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Med9 Interacts with eIF4E Dependent on the Carbon Source in *Saccharomyces cerevisiae*

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Abstract

The cap-binding protein eIF4E is a key protein for mRNA metabolism. The eIF4E biological role is defined by the specific protein it interacts with. The best characterized role of eIF4E is in promoting mRNA translation through its interaction with eIF4G. To seek for new interactors in the ascomycete *Saccharomyces cerevisiae*, we performed a genomic yeast two-hybrid screen using eIF4E as bait. In addition to the already reported p20 and Eap1, we identified Med9, a component of the RNA polymerase II Mediator complex. A physical interaction between eIF4E and Med9 was confirmed using recombinant proteins prepared in *E. coli* and further isolating the eIF4E—Med9 complex both by size-exclusion chromatography and by m^{7}GTP-Sepharose pull-down experiments. Surprisingly, the eIF4E W75A mutation, which impairs the interaction with eIF4G, p20, and Eap1 only slightly affected the interaction with Med9 in the two-hybrid system. We further performed random mutagenesis to identify the Med9 amino acids involved in eIF4E interaction. Mutants F65A/I66A and F65A/I66A/H68N did not interact with eIF4E. We also demonstrated that the interaction eIF4E—Med9 depended on the carbon source for cell growth and that it might happen within the nucleus. Finally, we found that the eIF4E—Med9 interaction is conserved in the yeast *Saccharomyces kudriavzevii.*

Keywords: eIF4E; Med9; mRNA metabolism; translation initiation; carbon source

1. Introduction

The cap-binding protein eukaryotic initiation factor (eIF) 4E is a key protein for mRNA metabolism. The most characterized activity of eIF4E is its involvement in promoting mRNA translation [1]. Approximately 50 eIF4E-interacting proteins (4E-IPs) have been reported to bind to different eIF4E paralogs in diverse species to mediate mRNA nucleus-cytoplasm export, translation, decay, or storage [2,3]. Each 4E-IP defines the process in which eIF4E gets involved. For example, when eIF4E makes a complex with eIF4G is able to drive translation initiation [4–7]; its interaction with the DEAD-box RNA helicase Rck/Xp54 enables eIF4E to involve in mRNA storage in cytoplasmic foci [8,9]; and interaction with human homeodomain protein 9 (HOXA9) HOXA9 within the nucleus mediates export of cyclin D1 and ornithine decarboxylase (ODC) mRNAs [10]. All eIF4E activities strictly depend on its association with 4E-IPs, therefore eIF4E behaves as a promiscuous and multifunctional wildcard at the crossroads between different RNA processes. Thus, eIF4E is a crucial interphase between gene expression and RNA metabolism [2,11].

The genes encoding eIF4E have undergone multiple duplications in many eukaryotes [11–14]. Fungal species contain one to seven paralogs of the *eIF4E* gene [14,15]. The ascomycete yeast *Saccharomyces cerevisiae* is endowed with a single, essential gene encoding eIF4E termed *CDC33* [16,17] that promotes mRNA translation initiation [18,19]. *S. cerevisiae* eIF4E interacts with four proteins, namely eIF4G1, eIF4G2 [20–22], eIF4E-associated protein (EAP) 1 [22,23], and p20 [21,22,24,25]. eIF4E interaction with eIF4G1 or eIF4G2 drives mRNA translation [22,26–28]. Eap1 is involved in translation inhibition of specific mRNAs encoding nuclear pore membrane proteins [29] or in lipid synthesis [30,31]. Eap1 promotes translation during nitrogen deprivation [32] and also triggers degradation of a subset of mRNAs [33,34]. p20 modulates the translation of specific mRNAs by both eIF4E- and eIF4E-independent mechanisms [35–37]. Recently, it was shown that the eIF4E/p20 complex stimulates the translation of reporter mRNAs with variable 5′-UTRs *in vitro* [38].

Here, we report that Med9, a component of RNA polymerase II transcriptional Mediator complex, is a novel interactor of eIF4E and that this interaction depends on the carbon source. The eIF4E—Med9 complex might be formed within the nucleus and is conserved in the yeast *Saccharomyces kudriavzevii*.

Results

2.We rationalized that in organisms with a single eIF4E ortholog interaction with diverse, so far unknown proteins is expected. Thus, other eIF4E-interactors might fulfill the diverse functions performed by eIF4E paralogs in other species. To search for new eIF4E interactors in *S. cerevisiae*, we cloned eIF4E as a bait into the vector pOBD and performed a yeast two-hybrid high-throughput screen using an activation-domain library [39] in which two *S. cerevisiae* genomes were tested, i.e., 10 000 – 12 000 prey clones. To rule out false positives, we performed several rounds of cell growing in selective medium (lacking both tryptophan and leucine) at high stringency (i.e., containing 30 mM 3-amino-1,2,4-triazole, 3AT). We recovered seven clones encoding positive eIF4E interactors, namely p20, EAP1, the ATPase-stabilizing factor family protein (STF2), the histone chaperone SPT2, the subunit of the proteasome RPN3, the monolysocardiolipin (MLCL), and Med9, a component of the RNA polymerase II Mediator complex. Among them, Med9 consistently showed the strongest interaction with eIF4E. To corroborate this interaction, we PCR-amplified Med9 from an independent, wild-type strain and performed again two-hybrid essays. As positive interactors, we used p20 and EAP1 cloned as prey onto the pOAD vectors (Figure 1A). Again, we observed clear interactions of eIF4E with Med9, p20 and EAP1, but not of Med9 with empty vector (negative control).

2.We next corroborated the eIF4E—Med9 interaction using recombinant proteins. We cloned Med9 and eIF4E as GST and His6X tag fusions, respectively, and expressed the proteins in the bacteria *E. coli*. The proteins were purified and the GST tag was removed from Med9 using the PreScission protease cleavage. Pure Med9 and eIF4E-His6X proteins were analyzed by gel filtration FPLC through a size exclusion S75 column. Individual Med9 and eIF4E-His6X eluted to the expected sizes, namely 18 kDa and 25 kDa, respectively. When both proteins were mixed in equimolar amounts, they underwent a shift in the elution profile and eluted as a complex (Figure 1B). We next conducted m^{7}GTP-Sepharose pull-down assays using the recombinant proteins GST-Med9 and eIF4E (Figure 1C). Whereas GST (lane 2) and GST-Med9 (lane 4) did not bind the resin, and GST did not copurify together with eIF4E (lane 8), GST-Med9 copurified along with eIF4E (lane 10), indicating a direct interaction. Altogether, these experiments proved a physical and stable interaction between Med9 and eIF4E.

2.3. Med9 and eIF4E Binding Site

Multiple proteins contact different regions of eIF4E dorsal surface. Most of them share the canonical eIF4E-binding motif YXXXLL◎ (where ◎◎is a hydrophobic amino acid) [5,27] and others interact via non-conserved binding sites [3,40,41]. Metazoan eIF4G and eIF4E-binding proteins (4E-BPs) physically bind the fragment 70S/TVXXW74 at the dorsal region of eIF4E, where W74 is critical for this interaction [5,42,43]. A second, non-conserved eIF4E-binding site present in eIF4G and 4E-

BPs wraps the lateral surface of eIF4E [22,44–48]. In yeast, eIF4E Val71 and W75, which are located on the convex dorsum of the protein, contact eIF4G1, p20 and EAP1 [22,49]. Thus, we asked what region of Med9 interacts with eIF4E. We randomly mutated the Med9 cDNA and further performed yeast two-hybrid assays to test interaction between both wild-type (Figure 2A, upper) or W75A mutant eIF4E (Figure 2A lower) and wild-type Med9, and the randomly generated Med9 mutants. We also tested p20 as a positive control. Among approximately 80 Med9 mutants, we observed that the mutations F65A/I166A and F65A/I66A/H68N did not interact with wild-type eIF4E nor eIF4E W75A. Is noteworthy that, while p20 (positive control) lost interaction with eIF4E W75A, cells expressing wild-type Med9 still showed growth with this mutant, suggesting a stronger interaction with eIF4E's dorsal surface. The Med9 mutations are shown in Figure 2B. *In silico* AlphaFold docking using the three-dimensional protein structures predicted the formation of a Med9—eIF4E complex via the analyzed amino acids (Figure 2C.). Further AlphaFold modeling utilizing the mutants eIF4EW75A and Med9 F65A/I66A/H68N indicated no interaction of the same regions of the proeins (not shown), in agreement with the two-hybrid experiments. We conclude that the Med9 region 65FIPH68 located at the ◎1 helix of the protein interacts with eIF4E.

2.4. Med9 and eIF4E Form a Complex in Glucose and Galactose but Not in Lactate

In a high-throughput analysis of protein complexes via co-immunoprecipitation assays, Ho *et al*. [50] isolated a protein complex containing Med9, eIF4E, porin1, GLK1, and GMP1 when cells were grown in galactose as carbon source. This complex formed in the presence of total RNA. Thus, we performed two-hybrid interactions in different carbon sources. We platted diploidic cells onto glucose, galactose, or lactate (Figure 3A) and cells were tested for interaction in the absence of adenine (Figure 3A, upper) or the lack of histidine containing 30 mM 3AT (Figure 3A, lower). Two independent transformations were tested with similar results. As expected, a strong Med9—eIF4E interaction happened in cells grown in glucose and galactose. A much weaker interaction was observed in lactate in the absence of adenine or the lack of interaction in the media minus histidine, showing a dependence on the carbon source.

We next analyzed protein localization using the *CDC33-GFP* and *CSE2-GFP* strains encoding eIF4E and Med9 in fusion with the green fluorescent protein at the carboxy-terminus (eIF4E-GFP and Med9-GFP, respectively) [51,52]. We grew the cells in glucose or galactose as carbon source and observed a significant shift in the cellular distribution of proteins dependent on the media used (Figure 3B). In glucose, eIF4E-GFP was mostly cytoplasmic and about 20% cells showed a nuclear and cytoplasmic localization. In contrast, up to 80% cells showed a nuclear and cytoplasmic localization when grown in galactose, indicating an eIF4E-GFP import toward the nucleus (Figure 3C, upper). In glucose, Med9-GFP was mostly nuclear, but became distributed both nuclear and cytoplasmic in up to 95% of cells when grown in galactose (Figure 3C, lower). When we grew the cells in lactate, both eIF4E-GFP and Med9-GFP showed mostly a cytoplasmic localization (Figure 3C). Altogether, these results suggest that the endogenous Med9—eIF4E complex might be formed both in the cytoplasm and within the nucleus when grew in glucose and galactose, but not lactate.

2.5. The Med9—eIF4E Complex is Conserved in Saccharomyces kudriavzevii

eIF4E interaction with specific 4E-IPs is conserved in different species, including 4E-BPs and 4E-T, but is not in other cases such as Bicoid or Maskin [3,53]. Thus, we asked whether or not the Med9—eIF4E interaction is conserved in species phylogenetically closed to *S. cerevisiae*. We cloned the ortholog proteins from *S. kudriavzevii*, *Zygosaccharomyces rouxii* and *Candida glabrata* (Figure 4A), all belonging to the ascomycetal Family *Saccharomycetaceae* [54], and assayed interaction in the two-hybrid system. We observed interaction in *S. kudriavzevii* (Figure 4B), which is the closest relative species to *S. cerevisiae*, but not in *C. glabrata* or *Z. rouxii*. The *S. cerevisiae* V71 and W75 eIF4E involved in eIF4G1, p20, and EAP1 [22,49] interaction, and the Med9 residues contacting eIF4E, are also present in the *S. kudriavzevii* orthologs.

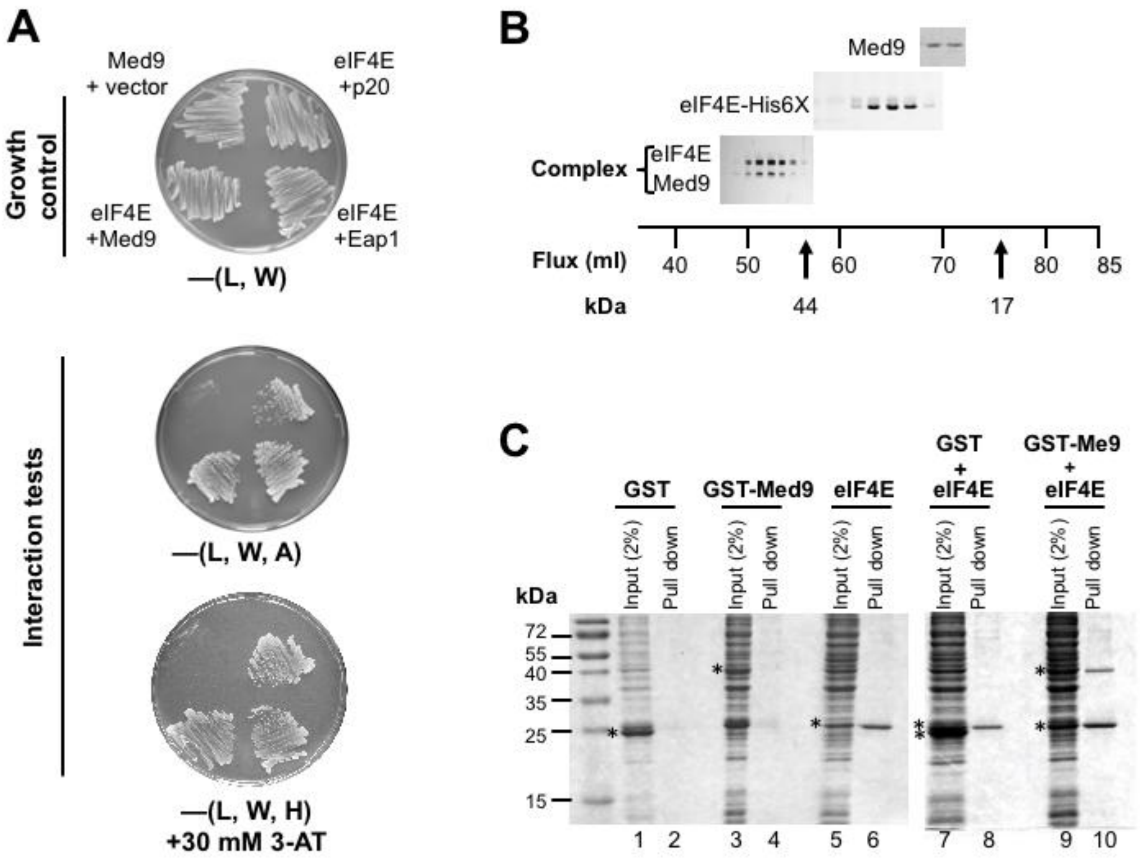

Figure 1. Med9 physically interacts with eIF4E. *A)* Interaction in the yeast two-hybrid system. "Bait" eIF4E is in the binding domain; "Prey" Med9, p20, and Eap1 are in the activator domain. Auxotrophic gene markers *Leu2*, *Trip1* and *His3*, and *Ade*, are used to grow in media lacking leucine (*L*), tryptophan (*W*), histidine (*H)*, and adenine (*a*), respectively. Cells coexpressing both bait and prays were grown in *–(L, W)* medium. To detect interaction between baits and prays, cells were replated onto media *–(L, W, H)* + 30 mM 3-amino-1,2,4-triazole (*3AT*) and *–(L, W, A)*. **B)** Gel filtration profiles of Med9, eIF4E-His6X or Med9—eIF4E-His6X complexes using a S75 size exclusion column. Isolated recombinant Med9 (2-10 mg/ml) and eIF4E-His6X (2-10 mg/ml) were mixed in equimolar amounts, subjected to chromatographic separation on a S75 size exclusion column. The eluted fractions were resolved in SDS-PAGE and detected by silver staining. Med9 alone and eIF4E-His6X alone are purified at 18 kDa and 26 kDa, respectively. Together, they purified forming a complex. **C)** m^7GTP-sepharose pull-down assays using total protein extracts of bacteria expressing GST, GST-Med9, eIF4E or the indicated coexpressed proteins. Proteins were resolved in SDS-PAGE and detected by Coomassie staining. Asterisks point the recombinant proteins. GST-Med9, but not GST, co pull-downed with eIF4E. *Input* was 2%; *Pull down* was 100%.

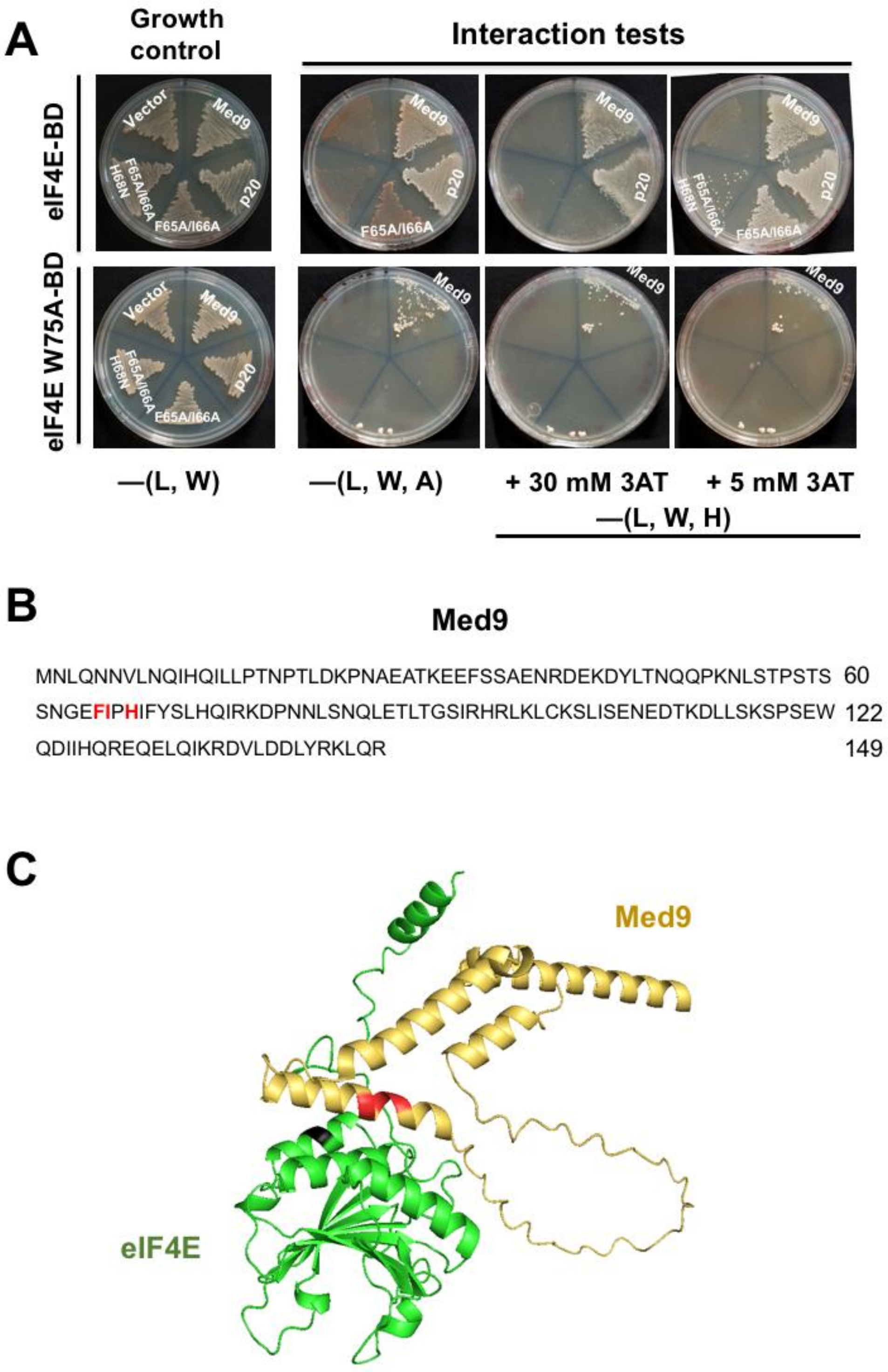

Figure 2. Med9 possesses a non-canonical eIF4E-binding site. ***A)*** Interactions between wild-type eIF4E and the eIF4E W75A mutant with wild-type and Med9 mutants in the yeast two hybrid system. *eIF4E* and *eIF4E W75A* mutant are expressed in the binding domain (BD). Wild-type *Med9* and the mutants *65FA/I66A/H68N* and *65FA/I66A*, *p20* (positive control) and empty *vector* (negative control) were expressed in the activator domain

(AD). *–(L, W)*, growth control; *–(L, W, A)*, and *–(L, W, H)*, interaction tests. *L*, leucine; *W*, tryptophan; *H*, histidine; *A*, adenine; *3AT*, 3-amino-1,2,4-triazole. ***(B)*** Sequence of the Med9 protein. The region 65FIPH68 when mutated towards 65AAPN68 failed to interact with eIF4E, and is highlighted in red. ***(C)*** AlphaFold3 prediction of interaction between eIF4E (*green*) and Med9 (*yellow*). The eIF4E W75 residue that interacts with eIF4G1 and p20 [22,49], and the mutation W75A are depicted in *black.* The eIF4E-interacting site 65FIPH68 of Med9 is shown in *red*.

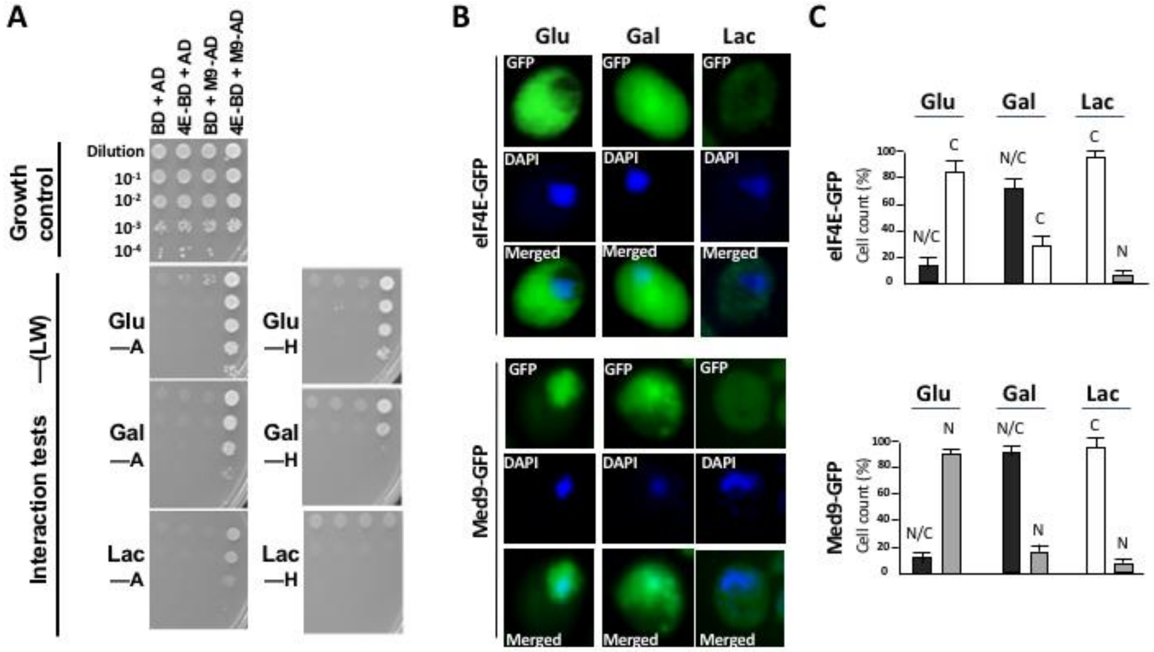

Figure 3. The interaction and localization of eIF4E and Med9 depends on the carbon source. ***A)*** Yeast two-hybrid system serial dilutions of the indicated interactions. Cells were grown in the indicated carbon sources. *BD*, binding domain; *AD*, activator domain; *Glu*, glucose; *Gal*, galactose; *Lac*, lactate. *–(L, W)*, growth control; *–(L, W, A)* and *–(L, W, H)*, interaction tests. ***B)*** Cellular distribution of eIF4E-GFP and Med9-GFP grown in glucose or galactose as carbon sources. Epifluorescence images are shown. ***C)*** Cell count of the indicated cellular distribution of the protein. *N/C*, nuclear and cytoplasmic localization; *C*, cytoplasmic localization; *N*, nuclear localization. Cell were observed at O.D.= 1.0.

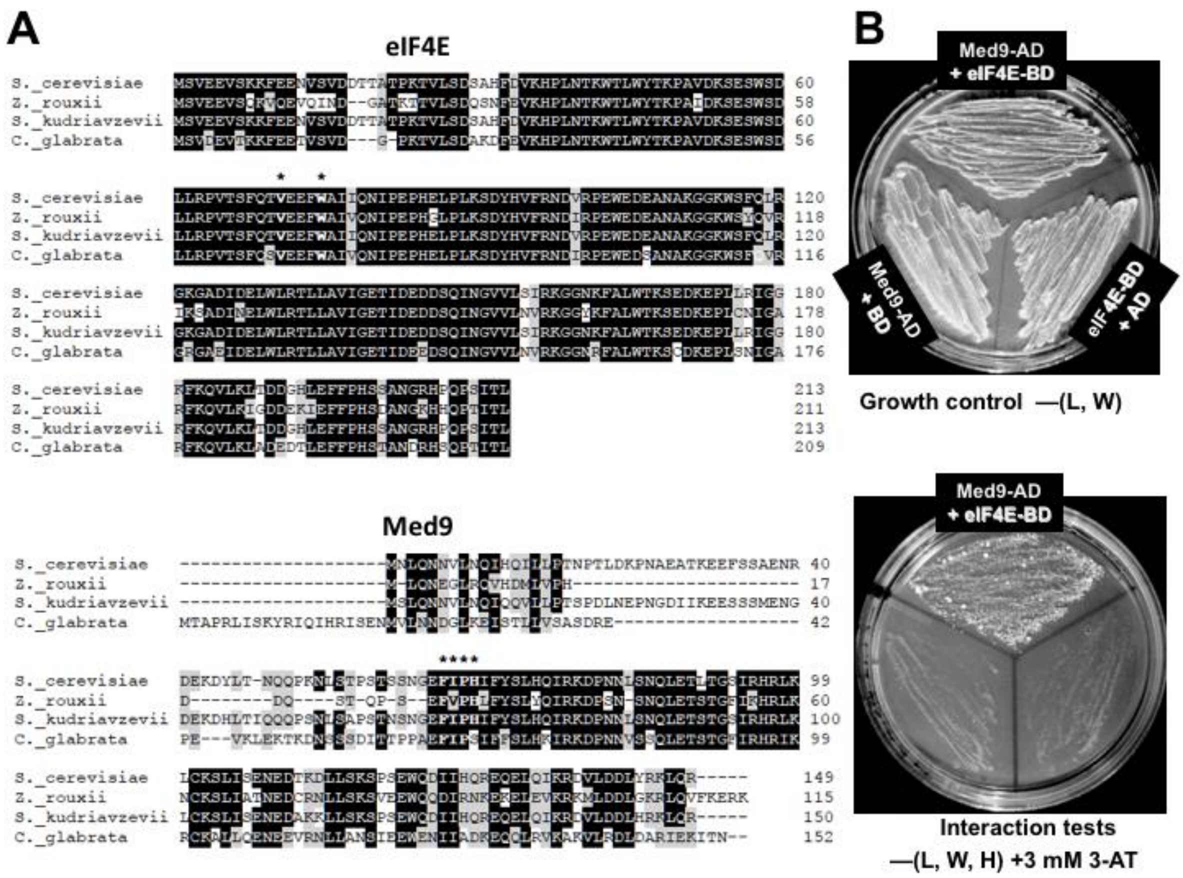

Figure 4. The interaction Med9—eIF4E is conserved in the yeast *Saccharomyces kudriavzevii*. *A)* Sequence comparison of full-length eIF4E (*left*) and Med9 (*right*) of the indicated species of the fungal Family *Saccharomycetaceae*. The V71 and W75 of *S. cerevisiae* eIF4E involved in eIF4G1, p20, and EAP1 [22,49] interaction, and the *S. cerevisiae* Med9 residues contacting eIF4E, are indicated by *Asterisks*. Residues identical in at least three sequences are shaded in black boxes. Conservative changes in at least three sequences are in grey boxes. Gaps are represented by dashes. ***B)*** Interaction assay in the two-hybrid system. Only *S. kudriavzevii* Med9—eIF4E interaction is shown. *Upper*, growth control. *Lower*, interaction test. Interaction with empty vectors was assayed as negative control. *AD*, activator domain; *BD*, binding domain.

3. Discussion

Ho *et al*. [50] described a protein complex containing Med9-Flag, eIF4E porin1, GLK1, and GMP1 formed in the presence of RNA. In a yeast two-hybrid genomic screening, we found Med9 as a novel interactor of eIF4E and further corroborated a physical Med9—eIF4E interaction; we also described the eIF4E-binding site and that the formation of the Med9—eIF4E complex depended on the carbon source. This interaction also happened in *S. kudriavzevii*. Protein localization suggested that this interaction might happen both in the cytoplasm and within the nucleus.

3.1. A Possible Nuclear Complex eIF4E—Med9

eIF4E has been mostly characterized as a cytoplasmic translational factor. However, in monkey, human, *Drosophila*, and mouse cells [10,55–61] a fraction of eIF4E lays within the nucleus, where it mediates mRNA import, export, or 3’end pre-mRNA processing via binding to different 4E interactors [11,62]. In *Xenopus* [63], *Drosophila* embryos and cultured S2 cells [46,64], *Giardia* [65], *Trypanosoma brucei* [66,67], and *S. cerevisiae* [68], eIF4E orthologs also have been detected in the nucleus, but their role there in these species is unknown. We described that eIF4E is both nuclear and cytoplasmic and that interacts with Med9, a component of the RNA polymerase II transcriptional Mediator complex [69–71] that also localizes to the cytoplasm.

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Interestingly, other transcription factors have been also reported to interact with eIF4E orthologs in different species. This is the case of human HOX9 [10] and PRH [72], mouse EMX2 [73] and PREP1 [74], and *Drosophila* Bicoid [75]. Thus, eIF4E regulation by transcription factors is a recurrent phenomenon that has evolved independently several times in different lineages [53]. In our genomic screening we also recovered the nuclear histone chaperone SPT2 as a potential eIF4E interacting protein. Our results didn't show colocalization or interaction of endogenous eIF4E and Med9. Since Med9 must displace the other eIF4E-interactors, namely eIF4G1, eIF4G2, Eap1, and p20, we might need a method with high sensitivity to detect the endogenous complex.

Because both GFP-fusion proteins localized in nucleus and the cytoplasm, and because the two-hybrid's interactions actually occur within the nucleus, our results suggest that the endogenous Med9—eIF4E complex might be formed both in the cytoplasm and within the nucleus when glucose or galactose, but not lactate, are used as carbon source. Indeed, since the two proteins are mostly cytoplasmic when grew in lactate as a carbon source, but no interaction was detected in the two-hybrid assays in this medium, the eIF4E—Med9 interaction cannot happen in the cytoplasm, but rather inside the nucleus.

3.2. Med9 Possesses a Non-Canonical eIF4E-Binding Site

Metazoan proteins bind eIF4E via canonical (YXXXLLϕ) [5,27] and non-conserved motifs that recognize the dorsal and lateral surfaces of eIF4E, respectively, in a bipartite interaction mode [22,44–48]. This is also the case of the yeast eIF4G1 and EAP1 [22]. We described that the Med9 region contacting eIF4E is rather different than the canonical motif. AlphaFold *in silico* docking predicted a non-bipartite mode of interaction between Med9 and eIF4E.

4. Materials and Methods

4.1. Yeast Culture, Manipulation and Molecular Biology

Protocols for yeast growth and manipulation, and yeast DNA and proteins isolation were from Xiao (2014) and Nosek and Thomaska (2013) [76,77].

4.2. Plasmid Construction

DNA manipulation, PCR-amplification, cloning and sequencing were done according to Green and Sambrook (2012) [78]. PCR-amplification of DNA fragments was carried out using Taq DNA Polymerase (ThermoFisher) and specific oligonucleotides. We cloned *S. cerevisiae* eIF4E (gene ID: *CDC33*) and Med9 (gene ID: *CSE2*) into the pGEX-6P2 (Amersham) and pET-28 or pET-30 (Novagen) vectors in-frame with the glutathione S-transferase (GST) or histidine (His6X) tags, respectively. The proteins were also cloned into the pRSET vector (ThermoFisher) without any tag. Different versions of *S. cerevisiae* eIF4E and Med9 cDNAs were subcloned onto the two-hybrid vectors pOAD and or pOBD2 [79] in-frame either with the activator domain (AD) or the DNA-binding domain (BD) sequence of GAL4, respectively, to create the "prey" plasmids eIF4E-AD and Med9-AD, and the "bait" plasmids eIF4E-BD and Med9-BD. Mutations in the Med9 and eIF4E cDNAs were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene) and further subcloned onto the indicated plasmids.

Med9 from the ascomycetes *Zygosaccharomyces rouxii* (gene ID: *ZYRO0C05412g*), *Saccharomyces kudriavzevii* (gene ID: *YNR010W*), and *Candida glabrata* (gene ID: *CAGL0I05742g*) were PCR-amplified using genomic DNA as template and further cloned in-frame with the GAL4 activator domain to create the respective "prey" plasmids. eIF4E from *Z. rouxii* (gene ID: *ZYRO0C03234g*), *S. kudriavzevii* (gene ID: *YOL139C*), and *C. glabrata* (gene ID: *CAGL0E01463g*) were PCR amplified using genomic DNA as template and further cloned in-frame with the GAL4 DNA-binding domain sequence of GAL4 to create the respective "bait" plasmids. The genomic DNA from *Z. rouxii* (cat. num. 2623D-5) and *S. kudriiavzevii* (cat. num. MYA-4449D-5) were purchased from ATCC. The genomic DNA from *C. glabrata* was a kind gift of Irene Castaño (IPICyT, Mexico).

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4.3. High-Throughput Screens and Interaction Assays in the Yeast Two-Hybrid System

We performed a high-throughput yeast two-hybrid screen using the eIF4E-binding domain (BD) construct as a "bait" against an activation-domain yeast library [39] according to [79]. Cells were transformed using the lithium acetate-polyethylene glycol method [80]. We screened approximately 12,000 clones of *S. cerevisiae* genes in the plasmid pOAD (prey, activator domain), representing two genomes, and platted onto thirty-two 384-well microtiter plates of –Leu medium using a robotic replicating tool (Biomek 2000, Beckman Coulter) [79]. Interactions between "bait" and "prey" proteins were detected following an interaction-mating method using the strains PJ69-4α [81] transformed with "baits" and PJ69-4a [82] transformed with "preys" containing the reporter genes *HIS3* and *ADE2* [79]. Diploid cells containing both bait and prey plasmids were grown in –(Trp, Leu) selective media (Clontech) and shown as controls for growth. Protein interactions were detected by replica-plating diploid cells onto –(Trp, Leu, Ade) or –(Trp, Leu, His) selective media (Clontech) containing either 5 mM or 30 mM 3-amino-1,2,4-triazole (3AT, Sigma-Aldrich). Growth was scored after 4-5 days of growth at 30 °C.

4.4. Production of Recombinant Proteins

The *GST Gene Fusion System* from *Amersham* and the *QIAexpressionist* (Qiagen) protocols were used to produce and purify recombinant protein. Briefly, *E.coli* BL21 (DE3) One shot (Invitrogen) <pGEXT-eIF4E>, <eIF4E-His6X>, <pGEXT-Med9>, or <Med9-His6X> cultures were induced with 0.5 mM IPTG for 3 hrs at 37 °C with shaking at 200 rpm. The cultures were centrifuged, and the pellets treated with lysozyme followed by French press cell disruption in the presence of and Complete Protease Inhibitor Cocktail EDTA-free (Roche). The resulting supernatant, containing solubilized GST-eIF4E, eIF4E-His6X, GST-Med9, or Med9-His6X were incubated with Glutathione-Sepharose 4B (GST-eIF4E and GST-Med9) or Ni-NTA-Agarose Superflow (Qiagen) (Med9-His6X and eIF4E-His6X) resins for 45 minutes at 4 °C. Subsequently, the sepharose was recovered and washed once with PBS. GST-eIF4E and GST-Med9 were cleavaged out using PreScission Protease (Merck) for 4 hours at 4 °C. Following cleavage, the supernatant containing eIF4E or Med9 was recovered.

4.5. FPLC Analysis

Fast protein liquid chromatography (FPLC) was performed according to Schutz et al. 2008 [83]. Two to ten micrograms/ml of recombinant proteins were diluted in column buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA) and further passed through a Superdex 75 (S75) gel filtration column (Amersham Pharmacia). The collected fractions were analyzed in SDS-PAGE and silver staining.

4.6. m^{7}GTP-Sepharose Pull Down

50-ml of bacterial cultures were grown 3 hr at 37 °C and then induced induced for 3 hr with 1 mM IPTG, further pelleted and resuspended in 2.5 ml cap binding buffer (CBB; 100 mM KCL, 20 mM HEPES pH 7.6, 0.2 mM EDTA, 10% glycerol, 0.1% Triton X100, Complete Protease Inhibitor Cocktail EDTA-free, 0.2 mM PMSF, 50 mM beta-glycerol phosphate, 7 mM beta-Mercapto ethanol, and 1 mM orthovanadate) on ice. Cells were lysed with lysozyme and sonication on ice. Lysates were pelleted and 1 ml supernatant was pre-cleared with 150 µl Sepharose CL 4B (GE Healthcare) preequilibrated 1 hr in CBB with gentle rotation at 4 °C. Beads were removed by centrifugation for 2 minutes at 2500 rpm. Lysates were further incubated either with 150 µl slurry of m^{7}GTP-Sepharose 4B beads (GE Healthcare) or Sepharose CL 4B (control) pre-equilibrated in CBB for 1 hr at 4°C with gentle rotation. The beads were collected by gravity on ice, washed three times with 750µl of CBB containing 1 mM GTP. Subsequently, the beads were recovered and resuspended in SDS-PAGE sample buffer and subjected to SDS-PAGE and Coomassie staining.

4.7. Fluorescence Microscopy

The *S. cerevisiae* strains *CDC33-GFP* and *CSE2-GFP* [51,52] (https://yeastgfp.yeastgenome.org/) encoding eIF4E-GFP and Med9-GFP, respectively, were grown to OD_{600} = 1-1.4 and imaged as described [51] using Zeiss Axioskop 2 mot plus fluorescence microscope, and a 100× objective with Zeiss axiocam MRc5 using Zen 3.4 software. Quantitative analysis for fluorescence quantitation of fluorescent proteins was performed using the Fiji (Image J) algorithm [84].

4.8. Protein Docking

Protein-protein docking were predicted by AlphaFold3 [85] using the Worldwide Protein Data Bank (PDB) *S. cerevisiae* eIF4E [22] and Med9 [86] 3D structures, codes 6FC1 and 1YKH, respectively. Images were visualized using PyMOL software (Schrödinger, L. & DeLano, W. (2020); *PyMOL* retrieved from http://www.pymol.org/pymol).

4.9. Sequences Alignment

Protein accession numbers are the following: *S. cerevisiae* eIF4E (ID: NP_014502.1) and Med9 (ID: P33308); *Z. rouxii* eIF4E (ID: C5DSV2) and Med9 (ID: C5DT46); *S. kudriavzevii* eIF4E (ID: JGEGQ4) and Med9 (ID: JGECA8); and *C. glabrata* eIF4E (ID: Q9P974) and Med9 (ID: Q6FQJ5). Sequences were obtained from UniProt.org (https://www.uniprot.org/) and OrthoDB.org (https://www.orthodb.org/), aligned using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/), and optimized by eye. The following conservative amino acids were considered: G and A; S and T; K, R and H; E, D, Q and N; and L, I, M, V, C, Y, F, and W.

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