19	- promotes a stable complex with 40S ribosome subunit positioned at the correct AUG codon. - acts on the dissociation of ribosomal subunits by binding to the 40S particle and stabilizing the pre-initiation complex	[1, 3]
eIF2	Subunits: alpha	46	- promotes the binding of Met-tRNAi to the 40S subunit of the ribosome in the presence of GTP - phosphorylation of serine 51 blocks protein synthesis. - is a rate limiting step in the protein synthesis process	[4-7]
	beta	48	- promotes the binding of Met-tRNAi to the 40S subunit of the ribosome in the presence of GTP - interacts with mRNA, eIF2B, eIF3 and eIF5 recognition of initial AUG codon	[4, 5, 8-11]
	gamma	52	- promotes the binding of Met-tRNAi to the 40S particle of the ribosome in the presence of GTP - Met-tRNAi binding - GDP/GTP binding - GTP hydrolysis	[4, 5, 12]

eIF	Subunits/ Isoforms	Molecular weight (kDa)	Characteristics and functions on translation process	References
eIF2B	Subunits: alpha beta sigma lambda epsilon	 29 39 54 66 84	eIF2B subunits are responsible for: - the exchange of GDP for GTP bound to eIF2 after each cycle of initiation - the availability of active form of eIF2-GTP - its epsilon and sigma subunits bind to the beta subunit of eIF2	9, 13-19
eIF3	subunits: a b c d e f g h i j k l m	 170 116 110 66 48 47 44 40 36 35 28 42 ?	- participates in multiple stages of initiation of translation - binds to the 40S ribosomal subunit and stimulates the binding of ternary complex eIF2-GTP-Met-tRNAi to form the 43S pre-initiation complex. - stimulates the binding of mRNA to the 43S pre-initiation complex - binds to the 40S ribosome particle and prevents the association of 60S subunit ribosome until start codon is recognized and all of the eIFs dissociate from the complex	20-24
eIF4A	isoforms: I II III	46	- functions as an RNA helicase that unfolds the secondary structure of the mRNA - facilitates binding of the small ribosomal subunit to mRNA	25-27
eIF4B		69	- stimulates eIF4A activity - can be related to ribosome binding	28-30

eIF	Subunits/ Isoforms	Molecular weight (kDa)	Characteristics and functions on translation process	References
eIF4E		24	- recognizes and connects the 5'cap structure of the mRNA to eIF4G - is a rate limiting step in the protein synthesis process and is regulated by phosphorylation - is regulated by 4EBPs activity, which in turn is phosphorylated by mTor	[25, 27, 31-35]
eIF4G	Isorforms: I II	171 176	- is a scaffold protein that interacts with the PABP and facilitates the functional association of the 3´end of the mRNA with its 5´end to promote translation - has binding sites on eIF4E, eIF4A, eIF3 and PABP and the carboxyl- terminal binds to the 40S subunit of the ribosome - functions to bring the necessary factors to unwind the mRNA secondary structure near the 5'cap of the RNA in the correct orientation	[7, 27, 36-38]
eIF4F		ND	- consists of eIF4A, eIF4E and eIF4G, interacts with the 5'cap and with the ribosome-associated eIF3 through eIF4G - performs a pivotal role, mediating the link between ribosome and mRNA	[27]
eIF5		49	- possesses a ribosome-dependent GTP hydrolysis activity - allows the joining of ribosome subunits 40S and 60S after hydrolysis of GTP bound to eIF2 - interacts with beta subunit of eIF2	[39-43]

eIF	Subunits/ Isoforms	Molecular weight (kDa)	Characteristics and functions on translation process	References
eIF5A	Isoforms 1 2	16.7	- interacts with RNA through a hypusine residue - interacts physically with components of the ribosome and elongation factor - specifically binds to actively translating ribosomes - may link the initiation and elongation steps of translation	[44-53]
eIF5B		175 (mouse)	- is a homologue of IF2 in eubacteria - has ribosome-dependent GTPase activity - mediates dissociation of eIFs 1, 1A and 3 and residual eIF2-GDP from the 40S subunit after hydrolysis of free GTP - allows the joining of ribosome subunits 40S and 60S after hydrolysis of free GTP	[2, 23, 54-58]
eIF6		26	- regulates the processes of ribosomal biogenesis and initiation translation - binds to the 60S subunit and prevents its association with the 40S subunit - acts to keep ribosomes dissociated - is a rate limiting factor of the protein synthesis process	[59-65]

The role of eIFs in the nucleus

Several eIFs localize in the nucleus of eukaryotic cells including yeast, plants and mammals; and they generally function in the processing and export of mRNA. Nucleolar localization of some eIFs has also been shown and is probably involved in the processing of rRNA and the exportation of ribosomes. The specific nuclear localization and possible nuclear functions of eIFs are described in Table 2. The majority of these proteins undergo active import into and export from the nucleus. Transport of macromolecules across the nuclear envelope is mediated by nuclear pore complexes (NPCs).[7] Soluble transport receptors, termed importins and exportins (also collectively called as karyopherins) bind to specific signals present within their target proteins and mediate the nucleocytoplasmic transport of cargo. The specific signals recognized by importins are called Nuclear Localization Signal (NLS); those recognized by exportins are termed Nuclear Export Signal (NES).[8, 9] The interaction of receptor–cargo molecules is regulated by the small GTPase Ran, which controls transport in each direction depending on its nucleotide-bound state. RanGTP concentrates in the nucleus, whereas the GDP-bound form is predominant in the cytoplasm. RanGTP controls substrate binding to importins and exportins in an opposing manner. Importins bind to their cargo in the cytoplasm and release it upon binding to nuclear RanGTP. Conversely, nuclear RanGTP promotes the binding of exportin to its cargo, resulting in the formation of a stable export complex. Export complexes are dissociated by the hydrolysis of Ran-bound GTP promoted by RanGAP in the cytoplasm.[8, 10] Several eIFs have single or multiple NLSs and/or NESs that can be recognized by different importins or exportins (Table 3).

Table 2. Localization and function of eIFs in the nucleus.

eIF	Nuclear localization	Possible nuclear function	Methodology	References
eIF1	- nucleoplasmic	ND	- IF	[21]
eIF1A	- nucleoplasmic - nucleolar	ND	- IF	[11]
eIF2α	- association with the nuclear membranes - nucleoplasmic - nucleolar - around the nucleolus - associated with strands of heterochromatin, - along putative nuclear filaments	- Apoptosis	- ICC - Mass spectrometry - IHC - IF - WB - Post-embedding electron microscopical immunogold	[12, 15, 18-20, 128, 193, 194]
eIF2β	- Nuclear - Nucleolar	- Possible DNA repair - mRNA binding	- ICC - WB - IF - Mass spectrometry	[21, 193-196]; Salton et al., manuscript in prep.
eIF2γ	- Nucleolar	ND	- Mass spectrometry	[193, 194]
eIF3a	-Nuclear	ND	- IF - Cell fractioning/WB	[42]
eIF3b	- Nuclear	Pioneer round of translation	- Cell fractioning/WB	[36, 39]
eIF3c	- Nuclear	ND	- Cell fractioning/WB	[39]
eIF3d	- Nuclear	ND	- Cell fractioning/WB	[39]

eIF	Nuclear localization	Possible nuclear function	Methodology	References
eIF3e	- Nuclear bodies - Colocalization with PML nuclear bodies - nuceloplasm - partly associated with cromatin	- Protein degradation - Nuclear trafficking - NMD	- IF	27, 30-32, 34, 36, 41
eIF3f	- Nuclear	- Ribosome degradation - Apoptosis	- IF - Cell fractioning/WB	38, 39, 41
eIF3i	- Inner side of the nuclear envelope	- Thermal shock response	- IF	197
eIF3k	-Nucleo-cytoplasmic	- Apoptosis	- IF	41, 198
eIF3l	- nucleolus	- cofactor of RNA Polymerase I		199, 200
eIF4AIII	- Nucleoplasmic - Nucleolar - Speckle splicing	- Exon junction complex - mRNA splicing - mRNA nuclear export - NMD - Several stress condition responses	- Mass spectrometry - IF - ICC	43, 44, 47-49, 193, 194, 201-203
eIF4E	- Speckle splicing - PML nuclear bodies	- mRNA nuclear export - mRNA splicing - Serum starvation response	- IF - Cell fractioning/WB IHC	12, 50-52, 55, 57-59, 128

eIF	Nuclear localization	Possible nuclear function	Methodology	References
eIF4G	- Nucleoplasmic - Nuclear foci	- mRNA processing - NMD - Heat shock response	- IF - Cell fractioning/WB	12, 64-66, 204
eIF5A	- Nucleoplasmic - Nucleolar - NPC associated	- mRNA binding -Nucleo-cytoplasmic transport - Apoptosis	- IF - Mass spectrometry	73-76, 79-81, 193, 194
eIF5B	- Transcriptional sites - Nucleolus	- Nuclear protein synthesis associated with transcription	- IF	82
eIF6	- Nucleolar	- Ribosome biogenesis - RNA processing	- Mass spectrometry - IF - Cell fractioning/WB	84, 86, 89, 90, 93, 193, 194

ND- possible nuclear functions of eIFs not determined, IF- immunofluorescence, ICC- immunocytochemistry, IHC- immunohistochemistry, WB- western blot, NPC- Nuclear Pore Complex, PML- Promyelocytic Leukemia protein.

Table 3. Evidence of the nuclear localization of eIFs.

eIF	Nucleus import: signal/ importin	Nucleus export: Signal/exportin	Interaction with predominantly nuclear proteins	References
eIF1	Passive diffusion	NES/Exportin 7	ND	[195]
eIF1A	Passive diffusion	NES/Importin 13	ND	[11]
eIF2α	NLS	ND	- DNA-PK	[13, 14, 22]
eIF2β	NLS NTS	NES/CRM1	- DNA-PK	[22], Salton et al., manuscript in prep.
eIF2γ	ND	ND	- DNA-PK	[22]
eIF3a	NLS/Sal3p/ Kap121p NLS/Kap123p	ND	ND	[42]
eIF3e	NLS/Kap123p/ Sal3p	NES/CRM1	- Protein COP9 signalosome - 26S proteasome	[24, 32, 205, 206]
eIF3f	ND	ND	CDK11p46	[38]
eIF3k	ND	ND	D3 cyclin	[198]
eIF4AIII	ND	ND	- Magoh and Y14 EJC complex - mRNA export factors TAP and with Aly/REF	[44-46, 201, 202]
eIF4E	importin αβ eIF4E-transporter	ND	- Sm and U1snRNP	[50, 207]
eIF4G	ND	ND	- Spliceosomal snRNPd - Cap Binding Complex - Sc-35 splicing factor - U1 snRNA	[64, 65]

eIF	Nucleus import: signal/ importin	Nucleus export: Signal/exportin	Interaction with predominantly nuclear proteins	References
eIF5A	NLS Passive diffusion	NES/CRM1 Exportin 4	ND	[73-76]
eIF6	ND	NES/CRM1	ND	[93]

ND- data about the specific approach not determined, NES- Nuclear export signal, NLS- Nuclear localization signal, NTS- Nuclear translocation signal.

eIF1 and eIF1A

As shown in Table 2, there is very little evidence of the nuclear localization of eIF1 and eIF1A, and no nuclear functions have been reported to date. eIF1 is so small that it may cross the NPC freely, requiring an active export mechanism for cytoplasmic exclusion. Mingot et al. considered the possibility that eIF1 could be confined in the cytoplasm via exportin 7 (Exp7), and mutagenesis experiments indicated that the specific residues Asp52, Asp53, Asp55, Lys56, Lys58, Lys64, Lys65 and Lys66 in this factor are critical for Exp7 binding and that they strongly reduced or even abolished the interaction of eIF1 to Exp7. eIF1A is small enough to enter nuclei efficiently by passive diffusion. An active export mechanism should also act to promote cytoplasmic localization because, in permeabilized cells, eIF1A was found in the nuclei and accumulated in bright nucleolar spots. However, when these nuclei were incubated with Importin 13 (Imp13), eIF1A was exported efficiently, and the nucleolar signal was lost.[11]

eIF2

eIF2 also exhibits nuclear localization and seems to be involved in events that occur in the nucleus of eukaryotic cells. The best characterized subunit in this sense is the α subunit. Several studies have shown nuclear and nucleolar distribution of eIF2α in different human cell types and in diverse situations of cellular stress. Similarly, a study that quantified eIFs in the nucleus by immunofluorescence showed that 25% of eIF2α was present in this compartment in HeLa cells.[12] The primary eIF2α sequence contains several NLSs, and one of them is found adjacent to the Ser15 phosphorylation site. [13, 14]

Lobo et al. found nuclear eIF2α in primary neuronal cells in culture as well as in two established cell lines: PC12 pheochromocytoma and rat pituitary GH4C1 cells. In the cytoplasm, this factor was present mainly in areas that are rich in ribosomes; and in the nucleus, in both nucleolar and extranucleolar areas.[15] Because the protein kinase activated by double-stranded RNA (PKR) phosphorylates eIF2α under specific conditions and inhibits global protein synthesis,[16, 17] this nucleolar distribution of eIF2α could be correlated with the nuclear distribution of PKR where it was found in the nucleolus, as well as being found diffusely in the nucleoplasm.[18] DeGracia et al. used an antibody specific to the phosphorylated form of eIF2α (eIF2α(P)) to study its regional and cellular distribution in normal, ischemic, and reperfused rat brains. eIF2α(P) initially accumulated in the cytoplasm of CA1 hippocampal neurons within the first 10 min of reperfusion, but it was found in the nuclei of selectively vulnerable neurons (SVNs) 1 h and 4 h after reperfusion.[19] In the nucleus, using post-embedding electron microscopy immunogold methods, aggregates of gold particles in the nucleus were concentrated within and around the nucleolus, associated with strands of heterochromatin, and along putative nuclear filaments.[20] The presence of eIF2α(P) in the nucleolus may reflect its association with

nascent ribosomal subunits, and the association of eIF2α(P) with chromatin may have important implications for transcription.[20]

Little is known about the cellular distribution of the beta subunit of eIF2. Results from our lab demonstrate that wild-type eIF2β fused to green fluorescent protein (GFP) has cytoplasmic and nucleolar distribution; however, a GFP fused mutant form of eIF2β lacking conserved lysines does not localize to the nucleus and is found only in the cytoplasm (Fig. 2). The nucleolar localization without a nuclear signal in the wild-type form and the limited cytoplasmic localization of the mutated form indicate that this cellular distribution pattern is not from cytoplasmic contamination. In a recent study, Salton et al. (manuscript in prep.) used immunofluorescence analysis to demonstrate that eIF2β translocates from the cytoplasm to the nucleus and accumulates in the nucleolus of human cells. After leptomycin B (LMB) treatment, wild-type eIF2β was detected exclusively in the nucleus and nucleolus, showing the involvement of CRM1 in the nuclear export of this molecule. (Salton et al., manuscript in prep.),[21] The amino-terminal region of eIF2β contains a putative nuclear exclusion signal (NES) for CRM1. Conserved motifs in this protein, polylysine stretches and C2-C2 zinc finger motifs, are essential for eIF2β nucleolar localization, which is RNA binding dependent (Salton et al., manuscript in prep.). Therefore, the eIF2β nucleolar function may be related to processes involving ribosomal RNA such as ribosomal biogenesis, the pioneer round of translation in the nucleus, or the associated to nonsense mediated mRNA decay (NMD) process.

In addition, Ting et al. showed that α, β and γ subunits of eIF2 purified from human cells interact with the DNA-dependent kinase (DNA-PK) and stabilize the formation of a complex of DNA, DNA-PKCs (DNA-PKCs is the catalytic subunit of DNA-PK) and Ku (Ku is a component of DNA-PK that binds to DNA).[22] Moreover, eIF2β is phosphorylated

by DNA-PK in vitro. Based on these data, it is possible that the eIF2 complex could act in a DNA repair mechanism[23] and function in transcription regulation.

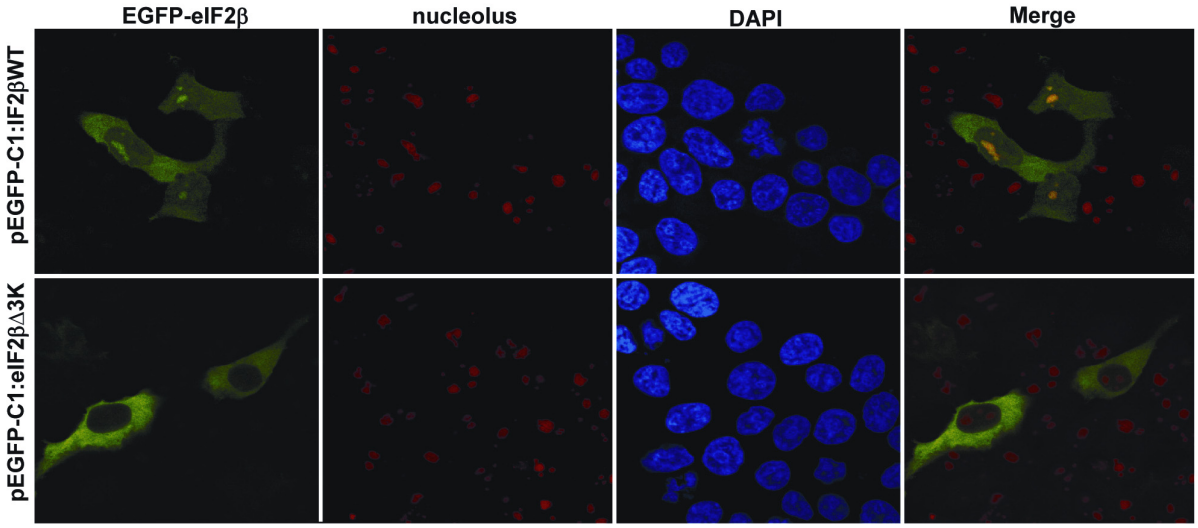

Figure 2. Subcellular distribution of different eIF2b forms fused to EGFP. Hek293 cells were transfected with empty vector pEGFP-C1, with plasmid expressing the wild-type eIF2β form fused to EGFP (pEGFP-C1:eIF2βWT) or with plasmid expressing the eIF2b without polylysine stretches fused to EGFP (pEGFP-C1:eIF2βΔ3K). Twenty-four hours after transfection, cells were fixed in 4% formaldehyde, permeabilized in 0.5% Triton X-100 and submitted to immunocytochemistry using anti-nucleolus human serum from Immuno Concepts (Sacramento, CA, USA) for 1 h, followed by anti-mouse Cy3-conjugated antibody (1:100) from Sigma Chemical (St. Louis, MO, USA) for 1 h. DAPI (1 µg/mL, Invitrogen) was used to stain the nucleus for 10 min Images were acquired from numerous fields using 60X objectives (oil immersion) on a confocal microscope (FluoView FV1000, Olympus, Japan).

eIF3 complex

Affinity purification and highly sensitive LC-MS/MS were used to determine the interactors of eIF3 from Saccharomyces pombe eIF3.[24] The group of 230 proteins found is called the eIF3 interactome and was found to be RNA independent because RNase treatment did not affect these interactions. Proteins interactions suggesting nuclear function

for the eIF3 complex and other components of the eIF3 interactome are described in Table 4.

The proteasome complex has been shown to associate with the translation machinery and could possibly function to remove proteins that are incorrectly folded.[25] The eIF3 interactome also contained the subunits of the 19S proteasome regulatory particle, molecular chaperones of the HSP70/40 family and the chaperonin-containing TCP1 complex (CCT), all of which mediate co-translational protein folding.[26] In this way, the recruitment of the eIF3 proteasome to elongating proteins coming out of the ribosomes may mediate the cotranslational degradation of proteins that cannot be properly folded by chaperones.[24]

Table 4. Components of eIF3 interactome.

eIF	eEF	Proteins suggesting nuclear function for eIF3 complex		Proteasome components	Molecular chaperones	References
		Proteins of ribosome biogenesis	**Proteins of nuclear translocation**			
eIF1A eIF2B eIF4A eIF4E1 eIF4G eIF5 eIF5A eIF5B	eEF1A eEF2 eEF3	- small subunit processosome: U3 snoRNP subunits, U3 protein complex, the UTP-B and UTP-C complexes - two proteins involved in modification of pre-rRNA: Fib1p and Gar1p - 7 helicases involved in the maturation of pre-40S and pre-60S ribosome - factors mediating nuclear export pre-40S and pre-60S ribosome: Rrp12p and SPBC16H5.08c	- transport proteins of the importins-β family: Kap123p and Sal3p	- subunits of the 19S proteasome regulatory particle	-HSP70/40 family - chaperonin containing TCP1 complex (CCT) complex	[24]

The eIF3e/INT6 subunit is found within nuclear bodies (NBs) containing the promyelocytic leukemia (PML) gene product.[27] PML organizes into nuclear matrix-associated multiprotein complexes known as PML NBs, which contain a number of proteins involved in transcriptional regulation, but neither DNA nor RNA polymerase II associates with PML NBs under normal conditions,[28, 29] suggesting that PML nuclear bodies are not transcription sites. The majority of PML NBs were stained by the antibodies to eIF3e/INT6, but this protein is also distributed in other nuclear compartments; some INT6 foci do not colocalize with PML NBs.[27] A mutant form of eIF3/INT6 lacking 8 amino-terminal amino acids was exclusively localized in PML NBs;[30] therefore, the amino-terminal region of eIF3/Int-6 may play a role in the subcellular localization of the protein. Watkins et al. showed that the eIF3e/INT6 signal was strongly nuclear in quiescent cells and that its nuclear localization was reduced in cycling cell populations mainly during G1 or early in the S phase for human fibroblasts. These results show that the nuclear distribution of eIF3e/INT6 occurs in a cell cycle-dependent manner.[31]

Mutational analyses showed that the eIF3e/INT6 protein contains two stretches of basic residues at positions 268 and 310 that constitute a bipartite NLS. In addition, a NES within the first 20 residues of the amino-terminal region was found, consisting of three leucine residues (positions 6, 14 and 18); replacement of any of these residues by alanine extinguished the function of the export signal.[32]

Many proteasome subunits contain the proteasome-COP9-initiation factor (PCI) domain.[33] This domain was found at eIF3e/INT6 in HeLa cells, and the mutation of a specific leucine leads to nuclear accumulation of eIF3e,[34] suggesting a relationship between proteasome activity and a regulation of the nuclear localization of eIF3e/INT6. Sha et al. have suggested that the eIF3e, in addition to Sal3p, importin regulates the

nuclear localization of the proteasome, which influences its correct function.[24] This idea is also supported by the observations that eIF3 and Sal3 are required for proper proteasome accumulation in the nucleus.[24, 35] The proposed model suggests that Sal3p regulates passage through the nuclear pore[24] and that eIF3e regulates the retention of the proteasome in the nucleus.[24, 35] Moreover, through the interaction between eIF3e and Kap123p importin, eIF3 may mediate nuclear trafficking of others cargo, such as ribosomes and ribosome biogenesis factors, whose nuclear functions are essential for cell viability.[24]

Morris et al. showed that INT6/eIF3 participates of quality control of specific mRNAS regulated by NMD since it is required for degradation of several cellular mRNAS.[36] By coimmunoprecipitation assay, they demonstrated that INT6/eIF3 interacts with both CBP80 and UPF2 and with eIF4GI. Therefore, silencing of INT6/eIF3 did not affect the general translation, but inhibits NMD process. In this way and concerning that INT6/eIF3 did not associate with eIF4E, these authors suggest that this protein, in combination with UPF2, is more committed to NMD process than to active translation.[36]

The eIF3f protein appears to be a Mov34 family member that is involved in the regulation of the proteasome, translation initiation, and transcription.[37] Shi et al. showed that eIF3f colocalizes with CDK11p46 in the nucleoplasm, directly interacts with the carboxyl-terminal domain of CDK11 (CDK11p46) and that its Mov34/JAB_MPN domain is important for this interaction.[38] Under apoptotic stimulation, the interaction of CDK11p46 and eIF3f is enhanced, and CDK11 can phosphorylate eIF3f, probably at Ser46, in vitro and in vivo.[38] More recently, identified Thr119 as another phosphorylation site of eIF3f for CDK11p46 during apoptosis in vivo showing that phosphorylated eIF3f may predominantly localize to the nucleus.[39]

Ribosome profiles and rRNA fragmentation assays showed that the overexpression of eIF3f induces the degradation of 28S rRNA and decreases the amount of 60S ribosomal subunit, suggesting that eIF3f may also function in ribosome degradation during apoptosis.[40] Shi et al. propose that the phosphorylation of eIF3f by CDK11p46 enhances its binding to different subfractions of the eIF3 complex during apoptosis and may regulate translation initiation in this way.[39] Phosphorylation of eIF3f could also regulate its role in ribosome degradation, which may contribute to apoptosis. Shi et al. suggest that the phosphorylated form of eIF3f joins to the nuclear eIF3 complex, which contains eIF3b and eIF3c during apoptosis.[39] A work that studied the subcellular distrbution of different subunits of eIF3 found that a, b, c, h and i subunits were present exclusively in the cytoplasm, in contrast the e, f and k subunits localized predomenantly in the nuclear compartiment.[41] In a subcellular fractioning experiment of apoptotic cells demosntrated that eIF3a is found predominantly in the cytoplamic fraction and eIF3b, c and f were localized in both cytoplasmic and nuclear fraction.[39] However, fluorescence microscopy of proteins fragments fused to GFP showed that at least two of four NLS in mammal eIF3a sequence are able to direct it the to the nucleus.[42] Also, Morris et al. showed that mammal eIF3b silencing inhibit both steady-state translation and pioneer round of translation.[36]

As previously suggested, it is possible that the entire eIF3 complex could undergo nucleocytoplasmic transport by way of NLS or NES within a specific eIF3 subunit, in this case eIF3e. As eIF3 interacts with factors of translation initiation and elongation, mRNA quality control, ribosomes (40S and 60S) and the proteasome (called the “translasome”), it is possible that this translasome is transported to the nucleus similarly to a single subunit.[24] Moreover, this could apply to other eIFs that are made of different subunits.

eIF4F complex

eIF4AI and II are cytoplasmic while eIF4AIII, which shares 70% identity with eIF4I/II, is a nuclear protein that localizes in speckle domains.[43] Recently, eIF4AIII was identified as a novel component of the EJC.[44] It associates with nuclear complexes containing the EJC proteins magoh and Y14 and with the mRNA export factors TAP and Aly/REF in vitro. Mutational analysis of human eIF4A-III[45] and the results of the crystal structure analysis of an exon junction core complex have been used to identy regions of eIF4AIII for EJC formation important for binding to EJC components.[45, 46] Association of eIF4AIII with mRNA export factors suggests that it might play a role in nuclear mRNA export and may provide a link between splicing and translocation to the cytoplasm.[44] Ferraiuolo et al. reported that eIF4AIII is recruited to the mRNA during splicing and functions in NMD because small interference RNA (siRNA) inhibits NMD against eIF4AIII but not against eIF4AI/II.[47] The helicase activity of eIF4AIII is required for remodeling the components of the EJC and may also contribute to the remodeling and translocation of mRNPs during transit through the NPC. Thus, it is possible that in the nucleus, eIF4AIII functions to promote the unwinding of secondary structures within pre-mRNA substrates to stimulate splicing and/or facilitate the assembly of EJC on spliced mRNAs, suggesting a fundamental role in the NMD mechanism.[47]

Some studies have demonstrated that eIF4AIII localizes to the nucleoplasm, and under cellular stress, it localizes to the nucleolus in plant cells.[48, 49] eIF4AIII subnuclear localization seems dynamic and very sensitive to cellular conditions. In Arabidopsis thaliana and Nicotiana tabacum, eIF4AIII rapidly relocalizes to the plant nucleus under hypoxia; the inhibition of respiration, transcription or phosphorylation; or ethanol treatment.[48, 49] In normal cell growth conditions, GFP-eIF4AIII was mainly nucleoplasmic,

but under stress conditions, it moved to the nucleolus and splicing speckles. Koroleva et al. proposed that changes in subnuclear localization could be related to eIF4AIII's role in the regulation of mRNA processing, export and turnover and subsequent processing steps and degradation by NMD as a response to cell stress.[48] Koroleva et al., in contrast, demonstrated that a specific treatment using the proteasome inhibitor MG132 caused increased concentration of eIF4AIII in the nucleolus, but it did not cause localization to speckles.[49] The initial accumulation of eIF4AIII in the nucleolus may represent an early response to the stress conditions reflecting the sequestration of mRNPs in different stages of maturation. The presence and differential dynamic properties of eIF4A-III in different regions and compartments of the nucleus may reflect different stages of EJC assembly and interactions with mRNA targets. The localization of plant EJC factors to the nucleolus suggests that one or more stages of mRNA processing, such as mRNA splicing, mRNA export, surveillance, or NMD, involve the plant nucleolus.[48, 49]

The eIF4E is predominantly cytoplasmic in mammalian cells and yeast; however, 12-33%[50-52] to 68%[12] is estimated to be localized in the nucleus under different analysis methods. eIF4E might be involved in nuclear mRNA processing because it is found in nuclear speckles that are considered storage and assembly sites for many splicing and transcription components and splicing sites.[53, 54] The nuclear distribution of eIF4E is sensitive to RNA polymerase II transcription inhibitors and the availability of 5'cap structures but not to RNase treatment, suggesting that retention of eIF4E in the speckles is not RNA dependent and is associated with proteins.[50] The localization of eIF4E in speckles is also regulated by the dual-specific kinases Clk/Sty that causes the dispersion of eIF4E nuclear speckles.[50] In this way, eIF4E might be associated with splicing factors, involved in splicing and/or mRNA export and may be required for the processing of a specific

subset of mRNA. Moreover, in cells overexpressing eIF4E, the total level of cyclin D1 mRNA does not change; however, its nuclear levels decrease, whereas its cytoplasmic levels increase. This activity indicates that eIF4E overexpression causes an increase in the nuclear export of cyclin D1 mRNA.[55]

PML NBs contain proteins involved in nuclear RNA metabolism, translation and ribosome assembly, such as eIF3/int-6[27], the ribosomal P-protein[56] and eIF4E[57]. PML is an important regulator of mammalian cell growth and apoptosis. In this context, Cohen et al. showed that PML directly binds eIF4E through the PML RING-domain and that eIF4E itself forms both a functional and structural basis for some PML nuclear bodies. Inhibition of eIF4E function by PML is a direct consequence of the direct interaction of PML RING with the dorsal surface of eIF4E, which drastically reduces the affinity of IF4E for its substrate, the 5'cap of mRNA.[58] It has been demonstrated that the PML exerts its transformation-suppressing functions, at least partially, by modulating the ability of eIF4E to bind the 5'cap. Cohen et al. suggest that nuclear eIF4E requires 5'cap-binding activity for its role in modulating the nucleocytoplasmic transport of cyclin D1 mRNA[55, 57] and propose the 5'cap-binding activity is required for the transformation activity of eIF4E because a mutant form of eIF4E that does not bind to the 5'cap with high affinity is unable to increase cyclin D1 protein levels;[58] it is therefore unable to transform cells.[58] PML modulates eIF4E function by reducing its affinity for the 5'cap substrate, thereby allowing PML to directly modulate the mRNA transport function of eIF4E and to suppress its transformation activity. It is likely that in vivo, nuclear eIF4E could be subject to regulation by PML in response to changes in cell growth or cellular conditions.[58] In this way, PML was the first factor shown to modulate nuclear eIF4E function.

Recently, other regulators of eIF4E in the nucleus were identified as 4E-binding protein (4E-BP).[59] These proteins are a family of translation suppressors that, in mammals, consists of 4E-BP1, 4E-BP2 and 4E-BP3. The first two are expressed in most tissues, whereas the latter has a restricted expression pattern.[60, 61] Generally, hypophosphorylation of 4E-BPs induces 4E-BPs to bind to eIF4E, preventing the interaction of eIF4E with eIF4G and consequently impairing cap-dependent translation.[62] Inversely, the phosphorylation of 4E-BPs by mammalian target of rapamycin (mTor) kinase releases eIF4E, which can then interact with eIF4G, enhancing translation.[63] Decreases in 4E-BP phosphorylation by serum-deprivation or rapamycin treatment increased the fraction of eIF4E in the nucleus from 1-20%, while serum starvation in conjunction with rapamycin caused an increase of 30% in nuclear accumulation of eIF4E.[59] However, accumulation of eIF4E in the nucleus in response to serum starvation and rapamycin treatments did not occur in MEFs in which 4E-BP1 and 4E-BP2 were deleted, indicating that 4E-BPs are required for eIF4E nuclear accumulation. A mutant 4E-BP1 that is constitutively bound to eIF4E causes a nuclear accumulation of eIF4E, even under normal growth conditions, whereas a 4E-BP1 mutant lacking the eIF4E binding site did not cause nuclear accumulation under stress conditions.[59] Therefore, eIF4E can be retained and released from the nucleus in response to regulators of the mTOR pathway in a 4E-BP–dependent manner; and more specifically, 4E-BP1 localizes partly to the nucleus and regulates eIF4E nuclear levels under stress conditions. Nuclear 4E-BP modulates eIF4E activity and releases it from the nucleus. Additionally, 4E-BP may regulate the export of eIF4E bound to certain mRNAs and/or may regulate the release of free eIF4E to the cytoplasm for translation.[59]

eIF4G is another eIF found in the nucleus of different cell types and organisms. In the nucleus, eIF4G seems sensitive to cell stress and has functions related to mRNA

processing. Quantification in human cells shows 38% of total eIF4G in the nucleus.[12] McKendrick et al. demonstrated that there is a nuclear pool of eIF4GI with several foci of higher concentrations superimposed on diffuse nucleoplasmic staining but not significant nucleolar localization.[64] Similar results are obtained when this protein is overexpressed. Quantitative analysis of cellular fractioning showed that approximately 22% of the eIF4G was nuclear at steady state.[64] This nuclear pool of eIF4G stably associated with cap binding complex (CBC), pre-mRNA, and the spliceosome. eIF4GI is stably associated with capped RNA throughout pre-mRNA splicing in vitro, and eIF4GII has a cellular distribution similar to eIF4GI.[64] These data suggest that in the nucleus, eIF4G does not interact with eIF4E but does interact with CBC, and eIF4G acts in coupling RNA-processing events in the nucleus prior to export and translation.

Kafasla et al. reported that isoforms of eIF4G, Tif4631 and Tif4632p are found in the nucleus, and pull-down assays showed that they interact with spliceosomal snRNPS.[65] In vitro Tif4631p and Tif4632p interacts with the proteins U1 snRNP's Snu71p and U2 snRNP's Prp11p via specific domains consisting of 453-647 residues in Tif4631p and 424-609 residues in Tif4632p. These domains are able to inhibit splicing in vitro, probably by sequestering splicing components. In microarray experiments, deleting Tif4631 resulted in the accumulation of certain pre-mRNAs that give rise to ribosomal proteins in vivo. It is proposed that eIF4G, by its binding to spliceosome components, may participate in nuclear RNA processing and could act in degradation pathways such as NMD by acting as a scaffold protein that keeps important protein components together.[65] Cellular stress conditions can induce nuclear translocation of PABP1 and eIF4G in a time- and temperature-dependent manner.[66] During heat shock, the global cap-dependent translation is inhibited and specific mRNAs are translated, frequently via internal ribosome entry site-

mediated sequences (IRES).[67, 68] General protein synthesis is inhibited under heat shock conditions, and the translation of specific mRNAs referred to HSP proteins is increased.[67] Ma et al. reported that, in heat shocked human cells, the complex between PABP1 and eIF4G was dissociated and both translocated to the nucleus with HSP27.[66] When a normal temperature was reestablished, PABP1 and eIF4G were gradually redistributed to the cytoplasmic compartment, colocalized with each other and accumulated with HSP27. These data suggest that nuclear translocation of PABP1 and eIF4G is associated with the induction and nuclear translocation of HSP27 under heat shock. Additionally, the sequestration of PABP1 and eIF4G in the nucleus after heat shock may be important to inhibit the translation of mRNA in general.[66]

eIF5A

Some studies have reported that eIF5A may be required to translate mRNAs encoding cell cycle proteins because its depletion in yeast and mammals caused a short inhibition of cell proliferation and arrest in the cell cycle at the G1 phase.[69, 70] In global protein synthesis, eIF5A acts in the interaction of translating ribosome and elongation factors.[71] There are two forms of eIF5A in human cells: constitutively expressed eIF5A1 and tissue-restricted eIF5A2.[72]

Several reports suggest that in addition to its cytoplasmic localization, eIF5A can enter the nucleus via passive diffusion because it does not contain a classic NLS and is only 16.7 kDa.[73, 74] However, the amino-terminal region is important for the nuclear localization of eIF5A.[74] Unlike the nuclear import mechanism, the nuclear export of eIF5A is active in mammalian cells and can be mediated by CRM1 because its nuclear export is blocked by leptomycin B[75] or by exportin 4 (Exp4)[76]. Also, hypusination of eIF5A is

involved in the interaction of eIF5A with Exp4. eIF-5A accumulates in nucleoli when Exp4 is absent, suggesting that Exp4 somehow reduces the nucleolar localization of eIF5A.[76] Jao and Yu have reported that energy depletion, heat shock, and inhibition of transcription, translation and polyamine synthesis did not affect the cellular distribution pattern of eIF-5A.[73]

Considerable evidence has been acquired to support the participation of eIF5A in the nucleocytoplasmic transport of specific RNAs. For example: 1) eIF-5A concentrates at the nucleoplasmic face of NPCs associated with intranuclear filaments, it binds to CRM1 protein in Xenopus oocytes and mammalian cells, and thus could participate in nucleocytoplasmic transport processes;[75] 2) inhibitors of the hypusination of eIF-5A cause the disappearance of specific mRNAs from polysomes;[77] 3) eIF-5A affects the decay of specific yeast mRNAs, acting after the decapping process and before these transcripts are degraded by the exonuclease Xrn1p.[78]

The amino-terminal region is important for hypusination and contains a consensus phosphorylation motif for CK2, which phosphorylates eIF5A in Zea mays, Arabidopsis thaliana and Saccharomyces cerevisiae.[79] Indeed, Ser2 is the phosphorylation target for CK2 in vivo in Zea mays protoplasts, and it probably plays a role in the intracellular distribution of Zea mays eIF5A because confocal microscopy analysis showed that an EYFP-tagged mutant form of eIF5A, which mimics phosphoserine, had higher levels of fluorescence in the nucleus compared to wild-type protein.[79] Because these serine residues are absent in human, insect, and Saccharomyces pombe eIF5A, a model was proposed for plants and Saccharomyces cerevisiae: eIF5A would enter the nucleus by passive diffusion, but its nuclear export, mainly via Exp4, would be regulated by phosphorylation. The phosphorylation of Ser2 blocks its nuclear export by preventing interaction with the

nuclear exporting complex, whereas phosphoserine 2 dephosphorylation would reactivate nuclear export of eIF5A. This mechanism of regulation could be related to the specific nuclear export of eIF5A bound to mRNAs.[79]

Taylor et al. have suggested that overexpression of eIF5A induces apoptosis and its suppression inhibits p53 expression.[80] In this way, eIF5A without hypusination would mediate apoptotic functions of eIF5A, whereas the hypusinated form would have a cell survival role. eIF5A is present almost exclusively in the hypusinated form in normally growing cells, but after treatments with interferon γ (IFN)γ/Tumor necrosis factor α (TNF-α) or actinomycin D, it was translocated to the nucleus, suggesting that eIF5A may have a function in the nucleus during apoptosis.[80, 81] Taylor et al. introduced the possibility that eIF5A is hypusinated and retained in the cytoplasm until an apoptotic stimulus triggers its translocation to the nucleus where it may have pro-apoptotic functions.[80] Alternatively, it was also proposed that nuclear translocation of eIF5A during apoptosis interferes with a cytosolic function of eIF5A that is critical for cell survival and thus contributes to apoptosis.[80]

eIF5B

Jin et al. demonstrated that eIF5B of polytene chromosomes in Drosophila sp. and ribosomes colocalize with RNA polymerase II and also localizes to the nucleolus.[82] Likewise, evidence that translational machinery is associated with active sites of transcription is provided.[82]

eIF6

eIF6 is a protein required for ribosomal subunit dissociation that prevents the interaction of 40S ribosomal subunits with 60S subunits through its binding to 60S ribosomes.[83] In the nucleolus, eIF6 is a component of the pre-ribosomal particles and is required for the biogenesis of 60S subunits, whereas in the cytoplasm it mediates translation.[84, 85] Ribosome biogenesis is a complex process that involves several trans-acting factors and leads to the formation of mature ribosomal subunits, 40S and 60S, in eukaryotic cells. Ribosomal assembly occurs in the nucleolus; however, the final steps of ribosomal maturation are cytoplasmic.[86] Deletion of the yeast eIF6 homologue, Tif6, leads to a loss of 60S ribosomal subunits that can be rescued by the ectopic expression of human eIF6.[87, 88]. Therefore, eIF6 might act in the biogenesis of the 60S subunit, rather than in its stabilization.[89] eIF6 is also nucleolar[84, 90] in mammalian cells; in addition its downregulation by siRNA did not affect the ribosomal biogenesis because only the cytoplasmic pool of eIF6 was reduced, whereas the nucleolar eIF6 remained at normal levels.[84] Mammalian eIF6 is needed for ribosome biogenesis because its total depletion results in lethality before embryonic implantation.[84] Cell fractionation and indirect immunofluorescence studies have previously shown that yeast eIF6 is distributed throughout the cytoplasm and nuclei of yeast cells,[89] and phosphorylation of Ser174 and 175 of yeast eIF6 regulates its cell distribution, causing a loss of cell growth and viability.[91, 92] When both Ser174 and 175 were mutated, yeast eIF6 phosphorylation was abolished, and the protein was constitutively nuclear.[91] Biswas et al. showed that mammal eIF6 was phosphorylated at Ser174 and 175 by a nuclear form of casein kinase 1 (CK1), and phosphorylation of these residues regulates the nuclear export of mammal eIF6.[93] Moreover, eIF6 nuclear localization is promoted by a Ca2+-activated calcineurin

phosphatase and high Ca2+ concentration causes a translocation of eIF6 from the cytoplasm to the nucleus.[93]

Pioneer round of translation and Nonsense Mediated mRNA Decay (NMD)

In eukaryotes, the nuclear membrane separates the nucleus material from the cytoplasm; therefore, it was assumed that transcription and mRNA processing occur into the nucleus and that translation is confined to the cytoplasm. However, recent evidence suggests that translation-like processes can also occur in the eukaryotic nucleus and could be coupled to the transcription process.[12]

The nuclear translation hypothesis is supported by the following evidence: amino acids can be incorporated into proteins in an isolated nucleus, components of the translation machinery are found in the nucleus and a nuclear NMD mechanism has been uncovered. This process is a surveillance and quality-control mechanism that selectively destroys mRNAs containing premature termination codons (PTCs) to prevent truncated proteins from being produced.[94-98] Because ribosomes are known to recognize termination codons, it was assumed that some type of nuclear translation occurs to proofread the mRNA to find PTCs and lead to mRNA degradation through NMD. This assumption is further supported by the incorporation of amino acids into proteins in the nucleus, localization of translation factors in the nucleus and nuclear sites of translation coupled to transcription.[12, 99] In this context, the presence and role of eIFs in the nucleus has become a very important issue for further exploration.

Iborra et al. found that components of the complexes involved in transcription, translation and NMD colocalize and copurify and that interactions between them are probably mediated by the carboxyl-terminal domain of the catalytic subunit of RNA

polymerase II.[100] Moreover, direct evidence for nuclear translation coupled to transcription in mammals has been shown.[12] Biotin- or fluorescent-labeled lysine analogues were used to label newly made proteins and indicate protein synthesis sites in the nucleus and nucleolus. This labeling was performed in permeabilized mammalian cells, and immunofluorescence analysis detected eIFs and ribosomal proteins in the nucleus.[12] Additionally, alteration of nucleotide triphosphate (NTPs) concentration modifies the labeled lysine incorporation in newly made peptides, and the inhibition of transcription by α-amanitin causes a decrease in nuclear translation.[12] Also, immunogold assays demonstrated that nascent biotin peptides and RNA labeled with 5-bromouridine 5′-triphosphate (Br-UTP) colocalized within the nucleus, indicating that nascent peptides colocalize with nascent transcripts.[12] In addition, the translation apparatus was found at sites of transcription and seems to occur cotranscriptionally.[82] Immunostaining and in situ hybridization against rRNA in polytene chromosomes of Drosophila showed that rRNA, about 20 ribosomal proteins, eIF5B and IF2 colocalize with the RNA polymerase II catalytic subunit that has a hyper-phosphorylated carboxyl-terminal domain (CTDp).[82] Taken together, the evidence for nuclear translation coupled to RNA polymerase II transcription were obtained using different methods,[12, 82, 100] minimizing the possibility that residual cytoplasmic protein synthesis or other artifacts are responsible for the observed nuclear protein synthesis.

However, this issue remains extremely controversial as several sources refute nuclear translation.[21, 101-103] A majority of the arguments against nuclear translation are related to technical and methodological limitations, such as cytoplasmic contamination, overpermeabilization of the cells and antibody specificity. Other arguments against nuclear

translation rely on the low concentration of translation machinery components and the possible absence of active ribosomes in the nucleus.[102 103, 104]

Therefore, the presence in the nucleus of several components of translation machinery, including eIFs, could contribute indirectly or directly with nuclear translation hypothesis. These presences could suggest that is not impossible that a nuclear pioneer round of translation associated with NMD occurs. Whether nuclear translation actually produces fully processed and functional proteins is another matter and is probably not a major role of the processes discussed here.

Some studies examining this issue have demonstrated that a few cycles of nuclear translation as a pioneer round of translation could occur in newly made mRNA. It is thought to function as a proofreading mechanism that can, if necessary, induce NMD.[99, 105, 106] The translation machinery, including nuclear ribosomes and translation factors, scans the mRNAs to detect PTCs; and the NMD machinery degrades messages containing PTCs. PTCs within the mRNA can be the result of transcription errors, improper splicing or nonsense and frameshift mutations.[107] The translation of these mRNAs can produce truncated proteins with dominant-negative or deleterious gain-of-function activities. NMD reduces the level of mRNAs harboring PTCs to 5-30% in both the nucleus-associated and cytoplasmic fractions of cells.[101] During RNA splicing, an exon-junction protein complex (EJC) associates with the mRNA and remains attached to the mature mRNA after splicing. This activity always occurs approximately 20-24 nucleotides upstream of the exon-exon junction, which can promote mRNA export and provide a signal needed to trigger an NMD response.[108] The NMD machinery comprises three trans-factors called up-frameshift proteins (UPF) that are found in Saccharomyces cerevisiae and higher eukaryotes (UPF1 to -3).[109, 110]

Different models of subcellular localization for this process have been suggested and indicate pioneer round of translation/NMD occurrence in the cytoplasm,[95, 111-115] nucleus-associated. [95, 99, 102, 106, 113, 116]

In this way, pioneer rounds and NMD could also be a nuclear surveillance mechanisms against mRNAs containing PTCs. A nuclear model for this process in presented in Fig. 3.[95, 99, 106, 117-119]

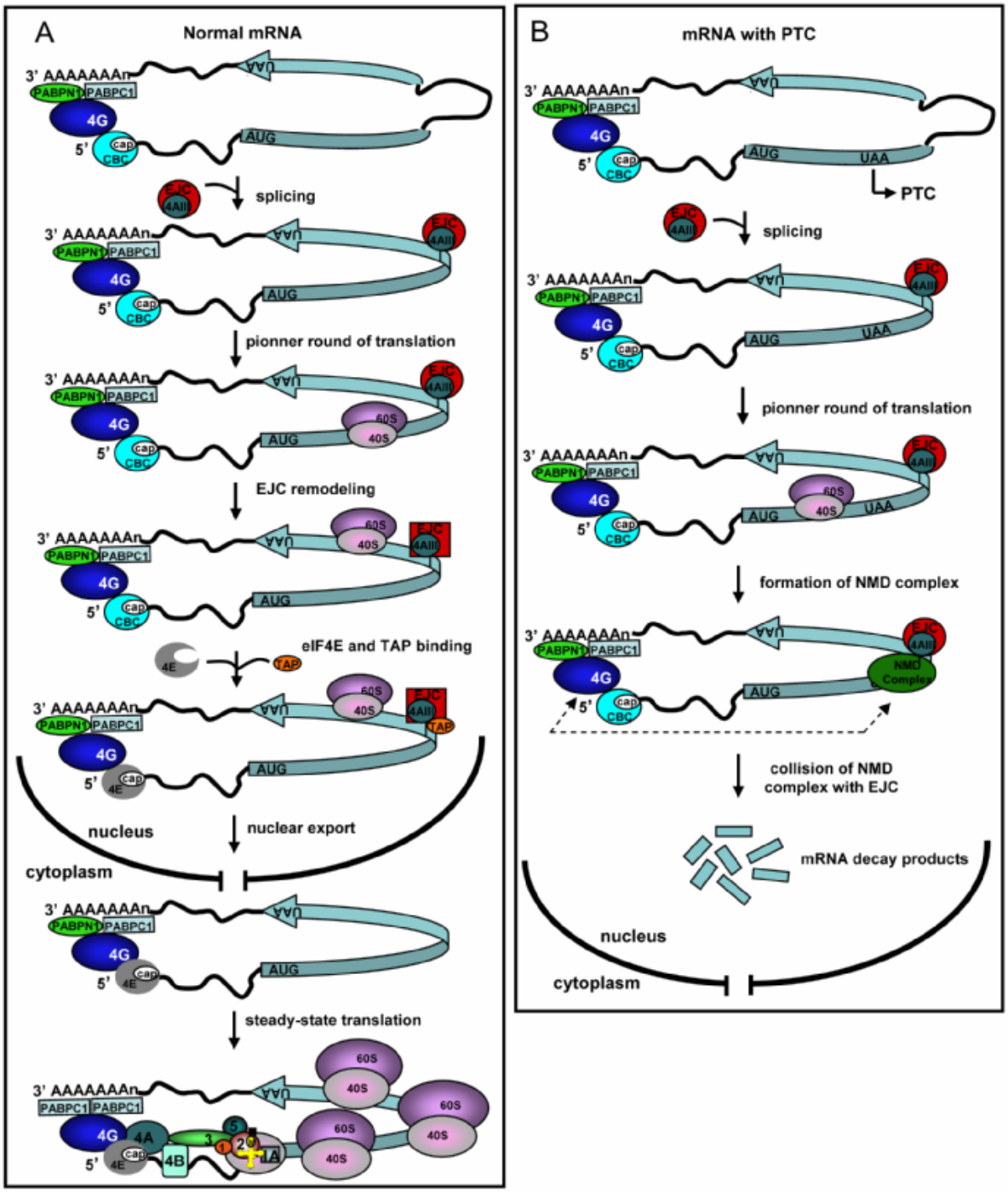

Figure 3. General model of the nuclear pioneer round/NMD. The mRNA 5′ terminus is bound to the heterodimer cap binding complex (CBC) with CBP80 and CBP20 subunits that are added during transcription. eIF4G binds to mRNA and eIF4AIII participates of the EJC complex. (A) The normal termination codon is generally in the terminal 3' exon of the mRNA and premature stop codons in internal exons. The ribosome scans the mRNA bound to CBC and removes EJCs. During translation of RNAs with no PTCs, the EJC is

remodeled and CBC is replaced by eIF4E. (B) However, if the ribosome encounters a PTC upstream to EJC, it pauses and several additional proteins are recruited to the mRNA and 40S ribosome subunit, initiating mRNA degradation by NMD. The eIF4G and eIF4AIII seem participate in the NMD process. PTC - premature stop codon, EJC - exon junction complex, NMD - nonsense mediated mRNA decay, 60S - large ribosomal subunit, 40S - small ribosomal subunit, PABPN1 - poly(A)-binding protein nuclear 1, PABPC1 - poly(A)-binding protein cytoplasmic 1, CBC - cap binding complex, TAP – mRNA nuclear export mediator, 4G –eIF4G, 4AIII - eIF4AIII, 4A – eIF4A, 4B – eIF4B, 4E – eIF4E, 2 – eIF2, 3 – eIF3, 1 – eIF1, 1A –eIF1A, 5 – eIF5, cap – 5'cap structure.

Cellular distribution of eIFs and cancer

Gene expression in eukaryotes is regulated at many levels including transcription, mRNA poly-adenylation and splicing, export of mRNAs from the nucleus to the cytoplasm, mRNA degradation and mRNA translation. Deregulation of any one of these processes can cause abnormal gene expression that can result in altered cell growth and can contribute to development of diseases, such as cancer.[120]

The regulation of mRNA translation is important for embryonic development, the regulation of cell growth and cell differentiation. Alterations at the translation level that occur in cancer can affect the translation of an individual mRNA or can lead to more global changes, such as an increase in the overall rate of protein synthesis, and the translational activation of a subset of mRNAs that play important roles in many essential cellular processes including proliferation, apoptosis, angiogenesis and DNA repair. These alterations may be due to variations in mRNA sequences that increase or decrease translational efficiency, to changes in the expression or availability of components of the translational machinery, and to activation of translation through aberrantly activated signal transduction pathways.[121]

Several studies have shown that the altered expression of many eIFs is associated with malignant transformation, cancer prognosis and the regulation of gene expression.[120, 122-125] Although eIFs are involved in malignant transformation, the molecular mechanisms of this process remain poorly understood. It is believed that the altered expression levels of eIFs up- or down-regulate the translation of various mRNAs encoding proteins important for different cellular processes such as cell cycle, proliferation, survival and apoptosis.[120] Moreover, eIFs functionally interact with oncogenes and are often primary targets of signal transduction pathways similar to proto-oncogenes that underlie most human cancers.[124, 125] Evidence also suggests that eIFs may be a new group of tumor suppressors.[122, 123] It is well known that several eIFs show altered expression in different tumor types.[120, 124, 125] Moreover, translation initiation can also be regulated by alteration of the phosphorylation status of the various eIFs.[2, 126, 127] Changes in the expression levels of specific eIFs are also associated with resistance to radiation and chemotherapy.[124]

As discussed in this review, various eIFs have additional functions to their regulatory roles in protein synthesis. These are related to their cellular distribution and are important for a variety of other cellular processes such as mRNA splicing and degradation, mRNA nuclear export and ribosomal biogenesis. Therefore, alteration of these functions could deregulate gene expression and consequently lead to cancer development. This idea is supported by evidence showing that some eIFs have altered cell distribution and impair different cellular process in several tumor types (Table 5).

Table 5. Cellular distribution of eIFs in different tumor types.

Alteration of cellular distribution of eIF	Tumor type	Possible effect on the cell	Reference
- increased level of eIF2α and eIF2α(P) in nucleus and nucleolus	- carcinomas of the colon, stomach and rectum carcinomas - bronchoalveolar carcinomas of lung and liver carcinoma - astrocytic, oligodendroglial tumors and meningiomas	ND	15, 128, 208
- nuclear distribution of eIF3e/INT6 of a cell cycle-dependent manner - in contrast to normal cell, cancer cells have only cytoplasmic localization	- Breast and colon carcinomas	- impaired chromosome segregation and altered genome stability contributing to tumorigenesis	132, 134
- increased level of eIF4E and eIF4E(P) in the nucleus	- astrocytic, oligodendroglial tumors and meningiomas	- transforming properties related to control of the specific mRNA transport	128, 135
- nucleolar overexpression of eIF6	- Head and neck carcinomas - colorectal cancer	- cell proliferation increased - ribosomal biogenesis increased	136-138

ND shows the not determined effect for the cell.

eIF2α

The levels of eIF2α and eIF2α(P) are significantly increased in carcinomas of the colon, stomach and rectum compared to cells from normal tissue.[18] Electron microscopy showed the cytoplasmic, nuclear and nucleolar localization of this subunit in gastrointestinal carcinomas and in normal cells from the gastrointestinal tract. These gastrointestinal carcinoma cells have an increased percentage of eIF2α located in the nucleus and nucleolus compared to normal cells, whereas less differentiated tumors have an increased percentage of nuclear localization of eIF2α compared to more differentiated tumors.[18] A study of three brain tumor types; meningiomas, oligodendroglial tumors and astrocytomas, found that eIF2α was present in the nucleus of cells from three tumor types, but in a higher proportion in meningiomas.[128]

eIF3e

Loss of function in human eIF3e/INT6 may contribute to tumorigenesis as supported by the following studies: The expression of eIF3e/INT6 is reduced in a proportion of mammary carcinomas and non-small cell lung carcinomas[129] and both human and mouse eIF3e/INT6 genes play critical roles in breast and lung tumorigenesis.[130] Miyazaki et al. also showed that human INT6 is frequently lost in primary breast carcinomas and loss of heterozygosity (LOH) occurs.[131] eIF3e/INT6 mutations in humans impairs chromosome segregation and consequently may alter genome stability, contributing to tumorigenesis.[132] Stable expression of a truncated eIF3e in NIH 3T3 cells, unlike the wild-type form, causes malignant transformation. These cells were characterized by their ability to form foci, increased proliferation and anchorage-independent growth, lack of contact inhibition and inhibition of apoptosis onset under serum starvation.[133]

In the same way, Traicoff et al. showed that the cellular distribution of eIF3e/INT6 is altered in specific cancers; in normal breast and colon epithelium, eIF3e/INT6 protein localized in the cytoplasm and nucleus, but in tumor tissue only cytoplasmic localization was observed.[134] Therefore, loss of regulation of eIF3e/INT6 cellular redistribution may be a significant feature of malignancy in human cells.[134]

eIF4E

Tejada et al. observed that eIF4E localized only in the cytoplasm of non tumoral neurons, whereas eIF4E was present in the nucleus and cytoplasm of cells in specific brain tumors.[128] Moreover, the nuclear localization of eIF4E differed between the types of brain tumors studied; it was more common in the nucleus of astrocytic and oligodendroglial tumors cell than of meningioma cells.[128]

In addition, analysis of phosphorylated eIF4E levels showed that meningiomas have higher levels of phosphorylated eIF4E than glioblastomas and astrocytomas. Levels of phosphorylated eIF4E strongly correlated with cyclin D1 expression in these tumors. In this way, eIF4E in the nucleus of these tumors should be regulating the nuclear export of cyclin D1 mRNA among others.[128] Therefore, phosphorylation of nuclear eIF4E becomes an important step in the control of specific mRNA transport that may be related to the transforming properties of eIF4E.[135]

eIF6

eIF6 is overexpressed in the nucleoli of both head and neck cancer and colorectal cancer, and this increase is associated with disease progression.[136, 137] In contrast, low eIF6

expression is associated with reduced disease-free survival in a study of 66 serious carcinomas.[138]

Cells that express only 25% of normal eIF6 levels, knocked down by interference RNA (RNAi), have normal nucleolar levels of eIF6 and normal ribosomal biogenesis; however, cytoplasmic levels of eIF6 were strongly reduced.[84] These results suggest that the contrast in eIF6 activity may be associated with differences in intracellular localization.

Conclusions

The topic of "nuclear translation" still remains controversial in the scientific world. However, increasing evidence shows that a process similar to translation may occur in the nucleus. Although its function is probably not involved in the production of proteins, this process is likely related to the processing of RNAs and NMD. In this context, the localization and roles of eIFs in the nucleus or subnuclear compartments become an essential field to be studied for the best understanding of how nuclear translation may function.

In this review, we discussed what is known about the subcellular localization and roles of the fundamental components of translation machinery, eIFs, in the nucleus. We observed that, besides their cytoplasmic localization, several eIFs are localized to the nucleus and nucleolus. The eIFs play an essential role in the cytoplasmic protein synthesis process that is regulated by several signaling pathways and affects several processes such as cell proliferation, survival and apoptosis. Similarly, in the nucleus, eIFs function in key processes that regulate gene expression, cell viability and death. These factors may work in a process such as mRNA splicing, protein degradation, ribosomal biogenesis, mRNA nucleocytoplasmic transport, NMD and apoptosis. The roles of some eIFs in mRNA

splicing and protein degradation suggest that these factors could act in the proofreading process to control the presence of PTCs in nuclear mRNA. However, direct evidence of eIFs working in the nuclear translation process remains unclear and requires further study.

Deregulation of the expression or phosphorylation status of many eIFs is associated with malignant transformation, cancer prognosis and deregulated gene expression.[23, 120, 122, 123, 127, 139] Several cell stress conditions modify the subcellular distribution of eIFs and consequently interfere with or regulate their nuclear function in these processes. eIFs are susceptible to deregulation in the nucleus that can result in loss of function and lead to drastic consequences for the cell. This idea is supported by evidence showing that tumorigenesis is closely related to the regulation of eIF nuclear localization and function.

In this way, eIFs could also act in the nuclear pioneer round of translation/NMD processes. Moreover, as in the cytoplasm, they participate in important mechanisms and are subject to intense regulation in the nucleus. This property in both cellular compartments indicates the importance of eIFs to the regulation of gene expression and strengthens the possibility of eIFs as effective therapeutic targets for cancer therapy.

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10. ANEXO II

10.1 APRESENTAÇÃO

Essa seção apresenta um artigo científico intitulado “Betacellulin Overexpression in Mesenchymal Stem Cells Induces Insulin Secretion In Vitro and Ameliorates Streptozotocin-Induced Hyperglycemia in Rats” publicado na revista Stem Cells and Development em fevereiro de 2011.

O controle adequado da glicemia no diabetes mellitus tipo 1 (DM1) permanece ainda difícil de ser alcançado, apesar da variedade tratamentos existentes. Atualmente, o transplante de ilhotas pancreáticas tem sido utilizado para o tratamento da DM1. Entretanto, embora seja efetivo para o tratamento da doença, não está disponível para todos os pacientes. Desta forma, a busca por fontes alternativas de células que produzam insulina é muito pertinente. O uso de células tronco mesenquimais (MSCs) no tratamento de diferentes patologias tem sido sugerido, incluindo o diabetes, uma vez que apresentam capacidade de se diferenciarem também em células secretoras de insulina. A proteína betacelulina (BTC) é um ligante do receptor do fator de crescimento epidermal que promove o crescimento e a diferenciação de células β-pancreáticas e que tem mostrado melhorar o metabolismo da glicose em modelos experimentais murinos.

Nesse trabalho foram analisados os efeitos *in vitro* da superexpressão de BTC em MSCs, bem como o efeito *in vivo* decorrente da terapia utilizando essas células em um modelo murino de diabetes.

Os resultados obtidos nesse estudo mostram que MSCs superexpressando BTC foram capazes de diferenciarem-se em células produtoras de insulina. Além disso, o transplante dessas células, em ratos com diabetes induzida por streptozotocina, causou uma diminuição significativa na hiperglicemia desses animais. Esses dados sugerem que a

superexpressão de BTC é capaz de guiar a diferenciação das MSCs em células produtoras de insulina e que a terapia celular com MSCs superexpressando BTC apresentam potencial terapêutico para tratar o diabetes.

As contribuições de cada autor nesse trabalho foram: Ana H. Paz participou da proposta da hipótese, do delineamento experimental, da realização de todos os experimentos, da análise e interpretação dos dados e da elaboração do artigo. Gabrielle Dias Salton participou do delineamento, da realização experimental e da interpretação dos dados referentes à construção dos plasmídeos e à transfecção das células; e da revisão do artigo. Ana Ayala Lugo participou da realização do estabelecimento de culturas de células tronco-mesenquimais. Cristiano Gomes participou da experimentação animal. Paula Terraciano participou da experimentação animal. Rosana Scalco participou da realização dos experimentos de radioimunoensaio. Claudia Cilene Fernandes Correia Laurino participou do delineamento experimental, da realização e da interpretação dos dados de transfecção e seleção de células; e revisão do artigo. Marlon R. Schneider, Eduardo Pandolfi Passos e Elizabeth Cirne-Lima participaram da proposta da hipótese, orientação do trabalho e revisão do artigo. Luise Meurer participou da proposta da hipótese, orientação do trabalho e da realização das análises patológicas.

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Betacellulin Overexpression in Mesenchymal Stem Cells Induces Insulin Secretion In Vitro and Ameliorates Streptozotocin-Induced Hyperglycemia in Rats

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Betacellulin (*BTC*), a ligand of the epidermal growth factor receptor, has been shown to promote growth and differentiation of pancreatic β-cells and to improve glucose metabolism in experimental diabetic rodent models. Mesenchymal stem cells (MSCs) have been already proved to be multipotent. Recent work has attributed to rat and human MSCs the potential to differentiate into insulin-secreting cells. Our goal was to transfect rat MSCs with a plasmid containing *BTC* cDNA to guide MSC differentiation into insulin-producing cells. Prior to induction of cell MSC transfection, MSCs were characterized by flow cytometry and the ability to in vitro differentiate into mesoderm cell types was evaluated. After rat MSC characterization, these cells were electroporated with a plasmid containing *BTC* cDNA. Transfected cells were cultivated in Dulbecco's modified Eagle medium high glucose (H-DMEM) with 10 mM nicotinamide. Then, the capability of MSC-*BTC* to produce insulin in vitro and in vivo was evaluated. It was possible to demonstrate by radioimmunoassay analysis that 10^4 MSC-*BTC* cells produced up to 0.4 ng/mL of insulin, whereas MSCs transfected with the empty vector (negative control) produced no detectable insulin levels. Moreover, MSC-*BTC* were positive for insulin in immunohistochemistry assay. In parallel, the expression of pancreatic marker genes was demonstrated by molecular analysis of MSC-*BTC*. Further, when MSC-*BTC* were transplanted to streptozotocin diabetic rats, BTC-transfected cells ameliorated hyperglycemia from over 500 to about 200 mg/dL at 35 days post-cell transplantation. In this way, our results clearly demonstrate that *BTC* overabundance enhances glucose-induced insulin secretion in MSCs in vitro as well as in vivo.

Introduction

Despite a wide variety of pharmacological treatments for type 1 diabetes, including insulin therapy, adequate control of blood sugar levels is often difficult, in part because pharmacological agents are not able to duplicate the glucose regulatory function of normal islets [1]. At the moment, transplantation of cadaveric pancreatic islets is the most preferred cell replacement available to treat type 1 diabetes [2]. However, the scarcity of transplantable human islets poses a major obstacle in the widespread use of this therapy [2]. This scenario has evoked a large-scale search for alternative sources of p-cells. Although embryonic stem (ES) cells may differentiate into nearly all cell types and be used to assemble functional organs, the source of ES cells presents ethical and legal concerns, and the tumorigenic tendency of ES cells is at the moment not under control [3]. Other cell types such as intestinal [4], hepatic [5–7], ductal, or pancreatic stem cells [8–10] have been studied as candidates to replace β-cells. However, scarcity of the source and the invasive procedures required to isolate and culture these cells have limited their use. Bone marrow-derived stem cells (hematopoietic and mesenchymal) carry significant potential for clinical applications, because they are easily accessible for an autograft and routinely collected from adults without ethical concern inherent to fetal embryonic tissues [11].

Mesenchymal stem cells (MSCs) can be isolated from bone marrow based on their ability to adhere to plastic substrates [12]. Under appropriate conditions, they differentiate into multiple mesenchymal cell types including cartilage, bone,

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adipose and fibrous tissues, and myelosupportive stroma [13]. Recent work has attributed to MSCs the potential to differentiate into cells of all 3 primary germ layers, including mesoderm, neuroectoderm, and endoderm; they are also reported to be immunoprivileged and immunosuppressive [11]. Rat [14] and human MSCs [15] have been shown to differentiate into insulin-secreting cells.

Davani et al. [16] have shown that human islet-derived precursor cells are capable of differentiating to adipocyte, condrocyte, and osteocyte, human islet-derived precursor cells also express MSC markers, indicating that these cells could be MSCs that are present in adult islets in situ. Betacellulin (*BTC*) is a multifunctional polypeptide growth factor belonging to the family of epidermal growth factor receptor (*EGFR*) ligands [17]. *BTC* was first described as a mitogen from a mouse pancreatic insulinoma cell line [18]. *BTC* is initially produced as a transmembrane protein that can be cleaved by metalloproteinase to release the mature circulating form [19]. Regarding the tissue distribution, *BTC* is expressed abundantly in the pancreas and intestine [20]. There are several evidences indicating that, among the 7 EGFR ligands, *BTC* exerts unique actions in pancreatic islet physiology. *BTC* converts pancreatic progenitors to insulin-secreting cells in vitro [21–23], sustains PDX1 expression, and induces β-cell differentiation in human ES cells [24].

In addition, the administration of this growth factor in vivo improves glucose metabolism by increasing the β-cell mass in diabetic animal models [22,25]. These results suggest that *BTC* plays an important role in regulating growth and differentiation of pancreatic endocrine precursor cells.

Considering the plasticity of MSCs and the action of *BTC* in differentiation and proliferation of β-pancreatic cells, our aim was to evaluate the in vitro and in vivo effects of *BTC* overexpression in rat MSCs.

Materials and Methods

Transgene construction

The *BTC*-coding sequence was amplified from mouse lung cDNA by polymerase chain reaction (PCR) using the primers: BTC 5′-GGC CCA GGA AGG GCA TAG AGA-3′ and BTC 5′-ATG AGT CAG GTC TTT TGT AGC TTG-3′ [26]. The product was inserted into the pCRII-TOPO cloning vector (Invitrogen), and after sequencing, cDNA was cloned into *Eco*RI site downstream of the cytomegalovirus promoter and upstream to the internal ribosome entry site (IRES) and the enhanced green fluorescent protein (EGFP) in the expression vector pIRES2-EGFP (Clontech). Correct orientation was checked by digestion with *Kpn*I and *Sma*I. The constructed vector was named pIRES2-BTC-EGFP.

Isolation and culture of bone marrow cells

Eight-week-old Wistar rats were purchased from the Centro de Reprodução e Experimentação de Animais de Laboratório–CREAL-UFRGS. The procedures were performed in accordance with the guidelines for animal experimentation of UFRGS University. Bone marrow cells were obtained from femurs and tibias. After isolation, 1×10^7 bone marrow-derived cells were cultured (37°C, 5% CO_2) in T25 culture flasks (TPP) with DMEM (Invitrogen) medium containing 15 mM HEPES, 15% inactivated fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin, and 100 mg/mL streptomycin antibiotic solution (Gibco). On the third day of culture, medium was changed and nonadherent cells were removed. Adherent cells gaining 80% of confluence were passaged with the use of 0.05% trypsin–EDTA solution (Gibco) and then maintained in Dulbecco's modified Eagle medium (DMEM) with 10% FBS (complete medium).

Differentiation assays

To characterize MSCs in accordance with the International Society for Cellular Therapy statement [27], 2 different experimental procedures were employed. Osteogenic differentiation was induced by culturing MSCs for up to 3 weeks in DMEM containing 10% FBS and 15 mM HEPES, supplemented with 10^{-8} M/L dexamethasone (Sigma), 5 μg/mL ascorbic acid 2-phosphate (Sigma), and 10 mM/L β-glycerolphosphate (Sigma). To observe calcium deposition, cultures were stained with alizarin red S stain (Nuclear).

Second, to induce adipogenic differentiation, MSCs were cultured with 10^{-8} M dexamethasone (Sigma), 5 μg/mL insulin, and 50 μg/mL indomethacin (Sigma). Adipocytes were easily discerned from the undifferentiated cells by phase-contrast microscopy. To further confirm their identity, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and stained with oil red (Sigma) on day 21 of adipogenic differentiation.

Flow cytometry

Approximately 1×10^6 MSCs were prepared. They were placed in sterile tubes and washed 2 times by centrifugation at 300 *g* for 5 min at 4°C. MSCs were then resuspended in PBS and incubated for 20 min at 4°C with phycoerythrin- or fluorescein isothiocyanate-conjugated antibodies against rat CD34, CD45, CD11bc, CD44, CD90, and CD29. All assays were conduced using antibody concentrations as recommended by the manufacturers. Phycoerythrin and fluorescein isothiocyanate mouse anti-rat IgG1, IgG2a, and IgM were used as isotype controls. Cells were collected and washed with PBS by centrifugation and fluorescence analysis was carried out with the BD FACS-Calibur flow cytometry system (Becton-Dickinson) with a one-laser system that is capable to detect 3 fluorochromes excited by the 488 nm laser in a multiparameter manner. Data samples were analyzed using Cellquest and PAINT-A-GATE software.

Cell transfection

MSCs were harvested by trypsinization and then pelleted via centrifugation. Excess media were removed to obtain a tight cell pellet, which was resuspended in 4°C DMEM without serum or antibiotics. Cells were quantified in Neubauer's chamber and 1×10^6 cells in pure DMEM were transferred to a 4-mm cuvette with 60 μg of pIRES2-*BTC-EGFP* or pIRES2-*EGFP* (empty vector used as negative control). After an incubation time of 5 min at room temperature, cells were electroporated using Gene Pulser XCell (Bio-Rad Laboratories) by applying the following parameters: 950 μF, 200 Ωm, and 350 V. After electroporation, cells were incubated for 5 min at room temperature and plated in H-DMEM complete medium supplemented with 10 mM nicotinamide (Acros). Twenty-four hours after electroporation, medium

was replaced and cells were analyzed under fluorescence microscopy. After 48 h, cells were submitted to the selection media using G418 (Geneticin; Gibco).

Immunocytochemistry assay

For *BTC* immunofluorescence, pIRES2-*BTC-EGFP*–transfected cells were fixed with 4% formaldehyde in PBS. The primary antibody, goat anti-mouse *BTC* (R&D Systems), was diluted 1:100 and incubated overnight at 4°C. A Cy3-conjugated rabbit anti-goat secondary antibody (Sigma) was used. Fluorescence was detected using fluorescence microscopy (Nikon Eclipse TE 2000-U; Nikon Instruments).

For insulin immunocytochemistry assay, pIRES2-BTC– and pIRES-*EGFP*–transfected cells were fixed with 4% formaldehyde in PBS. We used a 1:500 dilution of guinea pig anti-insulin (Dako) and Universal Dako LSAB Kit Peroxidase (Dako). Cells were analyzed under microscopy (Nikon Eclipse TE 2000-U; Nikon Instruments). The same antibody was used for immunohistochemistry of the grafts.

Insulin radioimmunoassay

The static incubation method was applied as described by Lumelsky et al. [28].

Briefly, 10^5 adherent cells at 4, 8, 12, and 16 days post-transfection were rinsed twice with Krebs-Ringer with bicarbonate buffer (120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM $MgCl_2$, 25 mM $NaHCO_3$, and 0.1% bovine serum albumin). An inhibitor of insulin secretion (Nifedipine; Sigma) was added to the medium at 30 min prior to medium replacement with Krebs-Ringer containing 2.7 mM glucose. Cells were incubated for 15 min at 37°C with Krebs-Ringer and then in buffer containing 5, 30, or 50 mM glucose for 1 h.

Insulin levels in cell supernatants were measured using an immunoradiometric assay kit (RIA 13k; Linco Research). To each polypropylene tube, anti-insulin, ^{125}I-Insulin, and 100 pL of the samples were added. Immune complexes were precipitated 24 h later with polyethylene glycol solution, and a gamma counter was used to determine the radioactivity in the precipitates. Four determinations were carried out in duplicate and the means and standard error were obtained.

RNA extraction and reverse transcription–PCR

Reverse transcription (RT)–PCR was performed to assess the expression of pancreatic β-cell–related genes (*PDX1, PAX4, INS1,* and *NKX6.1*).

Total RNA was isolated from nontransfected MSCs, MSCs at 14 days after transfection with pIRES2-*EGFP-BTC* or pIRES2-*EGFP*, and RINm5f cell line (used as a positive control for pancreatic genes expression). Total RNA was isolated using the SS III First Strand super mix (Invitrogen). Standard RT was performed using M-MLV Reverse Transcriptase (Sigma) according to the manufacturer's instructions. Reactions mixtures for PCR included 1 μg of cDNA, 0.2 μM of each antisense and sense primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Invitrogen). PCR was performed on a thermocycler Techne TC412 (Barloworld Scientific). Amplification conditions consisted of an initial denaturation step at 94°C for 1 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing for 1 min, extension at 72°C for 30 s, and final polymerization at 72°C for 10 min. *β-Tubulin* was used as an internal standard.

Diabetes animal model

Male Wistar rats housed at 22°C in an air-conditioned environment, with a 12-h light–dark cycle, and fed a regular unrestricted diet received a single intraperitonial injection of 70 mg/kg streptozotocin (Sigma) freshly dissolved in 0.1 M citrate buffer (pH 4.5).

Blood glucose was measured weekly with a glucometer (Optium Xceed; Abbott). Animals whose blood glucose level exceeded 300 mg/dL were considered diabetic.

Cell transplantation

Under anesthesia, a medial abdominal laparotomy was performed and the left kidney was exposed. About 4×10^6 cells in 50 μL of PBS were transplanted into the kidney parenchyma by direct insertion of a 24-gauge syringe into the kidney capsule at the upper pole and cells were gently discharged into the parenchyma. Blood glucose levels were monitored once a week (days 14, 21, 28, and 35) in samples obtained from the tail vein, using a glucometer.

At the end of the experiment (day 35), rats were euthanatized and kidneys and pancreas removed for hematoxylin and eosin staining for histological analysis.

Statistical analysis

Data are expressed as mean ± SE and statistical differences between groups were determined by repetitive measure ANOVA in SPSS version 16. Bonferroni post-hoc test was applied when necessary. Differences between groups were considered significant when $P < 0.05$.

Results

Phenotypic characteristics of expanded undifferentiated MSCs

Bone marrow MSCs were obtained from rats by plating bone marrow cell suspension in tissue culture dishes and propagation of adherent cells. The isolated cells developed into visible systematic colonies of adherent fibroblast-like cells at about 7–10 days after initial plating and became morphologically more homogeneous (Fig. 1A, B) with time in culture by depletion of hematopoietic and other bone marrow stromal cells. Rat MSCs are known to be positive for CD44, CD90, and CD29 and negative for CD34, CD45, and CD11bc. Flow cytometry was performed from the moment of MSC extraction to the third trypsinization step. By the third passage, the vast majority of cells stained for the markers CD44 (99.3%), CD90 (99.8%), and CD29 (99.5%) and only a small proportion manifested expression of the markers CD45 (0.9%), CD11b/c (0.52%), and CD34 (0.05%). According to this pattern of cell surface marker expression, the cell population at this time point (~24 days in culture) was quite uniform and can be considered bona fide MSCs.

The MSC differentiation potency was shown using protocols known to induce differentiation into bone and adipose cells. The results showed a clear potency for adipogenic differentiation as detected by oil red staining and for

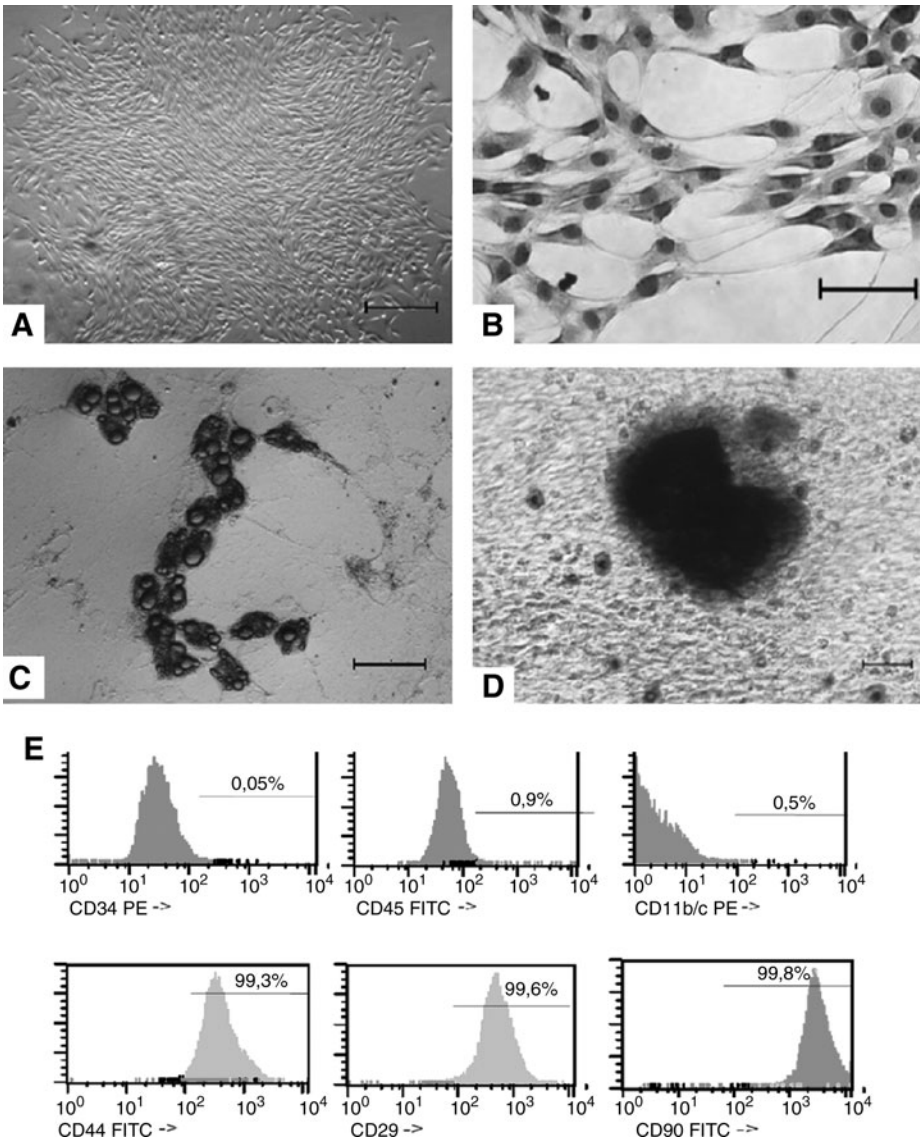

FIG. 1. Characterization of MSCs. **(A)** Phase contrast image of adherent fibroblast-like cell colony at 10 days after plating. **(B)** MSC morphology stained with hematoxylin and eosin. **(C)** Induced adipogenic differentiation evidenced by oil red O-stained fat vacuoles. **(D)** Induced osteogenic differentiation, stained with alizarin red. Scale bars represent 100 μm. **(E)** FACS analysis for CD34, CD45, CD11b/c, CD44, CD29, and CD90. MSC, mesenchymal stem cell.

osteogenic differentiation as detected by deposit of calcium (Fig. 1C, D, respectively).

BTC expression in vitro modified the morphology and gene expression pattern in MSCs. After characterization, MSCs within 12 passages were transfected by electroporation with the expression vector pIRES2-*BTC-EGFP* or the empty vector. The vector included neomycin resistance gene and an IRES sequence of the encephalomyocarditis virus in the multiple cloning site, permitting both *BTC* and *EGFP* cDNAs to be translated from a bicistronic mRNA.

The efficiency of transfection with this vector as evaluated by the number of GFP-positive cells was ~40%. Following selection, the surviving cells (termed *BTC*-MSCs or pIRES-MSCs, depending on the vector employed) were analyzed.

BTC-MSCs showed *GFP* expression and immunofluorescence for BTC (Fig. 2). No expression of *BTC* was detected in MSCs and pIRES-MSCs (data not shown).

Under inversed microscope, it was possible to observe within 10 days that the morphology of *BTC*-MSCs changed from spindle-like (Fig. 3B) to flat epithelial-like cells (Fig. 3A).

To assess the mRNA expression of typical β-cell genes (*PDX1, PAX4, INS1*, and *NKX6.1*), RT-PCR was applied on RINm5f insulinoma cells (used as positive control for pancreatic gene expression), MSCs (negative control), pIRES-MSCs, and BTC-MSCs. Transcripts of *PDX1, PAX4, INS1*, and *NKX6.1* were detected at 14 days after transfection in MSC-BTC but not in MSCs or MSC-pIRES, as expected (Fig. 4). The results strongly suggest that the overexpression of BTC may induce the expression of multiple islet genes involved in the β-cell differentiation pathway.

After 23 days of culture, different from pIRES-MSCs, some *BTC*-MSCs presented aggregating tendency and formed a 3D structure (Fig. 5A, B) similar to the structures described by other authors during specific differentiation protocols [29–31].

MSC-BTC *cells develop insulin expression and secretion*

To evaluate the functional differentiation of MSC-*BTC*, they were tested for insulin content and glucose-induced insulin secretion in vitro.

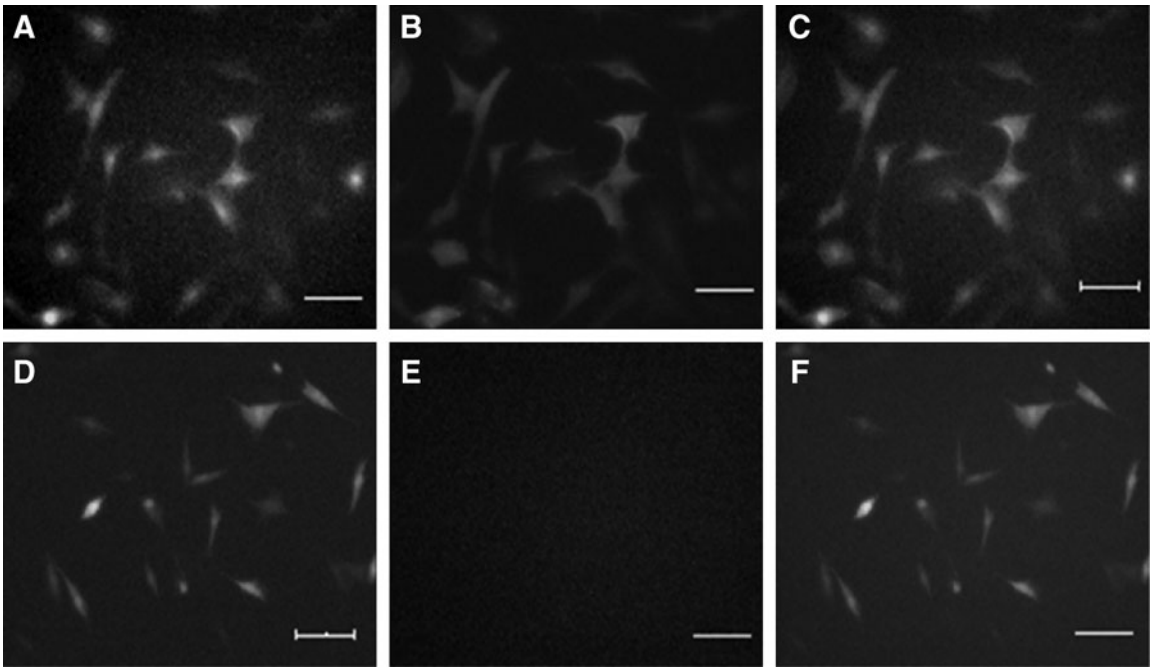

FIG. 2. Expression of BTC and GFP in BTC-MSCs and pIRES-MSCs. **(A)** BTC-MSCs that are GFP positive. **(B)** BTC-MSCs positive for -anti-BTC (diluted 1:100) traced with Cy3-conjugated rabbit anti-goat secondary antibody. **(C)** Merge of **A** and **B**. **(D)** pIRES-MSCs that are GFP positive. **(E)** pIRES-MSCs negative for the expression of BTC traced with Cy3. **(F)** Merge of **D** and **E**. Scale bars represent 100 μm. BTC, betacellulin; IRES, internal ribosome entry site; GFP, green fluorescent protein.

Immunocytochemistry analysis demonstrated positive staining for insulin (data not shown) in *BTC*-MSCs with the epithelial-like morphology after 12 days of culture. As expected, we could not detect positive stain for insulin in pIRES-MSCs.

To assess the cell's ability to produce and secrete insulin, RIA was used to measure insulin secretion from *BTC*-MSCs and pIRES-MSCs at different time points: 4, 8, 12, and 16 days after transfection. In vitro insulin secretion from *BTC*-MSCs was modulated by glucose and was first detected on day 8 and continued until day 16 posttransfection (Fig. 6). In contrast, in pIRES-MSCs, insignificant immunoreactive insulin was detected at all time points ($P = 0.01$).

Transplantation of *BTC*-MSCs reverts hyperglycemia in streptozotocin diabetic rats. Transplantation of *BTC*-MSCs under the renal capsule of streptozotocin diabetic rats was able to modulate glycemia in vivo as confirmed by the reduction of blood glucose levels, beginning 14 days

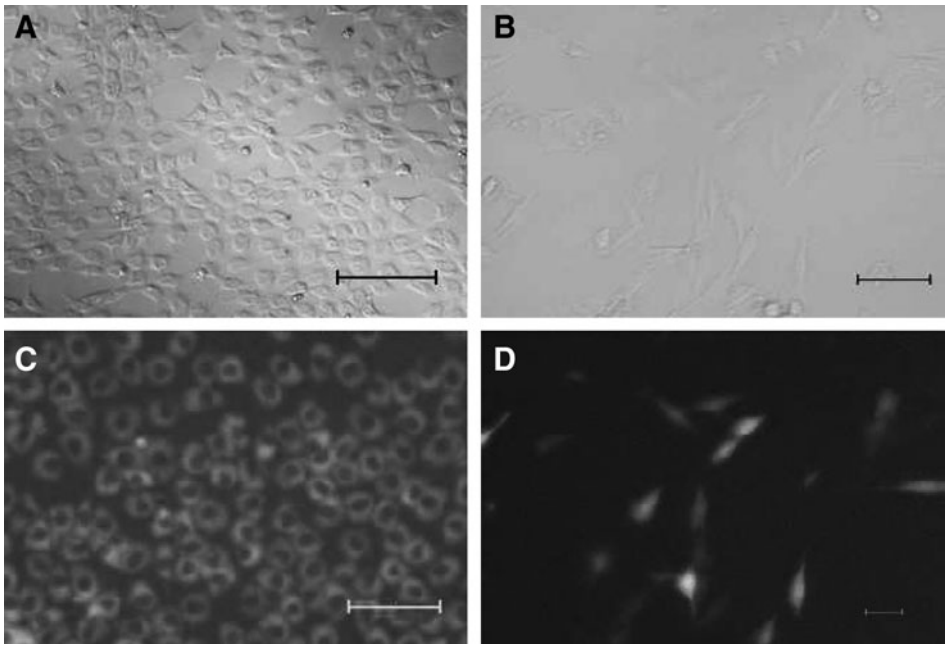

FIG. 3. Morphologic analysis of *BTC*-MSCs and pIRES-MSCs at 10 days after transfection. **(A)** Phase contrast: *BTC*-MSCs developed an epithelium-like morphology. **(B)** Phase contrast: pIRES-MSCs maintained a fibroblast-like morphology. **(C)** Fluorescence microscopy: *BTC*-MSCs epithelium-like cells are enhanced green fluorescent protein positive. **(D)** pIRES-MSCs in fluorescence microscopy. Scale bars represent 100 μm.

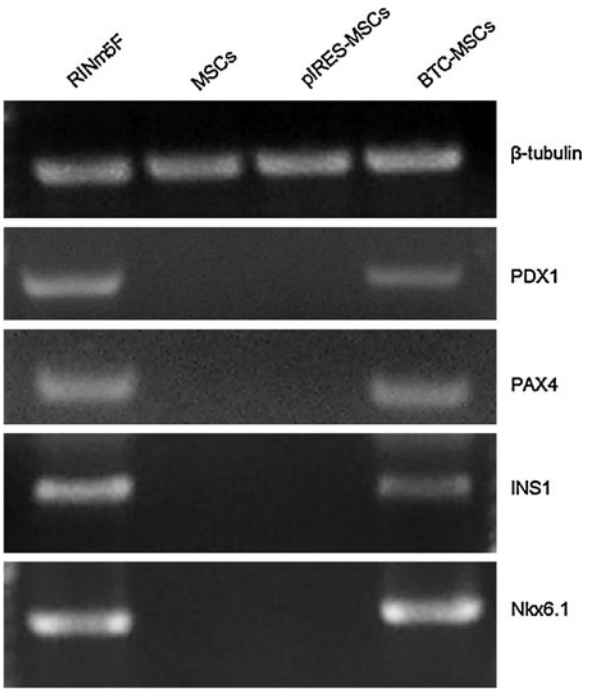

FIG. 4. Reverse transcription–polymerase chain reaction analysis of mRNA expression of the indicated genes in RINm5F, MSCs, pIRES-MSCs, and BTC-MSCs.

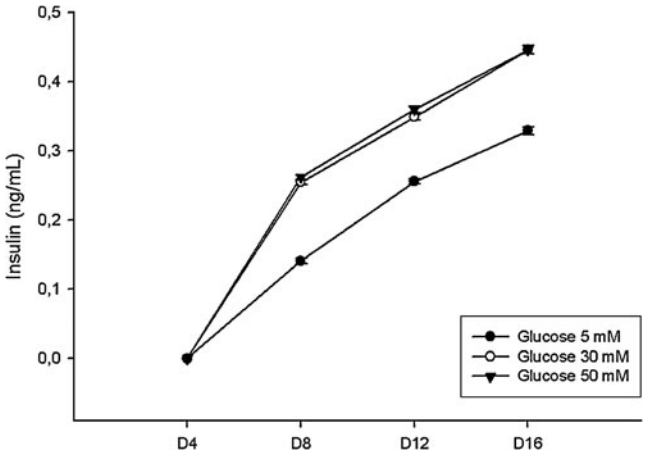

FIG. 6. In vitro glucose-induced insulin secretion in BTC-MSCs. The amount of insulin in the supernatant was determined by radioimmunoassay.

posttransplantation from over 500 to about 200 mg/dL at 35 days posttransplantation ($P = 0.001$) (Fig. 7A). In contrast, in streptozotocin (STZ) diabetic rats transplanted with control pIRES-MSCs, blood glucose levels continued to be high.

In parallel, streptozotocin (STZ) diabetic animals that received *BTC*-MSCs showed a less-severe weight loss (Fig. 7B) when compared with the pIRES-MSC animals ($P = 0.01$).

No gross tumor formation was seen in the animals and no tumor-like structures were observed among the grafts by histological analysis (Fig. 8). It was possible to observe the presence of insulin-positive cells in the renal subcapsular area of BTC-MSC–transplanted animals (Fig. 9).

Discussion

Although pharmacological therapy for diabetes has continued to improve, tight glucose control has not eliminated the complications of diabetes. Pancreas or islet transplantation can reconstitute the glucose-regulatory function of normal islets but is limited by an inadequate donor supply, need for immunosuppression, and loss of function of the transplanted islets [1].

In this regard, sources of pancreatic β-cells other than those from organ donors are needed. In the present study, we have devised a strategy to induce the formation of insulin-producing cells by overexpressing BTC in rat MSCs cultivated with nicotinamide. Previous studies have provided evidences that there are 2 pathways of pancreatic regeneration: (a) replication of pre-existing differentiated exocrine and endocrine cells and (b) proliferation and subsequent differentiation of duct epithelium to form new islets [32]. *BTC* is involved in both pathways; it is known to induce proliferation of insulinoma cells in vitro [33], improve glucose tolerance, enhance insulin secretion [34], reverse streptozotocin-induced hyperglycemia in mice [35], and induce endocrine differentiation of the exocrine cell line AR42J [23]. This differentiation potency can be explained because *EGFR* activation affects cell function on multiple levels, depending on the signaling pathways that are activated [18]. Our in vitro results indicate that the overexpression of BTC in rat MSCs may induce the expression of *PDX1* as well as

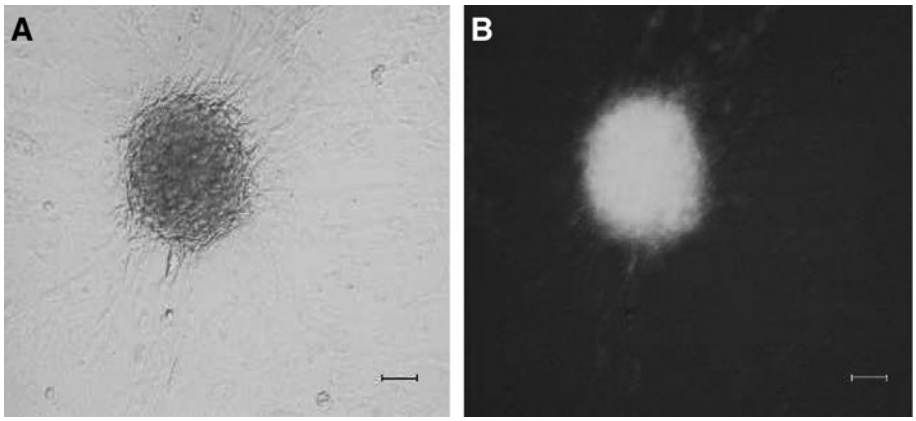

FIG. 5. *BTC*-MSCs tended to aggregate and formed a 3D structure similar to islet-like spheroids. **(A)** Phase contrast image of islet-like spheroids. **(B)** Fluorescence microscopy of islet-like spheroids. Scale bars represent 100 μm.

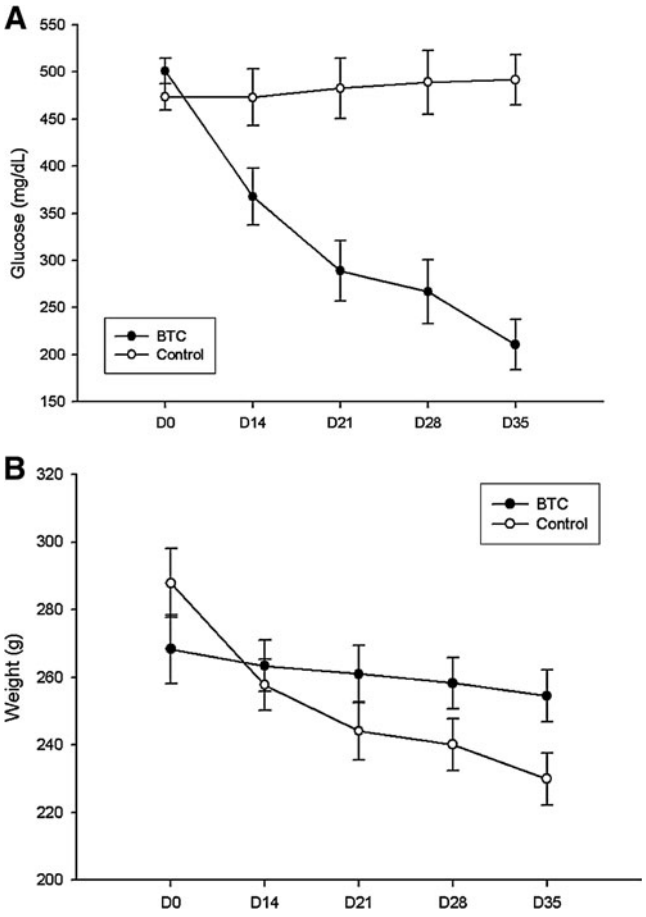

FIG. 7. Functional analysis of BTC-MSCs and pIRES-MSCs in vivo. **(A)** Blood glucose levels (mean ± SE): BTC-MSCs and pIRES-MSCs are significantly different from day 14 ($P = 0.001$). **(B)** Body weight (mean ± SE): BTC-MSC–transplanted animals had a less-severe weight loss when compared with pIRES-MSC–transplanted animals ($P = 0.01$).

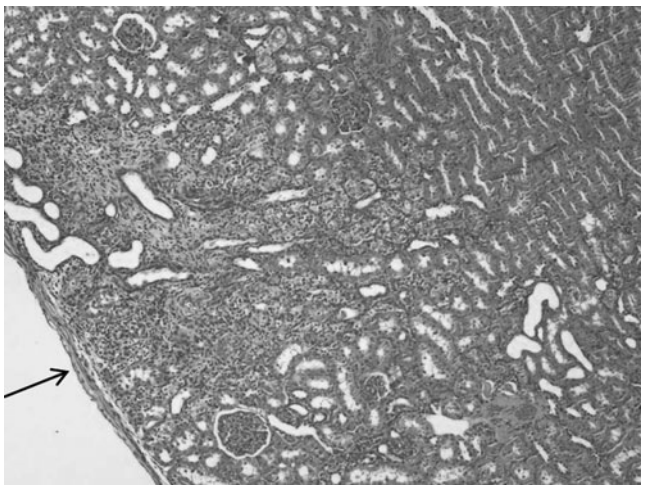

FIG. 8. Histological analysis of the graft area. Hematoxylin and eosin staining showing the presence of transplanted cells under the renal capsule (200×). Arrow indicates the graft area.

other important pancreas transcription factors, and the consequent production of insulin in agreement with Moriscot's conclusions about MSCs [36]. We observed that the morphology of *BTC*-MSCs changed from spindle-like to flat epithelial-like cells. This morphologic differentiation could be related to the findings of Gershengorn et al. [37] and Russ et al. [38], who believe in the theory of epithelial-to-mesenchymal transition where cell morphology differentiation is related to changes in gene expression from mesenchymal to epithelial patterns and vice versa.

In this way, others have observed similar morphologic differentiation trying to obtain endocrine cells from MSCs and attributed it to mesenchymal-to-epithelial transition [14]. Nevertheless, epithelial–mesenchymal transition theory is controversial and there are authors [39] who believe that this transition does not take place in cell culture.

The molecular mechanisms underlying the differentiation are not clear and it can only be speculated whether MSCs were induced to activate silent genes, or whether *BTC* overexpression enhanced the expression of already active genes.

It can be hypothesized that pluripotent stem cells reside in the adult bone marrow in a standby state and that these cells under certain circumstances may cross tissue (or germ-layer) boundaries to originate cells of a different lineage, challenging the notion of what it is believed—adult stem cells are committed to the germ-layer where they are derived from [15].

In our work during culture, the *BTC*-MSCs changed their undifferentiated status, started to produce BTC, and gained some transient elements that allow them to modify their gene expression profile. Nicotinamide—a poly(ADP-ribose) synthetase inhibitor—is also known to increase the indices of β-cells after pancreatomy [40] and it is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells [41]. Therefore, nicotinamide might play an important role in the endocrine differentiation observed in this study.

RT-PCR data showed the expression of 4 key pancreatic genes in BTC-MSCs (*PDX1, NKX6.1, Pax4,* and *INS1*) that are not expressed in MSCs.

The combined expression of *PDX1* and *NKX6.1* is found in the earliest pancreatic progenitor cells as well as in mature p-cells [42], indicating that BTC-MSCs have achieved expression of at least some typical β-cell genes.

The fundamental role of pancreatic p-cells is to secrete insulin in response to glucose variation. In the present study, we demonstrated the secretion of insulin from *BTC*-MSCs using an RIA detection technique.

Insulin secretion from BTC-MSCs was regulated by glucose; however, it occurred also in response to glucose concentrations above the physiological range. Although most of the response of normal islet occurs between 5 and 10 mM glucose, *BTC*-MSCs secreted most of the insulin in response to 30 mM glucose, indicating a possible difference between *BTC*-MSCs and p-cell, regarding the modulation of insulin release. Cell transplantation in vivo resulted in reduction of blood glucose levels, beginning 14 days posttransplantation. The glucose physiological values for rats range between 50 and 135 mg/dL [43]. In our work, diabetic animals that received bone marrow MSCs overexpressing *BTC* under the renal capsule presented on day 35 posttransplant reduced glycemic values compared with the control group. The

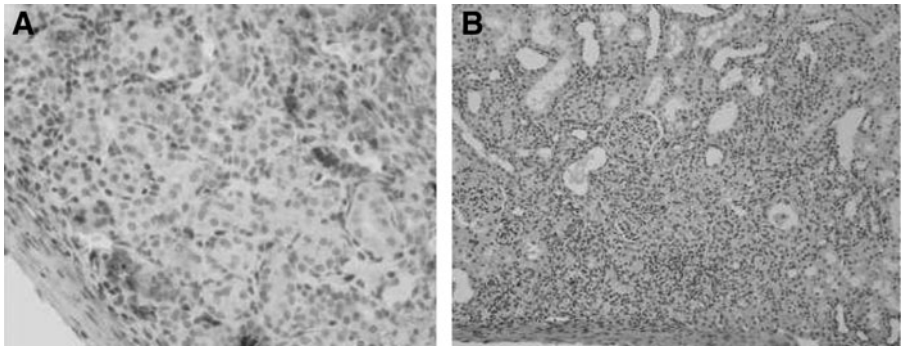

FIG. 9. Immunohistochemistry analysis of the transplanted kidney. **(A)** Insulin-positive cells in the border of tubule structures (400×). **(B)** Negative control with the omission of primary antibody (200×).

amelioration observed in the animals can be attributed to the insulin production by transplanted *BTC*-MSCs.

In parallel, no gross tumor formation was seen in the animals as found by Fujiwaka et al. [3]. Differently, in this work, we used adult stem cells that have a lower tumorigenic ability associated with a nonviral expression system to express BTC in a safer way.

Our findings are in conflict with an early report [14] by Li et al., who could not find differentiation by the expression of *BTC* alone. This can be attributed to the different protocols used and also the plasticity of the MSCs. Our cells presented all the characteristics required, as stated by the International Society for Cellular Therapy [27], confirming MSC identity and plasticity. On the other hand, in agreement with our results, Hisanaga et al. have reported the in vitro differentiation of murine MSCs by the addition of *BTC* to the medium [44].

BTC-MSCs were capable to secrete insulin in vitro. In vivo transplanted *BTC*-MSCs could ameliorate the glycemic values of diabetic rats. This concept is supported by the reduced weight loss in *BTC*-MSC-transplanted animals.

Regarding insulin production, strategies combining *BTC* overexpression and other soluble factors that mimic the in vivo microenvironment could be adopted to cause the differentiated cells to produce higher levels of insulin. The pancreatic islets in the adult are among the most vascularized of all organs in the body, with a unique dense glomerular-like angioarchitecture [45]. In this way, β-cells are exposed to other cell-derived molecules that can affect the physiological regulation of glucose-induced insulin secretion. It was demonstrated that endothelial-derived molecules, like endothelin-1, thrombospondin-1, and laminins, among others, can improve β-cell function [45–47]. These data show the importance of soluble factors secreted by islet vicinity, suggesting that the addition of soluble factors can also interfere in cell differentiation.

In agreement with Kobayashi et al. [48], we believe that, to create highly functional pancreatic β-cells such as those existing in the body, it may be necessary to reproduce the tissue structure of the pancreatic islets. Thus, the spontaneously formed 3D structures observed in our study could be a good start to improve necessary cell–cell communications.

In conclusion, our findings demonstrate the positive effects of *BTC* overexpression in MSCs, which can be used as a tool for cell therapy to revert hyperglycemia in a streptozotocin-induced diabetes animal model. Our results also highlight the therapeutic potential of adult bone marrow stem cells to treat diabetes.

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Author Disclosure Statement

All authors declare that there are no disclosures to be stated.

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11. ANEXO III

Curriculum vitae

Abril/2011

11.1 Curriculum Vitae

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Dados Pessoais

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Endereço profissional
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Formação Acadêmica/Titulação

2006 Doutorado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Importância de motivos conservados do fator de início de tradução 2β; (eIF2β) na síntese de proteínas e na distribuição subcelular desse fator em células humanas
Orientador: João Antonio Pêgas Henriques, Guido Lenz
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2003 - 2005 Mestrado em Genética e Biologia Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Construção de um vetor para terapia gência baseado no bloqueio da síntese proteica devido à expressão de eIF2 beta truncado, Ano de obtenção: 2005
Orientador: José Arthur Bogo Chies, Jomar Pereira Laurino
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1997 - 2002 Graduação em Ciências Biológicas Ênfase Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Estudo da relação entre os níveis plasmáticos do fator von Willebrand e o exercício físico
Orientador: Israel Roisenberg

Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

Atuação profissional

1. Hospital de Clínicas de Porto Alegre - HCPA

Vínculo institucional

2010 - 2010 Vínculo: Bolsa Extensão no País 2- CNPq , Enquadramento funcional: Bolsista , Carga horária: 40, Regime: Dedicação Exclusiva
Responsável direto: Elizabeth Cirne Lima
Outras informações: Projeto: Formação do Centro de Tecnologia Celular no Hospital de Clínicas de Porto Alegre. Atuação em trabalhos de pesquisa de terapia celular utilizando células tronco-mesenquimais.

2010 - Atual Vínculo: Celetista Formal , Enquadramento funcional: Biólogo , Carga horária: 40, Regime: Integral
Outras informações: Serviço de Hemoterapia- Banco de Sangue-Setor de Criobilogia e Unidade de Banco de Sangue de Cordão Umbilical e Placentário

2009 - Atual Vínculo: Colaborador , Enquadramento funcional: Pesquisador
Outras informações: Atuação em projetos de pesquisa do Laboratório de Embriologia e Diferenciação Celular do HCPA

2. Universidade Federal do Rio Grande do Sul - UFRGS

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1998 - 2002 Vínculo: Estagiário-bolsista, Carga horária: 20h
Estagiário/Bolsista de Iniciação Científica do CNPq no Laboratório de Hemostasia/Departamento de Genética.
Orientador: Israel Roisenberg
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Orientador: Lídia Fiuza
Outras informações: Atuação no projeto de pesquisa "Controle Biológico de doenças de plantas". Atuação em atividades de purificação de proteínas

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Bolsista de Iniciação Científica DTI do CNPq no Escritório de transferência de Tecnologia.
Responsável direto: Marli Elizabeth Ritter dos Santos
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Outras informações: Atuação em projeto de pesquisa relacionado à evolução dos povos nativo americanos a partir da análise do DNA mitocondrial.

Áreas de atuação

1. Biologia Molecular
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3. Terapia celular
4. Genética
5. Terapia gênica

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2010 Prêmio de Destaque para concorrer ao melhor trabalho de apresentação oral da 30 Semana Científica do HCPA, Hospital de Clínicas de Porto Alegre (HCPA)

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2002 Destaque do XIII Salão de Iniciação Científica da UFRGS, Pró- Reitoria de Pesquisa/ PROPESQ

Produção bibliográfica

Artigos completos publicados em periódicos

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The effect of ABO blood group on von Willebrand response to exercise.. Clinical and Applied Thrombosis/Hemostasis. , v.14, p.454 - 458, 2008.

Trabalhos publicados em anais de eventos (resumo)

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Diminuição da síntese proteica e do crescimento de células humanas induzido pela expressão de eIF2beta truncado In: 30 Semana Científica do Hospital de Clínicas de Porto Alegre, 2010, Porto Alegre.
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Animal Reproduction. Official Journal of the Brazilian College of Animal Reproduction, 2010. v.7. p.279 - 279

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Produção Técnica

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Produção bibliográfica

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Trabalhos publicados em anais de eventos	29
Apresentações de Trabalhos (Conferência ou palestra)	7

Produção Técnica

Produtos tecnológicos (outro)	1
Processos ou técnicas (terapêutica)	1
Curso de curta duração ministrado (extensão)	2
Curso de curta duração ministrado (outro)	1
Patentes	2

Eventos

Participações em eventos (congresso)	16
Participações em eventos (seminário)	6
Participações em eventos (simpósio)	5
Participações em eventos (oficina)	2
Participações em eventos (encontro)	1
Participações em eventos (outra)	6
Organização de evento (outro)	1