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Mapping eIF5A binding sites for Dys1 and Lia1: in vivo evidence for regulation of eIF5A hypusination

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Abstract The evolutionarily conserved factor eIF5A is the only protein known to undergo hypusination, a unique posttranslational modification triggered by deoxyhypusine synthase (Dys1). Although eIF5A is essential for cell viability, the function of this putative translation initiation factor is still obscure. To identify eIF5A-binding proteins that could clarify its function, we screened a two-hybrid library and identified two eIF-5A partners in *S. cerevisiae*: Dys1 and the protein encoded by the gene *YJR070C*, named Lia1 (Ligand of eIF5A). The interactions were confirmed by GST pulldown. Mapping binding sites for these proteins revealed that both eIF5A domains can bind to Dys1, whereas the C-terminal domain is sufficient to bind Lia1. We demonstrate for the first time in vivo that the N-terminal α-helix of Dys1 can modulate enzyme activity by inhibiting eIF5A interaction. We suggest that this inhibition be abrogated in the cell when hypusinated and functional eIF5A is required.

Key words: eIF5A; *DYS1*; *LIA1*; *YJR070C*; Two-hybrid; Hypusination

1. Introduction

The putative translation initiation factor 5A (eIF5A) is an intriguing protein. It was identified 25 years ago and implicated in protein synthesis but while subsequent work has revealed its importance in eukaryotic cells, its function remains unclear. eIF5A is the only protein that contains the essential amino acid hypusine, formed by a posttranslational modification of a specific lysine. Hypusination is a two-step process catalyzed consecutively by deoxyhypusine synthase (Dys1) and deoxyhypusine hydroxylase [1]. eIF5A and Dys1 are highly conserved throughout evolution, being found in all eukaryotic cells and archaea but not in eubacteria [2]. Yeast eIF5A is 63% and 35% identical to the mammalian and archaea proteins respectively. These similarities allow heterologous eIF5A to be functional in yeast [3]. Also, yeast Dys1 is 59% identical to the human protein and 44% to the archaeal homolog.

In *Saccharomyces cerevisiae*, eIF5A is encoded by the essential gene *TIF51A* in aerobic conditions [4]. eIF5A is a small abundant protein that localizes mainly in the cytoplasm [5,6]. The hypusine residue (residue 51 in *S. cerevisiae*) is essential for eIF5A function since the alteration of lysine 51 to arginine ($eIF5A^{K51R}$) blocks eIF5A hypusination and abolishes protein function in vivo [4]. The eIF5A protein comprises two well-defined domains: the N-terminal domain is folded in a β-roll architecture and the C-terminal domain is organized in a β-barrel structure. The N-terminal domain bears the hypusine residue in an exposed loop [7]. Dys1 is a tetramer of four identical subunits of 43 kDa encoded by the essential gene *DYS1* in *S. cerevisiae* and has a very high affinity for its substrate [8–10].

The importance of eIF5A in the cell is evident because it is essential for cell viability and highly conserved throughout evolution. In addition, the critical role of the hypusine residue is clear because this unique posttranslational modification is absolutely required for protein function in vivo. However, even after almost three decades of study, its function is still obscure. Initially, eIF5A was characterized as a translation initiation factor [11]. Nevertheless, its depletion in yeast resulted in a slight decrease (30%) in protein synthesis, arguing against eIF5A being considered a general translation initiation factor [12]. It was hypothesized that eIF5A might have a role in the translation of a specific subset of mRNAs, for instance those related to the G1/S cell cycle transition. This hypothesis was raised because eIF5A depletion results in an increase of G1-arrested cells [1,12]. Also, hypusine formation is essential for proliferation of mammalian cell lines [13–15]. eIF5A has also been associated with mRNA export acting as a cofactor of Rev, an HIV-1 shuttling protein [16]. However, the nucleocytoplasmic shuttling property of eIF5A has been questioned in a series of protein localization experiments [5]. Moreover, yeast cells deficient in the nuclear export machinery eIF5A localization remains mainly cytoplasmic, whereas Rev is retained in the nucleus [6]. eIF5A also participates in the mRNA decay process since eIF5A mutants accumulate transcripts at the restrictive temperature [6,17]. Thus, eIF5A has been associated with several steps of RNA metabolism but its function remains unclear.

In this study we sought to identify eIF5A-interacting proteins that could help us to understand its role in the cell. As expected, a two-hybrid screen of a cDNA library identified several clones containing *DYS1*. However, we also identified a new eIF5A-interacting factor, the protein encoded by the gene *YJR070C*, which we have called Lia1 (Ligand of eIF5A). We have mapped the eIF5A binding sites for both Lia1 and Dys1. Finally, we have shown that, in vivo, the N-terminal α-helix of Dys1 can regulate the interaction of Dys1 with its substrate eIF5A. This regulation could be under control of

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a cellular mechanism triggered to make hypusinated and functional eIF5A available in the cell.

2. Materials and methods

2.1. Plasmid construction

The plasmid expressing the bait protein lexA-eIF5A (pSV278, *TRP1*) was constructed as follows: *TIF51A* was PCR amplified and cloned into pBTM116 *Bam*HI site. GST–eIF5A expressing plasmid was achieved by cloning *TIF51A* gene into the vector pPS892 [18]; *TIF51A* was PCR amplified and inserted into pPS892 *Bam*HI–*Sal*I sites. For the expression of GST–eIF5A^{K51R} in yeast, a 281 bp *Sal*I–*Bst*EII fragment of the plasmid Yep352T-5A(K51R) [4], containing the *TIF51A*^{K51R} allele, was swapped with the corresponding fragment in the plasmid pPS892-*TIF51A* described above. Truncations in eIF5A were constructed as lexA fusion proteins using pSV278. The enzyme *Hin*cII was used for the deletion Δ1-42 (pSV429). The deletion Δ1-63 (pSV420) was obtained with *Eco*RV. To the deletion Δ1-90 (pSV421) *Mfe*I was used. The enzymes *Mfe*I and *Alu*I were used for the Δ90-152 deletion (pSV422). To express GST–Lia1 in yeast (pSV361), *LIA1* was subcloned from pGEX-*LIA1* (pSV356) into pPS892. Plasmid constructions were confirmed by DNA sequencing.

2.2. Two-hybrid screen

A two-hybrid screen was performed in the *S. cerevisiae* L40 strain that carries the *HIS3* and *lacZ* reporter genes [19]. Expression of the bait protein (lexA-eIF5A) in L40 was confirmed by western blot using anti-eIF5A. L40/lexA-eIF5A was transformed with a yeast cDNA library fused to the *GAL4* activation domain (pACT, *LEU2*, ATCC 87002) and plated on selective medium (−Leu/Trp/His) for candidate selection. The His$^+$ clones were further assayed for β-gal activity [20]. *LEU2* cDNA library plasmids from His$^+$/β-gal$^+$ transformants were isolated in *E. coli* HB101 strain (LeuB mutation). The *LEU2* plasmid was reintroduced by transformation into L40/lexA-eIF5A strain for plasmid linkage. The positive clones (His$^+$/β-gal$^+$) had the extremities of their *LEU2* plasmid sequenced and the gene present in each clone was identified using the *S. cerevisiae* Genome Database.

2.3. GST pulldown assay

Plasmids expressing GST–eIF5A, GST–eIF5A^{K51R}, GST–Dys1 and GST–Lia1 were introduced in the wild type strain FY23. The resulting strains were used for GST pulldown. Briefly, cells were grown at 30°C to OD_{600nm} = 0.4 and production of GST fusion proteins was induced with 2% galactose for 3 h. Cells were collected, washed twice in cold PBS and suspended in cold Buffer A (30 mM HEPES, pH 7.5, 100 mM KAc, 2 mM MgAc, 7 mM β-mercaptoethanol, 5 μg/ml of pepstatin, leupeptin, aprotinin and chymostatin). Cells were broken by vortex agitation with glass beads for 3 min, incubating the tubes on ice bath for 1 min after every 1 min agitation. Cell lysates were clarified by centrifugation at 20 000×*g* for 20 min at 4°C, and the total protein concentration was determined. Clarified lysates (2 mg) were incubated with 100 μl of glutathione-Sepharose beads (50% slurry) for 1 h, at 4°C. Beads were collected, washed five times with cold Buffer A and suspended in 30 μl of SDS–PAGE loading buffer. Proteins were separated by 12% SDS–PAGE, transferred to nitrocellulose membrane, stained with Ponceau-S and documented after rinsing. Finally, proteins were detected by western blot using the polyclonal anti-eIF5A, anti-Dys1 or anti-Lia1 and the ECL system (Amersham).

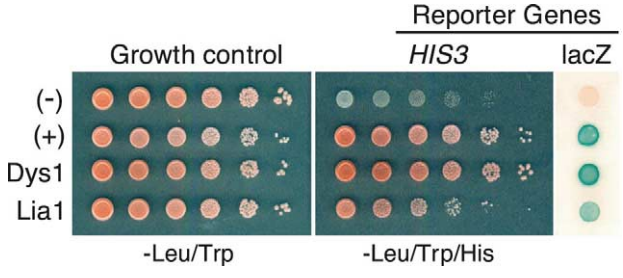

Fig. 1. eIF5A cellular partners revealed by two-hybrid. The strain L40/lexA-eIF5A containing pACT (negative control), the positive control L40-41 [21], pACT-Dys1 or pACT-Lia1 were grown on −Leu/Trp to OD_{600nm} = 0.5. Cells were collected by centrifugation and suspended in one tenth of the original volume. Ten-fold serial dilutions of these cell suspensions were spotted (4 μl) onto −Leu/Trp and −Leu/Trp/His plates and incubated at 30°C for 2 days. For β-gal activity filter assay, 3 μl of the concentrated cell suspensions were dropped onto a nitrocellulose filter laying on top of a −Leu/Trp plate and incubated 24 h at 30°C before the assay.

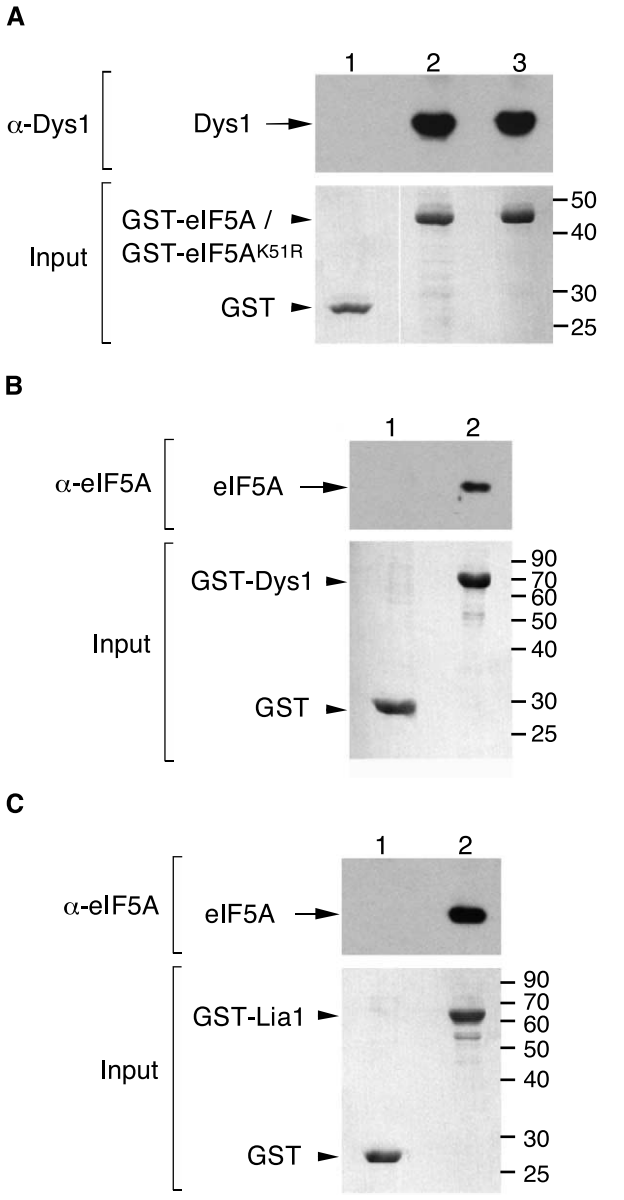

Fig. 2. Confirmation of eIF5A two-hybrid interactions by copurification. Western blot of GST pulldown assays (upper panels) and input of GST or GST fusion proteins (lower panels). A: Copurification of Dys1 with GST–eIF5A (lane 2) and GST–eIF5A^{K51R} (lane 3). B: Copurification of eIF5A with GST–Dys1 (lane 2). C: Copurification of eIF5A with GST–Lia1 (lane 2). GST negative control is shown in lane 1 of all panels. Arrowheads indicate GST or GST fusions and copurified proteins are indicated by arrows.

3. Results and discussion

3.1. Identification of eIF5A cellular partners: Dys1 and Lia1

In an attempt to understand the role of eIF5A in the cell, we searched for proteins that physically interact with eIF5A.

We screened a yeast two-hybrid cDNA library (1.1×10^6 transformants) and 14 positive clones were isolated. *DYS1* was the most frequent gene as it was present in 10 out of the 14 positive clones (Fig. 1). Although this interaction has not been described in *S. cerevisiae*, the physical interaction between eIF5A and Dys1 has been demonstrated in vitro for the human proteins [10]. The remaining four clones (30%) contained the gene *YJR070C*, suggesting that it encodes a specific and significant eIF5A-interacting protein. Here, we named the *YJR070C* gene *LIA1*, because it encodes a protein 'Ligand of eIF5A'. As shown in Fig. 1, the eIF5A interaction with Lia1 is not as intense as with Dys1. Lia1 is a protein of 325 amino acids (36 kDa), not essential for cell viability [22]. Lia1 has a cytoplasmic subcellular localization [23] and contains four to five HEAT-like repeats.

GST pulldown assays were performed to confirm these interactions using wild type yeast cells expressing the fusion proteins GST–eIF5A, GST–eIF5A^{K51R}, GST–Lia1 or GST–Dys1. The fusion proteins were purified by glutathione-Sepharose and the copurified proteins revealed by western blot. GST–eIF5A copurified Dys1 but not GST alone (Fig. 2A). This fusion protein (GST–eIF5A) is functional as it rescues the temperature-sensitive phenotype of an eIF5A mutant (data not shown). In a similar experiment, eIF5A was also copurified with GST–Dys1 protein (Fig. 2B). The copurification of Dys1 with the non-functional isoform eIF5A^{K51R} was as intense as with the wild type protein (Fig. 2A), indicating that this amino acid alteration does not impair eIF5A binding to the modifying enzyme, even though it cannot be hypusinated. GST–Lia1 but not GST alone copurifies eIF5A (Fig. 2C). On the other hand, GST–eIF5A copurifies small amounts of Lia1, assayed using anti-Lia1 (data not shown); this could be due to the fusion of Lia1 to GST or to the low expression of Lia1 in the cell.

The two-hybrid system is a valuable tool for defining protein function through the establishment of a protein interaction network with other well-known proteins. However, in this study the only protein found in the screen besides the expected Dys1 was the protein Lia1. Even though eIF5A is an essential protein, Lia1 is not required for cell viability. Overexpression of Lia1 does not suppress a temperature-sensitive allele of eIF5A and this allele is not synthetically lethal with a knockout strain of *LIA1* (data not shown). Due to the fact that Lia1 is not a well-characterized protein, further studies will be required to establish the functional relevance of this interaction.

3.2. Mapping eIF5A-binding sites to Dys1 and Lia1

To define which domain of eIF5A is required for the interactions with Dys1 and Lia1, three N-terminal deletions and one deletion of the C-terminal half of eIF5A (Fig. 3A) were created. The truncation sites are indicated in the predicted structure for the human eIF5A (Fig. 3B) [7]. Expression of the truncated lexA–eIF5A proteins, with the expected molecular sizes, was confirmed by western blot using anti-eIF5A (data not shown). The interactions were assayed by the expression of the two-hybrid *HIS3* reporter gene. The interaction of eIF5A with Dys1 was analyzed using one of the strongest interacting clones selected in the screen Dys1(K29) which does not contain the first 28 amino acids of Dys1. Binding of eIF5A to Dys1(K29) was not affected by truncation of either the N- or the C-terminal half (see Δ1-90 and Δ90-152, Fig.

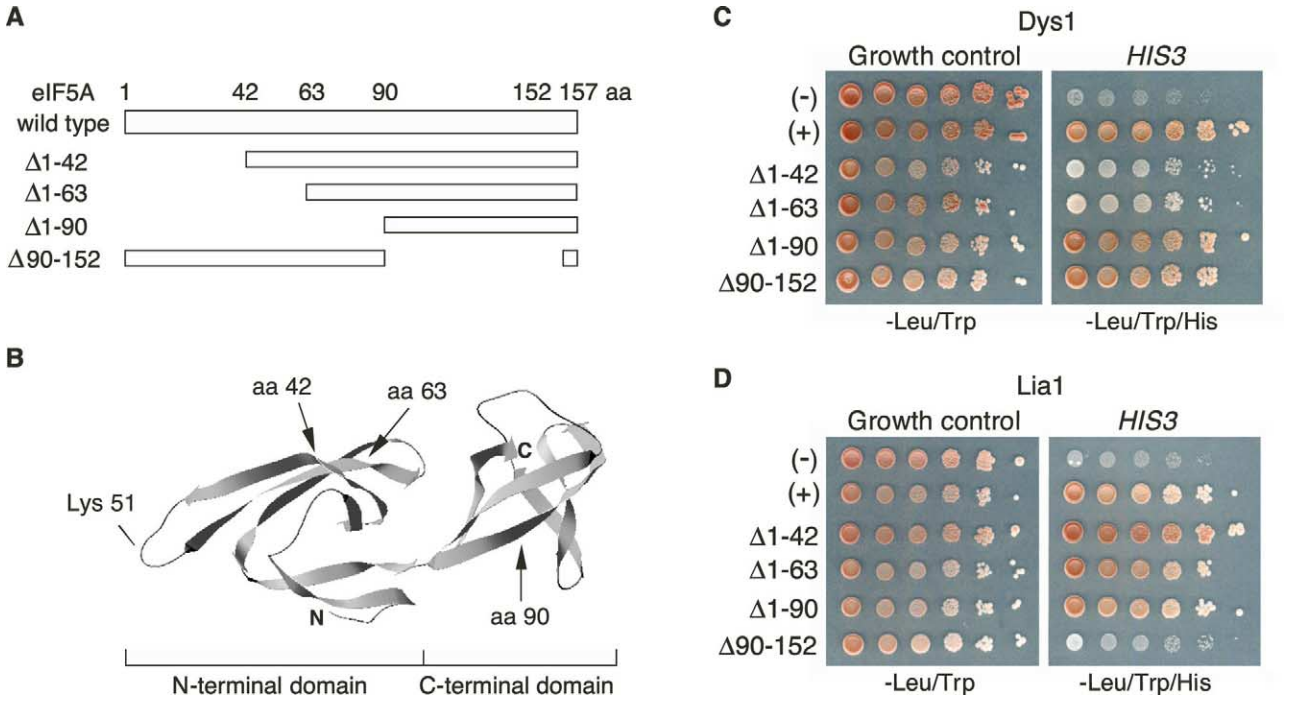

Fig. 3. Determining eIF5A binding sites for Dys1 and Lia1. A: eIF5A truncations scheme. B: Predicted tertiary structure for the human eIF5A [7]. Arrows indicate the position of the corresponding amino acid in the yeast protein and the truncation sites. C: L40 strains expressing vector alone (−), wild type eIF5A (+) or eIF5A truncations (Δ1-42, Δ1-63, Δ1-90 and Δ90-152) fused to lexA and Dys1 fused to Gal4-activation domain were tested exactly as described in Fig. 1, except that the expression of *HIS3* reporter was observed in selective medium containing 1 mM 3-aminotriazole. D: L40 strains expressing vector alone (−), wild type eIF5A (+) or eIF5A truncations fused to lexA and Lia1 fused to Gal4-activation domain were tested exactly as described in Fig. 1.

3C), suggesting that both domains of eIF5A can interact with Dys1. Thus, although the target site is located in the N-terminal domain, the modifying enzyme interacts with both domains of eIF5A. The truncation of eIF5A residues 1–42 or 1–63 weakens the interaction with Dys1 probably due to a conformational change in eIF5A structure, suggesting that the correct folding of the N-terminal domain of eIF5A is essential for efficient interaction with Dys1. The eIF5A N-terminal domain is sufficient for hypusination by Dys1 with a slight reduction of 24% when compared to hypusination efficiency of the native protein [8]. Although binding to the N-terminal domain is enough to permit hypusination, the interaction with C-terminal domain of eIF5A identified here might be important for the complete enzymatic activity.

The interaction with Lia1 was mapped to the C-terminal domain of eIF5A (Fig. 3D). None of the N-terminal truncations affected Lia1 binding to eIF5A. In contrast, the C-terminal deletion (Δ90-152) completely abolished the interaction. Although the N-terminal domain suffices for eIF5A hypusination [8], this domain is not functional by itself as it cannot substitute for the native protein in a knockout strain (data not shown). The significance of the eIF5A C-terminal domain for protein function is further highlighted by the fact that temperature-sensitive alleles of eIF5A with amino acid alterations in this domain have a more severe phenotype than alleles altered in the N-terminal domain [6,17]. Hence, the C-terminal domain must have an important role in eIF5A function and Lia1 might contribute to this function.

3.3. Dys1 N-terminal domain impairs eIF5A binding

During the analysis of the positive clones, we observed that the *DYS1* clones exhibited an irregular growth pattern, varying from very poor to normal growth (data not shown). Sequencing of all 10 *DYS1* cDNAs revealed several 5′-end truncations encoding Dys1 proteins starting at the following residues: aspartate 3 (D3), proline 9 (P9), leucine 12 (L12) and lysine 29 (K29). Interestingly, the protein encoded by a 82 nt deletion (K29) showed the strongest interaction with eIF5A, whereas a deletion of 24 nt (P9) or the entire gene (M1) resulted in a weakened interaction (Fig. 4A). Given that yeast Dys1 is 79% similar to the human protein, we used the crystal structure of the human Dys1 monomer [24] to locate the corresponding residues in these truncated forms of yeast Dys1. As shown in Fig. 4B, the truncated protein Dys1(K29) has the first N-terminal α-helix removed, whereas Dys1(P9) maintains this secondary structure. It was previously determined that the enzyme tetramer is formed by two pairs of tightly associated dimers which contain two active sites located at each dimer interface. The active site is buried inside a cavity and the N-terminal α-helix of a third monomer obstructs the active site entrance [24]. We found that clones containing the entire protein Dys1(M1) or a small truncation of Dys1(P9) have decreased interaction with eIF5A compared to the truncation that lacks the blocking N-terminal α-helix Dys1(K29). Therefore, the Dys1(K29) interaction probably reflects the total binding capacity to eIF5A since the entrance of the active site is uncovered.

Knowing that the N-terminal α-helix of Dys1 blocks eIF5A binding (Fig. 4A) and Dys1(K29) binds to both domains of eIF5A (Fig. 3C), we wondered if the inhibitory N-terminal α-helix interferes in the interaction of Dys1 with both eIF5A domains. To answer this question, we transformed the L40 strain with the *LEU2* plasmids expressing Dys1(M1) and the *TRP1* plasmids expressing eIF5A Δ1-90 or Δ90-152 and tested for the expression of the *HIS3* two-hybrid reporter gene. In Fig. 4C, we demonstrated that the interaction of Dys1 with the eIF5A C-terminal domain (Δ1-90) is not affected by the presence of the blocking α-helix. However, as expected, the Dys1 interaction with the eIF5A N-terminal domain (Δ90-152) is completely abolished in the presence of the blocking α-helix. These results demonstrate that each eIF5A domain has an independent binding site in the enzyme.

Here, we present for the first time in vivo evidence for the inhibitory effect of the Dys1 N-terminal α-helix on the interaction with its substrate eIF5A. This helix seems to modulate eIF5A Lys51 access to the active site [24]. These findings may suggest a mechanism where inactive Dys1 under negative control of the N-terminal α-helix would open the catalytic site only when hypusinated and functional eIF5A is needed in the cell, for instance, during cell cycle transition. Future work will address the question if this mechanism is under control of one of the signal transduction pathways governing cell proliferation.

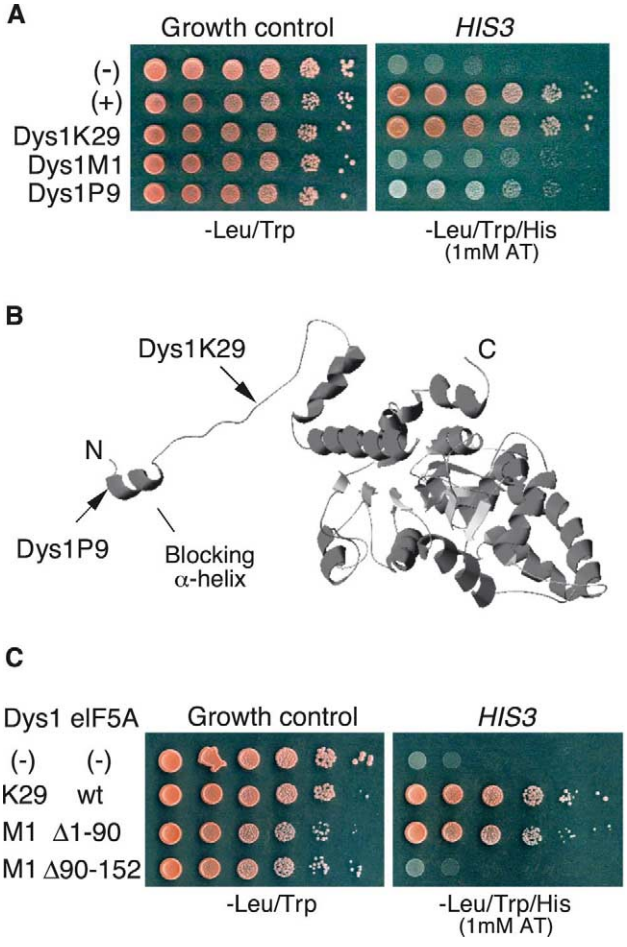

Fig. 4. In vivo inhibitory effect of the N-terminal α-helix on Dys1 interaction to eIF5A. A: Expression of *HIS3* reporter gene as described in Fig. 3C. Dys1(M1) = entire Dys1, Dys1(P9) and Dys1(K29) = truncated forms of Dys1. B: Tertiary structure of human Dys1 [24]. Location of the starting amino acid in the truncated forms of Dys1 is indicated by arrows. C: Interaction of Dys1(M1) to either eIF5A domains: N-terminal (Δ90-152) and C-terminal (Δ1-90).

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