Human-Like Eukaryotic Translation Initiation Factor 3 from *Neurospora crassa*

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Abstract

Eukaryotic translation initiation factor 3 (eIF3) is a key regulator of translation initiation, but its in vivo assembly and molecular functions remain unclear. Here we show that eIF3 from *Neurospora crassa* is structurally and compositionally similar to human eIF3. *N. crassa* eIF3 forms a stable 12-subunit complex linked genetically and biochemically to the 13th subunit, eIF3j, which in humans modulates mRNA start codon selection. Based on *N. crassa* genetic analysis, most subunits in eIF3 are essential. Subunits that can be deleted (e, h, k and l) map to the right side of the eIF3 complex, suggesting that they may coordinateentially regulate eIF3 function. Consistent with this model, subunits eIF3k and eIF3l are incorporated into the eIF3 complex as a pair, and their insertion depends on the presence of subunit eIF3h, a key regulator of vertebrate development. Comparisons to other eIF3 complexes suggest that eIF3 assembles around an eIF3a and eIF3c dimer, which may explain the coordinated regulation of human eIF3 levels. Taken together, these results show that *Neurospora crassa* eIF3 provides a tractable system for probing the structure and function of human-like eIF3 in the context of living cells.

Introduction

The regulation of protein synthesis in eukaryotes occurs predominantly during translation initiation. Translation initiation in eukaryotes is regulated by a number of eukaryotic initiation factors (eIFs) whose specific roles in this process remain unclear. In humans, eIF3 is the largest eIF, consisting of 13 non-identical protein subunits named eIF3a through eIF3m [1]. During cap-dependent translation, eIF3 functions as a structural scaffold for other eIFs and is crucial in the formation of the translation preinitiation complex (PIC) [2,3]. Similarly, eIF3 is required for genomic RNA recruitment to the small ribosomal subunit during viral internal ribosome entry site (IRES)-dependent translation [2,4,5,6]. Notably, altered expression levels of many subunits within eIF3—including eIF3a, b, c, f, h and m—have been linked to various cancers, although their roles in oncogenesis are not understood [7]. In zebrafish and worms, eIF3 subunits have been tied to developmental pathways that may require eIF3 to specifically recruit mRNAs to PICs [8,9,10]. Although the overall architecture of human eIF3 has recently been described [11], the specific functions of its subunits and its in vivo assembly pathway remain unclear [3,11,12].

The subunit composition of eIF3 varies dramatically among organisms, typically with eIF3 complexes missing subunits as species diverge from metazoa (Figure 1). Most genetic and biochemical studies of eIF3 have been performed with the yeast *Saccharomyces cerevisiae*, which contains only five stoichiometric subunits (eIF3 a, b, c, g and i) and the fission yeast *Schizosaccharomyces pombe* which contains two distinct, eight subunit complexes (eIF3 a, b, c, f, g, h, i, m or eIF3 a, b, c, d, f, g, h, i) [2]. The five subunits from *S. cerevisiae* eIF3 have been proposed to comprise the core of eIF3 in all eukaryotes [2]. However, the minimal stable core structure of human eIF3 is composed of eight subunits (a, c, e, f, h, k, l and m), only two of which are conserved with the *S. cerevisiae* eIF3 complex [12,13]. Thus, a genetically tractable model system with an eIF3 that more closely corresponds to that in humans would greatly aid studies of the assembly and function of this essential translation factor.

Results and Discussion

Essential and Non-essential Subunits in *Neurospora* eIF3

The filamentous fungus *Neurospora crassa* (Nc) is a morphologically complex, multicellular, model organism with at least 28 distinct cell-types [14]. The sequenced *Neurospora crassa* genome contains annotated orthologues of 10 eIF3 subunits, with eIF3j, k, and m remaining unannotated [15]. We conducted BLASTp searches of the *Neurospora* genome using human eIF3 query sequences and identified orthologues of eIF3k and m [16]. Using an eIF3j query from *Aspergillus niger*, we also identified an orthologue for eIF3j. Reciprocal BLASTp searches against the human database using *Neurospora* eIF3 subunit queries corroborated all 13 eIF3 subunit orthologues (Table 1) making *Neurospora* an attractive model system for studying human-like eIF3.

To assess the importance of eIF3 subunits to *Neurospora* viability, *Neurospora* knock-out strains were first propagated asexually.
Knock-outs that are null viable can be isolated as homokaryons, in which the strain only contains nuclei with the gene of interest deleted. Alternatively, knock-outs that are null lethal cannot be isolated as homokaryons, but instead can be maintained as heterokaryons in which nuclei from a compatible strain complement the null lethal phenotype through hyphal fusion [17].

Knock-out strains of 12 *Neurospora* eIF3 subunit orthologues were obtained from the Fungal Genetics Stock Center (FGSC) [18].

**Figure 1. The stoichiometric subunit composition of eIF3 varies across species.** Cladogram constructed using sequences of 18S rRNA from the listed organisms. The subunit composition of eIF3 from each organism is depicted using spherical models. Subunit count in the stoichiometric complex of the displayed organisms is as follows: *H. sapiens*, *D. rerio*, *M. musculus*, *C. elegans*, *D. melanogaster*, *N. crassa*, *D. discoideum* and *A. niger* (12); *A. thaliana* (11); *S. pombe* (Csn7b, left, or Int6, right, complexes) (8 each); *T. brucei* (8); *S. cerevisiae* (5). The size of the spheres used to depict eIF3 subunits are relative to their respective molecular weights. The tree was constructed using tools at www.phylogeny.fr [46].

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**Table 1. N. crassa eIF3 genes, identity to their human orthologues and knock-out phenotypes.**

| eIF3 subunit | N. crassa gene (NCU#) | UniProt Accession Number | % identity | KO phenotype |
|--------------|------------------------|--------------------------|------------|--------------|
|              | N. crassa              | Human                    |            |              |
| a            | 00040                  | Q7RWI1                   | 37         | lethal       |
| b            | 02208                  | Q7S464                   | 39         | lethal       |
| c            | 07831                  | Q75BD0                   | 39         | lethal       |
| d            | 07380                  | Q75212                   | 39         | lethal       |
| e            | 05889                  | Q7S519                   | 43         | viable       |
| f            | 01021                  | Q9F748                   | 32         | lethal       |
| g            | 08046                  | Q6MFP4                   | 38         | lethal       |
| h            | 07929                  | Q759Y9                   | 30         | viable       |
| i            | 03876                  | Q7RXH4                   | 52         | lethal       |
| j            | 07954                  | Q759Y1                   | 24         | viable       |
| k            | 09707                  | Q75291                   | 29         | viable       |
| l            | 06279                  | Q75B62                   | 51         | viable       |
| m            | 02813                  | Q7S6E1                   | 26         | lethal       |

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Methods). As anticipated, linear growth on water agar exacerbated the eIF3 knock-out phenotypes (Figure 2B, Table S2 in File S1), except for the ΔeIF3l strain, which grew better on water agar relative to wild-type Neurospora (Figure 2B, Table S2 in File S1). Taken together, these data reveal that many non-universally conserved subunits of *Neurospora* eIF3 are essential in human-like eIF3 complexes, whereas deletion of some subunits result in *Neurospora* eIF3 sub-complexes that maintain much of its biological function.

To further probe eIF3 subunit dispensability and genetic interactions, we constructed three double knock-out strains of *N. crassa*: deletion of subunits k and l (ΔeIF3kl), deletion of subunits h and k (ΔeIF3hk) and deletion of subunits j and l (ΔeIF3jl). We compared the linear growth of these double knock-out strains to each individual deletion, as well as to the predicted multiplicative effect of combining deletions (Figure 2C, Table S2 in File S1). Linear growth rates on sucrose minimal media or water agar were greater than predicted for both the ΔeIF3kl and ΔeIF3hk strains (Figure 2C, Table S2 in File S1), indicating synergistic epistatic effects. The ΔeIF3jl strain had linear growth rates similar to predicted rates (Figure 2C, Table S2 in File S1), indicating epistatic neutrality. Linear growth rates on water agar were also much greater than the predicted values for the ΔeIF3kl and ΔeIF3hk strains, indicating strong compensatory effects for the double knock-out (Figure 2C, Table S2 in File S1). These results show that the subunit pair of eIF3k and eIF3l and the subunit pair of eIF3h and eIF3j have structural or genetic synergy.

### Biochemical Properties of eIF3 and eIF3j Isolated from *Neurospora crassa*

To determine the structural integrity of the *Nc* eIF3 complex, subunits eIF3j, h, k or l were individually inserted with N- or C-terminal affinity tags into their respective knock-out background strains, at the *his-3* locus (Table S1 in File S1). Each tagged subunit rescued most or all of the linear growth phenotype observed with the respective knock-out strain (Figure 2B), with the exception of the C-terminally tagged eIF3k on water agar, indicating the recombinant constructs were functional *in vivo*. Using strains with tagged eIF3j, k or l, we isolated eIF3 complexes with 12 stoichiometric subunits (Figs. 3A, 4C and D), all corresponding to orthologues of human eIF3 subunits (a-m, excluding j). Analysis of this stoichiometric dodecamer by negative stain electron microscopy revealed that it has the same anthropomorphic features as human eIF3 (Figure 3B) [12,13]. Thus, like human eIF3, *Neurospora* eIF3 contains 12 stoichiometric subunits and is the first structurally human-like eIF3 to be purified and visualized outside of metazoa.

Notably, *Nc* eIF3j was not present in any of the purified eIF3 samples (Figs. 3A, 4C and D), similar to the reported behavior for *S. cerevisiae* eIF3j (Hcr1p) [23]. However, tagged Nc eIF3j was able to affinity purify a myriad of translation-related proteins including eIF3 subunits, ribosomal proteins and other translation initiation/elongation factors (Figure 3C and Table S3 in File S1), also as seen with *S. cerevisiae* [24] and *S. pombe* [25]. Taken together, these results suggest that *Neurospora* eIF3j, like its yeast counterparts, associates with the eIF3 complex in a weak manner. Together with the neutral epistasis seen with the ΔeIF3jl strain, these data indicate that *Nc* eIF3 functions in translation in *Neurospora crassa*.

### Assembly of eIF3k and l into eIF3 Complexes

Reconstitution of human eIF3 revealed that eIF3k, h and l are all required for the formation of a stable eight-subunit core composed of the subunits containing Proteasome Cop-9 Initiation factor 3 (PCI) or *M. oryzae* Pad1 N-terminal (MPN) domains [12].
Figure 2. Viable Nc elF3 knock-out strains display defects in conidiation and linear growth. (A) Growth of Nc elF3 single and double knock-out (KO) strains on Vogel's minimal media (MM) with 2% sucrose. Plates were spotted from frozen stocks of the indicated elF3 subunit single or double knock-out strain, grown in the dark and photographed on the indicated days. (B) Linear growth of Nc elF3 knock-out strains on Vogel's MM with 2% sucrose (black bars) or water agar (white bars). In all graphs linear growth is plotted as a fraction of wild-type growth on comparable media. Linear growth of wild-type (WT) on water agar as a fraction of MM with 2% sucrose is 0.63 (Table S2 in File S1). Linear growth of elF3 single knock-out strains is compared to WT, where the elF3 subunit knock-out is indicated below the bars (graph elF3 KO). Linear growth of elF3 knock-out compared to wild-type and the knock-out strain with an N-terminally tagged (N) or C-terminally tagged (C) recombinant elF3 subunit at the his-3 locus (graphs elF3h, j, k, l). The recombinant elF3 subunit and corresponding KO strain is indicated below each graph. (C) Linear growth of elF3 double knock-out
Consistent with human eIF3 reconstitutions, the ΔeIF3h strain exhibited appreciable defects in linear growth and developmental phenotypes (Figure 2A and B, Table S2 and Figure S1 in File S1). Surprisingly, the *Neurospora* ΔeIF3kl strain exhibited nearly wild-type linear growth rates and conidiation on sucrose minimal media (Figure 2A and B, Table S2 in File S1). These results suggest that subunits k and l are not required for assembly of the remainder of the eIF3 complex in *vivo*.

To better understand the role of eIF3k and l in eIF3 assembly, we used the ΔeIF3kl strain, and inserted either N-terminally tagged eIF3k (strain ΔeIF3kl, +N-k) or eIF3l (strain ΔeIF3kl, +N-l) at the *his-3* locus to assess the stability of the eIF3 complex in the absence of l or k, respectively (Table S1 in File S1). Neither N-terminally tagged subunit was able to affinity purify the remainder of the eIF3 complex (Figure 4A and B). Furthermore the level of tagged eIF3l in the ΔeIF3kl background was much lower compared to tagged k in the ΔeIF3kl background (Figure 4B), suggesting that subunit eIF3l is less stable in the absence of eIF3k (Figure S2 in File S1). Remarkably, mixing of lysates from the (ΔeIF3kl, +N-k) and (ΔeIF3kl, +N-l) strains—which contain free eIF3k and eIF3l—did not result in their assembly into the remaining 10-subunit complex (Figure S3 in File S1). However, the entire dodecameric complex could be affinity purified from a heterokaryon strain generated from the above two single-deletion strains (Figure 4A). These results indicate that subunits eIF3k and eIF3l require each other, as well as additional *in vivo* factors, to be incorporated into the dodecameric eIF3 complex.

Notably, a third strain containing a tagged h subunit in a ΔeIF3hl background (strain ΔeIF3hl, +N-h) yielded affinity-purified complexes lacking both eIF3k and l subunits (Figure 4C). Affinity purification of N-terminally tagged eIF3k in the absence of eIF3h (strain ΔeIF3hk, +N-k) yielded an eIF3k dimer (Figure 4D) indicating that eIF3k and l are either pre-assembled as a dimer and interact with eIF3 already containing the h subunit or

![Figure 3. Composition and structure of eIF3 assemblies.](image)

**Figure 3. Composition and structure of eIF3 assemblies.** (A) Coomassie stained purified *N. crassa* eIF3 with C-terminally tagged eIF3l. Subunits identified by tandem mass spectrometry are labelled. Subunits f and h co-migrate on the gel. The tagged subunit is indicated with an asterisk. (B) 2-D EM class averages of negatively stained *Neurospora* eIF3: A Human-Like eIF3 Complex.

![Figure 4. Affinity purification of elf3 knock-out strains reveal subunit interdependence during elf3 assembly.](image)

**Figure 4. Affinity purification of elf3 knock-out strains reveal subunit interdependence during elf3 assembly.** (A) Anti-FLAG affinity purifications from *N. crassa* extracts using N-terminally tagged eIF3l or eIF3k in the ΔeIF3kl double knock-out background (lanes 1 and 2). Affinity purification from *N. crassa* extract obtained from a heterokaryon strain (lane 3) made from strains used in lanes 1 and 2. N-terminally tagged k and l subunits expressed from the *his-3* locus are indicated above each lane. All strains are ΔeIF3kl double knock-outs. Contaminant bands in lanes 1 and 2 were identified as phenylalanyl tRNA synthetase beta chain (73 kDa) and tRNA ligase (58 kDa). (B) Anti-FLAG Western blot of the affinity purifications from the gel in (A). Each lane has the exact amount of total protein loaded in (A). Anti-FLAG affinity purifications from *N. crassa* extracts using N-terminally tagged eIF3h in ΔeIF3hl or ΔeIF3kl strains (C) or N-terminally tagged eIF3k in ΔeIF3hk strains (D). The tagged h or k subunits are indicated with asterisks. Arrows indicate the missing k and l subunits in the ΔeIF3hl strain (C) or a degradation product of eIF3a (D). All gels in panels (A), (C) and (D) are stained with Coomassie blue.
alternatively the eIF3k dimer binds to eIF3h and assembles with eIF3 as a trimer. These data corroborate the knock-out phenotypes, in which a ΔeIF3hk double deletion strain has essentially the same linear growth fitness as the ΔeIF3h single deletion (Figure 2C, Table S2 in File S1), i.e. both strains are functionally triple deletion strains. Taken together these data show that assembly of eIF3h into eIF3 occurs in the absence of eIF3k and eIF3l. Furthermore, the assembly of eIF3k and eIF3l into eIF3 is dependent on eIF3e-eIF3l interactions as well as on eIF3h.

The Right Side of eIF3 is Dispensable in vivo
When compared to the subunit positions in the structural core of human eIF3 [11], the eIF3 subunits that are dispensable in N. crassa (ε, h, k and l) are localized to the right arm and right leg of eIF3 (Figure 3B) [12]. Strikingly, our data show that at least three subunits (eIF3h, k and l; ΔeIF3hk strain) can be removed from the complex with the resulting strain remaining viable (Figures 2C and 4C), analogous to one of the two eIF3 complexes in S. pombe (Figure 1). Based on the recent cryo-EM structure of a mammalian PIC-like complex, eIF3 binds the platform of the human 40S ribosomal subunit with the right arm and leg positioned away from the ribosomal subunit interface [3]. Thus, subunits eIF3e, h, k, l and m-positioned away from the 40S subunit, may regulate translation initiation by serving as a binding platform for translation or regulatory factors. Consistent with this model, the predominant isoform of eIF3h in zebrafish regulates the translation of proteins involved in development [8]. A similar role for eIF3h has also been observed in Arabidopsis [26,27,28] and S. pombe [29]. In N. crassa, deletion of eIF3h also results in developmental defects in conidiation (Figure S1 in File S1, Figure 2A). Thus, the links between eIF3h and development are conserved among species spanning a large swath of eukaryotic phylogeny. Importantly, our results indicate that defects in eIF3h likely impact the function of subunits eIF3k and eIF3l as well. Future experiments will be required to determine how subunits eIF3h, k and l work together to regulate the translation of specific mRNAs.

Neurospora eIF3 Reconciles a Model for Human-like eIF3 Assembly
All four dispensable subunits, including subunits in double knock-outs (ΔeIF3hk and ΔeIF3dk) of the Neurospora dodecameric complex, were essential for the stable formation of the reconstituted human octameric core (subunits a, c, e, f, h, k, l and m) in Escherichia coli [12]. Similarly, reconstitution of functional human eIF3 in HeLa in vitro translation extracts or insect cells required subunits e and h [30,31]. By contrast, the viability of Neurospora eIF3e, h, k, l and m knock-out strains suggest that other subunits not present in the PCI-MPN core (e.g. subunits b, c, d, g, i) or molecular chaperones might contribute to the in vivo stability of eIF3 compared to the reconstituted systems. The co-dependence of eIF3k and eIF3l assembly into eIF3 provide the first direct evidence for subunit interdependence in endogenous eIF3 assembly, and also suggest that cellular factors may contribute to the process. Notably, the co-dependence of k and l is phylogenetically supported by the fact that k and l are present or absent in a pair-wise manner in other eukaryotic genomes (Figure 1 and [2,32]).

Models that define the core of eIF3 differ depending on whether they rely on minimal subunit composition using phylogenetics (Figure 1) [2], on cell viability (Table 1), or on structural information [12,23,30,33,34]. By comparing all three models of the core of eIF3 (Figure 5A), we propose that eIF3 assemblies on a dimer composed of subunits a and c (Figure 5A). The remaining subunits would then be assembled onto the eIF3a/eIF3c (ac) dimer either as single subunits or sub-complexes (Figure 5B). In support of this assembly model, we recently demonstrated the ac dimer to be a minimal eIF3 sub-complex capable of high affinity binding to the hepatitis C virus internal ribosome entry site (HCV-IRES) [35]. Our phenotypic analyses of eIF3e, h, k or l knock-outs suggest that these subunits likely assemble onto the ac dimer [12] in an ordered manner (Figure 4), but independently of the remaining essential subunits (b, d, f, g, i and m) (Figure 5B). In particular, subunits eIF3k and l are assembled into eIF3 as a dimer (Figure 4D), dependent on the incorporation of eIF3h into eIF3 (Figure 4C). Ordered assembly of subunits onto the ac dimer could allow the levels of eIF3 to be regulated by simply regulating the expression of subunits a and c, as observed in human cells [36].

Conclusion
We have used the power of N. crassa genetics and biochemistry eIF3 to map the structure and function of its compositionally human-like eIF3 translation initiation factor in vivo. Four subunits within the stable dodecameric complex (ε, h, k and l) and the eIF3 orthologue are dispensable for growth, and three double knock-out strains (ΔeIF3hk, ΔeIF3hk and ΔeIF3dk) are also viable. We also show the strong interdependence of eIF3h, k and l assembly into eIF3, which has important implications for the mechanism by which eIF3 regulates translation of specific mRNAs in animals and plants [8,26,27]. When compared with phylogenetic and reconstitution data, the results with N. crassa eIF3 allow us to redefine the core of eIF3 as the eIF3a/eIF3c dimer, onto which individual subunits or eIF3 subcomplexes assemble, possibly with the aid of in vivo factors. N. crassa eIF3 should therefore serve as a useful genetic and biochemical system for unravelling the assembly of human-like eIF3 and its roles in regulating translation in living cells.

Materials and Methods

Creation of Neurospora crassa eIF3 Expression Constructs
All eIF3 cDNAs were prepared from wild-type Neurospora total RNA (Trizol extracted) using the M-MLV reverse transcriptase (Invitrogen) with poly-dT reverse primers (first strand) and subsequent PCR amplification with eIF3 gene specific primers (second strand). PCR was used to introduce in-frame PreScission protease cleavage sites either upstream (N-terminally tagged) or downstream (C-terminally tagged) of the eIF3 gene, allowing the option of removing the affinity tag by proteolytic cleavage with PreScission protease. All constructs were cloned into the Acl and Pac restriction sites of plasmid pCCG::N-FLAG::HAT (N-terminally tagged; FJ457007) or into the XbaI and PacI restriction sites of plasmid pCCG::C-Gly::HAT::FLAG (C-terminally tagged; FJ457005) [37]. Plasmids were obtained from the FGSC [18]. Both constructs add FLAG and HAT affinity tags, separated by glycine linkers, to the terminus of the eIF3 subunit. The N-terminal affinity tags chosen were FLAG-3xGly-HAT-5xGly, and the C-terminal affinity tags were 10xGly-HAT-5xGly-FLAG. The plasmid constructs were designed to target the his-3 locus and expression was controlled by the constitutive promoter CCG-1. Plasmid constructs were transformed and selected using Escherichia coli.

N. crassa Strains
All strains used in this study are summarized in Table S1 in File S1. Knock-out strains, obtained from the Fungal Genetics Stock Center (FGSC) are indicated in Table S1 in File S1 [18]. His-3 auxotrophs of eIF3h, j, k or l were created by crossing with the his-3 strain of the opposite mating type and selecting hygromycin
resistant progeny on Vogel’s minimal media, 2% agar, 2% sucrose supplemented with 100 μg/mL histidine and 200 μg/mL hygromycin B. Strains isolated from the cross that were not histidine auxotrophs were used in phenotypic analyses (described below), whereas auxotrophic strains were used to insert tagged eIF3 subunits. Strains expressing N- or C-terminally tagged eIF3 constructs (subunits h, j, k or l) were generated by electroporating 1 μL of 100 ng/μL plasmid DNA of the tagged eIF3 construct into 90 μL of conidia (16109 conidia/mL in 1 M ice cold sorbitol) from their respective his-3 eIF3 knock-out strains to recreate the wild-type complex containing an affinity tag. Transformants were purified by back crossing with the his-3 strain and selecting on Vogel’s minimal media, 2% agar, 2% sucrose supplemented with 200 μg/mL hygromycin B or by purifying microconidia using a protocol modified from http://www.fgsc.net/fgn37/ebbole1.html. Microconidia purification was done by plating a small amount of conidia from the transformant onto water agar (Vogel’s minimal media, 2% agar) and growing at 30°C in the dark for 4 days, followed by at least one week at room temperature in the light. Microconidia were harvested by scraping the water agar plates with 2 mL water, filtering the collected water through a 5 μm filter to remove macroconidia. Microconidia were pelleted by centrifugation, resuspended in 50–100 μL of water and plated on Vogel’s minimal media, 2% agar, 2% sucrose plates. Plated microconidia were germinated for 15–30 hours at 30°C.

Figure 5. Redefining the core of eIF3 and proposed models for assembly. (A) Venn diagram highlighting common subunits among three definitions of the eIF3 core complex: 1) the phylogenetically conserved complex from S. cerevisiae, 2) the reconstituted human PCI-MPN core complex and 3) the biologically essential subunits from N. crassa eIF3. (B) Proposed models for human-like eIF3 assembly. Individual subunits or sub-complexes assemble onto an ac dimer. Solid and dashed arrows represent assembly and alternative assembly interactions and are drawn to reconcile the compositional breadth of eIF3 sub-complexes across species as well as biochemical data [12,47].

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picked under a dissecting microscope and transferred to individual slants containing Vogel’s minimal media, 2% agar, 2% sucrose.

Double knock-out strains were created by crossing single knock-outs of opposite mating type. Double knock-outs that contained tagged eIF3 subunits at the his-3 locus were either made by crossing one knock-out with another strain that contained the other knock-out and tagged subunit or by creating a his-3 auxotroph of the double knock-out and transforming in the tagged subunit as described above.

N. crassa Strain Validation

Neurospora genotypes were assessed first using PCR with primer pairs that included either a reverse primer that binds in the hygromycin cassette, which is inserted in the reverse orientation, along with a forward primer in the in the 5' flanking region (hyg+5') or a forward primer that binds in the hygromycin cassette along with a reverse primer in the in the 3' flanking region (hyg+3'). These primer pairs provided a quick way of identifying whether or not the correct knock-out cassette was present. We further assessed strain purity (homokaryon vs. heterokaryon) by Southern blotting. Probes were made using the DIG Probe synthesis kit (Roche). Probes were made using the same primer pairs used for PCR identification and thus contained a flanking region of the complete hygromycin cassette. This enabled the identification of both wild-type and knock-out cassettes using a single probe. Genomic DNA (gDNA) preparations were digested overnight with restriction enzymes to test for specific knock-out cassettes (KO/enzyme/probe): (eIF3e/EcoRI/hyg+5') and hyg+3', (eIF3h/XhoI/hyg+5'), (eIF3j/XhoI/hyg+5' and hyg+3'), (eIF3k/Msal/hyg+3'), (eIF3l/EcoRI/hyg+3') and resolved on 0.8% agarose gels overnight at ~17 mA. In all cases wild-type gDNAs were analyzed in parallel. Blotting was done using the Easy Hyb system (Roche). Tagged eIF3 genes at the his-3 locus were identified by PCR using forward and reverse primers that bind to the N-terminal or C-terminal tags, respectively, along with a gene-specific reverse primer. Expression of the protein was confirmed by Western blot analysis of whole cell extracts using anti-FLAG monoclonal antibody HRP conjugate (Sigma).

Phenotype Assays

Lethal eIF3 knock-out strains were validated by crossing heterokaryons obtained from the FGSC (see Table 1) with wild-type Neurospora. Reciprocal crosses were done using the wild-type strain as either the male or female. Ascospores from each cross were harvested and germinated on Vogel’s minimal media, 2% agar, 2% sucrose supplemented with or without 200 μg/mL hygromycin B. No ascospores germinated on media containing hygromycin. Conidia harvested from germinated ascospores grown on non-hygromycin media subsequently failed to germinate when transferred to slants that contained Vogel’s minimal media, 2% agar, 2% sucrose supplemented with 200 μg/mL hygromycin B.

Conidia harvested using water from slants that had been grown at 30°C in the dark for 2–3 days and allowed to conidiate in the light for an additional 7–8 days. All strains within an independent race tube experiment used conidia of equal age. All race tube data were collected and analyzed in parallel with wild-type samples each time. After spotting, the race tubes were incubated at 30°C in the dark for 16–18 hours before taking the initial measurement. Subsequent measurements were typically taken in 6–8 hour increments over the course of 2–3 days. Linear growth was normalized to the first time point. Three technical replicates of each biological replicate were combined and plotted against time (hours) and fit with a linear function using Sigmaplot. The linear growth rate (slope) and errors on the slope of the linear fits were normalized as a fraction of the linear growth of wild-type. The presented data represent the combination of at least two independently isolated isogenic biological replicates, each with a minimum of three technical replicates. Knock-out strains transformed with empty vectors, which express only the HAT-FLAG affinity tag, had the same linear growth as their respective untransformed knock-out strain.

For double knock-out and epistatic analysis, the predicted double knock-out linear growth phenotypes were calculated by multiplying the relative linear growth rates of the individual knock-outs. Predicted double knock-out errors were calculated using the root of the sum of squares of the standard errors of the individual knock-outs.

Isolation of eIF3 Complexes or eIF3j from N. crassa Lysates

Strains containing tagged eIF3 subunits were grown in liquid culture (Vogel’s minimal media, 2% sucrose) for 40–46 hours from an initial conidial density of OD₅₅₀ = 0.04. Mycelia were collected using a Büchner funnel with filter paper, rinsed briefly with deionized water and immediately transferred to dry ice. Frozen biomass was ground up using a mortar and pestle in liquid nitrogen. Lysis buffer (TBS, 0.5% Triton X-100, 10% glycerol and protease inhibitors) was added directly to the powered biomass (~10–15 mL lysis buffer per gram of biomass) and further ground with the mortar and pestle until the lysate was an even consistency. The lysate was cleared by centrifugation and filtered with a 0.2 μm syringe filter. Anti-FLAG affinity beads (Sigma) were added directly to the lysate for 4–20 hours at 4°C, collected by centrifugation and washed 5 times each with ~10 bed volumes of wash buffer (TBS, 10% glycerol). The complex was eluted using FLAG peptide (Sigma). The eluate was washed three times with ~10 volumes of wash buffer containing 1 mM DTT though an Amicon Ultra 0.5 mL Ultracel 30k centrifugal filter to remove the FLAG peptide.

Analysis of eIF3j Interacting Proteins by LC MS/MS

N. crassa strains containing N- or C-terminally tagged eIF3j were grown and eIF3j and its interacting proteins were purified as indicated above. Strains that expressed only the affinity tag at the his-3 locus were used as negative controls to determine Neurospora proteins that bind non-specifically to the anti-FLAG affinity resin. Total eluates (~5–25 μg of total protein from eIF3j eluates, negative controls typically yielded <1 μg of total protein) were denatured, reduced and acetylated before digesting with trypsin. Trypsin digestions were desalted as previously described [25] and used for LC MS/MS analysis as previously described [38]. Table S3 in File S1 lists interacting proteins that were identified in the eluates from four replicates (two each with N- and C-terminally tagged eIF3j). Proteins listed in Table S3 in File S1 occurred in at least two of the four replicates and a minimum of four peptides
total. Proteins identified from negative controls were excluded in Table S3 in File S1.

Electron Microscopy

Protein samples were diluted to a final concentration of 50 nM in buffer 20 mM Hapes, pH = 7.4, 120 mM KCl, 0.5 mM EDTA, 1 mM DTT, and 3% trehalose. 400 mesh continuous carbon grids were plasma cleaned in a 75% Ar/25% O₂ atmosphere for 20 seconds in a Solarus plasma cleaner (Gatan, Inc). Sample aliquots of ∼4 µL were placed onto the grids, negatively stained with a 2% uranyl acetate solution and blotted to dryness. Data were acquired using a Tecnai T12 electron microscope operating at 120 keV using the Leginon automated data collection software [39] on a Titan 3×4K pixel CCD camera (1.5 µm pixel size) at a nominal magnification of 50,000X (2.18 Å/pixel). Images were collected in low-dose mode with a dose of ∼20 e-/Å² and a defocus range from -0.5 to -1.5 µm. Two-dimensional data processing was performed using programs and utilities contained within the Appion processing environment [40]. Particles were initially extracted using a difference of Gaussians (DoG) particle picker [41]. After contrast transfer function (CTF) estimation using CTFFind [42], particle image stacks were generated by extracting selected particles with a box size of 192×192 using the “batchboxer” program [43] with an estimated CTF confidence cutoff above 80%. The data were then binned by a factor of two for normal processing. Each particle was normalized using the XMIPP normalization program [44] using a cutoff of 4.5σ of the mean pixel value. The stack was subjected to several rounds of reference-free two-dimensional classification using IMAGIC [45] iterative multivariate statistical analysis and multi-reference alignment analysis (MSA-MRA). Overlapping particles or dust were removed and only those classes belonging to properly assembled complexes were kept. A new stack was generated with all the particles within these classes and subjected to final rounds of MSA-MRA.

Supporting Information

File S1 | File S1 includes Figures S1, S2 and S3. File S1 also includes Tables S1, S2 and S3. (DOCX)

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Author Contributions

Conceived and designed the experiments: MDS, JHDC. Performed the experiments: MDS YG JQA JMV AN. Analyzed the data: MDS JQA JHDC. Contributed reagents/materials/analysis tools: MDS YG AN. Wrote the paper: MDS JHDC.

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