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Malignant transformation by the eukaryotic translation initiation factor 3 subunit p48 (eIF3e)

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Abstract Several components of translation, e.g. eIF4E and PKR, are implicated in cancer. The e-subunit (p48) of mammalian initiation factor 3 is encoded by the Int6 gene, a common site for integration of the mouse mammary tumor virus genome, leading to the production of a truncated eukaryotic initiation factor-3e (eIF3e). Stable expression of a truncated eIF3e in NIH 3T3 cells causes malignant transformation by four criteria: foci formation; anchorage independent growth; accelerated growth; and lack of contact inhibition. Stable expression of full-length eIF3e does not cause transformation. The truncated eIF3e also inhibits the onset of apoptosis caused by serum starvation.

Key words: Protein synthesis; Translation initiation; eIF3; Int6; Malignant transformation

1. Introduction

Much evidence supports the idea that protein synthesis is involved in the regulation of cell proliferation, and that its dysregulation contributes to the loss of cell cycle control [1]. Mutations in genes that affect translational control may be oncogenic. Translation is also a target of signal transduction pathways; perturbations within these pathways may affect translational components that contribute to the malignant state of the cell. Two well-studied examples are eIF4E and PKR. eIF4E is the m^7GTP cap binding initiation factor in the eIF4F complex. Lazaris-Karatzas and coworkers [2] showed that overexpression of eIF4E in NIH 3T3 cells results in transformation and activation of the *ras* oncogene. Suppression of eIF4E activity in transformed cells by overexpression of the eIF4E binding proteins 4E-BP1 or 4E-BP2 results in partial reversal of the transformed phenotype [3]. Elevated eIF4E levels prevent apoptosis in NIH 3T3 cells subjected to serum starvation [4], whereas overexpression of 4E-BP1 or rapamycin treatment increases the susceptibility to apoptosis [5]. Thus, failure to down-regulate eIF4E may result in loss of growth control. It is noteworthy that overexpression of eIF4E mRNA or protein has been observed in a wide variety of transformed cell lines and solid tumors [6].

Overexpression of a dominant negative mutant form of the eukaryotic initiation factor-2α (eIF2α) kinase, PKR, causes malignant transformation of NIH 3T3 cells [7]. Overexpression of TRBP [8] or p58 [9], inhibitors of PKR, also causes malignancy. This suggests that PKR may act as a tumor suppressor through its down-regulation of protein synthesis by eIF2α phosphorylation. However, the issue is clouded by the fact that PKR has targets outside of translation. The problem was addressed by overexpression of eIF2α and an eIF2α mutant form where its phosphorylation site, Ser-51, was substituted by Ala [10]. Cells transformed with the mutant form became malignant whereas cells overexpressing the wild type factor did not. As with overexpressed eIF4E, the failure to down-regulate translation appears to contribute to dysregulation of cell proliferation. An apparent contradiction to the involvement of eIF2α phosphorylation is the elevated level of PKR found in some cancers [11,12]. Lower levels of PKR in cancer cells would be expected if PKR acts as a tumor suppressor. Also arguing against a role for PKR in cancer is the finding that the PKR double knockout mouse shows no predisposition to cancer [13]. However, in this case, eIF2 phosphorylation may occur through the action of other eIF2 kinases.

Another translation initiation factor implicated in cancer is eIF3. eIF3 is a high molecular weight complex of 11 or more protein subunits that plays an important role in the initiation pathway. The factor helps dissociate 80S ribosomes into ribosomal subunits; it promotes the binding of Met-$tRNA_i$ and mRNA to the 40S subunit; and it binds directly to eIF1 [14], eIF4B [15], eIF4G [16] and eIF5 [17], perhaps playing an organizational role on the surface of the ribosome. The eIF3 a-subunit (p170) is present at high levels in human mammary carcinomas and esophageal squamous cell carcinomas [18]. Furthermore, a yeast strain expressing a mutant form of *TIF32*, encoding the homolog of human eIF3a, is blocked at the G1 phase of the cell cycle [19]. Finally, overexpression of eIF3h (p40) occurs in some human breast and prostate cancers [20].

The eIF3 e-subunit (p48) also has been implicated in the regulation of cell proliferation. eIF3e is encoded by *Int6*, a common site of integration for the mouse mammary tumor virus (MMTV) [21]. Viral genome integration occurs within introns of *Int6*, in opposite translation orientation to *Int6*, resulting in truncation of *Int6* by an in-frame stop codon within the viral long terminal repeat (LTR) [22]. *Int6* is conserved throughout evolution with homologous sequences in *Drosophila*, *Caenorhabditis elegans*, budding and fission yeast, mice, and humans [23]. The eIF3e homolog in *Arabidopsis* has

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Abbreviations: FCS, fetal calf serum; eIF, eukaryotic initiation factor; LOH, loss of heterozygosity

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been shown to interact directly with the eIF3c (p110) homolog and the COP9 signalosome [24]. The eIF3e homolog is not essential for growth in budding [25] nor fission [26] yeast and Δ*int6* strains show only moderate inhibition of protein synthesis and no change in polysome profiles. Yeast eIF3e has been localized to the cytoplasm of cells by both in vitro and in vivo methods [27]. Flag-tagged eIF3e overexpressed in COS-7 cells localizes to the nucleus; however, overexpression of the Tax oncoprotein of the human T-cell leukemia virus causes eIF3e to appear in the cytoplasm [28]. Guo and Sen [29] later showed that the clone used to show nuclear localization lacked the nine amino-terminal amino acids required for export. More specifically, eIF3e has been seen associated with the Golgi apparatus, although no post-translational modification of eIF3e has been observed. The *Int6* gene has been localized to human chromosome region 8q22-q23, a 45 kb region containing 13 exons. *Int6* also has been implicated in human cancers: 28% of primary human breast carcinomas exibit a loss of heterozygosity (LOH) [30]. Another study revealed that 37% of primary human breast tumors and 31% of non-small cell lung carcinomas have reduced Int6 mRNA expression [31]. In addition to eIF3e's interaction with the Tax oncoprotein cited above, eIF3e has been shown to interact with the interferon-induced protein p56 [29]. Interaction of eIF3e with the interferon system strengthens the idea that eIF3e is involved in regulation of translation. Guo and Sen also showed that the interaction of eIF3e with p56 inhibits translation [32].

The findings above suggest that eIF3e is important in the regulation of cell proliferation. The role of the e-subunit in eIF3 and in translation initiation is not known. The consequences of expressing a severely truncated form of eIF3e, such as occurs in the MMTV genome insertions into *Int6*, also have not been described. We therefore have constructed long-term transfected NIH 3T3 cell lines that express either the full-length or truncated forms of eIF3e. We show that expression of truncated eIF3e is sufficient to cause the malignant transformation of NIH 3T3 cells.

2. Materials and methods

2.1. Construction of pMV7:p48 and pMV7:p48T

DNA encoding full-length eIF3e was amplified from pTZp48 [33] by PCR with custom primers to give a full-length product, or a product truncated after exon 4 (amino acid 157). The primers incorporated a 5′ *Eco*RI site and 3′ *Hin*dIII site for cloning into the *Eco*RI and *Hin*dIII sites of pMV7 [34] to yield pMV7:p48 and pMV7:p48T respectively.

2.2. Stable expression of eIF3e and truncated eIF3e

Stable cell lines were produced following the methods of Kirschmeier et al. [34]. Briefly, Ψ2ϕ cells were transfected with *Xho*I-linearized pMV7:p48 and pMV7:p48T using the calcium phosphate method, followed by selection with G418 for 10 days. The virus containing supernatant was used to infect NIH 3T3 cells. Viral-infected cells were grown in G418 selection media until colonies were of suitable size to select (10–14 days). 24 colonies were selected from the pMV7:p48T cells and identified as p48T1-24 and the remainder were pooled and identified as p48TP. The pMV7:p48 cells were pooled and identified as p48P.

2.3. Northern blot analysis

PolyA$^+$ mRNA was isolated following the protocol provided by the Oligotex mRNA Direct kit (Qiagen). RNA (2 μg) was run on a 1.2% denaturing agarose gel and transferred to nitrocellulose. The blot was probed with a ^{32}P-labeled eIF3e probe and a neomycin probe following the protocol provided with the Random Prime kit (LifeTech) and Northern Hybridization Solution (Stratagene).

2.4. Growth rate and foci formation

The long-term transfected cells (1×10^5) were plated on 35 mm plates. Cells were fed every other day with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and were counted every 24 h with a hemocytometer. For foci formation, cells were grown 7 days past confluence and stained with 0.1% methylene blue and photographed.

2.5. Anchorage independent growth

5×10^4 cells in 0.35% agar containing 20% FCS were overlaid onto 0.5% agar in a 35 mm plate. Two days post plating, 2 ml of 20% FCS–DMEM was added. Colonies were photographed 3 weeks after plating.

2.6. Apoptosis assay

Cells were cultured on chamber slides under normal growth conditions until 80% confluent, then cells were cultured for 48 h in DMEM lacking serum. Cells were fixed, permeabilized, and stained with 0.1 μg/ml TRITC-labeled Phalloidin and 2 μg/ml DAPI. Cells were observed with a fluorescent microscope and the appropriate filter sets. Apoptosis is indicated by chromatin condensation, resulting is smaller more intense nuclear staining.

3. Results

NIH 3T3 cell lines stably expressing eIF3e (called p48P) and a truncated version of eIF3e (called p48T) were constructed as described in Section 2. All p48T clones were characterized, and an extensive study is reported for two of the cloned cell lines, p48T1 and p48T2.

Cell lines from pooled clones were examined for the expression of eIF3e (p48P) and truncated eIF3e (p48TP) mRNAs as described in Section 2. Fig. 1 shows the Northern blot of control 3T3 cells along with p48T1, p48T2, p48TP and the full-length p48P probed with a fragment of eIF3e cDNA. In

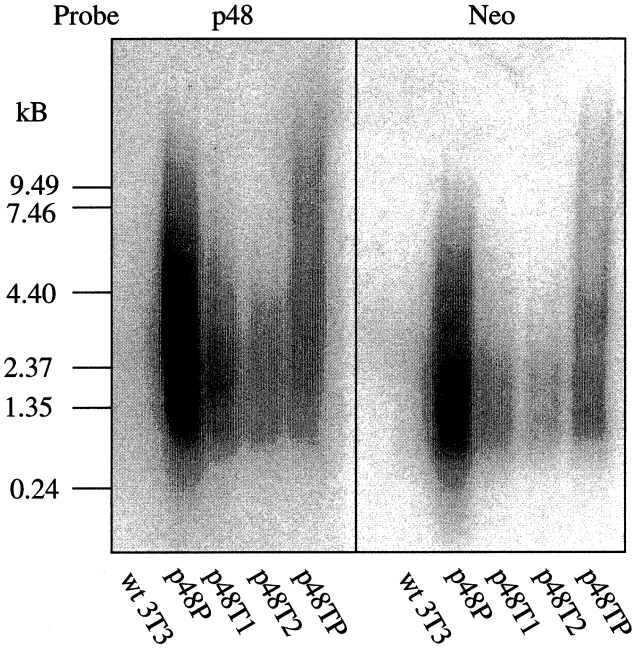

Fig. 1. Northern blot analysis of eIF3e transcripts. Poly(A) RNA from each cell line was prepared and subjected to Northern blot analysis as described in Section 2. The blot was treated with an eIF3e probe and a Neo probe and exposed to an X-ray film. RNA size markers are indicated on the left.

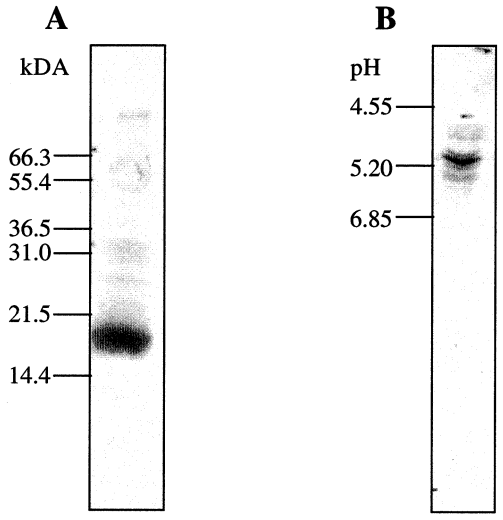

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focusing (IEF) of truncated eIF3e. Truncated eIF3e was expressed with the TNT Kit (Promega) and [^{35}S]methionine following the protocol provided. A: The TNT product was examined by 15% SDS–PAGE and the gel was exposed to X-ray film overnight. Molecular weight markers are indicated on the left. A single major band corresponding to truncated eIF3e is observed on the autoradiogram. B: The TNT product was run on a pH 3.5–10 IEF gel and exposed to X-ray film overnight. IEF markers are identified on the left and the major band corresponding to truncated eIF3e is observed on the autoradiogram.

the wild type 3T3 lanes hybridization of the probes is not observed; however, after longer exposures of the eIF3e-probed membrane, a faint band slightly smaller than the 1.35 kb marker is seen, indicating the endogenous mRNA (data not shown). The cells stably expressing full-length and truncated eIF3e react strongly with both the eIF3e probe and the Neo probe. The larger than expected bands are due to incorporation of the viral LTRs and Neo into the mRNA [34]. Given the high mRNA expression, we attempted to demonstrate enhanced expression levels of full-length and truncated eIF3e proteins. To confirm the integrity of the cDNA, a TNT system (Promega) was employed, resulting in a protein both of the expected size and p*I* (Fig. 2). Western blots of proteins derived from the cell lines showed a band at 48 kDa for all cell lines as expected for the endogenous eIF3e (data not shown). However, a band representing the truncated protein (19 kDa) was not seen with the various p48T cell lines (note: during preparation of this manuscript Rasmussen et al. [35] also failed to observe a truncated product in stable cells expressing Int6-short). A second attempt was made to identify the truncated protein based on its relatively acidic p*I* (4.98), but again, no band was observed. Apparently, the truncated form is unstable and does not accumulate at any detectable level in the cells. Alternatively, any amino-terminal modifications would make the protein undetectable to the antibody, due to the fact that the antibody was raised against a peptide representing the 19 amino-terminal residues of eIF3e.

The phenotypic characteristics of the long-term transfected cells were examined. Wild type NIH 3T3 cells exhibited a doubling time of 28.2 h (Table 1), similar to data previously reported [2]. Wild type cells showed normal contact inhibition, no foci formation (Fig. 3A), normal morphology (Fig. 3B), and no growth in soft agar (Fig. 3C). Wild type cells were also not able to inhibit apoptosis, with 63% of cells being apoptotic after 48 h on reduced serum (Fig. 4). The p48P cell line had a prolonged doubling time of 33.6 h, reduced confluency (50%) compared to wild type, no foci formation, normal morphology, and no growth in soft agar. The p48P cell line was not resistant to apoptosis, with 58% of cells being apoptotic in the medium deprived of serum. The p48T clones all exhibited decreased doubling times (21.9, 22.7, and 21.3 h), as well as increased saturation densities (178%, 168%, and 209%) compared to wild type cells for p48T1, p48T2 and p48TP respectively. The three p48T cell lines formed foci on plates, exhibited aberrant morphologies, and were able to form colonies in soft agar. The ability to grow in soft agar often correlates with tumorigenicity in mice. The p48TP cells were resistant to apoptosis, with only 16% of cells being apoptotic after 48 h with reduced serum.

4. Discussion

The data above indicate that truncation of eIF3e causes cell malignancy, but the mechanism whereby this form of eIF3e influences cell proliferation is not obvious. Two general possibilities can be identified: (1) eIF3e functions exclusively in the eIF3 complex as a component of translation; and (2) eIF3e has one or more additional functions independent from eIF3. Considering the first possibility, the involvement of the translational machinery is consistent with the well-described oncogenic properties of several other initiation factors, most notably eIF4E. In the case of eIF3, the yeast eIF3 is comprised of five essential core proteins homologous to mammalian eIF3a (p170), eIF3b (p116), eIF3c (p110), eIF3g (p44), and eIF3i (p36). This yeast core possesses the major activities attributed to eIF3 [36]. Several other mammalian subunits, including eIF3e and eIF3j [37], have homologous yeast proteins which are not essential, yet affect initiation whenever inactivated or deleted. The five core subunits may serve as a scaffold for docking of other regulatory proteins. Since failure to down-regulate translation corresponds with malignancy, one can hypothesize that the normal function of eIF3e may

Table 1
Growth properties of cell lines

Cell line	Doubling Time (h)	Standard deviation	Maximum density ($\times 10^4$ cells)
Wild type 3T3	28.2	2.1	335
p48P	33.6	0.5	166
p48TP	21.3	1.2	700
p48T1	21.9	2.2	595
p48T2	22.7	0.3	565

Cell lines were grown on 35 mm plates and fed every other day with DMEM containing 10% FCS. Cells were trypsinized and counted daily with a hemocytometer. Data are from at least three experiments done in duplicate. Maximum density is the number of cells observed after no further growth has occurred for 3 days.

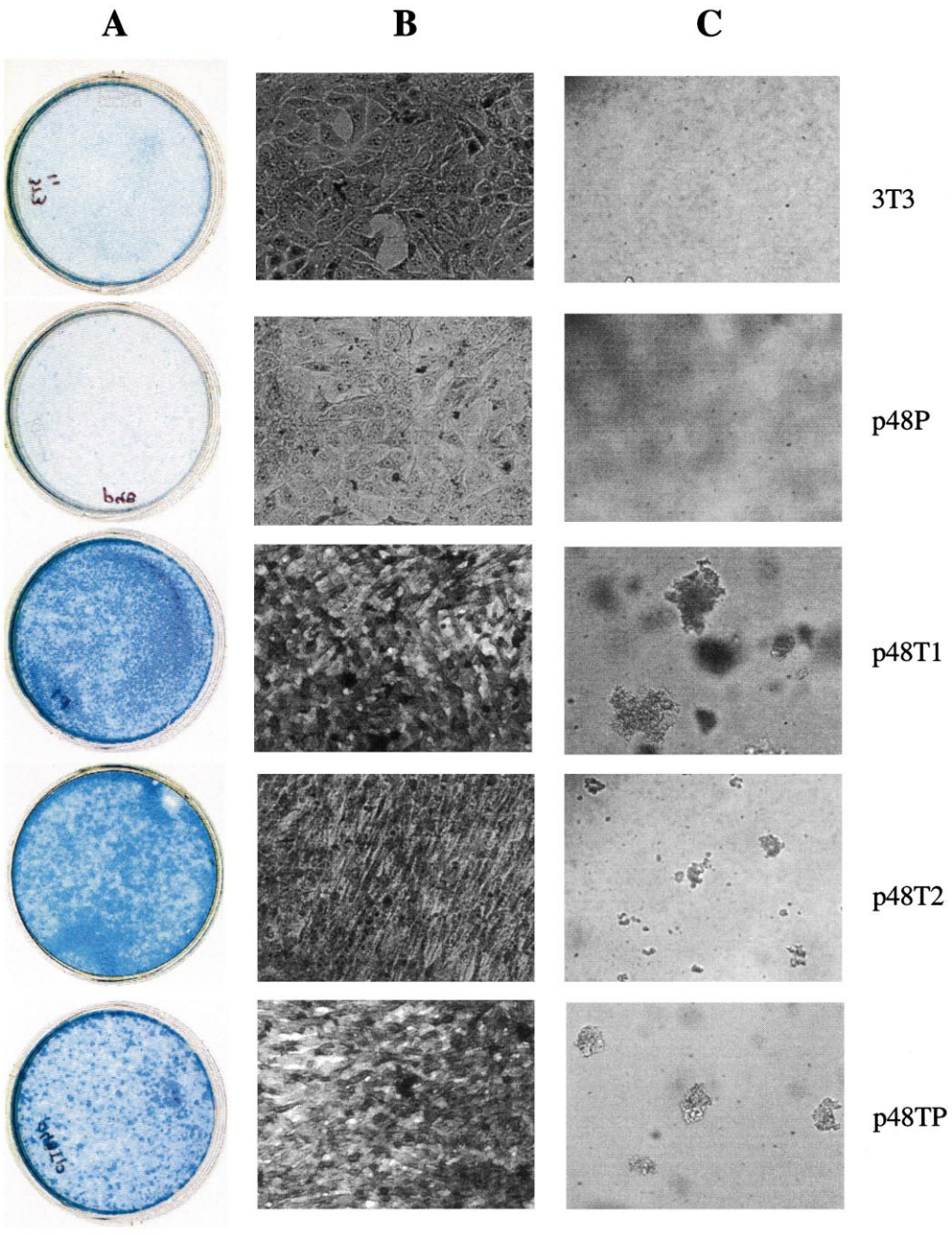

Fig. 3. Phenotypes of cell lines. Cells were grown to confluency and maintained for 1 week with the addition of fresh media every other day. Cells were fixed in 10% formaldehyde and stained with 0.1% methylene blue. B: Magnified view of the same cells in column A, exhibiting the aberrant growth of cells expressing truncated eIF3e. C: Cells were grown in soft agar as described in Section 2. Colonies were photographed after 3 weeks.

be to down-regulate eIF3 activity. This view is supported by the observations that cells overexpressing eIF3e results in a slower growth rate and the finding that LOH of the eIF3e (*int6*) locus occurs is numerous cancer cells. This assumes that the LOH of eIF3e is the critical factor in the transformation (other genes may be affected) and that LOH results in decreased protein expression (only mRNA levels have been examined). It is likely that the truncated form of eIF3e exhibits a loss of function due to the fact that about two-thirds of the protein is missing. This inactive truncated mutant may displace active eIF3e from the eIF3 complex, eliminating the down-regulatory effects of intact eIF3e. However, immunoprecipitation of eIF3a from lysates of cells expressing the truncated eIF3e showed normal amounts of full-length eIF3e in the eIF3 complex (data not shown). If full-length eIF3e remains bound to eIF3 it should maintain its ability to interact with p56, a protein whose expression is induced by interferon, and that functions to inhibit protein synthesis. The down-regulation of protein synthesis due to the interaction of eIF3e with p56 does not result from the removal of eIF3e from the eIF3 complex [29]. eIF3e may simply act as a docking site for p56, rather than directly regulating protein synthesis via its interaction with p56. The free truncated eIF3e may interfere with this interaction, preventing down-regula-

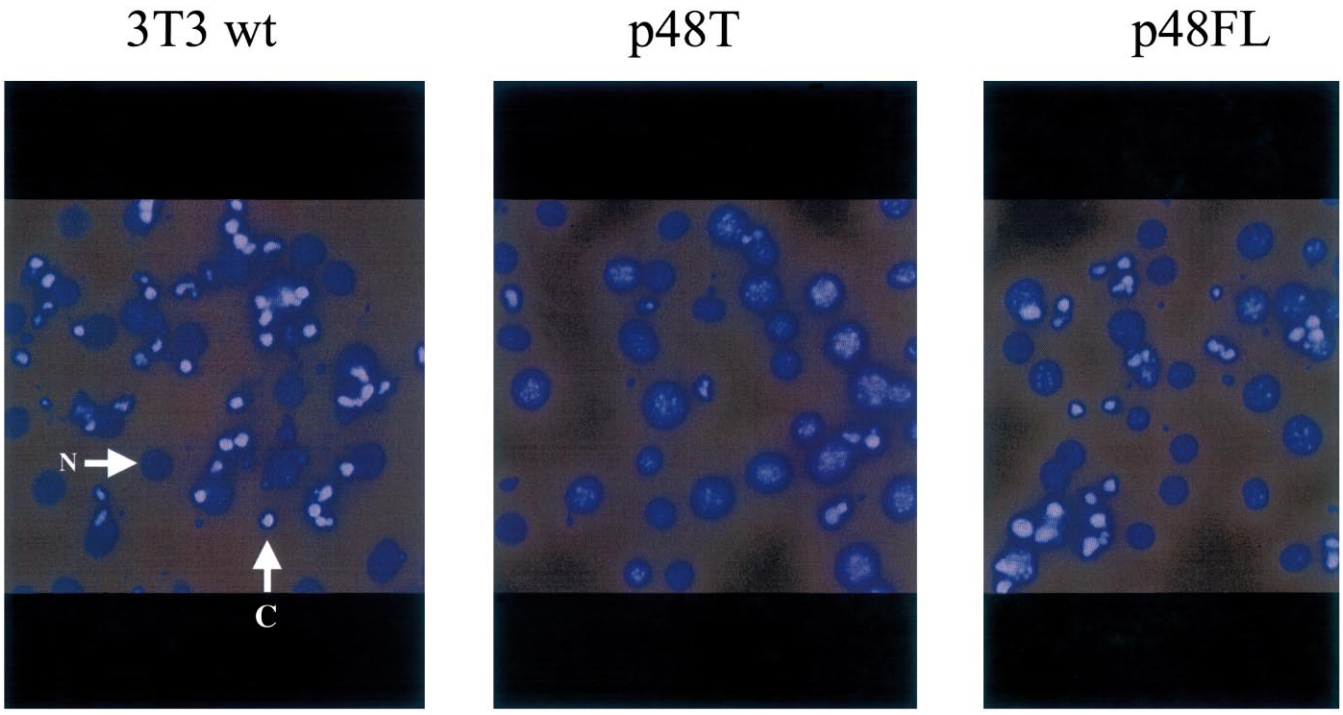

Fig. 4. DAPI staining of cell lines to visualize apoptotic cells. Control NIH 3T3 cells (3T3 wt), stable pooled clones expressing the full-length eIF3e (p48P) and the truncated eIF3e (p48TP) were deprived of serum for 48 h, stained with DAPI and visualized using a fluorescent microscope as described in Section 2. Apoptosis is indicated by the presence of condensed chromatin (C), and normal cells are indicated by diffuse chromatin (N).

tion of translation. This possibility is amenable to experimental verification.

In fission yeast, cells lacking eIF3e (ΔInt6) have a slow growth phenotype in minimal medium that was corrected by expression of full-length human or yeast eIF3e or a C-terminal fragment (which would be lacking in the p48T cell line), but was not corrected by an N-terminal fragment corresponding to the predicted MMTV disrupted products. The loss-of-function hypothesis could explain the lack of correction by the N-terminal fragment, but the slow growth phenotype of ΔInt6 indicates a positive regulatory role for eIF3e. The slow growth phenotype may be a result of its function outside eIF3 complex (see below). Bandyopadhyay et al. [26] showed that the effect of ΔInt6 on polysome profiles is minimal, indicating that the negative growth effect of ΔInt6 is not due to a global decrease in protein synthesis.

Examining the function of eIF3e outside the eIF3 complex, we note that eIF3e interacts with the COP9 complex independent of its interaction with eIF3 [38]. The COP9 signalosome, a protein complex highly conserved between plants and animals, may be involved in regulating protein stability via the ubiquitin-modulated degradation pathway and may be in the c-Jun signaling pathway (reviewed in [39]). The potential activity of truncated eIF3e in the COP9 complex has not been addressed experimentally, although one can imagine many mechanisms involving protein stability and signaling. Either a loss of function or a dominant negative activity caused by eIF3e truncation could affect these pathways, resulting in loss of growth control. Further research into the function of eIF3e and its interactions with other protein complexes in the cell is required for a full understanding of its oncogenic properties.

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