ROLE OF EIF3A EXPRESSION IN CELLULAR SENSITIVITY TO IONIZING RADIATION TREATMENTS BY REGULATING SYNTHESIS OF NHEJ REPAIR PROTEINS

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Dedication

This thesis is dedicated to my lovely princess

Lugin Hesham Elshabi
Acknowledgment

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Translation Initiation in protein synthesis is a crucial step controlling gene expression that enhanced by eukaryotic translation initiation factors (eIFs). eIF3a, the largest subunit of eIF3 complexes, has been shown to regulate protein synthesis and cellular response to cisplatin treatment. Its expression has also been shown to negatively associate with prognosis. In this study, we tested a hypothesis that eIF3a regulates synthesis of proteins important for repair of double strand DNA breaks induced by ionizing radiation (IR). We found that eIF3a up-regulation sensitizes cellular response to IR while its knockdown causes resistance to IR. We also found that eIF3a over-expression increases IR-induced DNA damage and decreases Non-Homologous End Joining (NHEJ) activity by suppressing expression level of NHEJ repair proteins such as DNA-PKcs and vice versa. Together, we conclude that eIF3a plays an important role in cellular response to DNA-damaging treatments by regulating synthesis of DNA repair proteins and, thus, eIF3a likely plays an important role in the outcome of cancer patients treated with DNA-damaging strategies including ionizing radiation.

Ahmed Safa, PhD, Chair
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Introduction

Eukaryotic initiation factors (eIFs) are a family of proteins that play important roles in mRNA translation and protein synthesis. Recent growing evidence suggest that eIFs do not just participate in translation initiation of global mRNAs but may also regulate synthesis of a subset of proteins [1, 2]. These regulatory functions have been thought to contribute to the potential oncogenic role of eIFs. Indeed, many eIFs were found to have increased expression in human tumors and have been shown to have oncogenic activity. One of these eIFs, eIF3a, has been found to overexpress in many human cancers including cancers of breast [3], cervix [4], esophagus [5], stomach [6], lung [7], bladder [8] and it was thought to be a proto-oncogene. Indeed, knocking down eIF3a reversed the malignant phenotype of human cancer cells [9] while over-expressing ectopic eIF3a transformed NIH3T3 fibroblast cells [10] in vitro. Most recently, it was found that over-expressing ectopic eIF3a transformed normal intestinal epithelial cells, which developed into tumors in vivo [11].
Figure 1. Overview of translation initiation in eukaryotic. eIF3 has multiple functions in translation initiation starting from the dissociation of the 60s and 40s subunits from each other. eIF3a promotes the separation of the two subunits by binding of the eIF3a to the 40s subunit and participating in the recruitment of eIF1A and the ternary complex to 40s subunits to form the 43s preinitiation complex (PIC). It is also required for the mRNA recruitment to PIC for scanning of the start codon AUG. 60s subunit will bind again to the 40s subunit to form the 80s initiation complex.
Interestingly, it has also been observed that eIF3a expression associates with clinical outcome and prognosis of esophageal, cervical, lung, bladder, ovarian, and oral cancer patients [4, 5, 8, 12-14]. While patients with lower eIF3a level had poor prognosis, patients with higher eIF3a level had better prognosis. Indeed, eIF3a knockdown resulted in cellular resistance to cisplatin, a common constituent in chemotherapeutic regimens for treating these cancers possibly by regulating nucleotide excision repair (NER) via increasing the synthesis of NER proteins [15]. It has also been shown that eIF3a up-regulation increases cellular sensitivity to anticancer drug doxorubicin, which causes DNA double strand breaks (DSBs) by inhibiting topoisomerase II [14].

Extensive DSBs induced by various exogenous and endogenous factors are one of the most fatal forms of DNA damages [16, 17] and are taken advantage of for treating human cancers in the form of chemo and radiation therapy. However, cells with efficient repair of DSBs are able to survive these treatments that cause DSBs using two two major mechanisms of repair of DSBs, homologous recombination (HR) and nonhomologous end-joining (NHEJ) [18, 19]. While HR repairs the damages using undamaged and symmetrical chromosome as a template during S or G phase of cell cycle [16], [20], NHEJ repairs DSBs throughout all cell cycle phases and is the pathway in repairing ionizing radiation (IR)-induced DSBs [21-23]. The major proteins important in NHEJ repair of DSBs include Ku (Ku70, Ku80) and DNA-PKcs to form the DNA-PK holoenzyme [24-27]. Because IR is a common and an important strategy for treating many types of human cancers [28-30], it is of interest to investigate if eIF3a possibly contributes repair of DSBs by regulating synthesis of proteins important for these repairs and, ultimately, cellular response to IR treatments that cause DSBs. Here, we found that eIF3a plays an important role in cellular
response to IR treatments by regulating NHEJ repair via inhibiting synthesis of NHEJ repair proteins including Ku70, K80, and DNA-PKcs. All of the observation apparently showed that changing the level of eIF3a has an additional non canonical function in suppressing translation of some crucial repair proteins.
Figure 2. Patterns of eIF3a in regulating the cellular response to ionizing radiation treatments. eIF3a regulates NHEJ repair activity that repairs the DNA double strand break induced by ionizing radiation. Changing the level of eIF3a enhance or suppress the cellular sensitivity to IR treatments by regulating the DNA repair pathways.
Materials and Methods

A. Materials.

Cell culture mediums and reagents (DMEM, fetal bovine serum, and trypsin-verse mixture) were purchased from BioSources International (Camarillo, CA), Media Tech (Herndon, CA), or Cambrex (Walkersvill, MD). All electrophoresis reagents, polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad (Hercules, CA). SYBR Green PCR master Mix for real-time PCR was purchased from Applied Biosystems by life technologies (Warrington, UK) and the high capacity cDNA reverse transcription kit from Applied Biosystems (Foster City, CA). All primers was used from Invitrogen. Metafectene® pro was purchased from Biontex (San Diego, CA). All other chemicals and reagents were of molecular biology grade from Sigma (St. Louis, MO) or Fisher Scientific (Chicago, IL).

B. Cell culture.

Human lung cancer cell line H1299 and rat intestinal epithelium cell line RIE were cultured at 37°C with 5% CO2 in RPMI 1640 and DMEM medium respectively, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 400 μg/ml G418.
C. Survival assay.

Colony formation survival assay was performed following IR treatments as previously described [31]. Briefly, 100 cells/well were seeded in 6-well plates and using of 2ml DMEM for RIE cells and PRMI for H1299 cultured for 24 hrs followed by treatments with different doses of IR and continuous culture for 10-14 days with media changed every 2-3 days. At the end of the study, cells were washed with PBS and the colonies were stained with 0.05% (w/v) crystal violet in phosphate-buffered saline (PBS) containing 20% methanol for 15 minutes at room temperature and counted manually.

D. Western blot analysis.

Western blot analysis to measure the amount of protein expression western blot analysis was performed using 2% SDS –PAGE as previously described [32, 33]. Briefly, 24 hours after seeding the cells the old media was removed and the cells were washed with cold PBS and collected in small Eppendorf tubes after scraping them by scraper to remove all of the cells from the plate. To perform the protein concentration measurement the cells were span down for 15 seconds at 12600 RPM and lysed with lysis buffer TNN- sodium dodecyl sulfate (SDS) buffer (50 mM Tris-HCl (ph 7.5), 150 mM NaCl, 0.5 % NP-40, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO3, with 1 mM DTT and 10 mM PMSF) incubated for 30 minutes on ice followed by sonication and centrifugation at 4 °C to collect the cells supernatant and discard the pellets. The protein concentration of cell lysates was determined using BioRad protein assay kit. For Western blot analysis, equal amount of proteins from different cells were separated by SDS-PAGE, transferred to PVDF membranes, and probed with antibodies specific to eIF3a, γ-H2AX, Ku70, Ku80, DNA-
PKcs, and actin control followed by probing with HRP-conjugated secondary antibody and ECL. The signals are captured on x-ray films.

E. Immunofluorescence Imaging.

Immunofluorescence staining was performed by culturing RIE and H1299 cells on glass coverslips, which were washed twice with cold PBS and fixed with an acetone/methanol mixture (1:1) for 10 min and blocked at 4 °C for 30 min with a blocking solution (1% BSA and 1% normal horse serum in PBS). The coverslips were then probed with the primary antibody γ-H2AX-specific antibody for 30 min at 4°C followed by incubation with FITC-conjugated secondary antibody goat anti-mouse IgG at room temperature for 30 minutes and washed twice with PBS. The cells were counter stained with DAPI before viewing on a confocal microscope.

F. Real-time RT-PCR.

Real-time reverse transcription (RT)-PCR was performed as described previously [32]. Briefly, total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) and real-time RT-PCR were performed using Power SYBR Green RNA-to-CT 1-Step kit (Applied Biosystems) Data were normalized to an internal control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer pairs used were; 5'-CATGGCAACTCCAGAGCAG (forward) and GCTCCTTTAAACTCATCCACC (reverse) for Ku70. AGAAGAAGGCCAGCTTTGAG (forward) and AGCTGTGACAGAACTTCCAG (reverse) for Ku80.
CCGGACGGACCTACTACGACT (forward) and AGAACGACCTGGGCATCCT (reverse) for DNA-PKcs.

G. Comet assay.

Comet assay was performed by embedding cells in low melting agarose (Trevigen) on a microscope slide which allowed to set for 1 hour at 4°C followed by incubation the slides in a pre-chilled lysis neutral pH solution (Trevigen, 4250-050-01) at 4°C for 30 min in the dark, then electrophoresis in TBE buffer for 2 hours using a Comet assay kit (Trevigen, Gaithersburg, MD) instructed by the manufacturer. To measure the DNA Double-strand break (DSB) damage following IR treatments, comets tails were observed by Zeiss Axiovert 25 fluorescent microscope equipped with a camera, and analyzed with CometScore V1.5 (TriTek, Sumerduck, VA) after staining the cells with SYBR Green I and scoring 120 cells in each sample to measure the olive tail moment.

H. Host cell reactivation-based NHEJ assay.

For host cell reactivation NHEJ assay, pGL3 reporter plasmid with firefly luciferase gene driven by CMV promoter was used, first by linearization of the plasmid using HindIII digestion. The TK-Renilla luciferase plasmid was used as a control and co-transfected with the linearized pGL3 plasmid followed by determination of both firefly and Renilla luciferase activity. Expression of firefly luciferase is dependent on repair of the plasmid to re-generate the circular plasmid via NHEJ.
I. Protein stability assay.

Cells were treated with 10µ/ml Cycloheximide in a time course experiment and harvested at several time points followed by collection of cells for western blotting analysis. Whole-cell lysates were prepared as described previously [34]. Scanned images were quantified using ImageJ (NIH, USA) software.

J. Pulse labeling and Immunoprecipitation.

Pulse labeling and pulse chase experiments were performed as previously described [9, 32]. Briefly, cells were washed with PBS twice, methionine-free media, and the pulse labeled with [35S] methionine (10 µCi/µl) in methionine-free media for 2 hours. The cells were then washed with PBS three times, collected for cell lysate preparation and immunoprecipitation.

Immunoprecipitation was performed as previously described [32]. Briefly, the cell lysates were first mixed with mouse IgG and incubated for 2 hrs at 4°C followed by addition of 50% protein G-agarose slurry and incubation for 3 hrs at 4°C to remove nonspecifically-bound proteins by centrifugation. The supernatant was transferred to a new tube and incubated with 5µg of primary antibodies against Ku70, Ku80 and DNA-PKcs at 4°C for 2 hr. Finally, 50µl of 50% protein G-agarose beads was added to the mixtures, incubated at 4°C overnight and then washed with lysis buffer 5 times and separated with SDS-PAGE.
Results

A. Role of eIF3a in cellular response to IR treatment.

To determine the potential role of eIF3a in cellular response to IR, we first knocked down eIF3a expression using siRNA in H1299 cells, which have high level of endogenous eIF3a (Fig. 3A) followed by analysis of cellular response to IR using colony formation assay. As shown in Fig. 3A and 3C, H1299 cells with eIF3a knockdown (H1299/Si) are significantly more resistant with a 2-fold increase in relative resistance factor (RRF) than the control H1299 cells transfected with scrambled siRNA (H1299/Scr). To confirm this observation, we performed a reverse experiment by taking advantage of the previously established stable RIE cells with eIF3a over-expression (RIE/eIF3a) (see Fig. 1C) [11] and tested its response to IR in comparison with control cells transfected with empty vector (RIE/Vec). As shown in Fig. 3B and 3C, RIE/eIF3a cells with eIF3a over-expression are remarkably more sensitive than the control RIE/Vec cells to IR with a 2-fold reduction in RRF. Thus, eIF3a expression may affect cellular response to IR treatments.
Figure 3. Effect of eIF3a expression on cellular response to IR. H1299 with transient eIF3a knockdown (A) and RIE cells with stable eIF3a over-expression (B) as well as their respective controls were subjected to Western blot analysis of eIF3a expression and colony formation assay following treatment with IR. Panel C shows summary of eIF3a effect on cellular sensitivity to IR treatments. Relative resistance factor (RRF) was derived by dividing the IC50 of the test cells by that of their control cells. (n=3, *p<0.05, **p<0.01).
B. Effect of eIF3a on $\gamma$H2AX recruitment induced by IR.

To investigate how eIF3a affect cellular response to IR, we tested the hypothesis that eIF3a may regulate repair of DSB induced by IR. For this purpose, we first tested the effect of eIF3a on $\gamma$-H2AX expression, a marker for DSB [35], following IR treatment. As shown in Fig. 4A, $\gamma$-H2AX level was detected in either H1299/Si or the control H1299/Scr cells. However, $\gamma$-H2AX expression drastically increased following IR treatment in these cells at 20 min after IR treatment. Interestingly, at 6 hours after IR, $\gamma$-H2AX in H1299/Si cells returned essentially to the basal level while remained at high level in the control H1299/Scr cells. This finding suggests that eIF3a expression may suppress repair of DSB induced by IR. We also performed similar experiments using RIE/eIF3a and RIE/Vec cells and found that eIF3a over-expression clearly delays DNA repair as indicated by the delayed disappearance of $\gamma$-H2AX (Fig. 4A), consistent with the findings using H1299/Si and H1299/Scr cells.

To verify above findings, immunofluorescence staining of $\gamma$-H2AX in the nuclei of these cells was detected at 2 and 6 hours after IR exposure. As shown in Fig. 4B, the punctate staining of $\gamma$-H2AX in the nuclei of H1299/Si cells disappeared at 6 hrs following IR while maintaining a high level in the control H1299/Scr cells. RIE/eIF3a cells retains high level of $\gamma$-H2AX whereas the control RIE/Vec cells lost $\gamma$-H2AX staining at 6 hrs following IR. These observations are consistent with the results shown using Western blot analysis. Thus, it is possible that eIF3a expression may inhibit repair of DSB induced by IR.
**Figure 4.** Effect of eIF3a on IR-induced γ-H2AX. Western blot (A) and immunofluorescence staining (B) analyses of γ-H2AX in H1299 cells with transient eIF3a knockdown and RIE cells with stable eIF3a over-expression as well as their respective controls following IR treatments.
C. Effect of eIF3a on DNA damage induced by ionizing radiation (IR).

In above studies, we used a γ-H2AX, a DNA damage marker, to evaluate DNA damage and repair. To directly evaluate DNA damages induced by IR in the presence of different levels of eIF3a, we performed neutral comet assay. As shown in Fig. 5A, H1299/Si cells clearly have significantly lower Olive tail moment than the control H1299/Scr cells at both 2 hrs and 6 hrs following IR treatment. Consistently, RIE/eIF3a cells have significantly higher Oliver tail moment than the control RIE/Vec cells (Fig. 5B) following IR treatment. Thus, eIF3a expression likely inhibit repair of DSB induced by IR and cells with high levels of eIF3a will retain higher levels of DSBs following IR while cells with lower eIF3a retains lower level of DSBs possibly due to activated repair of DSBs in the absence of eIF3a.
Figure 5. Comet Assay. The effect of eIF3a on the level of DSBs induced by IR were tested in H1299 cells with transient eIF3a knockdown (A) and RIE cells with stable eIF3a over-expression (B) as well as their respective control cells. The histograms show the summary of quantitative analysis of Olive tail moment in these cells. (n=3; *p<0.05, **p<0.01).
D. Role of eIF3a in NHEJ repair of DSB.

To determine if eIF3a regulates repairs of DSBs, we next performed host cell reactivation (HCR) assay of NHEJ activity since NHEJ is the main repair pathway of IR-induced DNA damages and it is independent on cell cycle stages as discussed in Introduction. As shown in Fig. 6A, eIF3a knockdown significantly increased the NHEJ activity in H1299 cells by ~2 fold compared with the control H1299/Scr cells. Consistently, eIF3a over-expression significantly reduced the NHEJ activity in RIE cells by ~70% compared with the control RIE/Veg cells (Fig. 6B). These finding suggest that eIF3a expression may play an important role in suppressing NHEJ repair of DSBs.
Figure 6. Role of eIF3a in NHEJ repair of IR-induced DSB. Host cell reactivity assay using reporter constructs were performed using H1299 cells with eIF3a knockdown (A) and RIE cells with stable eIF3a over-expression (B) as well as their respective control cells.
E. eIF3a regulates synthesis of NHEJ repair proteins.

Because eIF3a is known to regulate synthesis of proteins, we hypothesized that eIF3a may regulate NHEJ repair of DSBs by regulating synthesis of NHEJ repair proteins. To test this hypothesis, we first performed a Western blot analysis of major proteins involved in NHEJ in H21299/Si, H1299/Scr, RIE/eIF3a, and RIE/Vec cells. As shown in Fig. 7A-B, eIF3a knockdown in H1299 cells drastically increased the expression of DNA-PKcs, Ku70, and Ku80 proteins compared with the control H1299/Scr cells while eIF3a over-expression in RIE cells drastically reduced the expression of these proteins compared with the RIE/Vec control cells. Interestingly, real time RT-PCR analyses showed no change in the mRNA level of these genes, suggesting that the effect of eIF3a on the expression of DNA-PKcs, Ku70, and Ku80 is likely at the protein not mRNA level.
Figure 7. Effect of eIF3a on expression genes encoding proteins important for NHEJ repair. Western blot and real-time RT-PCR analyses were performed to determine the effect of eIF3a on the expression of Ku70, Ku80, and DNA-PKcs genes in H1299 cells with transient knockdown (A) and RIE cells with stable eIF3a over-expression (B) as well as their respective control cells.
F. Effect of eIF3a on the synthesis rate and half-life of NHEJ proteins.

To eliminate the possibility that eIF3a may influence the stability and the half-life of these NHEJ proteins, we next performed pulse labeling and protein stability assay to determine the eIF3a effect on synthesis and degradation rate of these DNA repair proteins, respectively. As shown in Fig. 8, eIF3a knockdown in H1299 cells using siRNA or eIF3a over-expression in RIE cells had no effects on the half degradation of these DNA repair proteins. However, the synthesis of Ku70, Ku80, and DNA-PKcs was dramatically increased by eIF3a knockdown in H1299 (Fig. 8A) and reduced by eIF3a over-expression in RIE cells (Fig. 8B). Thus, eIF3a likely inhibits synthesis of Ku70, Ku80, and DNA-PKcs proteins, leading to reduced repair of DSB by NHEJ pathway.
Figure 8. Effect of eIF3a on the synthesis and degradation of Ku70, Ku80, and DNA-PKcs. Pulse labeling and pulse-chase in combination with immunoprecipitation were performed to determine the synthesis (insets) and degradation, respectively, of Ku70, Ku80, and DNA-PKcs in H1299 cells with transient eIF3a knockdown (A) and RIE cells with stable eIF3a over-expression (B) as well as their respective control cells.
Conclusions and Discussion

Appropriate combinations of radiation with chemotherapeutic drugs have resulted in remarkable outcome in cancer treatments. However, resistance to both radiation and anticancer drugs frequently occurs, causing failure in successful treatment or cure of human cancers. In this study, using cell line models we show that eIF3a may play important roles in cellular response to radiation by regulating NHEJ repair of DSB induced by radiation due to eIF3a suppression of synthesis of NHEJ proteins, Ku70, Ku80, and DNA-PKcs. These findings are consistent with previous observations that lower eIF3a expression associates with poor prognosis of several cancers [3-8].

It is noteworthy that the difference in γ-H2AX protein level between cells with different eIF3a levels was observed very early (20 min) following IR treatments. Although eIF3a may protect cells from IR-induced DNA damage, it is more likely that the DSBs induced by IR can be much more quickly repaired in cells with low eIF3a level than cells with high eIF3a level considering that the basal level of DNA repair proteins are very different between these cells under eIF3a regulation.

Together with previous findings [14, 15, 36], it is now clear that eIF3a likely plays an important role in regulating synthesis of DNA repair proteins. The fact that eIF3a does not contribute to cellular response to non-DNA-damaging drugs such as vinca alkaloid [14], suggests that eIF3a regulation of cellular response to DNA-damaging treatments is not an unspecific event in cellular response to anticancer drugs. Whether eIF3a also regulates the synthesis of proteins important for other DNA repair pathways such as homologous recombination for repair of DSBs remains to be determined. Nevertheless, the
findings that eIF3a may suppress the synthesis of DNA repair proteins and contribute to the increased sensitivity of cancer cells to DNA-damaging treatments suggest that eIF3a may be developed as a biomarker for precision medicine prescription. Patients with high level of eIF3a may benefit from DNA-damaging drugs and radiation whereas such treatments may not benefit as much for the patients with low level of eIF3a.

In addition to the regulation in synthesis of DNA repair proteins, eIF3a has also been observed to possibly regulate the synthesis of p27, ribonucleotide reductase, and tubulin [9, 32]. In most of these cases, it was found that eIF3a suppresses the synthesis of these proteins. These findings are against the intuition and belief that eIF3a facilitates translational initiation as a subunit of eIF3 complex and its increased expression would increase protein synthesis. The fact that eIF3a suppresses the synthesis of some proteins while increase the synthesis of other proteins suggests that eIF3a may have an additional regulatory non-canonical function. How eIF3a regulates protein synthesis with its non-canonical activity remains unknown. However, the previous finding that eIF3a can bind to the 5’-UTRs of RPA2 mRNA suggests that eIF3a may bind to these mRNAs and suppress the translation of these mRNAs. It also remains to be determined whether this non-canonical activity requires other eIF3 subunits such as eIF3b. Recently, eIF3i has also been shown to be able to regulate the synthesis of COX2 suggesting that other eIF3 subunits may also have non-canonical activity although it is not clear if they work together. Clearly, more works are needed to investigate further the non-canonical function of eIF3 subunits in regulating protein synthesis.
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Curriculum Vitae

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**Education**

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**Training Experience**

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Rima Tumia, Zizheng Dong, Jian-Ting Zhang. Role of eIF3a in Cellular Sensitivity to Ionizing Radiation. Poster Presentation. 10th annual meeting of Great Lakes Drug Metabolism and Disposition Group Meeting. May 7 -8, 2015 Ann Arbor, Michigan United States.
Attended the 9th annual meeting of Great Lakes Drug Metabolism and Disposition Group Meeting. May 2014 Indianapolis, IN.
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Participation in CSL Behring Vaccine's day 10-11 Feb 2011 Damascus –Syria.
Participation in the 4th Medical Drug Fair 2004 Tripoli.
Participation in the National Day of Medical Herbs 23-24 Apr 2004

**Honors and Awards**

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