Yeast Eap1p, an eIF4E-associated protein, has a separate function involving genetic stability

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A rate-limiting step during translation initiation in eukaryotic cells involves binding of the initiation factor eIF4E to the 7-methylguanosine-containing cap of mRNAs. Overexpression of eIF4E leads to malignant transformation [1–3], and eIF4E is elevated in many human cancers [4–7]. In mammalian cells, three eIF4E-binding proteins each interact with eIF4E and inhibit its function [8–10]. In yeast, *EAP1* encodes a protein that binds eIF4E and inhibits cap-dependent translation *in vitro* [11]. A point mutation in the canonical eIF4E-binding motif of Eap1p blocks its interaction with eIF4E [11]. Here, we characterized the genetic interactions between *EAP1* and *NDC1*, a gene whose function is required for duplication of the spindle pole body (SPB) [12], the centrosome-equivalent organelle in yeast that functions as the centrosome. We found that the deletion of *EAP1* is lethal when combined with the *ndc1-1* mutation. Mutations in *NDC1* or altered *NDC1* gene dosage lead to genetic instability [13,14]. Yeast strains lacking *EAP1* also exhibit genetic instability. We tested whether these phenotypes are due to loss of *EAP1* function in regulating translation. We found that both the synthetic lethal phenotype and the genetic instability phenotypes are rescued by a mutant allele of *EAP1* that is unable to bind eIF4E. Our findings suggest that Eap1p carries out an eIF4E-independent function to maintain genetic stability, most likely involving SPBs.

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Results and discussion

During G1, yeast cells contain a single SPB that must be duplicated to form a bipolar mitotic spindle. The *Saccharomyces cerevisiae NDC1* 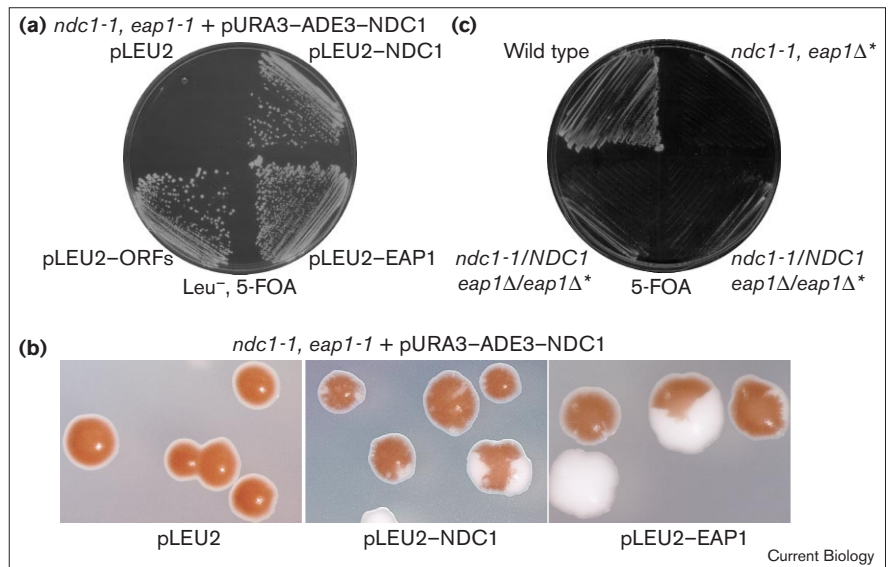gene is required for a late step in SPB duplication [12]. Yeast cells containing the cold-sensitive *ndc1-1* mutation initiate SPB duplication at the non-permissive temperature, but the newly synthesized SPB is not inserted into the nuclear envelope. All of the chromosomes remain associated with the pre-existing, functional SPB, leading to monopolar spindle phenotypes. *NDC1* encodes an essential 74 kDa membrane protein that localizes to both SPBs and nuclear pore complexes (NPCs) [12,15]. The *ndc1-1* mutation corresponds to a missense mutation (G to A) in the codon for amino acid 118, resulting in a change from a serine to an asparagine residue in a predicted transmembrane domain (data not shown). Yeast cells are also sensitive to changes in *NDC1* gene dosage; both increased and decreased *NDC1* gene dosage lead to genetic instability [14]. Here, we sought to identify genes with which *NDC1* interacts by screening for mutations that are lethal in combination with the *ndc1-1* allele.

We mutagenized an *ndc1-1* yeast strain and screened for a second mutation that caused it to require a plasmid-borne copy of *NDC1* at the permissive temperature (23°C). The plasmid harboring *NDC1* also contained the *URA3* and *ADE3* genes, allowing plasmid dependency to be assessed using two criteria: a failure to grow in the presence of 5-fluoroorotic acid (5-FOA), and a failure of colonies to sector (see Supplementary material for details of the synthetic lethal screen). We identified a single yeast strain that showed a specific requirement for *NDC1* (Figure 1a,b). A recessive mutation in the *EAP1* gene was responsible for the synthetic lethal phenotype (Figure 1a,b). The *eap1-1* mutation corresponds to an insertion of a thymine after the forty-third base, which results in a premature stop codon at amino acid 17. In agreement with a previous study [11], we found that *EAP1* is not essential. We also deleted the entire *EAP1* open reading frame (ORF) in the original *ndc1-1* yeast strain, and observed the synthetic lethal phenotype (data not shown).

We tested whether the loss of Eap1p function specifically affects Ndc1p, or whether the effect is more general. Mutations in either *MPS2* or a specific allele of *MPS1*, *mps1-737*, lead to defects in SPB duplication similar to those observed in *ndc1-1* yeast strains [16]. However, the *eap1Δ* allele was not lethal when combined with either the *mps2-1* allele or the *mps1-737* allele (data not shown). To extend the analysis of *EAP1*, we used the previously reported observation that *NDC1* is haploinsufficient, meaning that diploid yeast cells containing a single chromosomal copy of *NDC1* (*NDC1/ndc1Δ*) are not viable [14]. Diploid cells heterozygous for the *ndc1-1* mutation

Figure 1

A mutation in *EAP1* is synthetically lethal with *ndc1-1* and makes it haploinsufficient. **(a)** The mutagenized *ndc1-1* yeast strain, which requires a plasmid-borne copy of *NDC1* (*ndc1-1, eap1-1* + pURA3–ADE3–NDC1; noe581), was transformed with a *LEU2* centromeric vector (pLEU2; pRS315), the *LEU2* vector containing *NDC1* (pLEU2–NDC1; pRS315–NDC1), the *LEU2* vector containing all four ORFs (*YKL207W*, *YKL206C*, *LOS1* and *EAP1*) from the original rescuing genomic library clone (pLEU2–ORFs; pRS315–ORFs), or the *LEU2* vector containing the *EAP1* ORF alone (pLEU2–EAP1; pRS315–EAP1). The transformed yeast strains were streaked onto a Leu⁻, 5-FOA-containing plate to test for rescue of the *ndc1-1, eap1-1* synthetic lethal phenotype. **(b)** The ability of these transformed yeast strains to lose the wild-type copy of *NDC1* (pURA3–ADE3–NDC1) was also examined using a colony sectoring assay. The *EAP1* ORF restored both growth on 5-FOA and sectoring. **(c)** A diploid yeast strain that was heterozygous for the *ndc1-1* allele and homozygous for the *eap1Δ* allele (*ndc1-1/NDC1, eap1Δ/eap1Δ*; HC45-19b/HC38-3d) and contained a plasmid-borne copy of *NDC1* was streaked onto a 5-FOA-

containing plate to induce loss of the *NDC1*-containing plasmid. As controls, a wild-type yeast strain (HC14-10c) and a *ndc1-1, eap1Δ* haploid yeast strain (HC45-19b) were included (for more controls, see [14]). Asterisks indicate yeast strains that initially

contained the pURA3–ADE3–NDC1 plasmid (pALR10–NDC1). The heterozygous diploid yeast strain was unable to grow in the absence of the plasmid-borne copy of *NDC1*, showing that the *ndc1-1* allele becomes haploinsufficient in the absence of *EAP1*.


(*NDC1/ndc1-1*) are viable at the restrictive temperature, suggesting that *ndc1-1* is not a complete loss-of-function allele [14]. However, *ndc1-1/NDC1* heterozygous yeast strains that lack *EAP1* were not viable, even at the permissive temperature (Figure 1c). Therefore, the deletion of *EAP1* causes the *ndc1-1* allele to become haploinsufficient. Taken together, these results suggest that *EAP1* is specifically required in *ndc1-1* yeast strains.

Because yeast cells are sensitive to altered Ndc1p levels [14], it is likely that cells mutant for genes that affect Ndc1p expression or function would exhibit phenotypes similar to those observed in yeast strains mutant for *NDC1*. Given the genetic interactions between *EAP1* and *NDC1*, we characterized *eap1Δ* yeast strains (both W303 and S288C-derived strains) in detail. We found that haploid *eap1Δ* yeast strains incubated at lower temperatures begin to yield diploid and, occasionally, tetraploid clones. The *eap1Δ* yeast strains maintained a stable haploid DNA content at 37°C, but they began to show diploid DNA contents even at 30°C (Figure 2). This is similar to genetic instability phenotypes observed in either *ndc1-1* cells at the non-permissive temperature [13] or in cells overexpressing *NDC1* [14], both of which lead to the formation of monopolar spindles. Unlike *ndc1-1* yeast strains, synchronized cultures of *eap1Δ* cells did not exhibit a uniform arrest phenotype when shifted to 15°C, based on analysis of DNA content and bud morphology (data not shown).

Our experiments suggest that *eap1Δ* yeast strains became diploid at a rate of 3.5% per population doubling at 15°C, although this rate may actually be higher because of decreased viability (data not shown). Over time, however, the *eap1Δ* cells with increased ploidy began to represent a large part of the population.

Eap1p was recently identified as a novel eIF4E-interacting protein that inhibits cap-dependent translation *in vitro*. Because cells are sensitive to Ndc1p levels, it seemed possible that the phenotypes we observed in *ndc1-1* yeast strains that lack *EAP1* may be due to a loss of Eap1p function in translation. However, we did not find Ndc1p levels to be noticeably altered in an *eap1Δ* strain, when monitored by western blot analysis (data not shown). Eap1p is one of four yeast proteins, including eIF4G1, eIF4G2 and p20, that bind eIF4E *in vivo*. Like the mammalian 4E-binding proteins (4E-BPs), these yeast proteins each contain a canonical eIF4E-binding motif [11,17,18]. When this binding motif is mutated in *EAP1* (*eap1-Y109A*), the interaction between Eap1p and eIF4E is disrupted [11]. We used the *eap1-Y109A* allele to more directly address whether the *ndc1-1, eap1Δ* synthetic lethal phenotype or the *eap1Δ* genetic instability phenotypes are linked to Eap1p's function in translation. We transformed an *ndc1-1, eap1Δ* yeast strain and an *eap1Δ* yeast strain with a plasmid containing the *eap1-Y109A* allele [11] to test whether these phenotypes could be rescued by an allele of *EAP1*
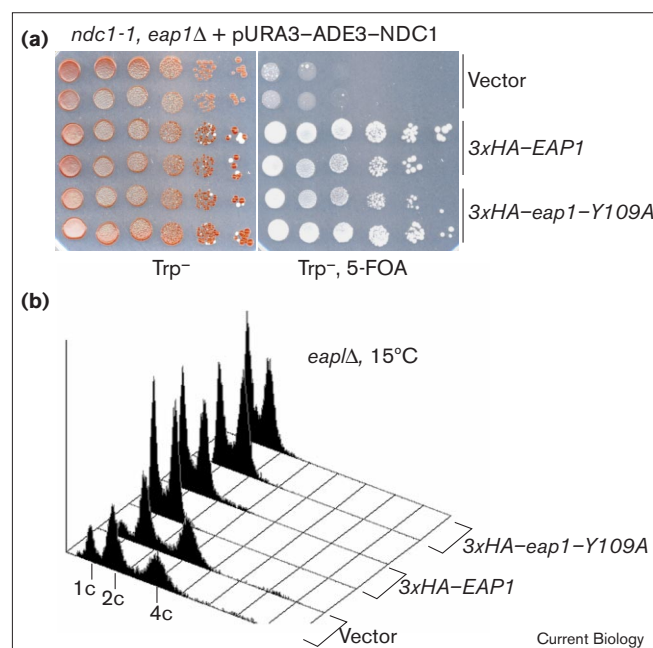
Figure 2
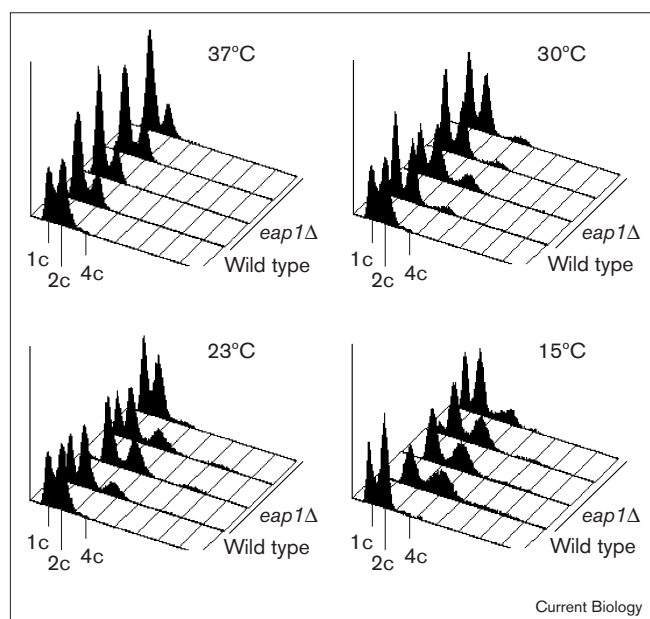
Deletion of *EAP1* leads to genetic instability phenotypes. We deleted the entire *EAP1* ORF in an S288C-derived yeast strain, BY4733 [28], that was transformed with a plasmid containing both the *EAP1* and *URA3* genes (pRS316–EAP1). The resulting *eap1Δ* yeast strain (ASW4) was streaked onto YPD plates at four different temperatures (37°C, 30°C, 23°C, or 15°C) and allowed to grow. The cells were then streaked onto 5-FOA-containing plates at their respective temperatures; this step selected for *eap1Δ* yeast strains that had lost the *EAP1*-containing plasmid. Following growth on the 5-FOA-containing plates, the yeast strains were streaked onto YPD plates at their respective temperatures and allowed to grow. Individual colonies were used to inoculate YPD liquid media and the cultures were grown at their respective temperatures, except the 15°C cells, which were grown at 23°C. Following overnight growth, the cultures were prepared for flow cytometry to examine their DNA contents. The original parent yeast strain (wild type; BY4733) and four separate isolates of the *eap1Δ* yeast strain are shown for each temperature. At temperatures below 37°C, the *eap1Δ* yeast cultures showed diploid DNA contents.

Figure 3

EAP1 carries out an eIF4E-independent function in the maintenance of genetic stability. **(a)** An *ndc1-1, eap1Δ* yeast strain containing a plasmid-borne copy of *NDC1* (HC45-6d) was transformed with a *TRP1*-marked plasmid (pRS314), the *TRP1* plasmid containing the *EAP1* allele *3xHA−EAP1* ([11]; pRS314−3xHA−EAP1), which encodes a hemagglutinin-tagged version of Eap1p, or the *TRP1* plasmid containing the *3×HA−eap1-Y109A* allele [11] (pRS314−3×HA−eap1-Y109A). The plasmid containing *NDC1* also contained the *URA3* and *ADE3* genes, allowing us to use both 5-FOA sensitivity and sectoring phenotypes to assess a requirement for *NDC1*. The resulting transformants were serially diluted 10-fold and plated to either Trp⁻ control plates or to Trp⁻, 5-FOA-containing plates to test for rescue of the synthetic lethal phenotype. The *eap1-Y109A* allele fully rescued the *ndc1-1, eap1Δ* synthetic lethal phenotype, as seen by the ability of the yeast strains to sector (Trp⁻ plate) and grow in the presence of 5-FOA. **(b)** An *eap1Δ* yeast strain (ASW3) containing a *URA3*-marked plasmid carrying *EAP1* (pRS316−EAP1) was transformed with either a *TRP1*-containing plasmid (pRS314), the *TRP1* plasmid containing 3×HA−EAP1 (pRS314−3×HA−EAP1), or the *TRP1* plasmid containing 3×HA−eap1-Y109A (pRS314−3×HA−eap1-Y109A). Two individual transformants for each of the three plasmids were streaked onto 37°C Trp⁻ plates and allowed to grow. They were then streaked onto 15°C, Trp⁻, 5-FOA-containing plates to select for loss of the *EAP1*-containing plasmid. This was followed by a second streaking onto 15°C, Trp⁻ plates. Individual colonies were then used to inoculate Trp⁻ liquid media and grown at 23°C until they reached mid-log phase, followed by flow cytometry to measure their DNA contents. Both the *3×HA−EAP1* allele and the *3×HA−eap1-Y109A* allele rescued the genetic instability phenotypes, whereas the vector alone did not.

that is defective in its interaction with eIF4E. Surprisingly, we found that the *eap1-Y109A* allele fully rescued the *ndc1-1, eap1Δ* synthetic lethal phenotype and the genetic instability phenotypes in *eap1Δ* yeast strains (Figure 3). These findings suggest that the binding of Eap1p to eIF4E is not necessary for its role in maintaining genetic stability.

An increased understanding of the mechanisms by which yeast cells exhibit genetic instability may provide insights into how cancer cells arise (reviewed in [16]). It has been known for some time that transformed cells exhibit genetic instability, and many studies suggest that genetic instability may play a more direct role during cellular transformation [19–22]. The haploinsufficient nature of the *NDC1* locus serves as a model for a tumor suppressor gene in which a single, inactivating mutation can immediately give rise to aneuploid surviving cells that exhibit genetic instability [14]. Recently, some cancer-related genes have been shown to fit this one-hit model for cellular transformation [23–27]. It is of interest that the deletion of *EAP1* causes the *ndc1-1* allele to become haploinsufficient. *EAP1* might serve as a model for a penetrance gene in which

mutations can lead to different phenotypes depending on genetic background.

Although Ndc1p localizes to both SPBs and NPCs, we have not detected any NPC-related defects in *ndc1-1* yeast strains [15], and the recessive nature of the *ndc1-1* allele suggests that it is not a complete loss-of-function allele. The genetic interactions between *NDC1* and *EAP1* are likely to be linked to SPB function. The genetic instability phenotypes in *eap1Δ* yeast strains also suggest a defect in SPB function. Of particular interest is the observation that the interaction between Eap1p and eIF4E is not required for the role of Eap1p in maintaining genetic stability. Further studies will be required to uncover the eIF4E-independent mechanism by which *eap1Δ* yeast strains exhibit increased ploidy.

Supplementary material
Additional methodological detail and figures showing that a single extra copy of *ndc1-1* suppresses the cold-sensitive phenotype in *ndc1-1* haploid yeast strains, and that the *ndc1-1, eap1-1* synthetic lethal phenotype is rescued by a low-copy plasmid containing *ndc1-1*, are available at http://current-biology.com/supmat/supmatin.htm.

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