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G2E3 IS A NUCLEO-CYTOPLASMIC SHUTTLING PROTEIN WITH DNA DAMAGE RESPONSIVE LOCALIZATION

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Abstract

G2E3 was originally described as a G2/M-specific gene with DNA damage responsive expression. The presence of a conserved HECT domain within the carboxy-terminus of the protein indicated that it likely functions as an ubiquitin ligase or E3. Although HECT domains are known to function in this capacity for many proteins, we demonstrate that a portion of the HECT domain from G2E3 plays an important role in the dynamic subcellular localization of the protein. We have shown that G2E3 is a nucleo-cytoplasmic shuttling protein with nuclear export mediated by a novel nuclear export domain that functions independently of CRM1. In full-length G2E3, a separate region of the HECT domain suppresses the function of the NES. Additionally, G2E3 contains a nucleolar localization signal (NoLS) in its amino terminus. Localization of G2E3 to the nucleolus is a dynamic process, and the protein delocalizes from the nucleolus rapidly after DNA damage. Cell cycle phase-specific expression and highly regulated subcellular localization of G2E3 suggest a possible role in cell cycle regulation and the cellular response to DNA damage.

Keywords

nucleolus; DNA damage; ubiquitin ligase; HECT; CRM1; leptomycin B; nuclear export

INTRODUCTION

Cell cycle phase-specific expression of genes is a principal mechanism controlling cell division. In previous work, we identified numerous genes that are specifically expressed in G2-phase and mitosis, suggesting a role for the encoded proteins in mitotic regulation [1]. Many of these proteins are known to function in cell cycle regulation including cyclin B1 [2], Plk1 [3], Aurora-A [4], Cks2 [5], topoisomerase IIα [6], and Rab6-kinesin [7], while others are still uncharacterized. In addition, we demonstrated that many of these genes are down-regulated in response to genotoxic agents, which may contribute to G2 DNA damage checkpoint function. Included among the genes identified in this screen were several proteins that are known or predicted to function in the ubiquitination of other proteins, including two ubiquitin conjugating enzymes and a component of the anaphase promoting complex (APC/C). Also among the G2/M-specific proteins identified in this screen was a protein with a c-

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terminal HECT domain that we have named G2E3. HECT domains function as ubiquitin ligase catalytic domains and are named for their similarity to E6-associated protein, an ubiquitin ligase involved in degradation of p53 in cells infected with tumorigenic papillomaviruses.

We were particularly interested in characterizing G2E3 since ubiquitin mediated protein degradation serves as a major regulatory mechanism in the cell division cycle and checkpoints. For example, degradation of cyclin B1 and securin [8] following ubiquitination by the ubiquitin ligase APC/C is essential for completion of mitosis. Similarly, degradation of cyclin-dependent kinase inhibitors p21 and p27 following ubiquitination by the SCF complex is required for entry into S-phase [9,10]. Oscillations of APC/C and SCF complex activity, therefore, serve as another major mechanism that contributes to control of the cell cycle [11]. Although these two ubiquitin ligase complexes are central to cell cycle control, other ubiquitin ligases also participate in cell cycle and checkpoint regulation. For example, the ubiquitin ligase CHFR is necessary for function of the mitotic stress checkpoint which leads to arrest in early mitosis in response to microtubule inhibitors [12], a checkpoint also referred to as the antephase checkpoint [13]. Similarly, Mdm2-mediated ubiquitination of p53 [14] plays a critical role in the G1 DNA damage checkpoint. Ubiquitin-mediated degradation will undoubtedly regulate many other aspects of cell cycle and checkpoint function that are not yet understood, and we predict a role for G2E3 in these processes.

Many cell cycle regulatory genes are compartmentalized within the cell as a means of additional gene regulation. Nucleo-cytoplasmic exchange has been described for several molecules that regulate the cell cycle and stress responses including Cdc25C [15], cyclin B1 [16], and p53 [17]. A variety of mechanisms are employed to control migration of proteins between the nucleus and cytoplasm. For example, Cdc25C is retained in the cytoplasm by binding to 14-3-3 proteins [18] and exported from the nucleus in a CRM1-dependent manner [15] to block its accumulation in the nucleus during interphase. Cyclin B1 is exported from the nucleus during interphase, but this export is inactivated by phosphorylation prior to mitosis to allow its nuclear accumulation [19]. The shuttling of p53 between nucleus and cytoplasm is controlled by cytoplasmic retention and nuclear import [20], as well as regulated nuclear export that is activated by ubiquitination [21]. Nuclear export plays a major role in controlling the subcellular localization of several proteins, but the mechanisms employed are incompletely characterized. Nuclear export by CRM1/Exportin 1 is the mechanism that is most completely characterized, due in large part to the availability of a potent inhibitor known as leptomycin B (LMB). This export factor binds to a leucine rich element ($LX_{2-3}LX_{2-3}LXL$) that was first identified in the HIV-1 protein Rev and the protein kinase A inhibitor PKI [22,23]. To date, several other nuclear export mechanisms have been described [24-28] which function through other members of the karyopherin family, but little is known about these CRM1-independent nuclear export mechanisms.

The HECT domain protein G2E3 is identified in GenBank as KIAA1333. In this work, we demonstrate that G2E3 accumulates in the nucleus of most cell types and specifically within the nucleolus of some cells. Nucleolar localization is highly regulated and this protein re-localizes to the nucleoplasm in response to DNA damage. We have defined the sequence responsible for directing the protein to the nucleolus and directing nuclear export of the protein in a CRM1-independent manner. Furthermore, we demonstrate that nucleolar localization is a dynamic process that occurs very rapidly. Although the HECT domain of G2E3 may function as a catalytically active ubiquitin ligase domain, this region of G2E3 also plays an important role in controlling the protein's subcellular trafficking. This unexpected function of a HECT domain has not previously been demonstrated.

MATERIALS AND METHODS

Cell Culture

All cell lines were obtained from the ATCC. HeLa (human cervical cancer derived), Cos-7 (African Green Monkey kidney derived), SiHa (human cervical cancer derived), HEK293T (human embryonic kidney derived), and BSC-40 (African Green Monkey kidney derived) cells were cultured in DMEM supplemented with 10% FBS, 100 I.U. penicillin, and 100μg/mL streptomycin. WI-38 primary foreskin fibroblasts were cultured in EMEM with 10% FBS, 100 I.U. penicillin, and 100μg/mL streptomycin. All cell lines were maintained under standard environmental conditions. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Plasmids

All primers used for preparations of clones and mutants are listed in Supplementary Data. The full sequence of all constructs prepared for these experiments was confirmed by nucleotide sequencing to ensure that no unexpected mutations were introduced during construct preparation. Full-length G2E3 was generated by RT-PCR using HeLa mRNA, RACE cDNA library synthesis kit (Stratagene), Advantage HF2 high fidelity PCR kit (Clontech) and G2E3 primers (G2E3GFP5 and G2E3GFP3). The cDNA was cloned into pEGFP-C3 (Clontech) after digestion with XhoI and KpnI. All other deletion mutants were cloned into pEGFP-C3 using XhoI and KpnI. G2E3 c-terminal deletion mutants were cloned using G2E3GFP5 along with either G2E3-237R (2-237 mutant), G2E3-363R (2-363 mutant), G2E3-496R (2-496 mutant), G2E3-654R (2-654 mutant), and G2E3-674R (2-674 mutant) primers. N-terminal deletion mutants were generated using G2E3GFP3 along with either G2E3-287F (287-706 mutant) or G2E3-80F (80-706 mutant). A mutant containing amino acids 287-496 was generated using G2E3-287F and G2E3-496R primers. To facilitate preparation of a mutant lacking amino acids 18-35 (2-17/36-706 mutant), two separate PCR products were prepared and cloned adjacent to one another in-frame in the pEGFP-C3 vector. A fragment encoding amino acids 2-17 was generated by PCR using the G2E3GFP5 and G2E3-17R primers and cloned using KpnI and XhoI. A second PCR product was generated using the G2E3-36F and G2E3-706XbaR primers. This fragment was cloned into the pEGFP-C3 plasmid already encoding amino acids 2-17. The resulting plasmid encodes amino acid 2-17 fused in-frame with amino acids 36-706 with an intervening KpnI site (that encodes a GT dipeptide). Although we predicted that addition of two amino acid residues was unlikely to alter protein localization, we separately prepared a control construct encoding amino acids 2-35 fused to amino acids 36-706 with an intervening KpnI site. This control is essentially wild-typed G2E3 with a GT dipeptide between amino acids 35 and 36. This control was prepared in the same way using the primer pairs G2E3GFP5 with G2E3-35R and G2E3-36F with G2E3-706XbaR. A mutant with K30A and K31A mutations was prepared in the same way with the exception that the cDNA fragment encoding amino acids 2-35 was replaced by a fragment generated using the G2E3-GFP5 and G2E3-K30/31A-35R primers. cDNAs encoding NYD-SP6 and murine Nucleolin were obtained from OpenBiosystems and amplified by PCR using the primers NYD-GFP5 and NYD-GFP3 or nucleolin5 and nucleolin 3, respectively and subsequently cloned into pEGFP-C3.

To generate GFP-Cdc25C, a Cdc25C cDNA obtained from OpenBiosystems was amplified using the Cdc25C-F and Cdc25C-R primers and cloned into pEGFP-C3 after digestion with KpnI and BamHI. Using a strategy similar to that for the GFP-GST constructs, G2E3 fragments were generated by PCR and cloned into this GFP-Cdc25C construct after digestion with BamHI and XbaI. Fragments were generated using the following primer pairs; G2E3-491F with G2E3-25C-706R, G2E3-491F with G2E3-589R, G2E3-589F with G2E3-25C-706R, G2E3-364F with G2E3-405R, G2E3-406F with G2E3-447R, G2E3-448F with G2E3-490R,

G2E3-491F with G2E3-520R, G2E3-521F with G2E3-544R, and G2E3-556F with G2E3-589R.

A Rev cDNA obtained from Dr. John Kappes was amplified using the Rev-GFP5 and Rev-GFP3 primers and cloned into pEGFP-C3 after digestion with XhoI and HindIII. The Rev1.4 mutation was generated using two-stage PCR mutagenesis using primer pairs Rev1.4R along with Rev-GFP5 and Rev1.4F along with Rev-GFP3. The resulting GFP-Rev construct lacked a stop codon allowing the cloning of the G2E3 NES (amino acids 341-544) downstream and in-frame.

Immunoblotting

For analysis of protein expression, cells were harvested and then lysed using mammalian cell lysis buffer (MCLB) prepared as previously described [1]. The lysates were cleared by centrifugation, and proteins were quantitated using the Bradford assay (Bio-Rad). For immunoblotting, the proteins were separated on SDS-polyacrylamide gels of the indicated concentrations. After electrophoresis, blots were transferred by electrophoresis to nitrocellulose membranes (Osmonics) and immunoblotting was performed according to standard methodology. Antibodies for immunoblotting include anti-GFP (Becton-Dickinson), and anti-actin (Sigma). Secondary antibodies were horseradish peroxidase conjugated anti-mouse (Jackson Labs).

Immunofluorescent Staining

HeLa or BSC-40 cells were fixed (4% paraformaldehyde, 100 mM $MgCl_2$, PBS) for 10 minutes then permeablized in PBS + 0.5% Triton-X for 15 min. at 4°C. Antibodies to c-myc (Santa Cruz), nucleolin (Santa Cruz), PML (Medical and Biological Laboratories), SC-35 (Sigma), β-tubulin (Sigma), and SUMO-1 (Zymed Laboratories) were diluted in 50% goat serum and incubated with fixed cells for 2 hr at 37°C. Slides were washed 3 times in wash buffer (PBS + 0.1% Tween-20) then incubated with Texas Red-conjugated secondary antibodies (Molecular Probes) for 1 hr at 37°C. Following additional washes, slides were stained with 0.1 μg/mL DAPI (Sigma) and mounted.

Fluorescence Microscopy

Twenty-four hours following transfection, cells were treated as indicated or left untreated, then fixed and stained with DAPI. DAPI, E-GFP and Texas Red were visualized on an Olympus AX70 upright fluorescent microscope with a Speicher filter set and a Zeiss Axiocam color camera. Images were assembled in Adobe Photoshop.

For fluorescence recovery after photobleaching (FRAP), HeLa cells transiently expressing G2E3-GFP were incubated at room temperature in media supplemented with 25 mM HEPES. Photobleaching was performed with a Leica DMIRBE inverted epifluorescence/ Nomarski microscope outfitted with Leica TCS NT Laser Confocal optics (Leica. Inc.; Exton, PA). Time lapse images were taken before, immediately following and at indicated times after photobleaching.

Nucleolar Relocalization Assays

HeLa cells were seeded in two well chamber slides (Lab-Tek) at a confluency of approximately 100,000 cells/well. Twenty-four hours following transient transfection of the indicated construct, cells were treated with 1 μM doxorubicin (Sigma) or 10 μM etoposide (Sigma) for 2 hr, 3 μM nocodazole for 4 hr, or 10 μM MG-132 (Sigma) overnight. Heat shock was performed by incubating cells at 42°C for 1 hr. For irradiation treatments, cells were exposed

5 Gy γ-radiation from a cesium source followed by incubation for 2 hr. After treatment, medium was aspirated and cells were fixed in 4% buffered paraformaldehyde and then stained with DAPI (Sigma).

Nuclear Export Assays

Cos-7 cells were seeded into chamber slides at a density of 100,000 cells/well then transfected with the indicated construct. Twenty hours later, cells were mock treated or treated with 10 ng/mL leptomycin B (Sigma) for 3 hours. Cells were then fixed, stained with DAPI and mounted for imaging. For quantitation, a blinded observer scored 200 cells in triplicate for nuclear or cytoplasmic localization. Localization was defined based on the area of greatest accumulation of the protein.

Heterokaryon Assay

100,000 Cos-7 cells were seeded into one well of a chamber slide. Following attachment, the cells were transiently transfected with GFP-G2E3 or the nuclear export deficient mutant GFP-G2E3$^{(2-363)}$. Four hours later, an equal number of NIH-3T3 cells were seeded along with the transfected Cos-7 cells. Eighteen hours later, cells were treated with 50 μg/mL cyclohexamide (CHX) (Sigma) for 30 min. Cell fusion was conducted by addition of 50% w/v PEG 8000 in DMEM for 2 min. at 37°C. The cells were then washed twice in PBS and incubated 2.5 hours in media along with 50 μg/mL CHX. Cells were fixed in 4% paraformaldehyde and stained with DAPI. NIH-3T3 cells were identified and distinguished from Cos-7 cells by the presence of characteristic mouse satellite DNA.

RESULTS

G2E3 Primary Structure

Many genes with maximal expression in G2-and M-phase were identified in a microarray screen [1] and many of these were down-regulated in response to DNA damage. One of these genes was represented by two independent probe sets corresponding to ESTs AA233231 and AA281251. We used sequence data from these ESTs to design primers to clone the full-length cDNA by RT-PCR. We constructed a full-length cDNA from these PCR products which is in agreement with the GenBank sequence for FLJ20333. The encoded protein sequence (Figure 1B) was analyzed for domain structure using SMART (http://smart.embl-heidelberg.de/) and PFAM (http://pfam.wustl.edu/hmmsearch.shtml) to identify the domain structure (Figure 1A). Three PHD (plant homeodomain) domains in the n-terminus of the protein are referred to as PHD1, PHD2, and PHD3 as indicated in the diagram. A HECT (homologous to E6-associated protein) domain located in the c-terminus of the protein suggested that the protein might function as an ubiquitin ligase. Three putative nuclear localization signals: amino acids 18-35, 85-101, and 341-363, were identified. Based on G2-specific expression and the presence of a domain like that of many E3s, we refer to it as G2E3 (G2 Specific E3-like protein).

G2E3 is a Nuclear Protein with Variable Nucleolar Accumulation

We began our examination of G2E3 by looking at its subcellular localization using a GFP-tagged expression construct. This construct was transiently expressed in a variety of cultured human and simian cell lines including HeLa, SiHa, primary fibroblasts (WI-38), Cos-7, and BSC-40 (Figure 2A). Additionally, a myc-tagged G2E3 was expressed in BSC-40 cells and stained by indirect immunofluorescence to ensure that the GFP tag does not interfere with localization of the protein. Both the GFP- and myc-tagged G2E3 were found to localize primarily to nuclei with minimal diffuse cytoplasmic localization in each transformed cell line. Primary fibroblasts exhibited less nuclear enrichment of the protein and a more pancellular

distribution. Although we have made several attempts to raise an antibody to G2E3, only one of these antisera recognizes the protein by immunoblotting. However, it is ineffective for use in immunofluorescence of fixed cells so we are unable to compare the localization of GFP-tagged G2E3 with that of the endogenous protein.

Differences in subnuclear localization were seen between cell lines. Most notably, GFP-G2E3 accumulated within subnuclear structures in HeLa cells. In order to identify these substructures, HeLa cells expressing GFP-G2E3 were stained by indirect immunofluorescence for a variety of markers for nuclear subdomains (Figure 2B). Nucleolin was used as a marker for the nucleolus, the site of ribosome biogenesis (reviewed in [29]). PML bodies appear as nuclear speckles and are known to regulate transcription and the cell cycle (reviewed in [30]. SUMO is an ubiquitin-like protein that is known to be conjugated to PML, SP100 and other nuclear proteins (reviewed in [31]). SC-35 is a marker for spliceosomes, sites of mRNA splicing and maturation (reviewed in [32]). G2E3 was found to consistently co-localize with nucleolin within nucleoli but not with SUMO, PML, or SC-35 indicating that G2E3 does not associate with SUMO bodies, PML bodies, or spliceosomes.

Because G2E3 contained a conserved HECT domain, we wished to determine if function of this putative ubiquitin ligase catalytic domain had any impact on localization of G2E3. We mutated the conserved cysteine reside (C666) to alanine and examined its localization in HeLa and Cos-7 cells. GFP-C666A localized to the nucleus and nucleolus of HeLa cells and nuclei of Cos-7 cells, indicating that HECT function is not required for localization of G2E3 (Figure 2C).

Mutagenesis to Define Regions Regulating G2E3 Nuclear and Nucleolar Localization

A series of G2E3 deletion mutants fused to EGFP were prepared to identify regions of the molecule that determine the subcellular localization of the protein and are named according to the amino acid residues present in the molecule (Figure 3A). Each was expressed in HeLa cells and the localization of the protein was determined by fluorescence microscopy. GFP-G2E3$^{(2\text{-}237)}$ (Figure 3B) did not concentrate in nuclei but rather gave a pancellular distribution while GFP-G2E3$^{(2\text{-}363)}$ (Figure 3B) localized almost exclusively in the nucleus, demonstrating that sequences between 237 and 363 are important for nuclear localization. As discussed below, a putative nuclear localization signal (NLS) was found in this region of the protein. Paradoxically, mutants that extend further into the HECT domain, including GFP-G2E3$^{(2\text{-}496)}$ and GFP-G2E3$^{(2\text{-}654)}$ lacked nuclear localization (Figure 3B), suggesting that sequences downstream from amino acid 363 antagonize the nuclear accumulation of the protein. Full-length GFP-G2E3 as well as mutants with very small c-terminal deletions (such as GFP-G2E3$^{(2\text{-}674)}$) are localized to the nucleus and concentrated in the nucleolus. Two additional mutants containing amino acids 287-496 and 287-706, were pancellular and nuclear in distribution, respectively (Figure 3B). This confirms that sequences between amino acids 496 and 706 play a role in nuclear accumulation of the protein. A deletion mutant lacking only the first 79 amino acids (GFP-G2E3$^{(80\text{-}706)}$) localized primarily to the nucleus but not the nucleolus (Figure 3B), indicating that the n-terminal region contains sequences necessary for nucleolar targeting.

We attempted to determine which, if any, of the three putative NLSs were important for nuclear localization of G2E3. While amino acids 341-363 were found to be sufficient to direct nuclear localization of a heterologous protein, they were not required for nuclear accumulation of G2E3 (data not shown). Neither of the remaining two putative NLS sequences, 18-35 nor 85-101, were capable of mediating nuclear import. While amino acids 341-363 appear to play a role in nuclear localization of G2E3, these data suggest that either additional non-classical nuclear

import sequences are present within G2E3 or that it is brought into the nucleus by way of an interacting protein.

Residues 18-35 Function as a Nucleolar Localization Sequence

A deletion mutant lacking only the first 79 amino acids, GFP-G2E3$^{(80-706)}$, localized to HeLa nuclei but not the nucleoli (Figure 3B), indicating that sequences within the n-terminal 79 amino acids are essential for nucleolar localization. Nucleolar localization signals (NoLSs) are frequently rich in basic residues and often include the sequence K/R-K/R-X-K/R [33], so we assessed the effect of deletion of the basic region in the n-terminus of G2E3 that included this consensus sequence. This mutant, GFP-G2E3$^{(2-17/GT/36-706)}$, had the first 17 amino acids fused to amino acids 36-706 with an intervening glycine-threonine dipeptide (encoded by a KpnI site used for cloning purposes). This mutant failed to accumulate in the nucleolus (Figure 3B), indicating that this positively charged region of G2E3 is indeed essential for nucleolar targeting of the protein. The localization pattern of a control protein containing the full G2E3 sequence with the same dipeptide insertion (GFP-G2E3$^{(1-35/GT/36-706)}$) was indistinguishable from the wild-type protein indicating that the deletion of amino acids 18-35, not the dipeptide insertion, was responsible for loss of nucleolar localization. To further confirm the requirement for this basic region in nucleolar targeting, we prepared a point mutant with two lysine to alanine mutations (K30A/K31A). As expected, this mutant, GFP-G2E3$^{(K30A/K31A)}$, localized to the nucleus but failed to accumulate in the nucleolus (Figure 3B).

Even though there are clear sequence requirements for G2E3 accumulation in HeLa nucleoli, we considered the possibility that nucleolar accumulation might be merely a result of massive accumulation of the over-expressed protein. Therefore, we compared the amount of expression of G2E3 constructs that accumulate in nucleoli (full-length and 2-674) with those that do not localize to nucleoli (all others). Although the mutant containing amino acids 2-674 is localized to the nucleolus (Figure 3B), it is very poorly expressed (Figure 3C) demonstrating that there is no correlation between expression level and nucleolar accumulation in HeLa cells. This suggests that the accumulation in the HeLa nucleolus is a specific effect rather than an effect of massive over-expression of G2E3.

Nuclear Export is Mediated by a Novel CRM1-Independent Mechanism

Since many nuclear proteins are known to actively shuttle through the nucleus, we sought to determine if G2E3 was also a nucleo-cytoplasmic shuttling protein. To do so, we employed an interspecies heterokaryon assay, an experimental approach that has been previously employed to analyze shuttling of several proteins including mdm2 [34], WT1 [35], Smad1 [36], and the tumor suppressor adenomatous polyposis coli [37]. We transfected either GFP-G2E3 or GFP-G2E3$^{(2-363)}$ into Cos-7 cells and then assessed the ability of each protein to be re-localized to the nucleus of a fused NIH3T3 cell (Figure 4A). Both of these constructs were shown previously to reside almost exclusively in the nucleus (Figure 3B). To block any new protein synthesis, cells were treated with cycloheximide. After fusion, GFP-G2E3 was found in NIH3T3 nuclei, indicating that the protein was exported from the Cos-7 nucleus and then imported into the NIH3T3 nucleus. In contrast, GFP-G2E3$^{(2-363)}$ was found only within Cos-7 nuclei. These results indicate that G2E3 is a nucleo-cytoplasmic shuttling protein and that its export is dependent upon sequences in the carboxy-terminal half of the protein.

In order to map the putative nuclear export sequence, we prepared constructs directing expression of GFP-Cdc25C fused to portions of the G2E3 HECT domain (as shown schematically in Figure 4B). Cdc25C is known to actively shuttle into the nucleus and be rapidly exported by a CRM1/Exportin 1-mediated process [15], a process that is inhibited by the drug leptomycin B (LMB). As previously reported [15], GFP-Cdc25C localized primarily

to the cytoplasm but accumulated within the nucleus after inhibition of CRM1 (Figure 4C, labeled None) with LMB. The pattern of localization for GFP-Cdc25CG2E3$^{(589-706)}$ was indistinguishable from GFP-Cdc25C (Figure 4C, labeled 589-706). In contrast, GFPCdc25C-G2E3$^{(491-706)}$ and GFP-Cdc25C-G2E3$^{(491-589)}$ were localized to the cytoplasm both before and after LMB treatment (Figure 4C, labeled 491-706 and 491-589). This result demonstrated the presence of a sequence that blocks nuclear accumulation of chimeric protein within amino acids 491-589 from G2E3. We further mapped this putative CRM1-independent nuclear export domain to residues 364-544 using the same Cdc25C-GFP fusion construct. Interestingly, even when subdivided, several fragments from 364-544 can antagonize nuclear accumulation of the chimeric protein (Figure 4D). Since GFP-Cdc25C (labeled None) is primarily retained in the nucleus after LMB treatment, there is only a small fraction of cells with cytoplasmic protein. In contrast, fusion constructs containing some fragments of the putative G2E3 nuclear export domain (for example amino acids 364-405, 448-490, and 521-544) have almost exclusively cytoplasmic protein indicating that they retain the ability to mediate nuclear export of the chimeric protein. This large region that functions in nuclear export appears to be unlike previously described nuclear export sequences (NESs) so we have referred to it using the generic term “nuclear export domain” rather than calling it an NES.

To test the function of the nuclear export domain in an independent assay, we utilized Rev1.4, the mutant of HIV-1 Rev that is deficient in nuclear export [38]. Rev functions in nuclear export of viral mRNAs by way of a CRM1-dependent mechanism. For the purpose of its use as an experimental tool to test potential nuclear export sequences, the leucine-rich binding site for CRM1 was inactivated by mutation of leucine residues to generate Rev1.4 [38], a mutant that Henderson, *et al* showed to be useful in defining both CRM1-dependent and CRM1-independent NESs. As previously shown, wild-type GFP-Rev localizes primarily to the cytoplasm and nucleolus and GFP-Rev1.4 (with an inactivated NES) accumulates within nucleoli and the nucleoplasm (Figure 4E). We prepared a construct directing expression of GFP-Rev1.4 fused to amino acids 364-544 from G2E3 to test whether this region can mediate nuclear export of GFP-Rev1.4. As expected, GFP-Rev1.4-G2E3$^{(364-544)}$ was localized to the cytoplasm (Figure 4E), confirming that G2E3 residues 364-544 can direct nuclear export of Rev1.4. As with the GFP-Cdc25C constructs, the effect on nuclear accumulation exerted by G2E3 amino acids 364-544 is insensitive to LMB, confirming that it is a CRM1-independent process. Although GFP-Rev1.4-G2E3$^{(364-544)}$ was excluded from the nucleoplasm, accumulation was observed in the nucleolus, indicating that the fusion protein did not fail to enter the nucleus, but rather was actively exported from the nucleus.

G2E3 Rapidly Delocalizes from Nucleoli Following DNA Damage

G2E3 is transcriptionally down-regulated following γ-irradiation [1], so we sought to determine if DNA damage or other stimuli altered the subcellular localization of the protein. HeLa cells expressing GFP-G2E3 were either mock-treated or treated with doxorubicin, a DNA intercalating agent that induces double stranded DNA breaks. After treatment for two hours, the cells were fixed and stained with DAPI and then analyzed by fluorescence microscopy. This treatment resulted in a rapid delocalization of the protein from nucleoli to the nucleoplasm, often with enrichment in the perinucleolar region (Figure 5A, top two rows). In order to determine whether nucleolar delocalization after DNA damage is a generalized phenomenon (perhaps resulting from nucleolar breakdown) or a specific response for G2E3, we expressed two other nucleolar proteins and analyzed the effect of doxorubicin treatment on their subcellular localization. For these experiments, we used NYD-SP6 and nucleolin. NYD-SP6 [39] is a protein with significant homology to G2E3 and nucleolin is a well described nucleolar protein. GFP-NYD-SP6 is enriched in nucleoli, but also present in the nucleoplasm in untreated cells. This subcellular distribution of protein is not altered by treatment with doxorubicin

(Figure 5A, middle two rows). Nucleolin is almost exclusively nucleolar in untreated cells, and like NYD-SP6, this pattern of localization is not altered by genotoxic stress (Figure 5A, bottom two rows). These data demonstrate that delocalization in response to DNA damage is not a generalized phenomenon shared by all nucleolar proteins, but rather a specific effect for G2E3 and a limited number of other proteins.

In order to determine if this nucleolar delocalization was specific for doxorubicin or a general response to either DNA damage or cellular stress in general, we treated cells with γ-irradiation, etoposide, nocodazole, or 42°C heat shock (Figure 5B). Treatment with both DNA damaging agents, γ-irradiation and etoposide, caused nucleolar delocalization of G2E3, but nocodazole and heat shock (which do not damage DNA) did not alter nucleolar localization of the protein. This response to genotoxic agents suggests a possible role for G2E3 in the DNA damage response. We quantitated the fraction of cells with nucleolar versus non-nucleolar G2E3 and found that approximately 80% of untreated cells had predominant G2E3 accumulation within nucleoli while only 10% of those treated with doxorubicin had this localization pattern (Figure 5C).

Because G2E3 relocalizes rapidly following DNA damage, we tested whether the protein localized to nucleoli in a static manner or if it was actively shuttling through this nuclear subdomain. To do so, we utilized fluorescence recovery after photobleaching (FRAP) (Figure 6). As shown by the arrows, bleaching of one nucleolus resulted in loss of GFP fluorescence, but, within 10 seconds, full recovery was observed. The experiments shown here were conducted at room temperature, since fluorescence recovery in nucleoli is so rapid at 37°C that photobleaching is very difficult to observe. These data indicate that G2E3 shuttles through the nucleolus but accumulates in the HeLa nucleoli since the import rate is greater than the export rate. The failure of G2E3 to accumulate in the nucleoli of other cells may indicate that the rate of nucleolar export exceeds the rate of nucleolar import in those cells.

DISCUSSION

G2E3 was among a set of genes previously identified with G2/M-specific expression and altered expression during a G2 DNA damage checkpoint response [1]. In this study, we characterize the subcellular localization of this protein and identify those sequences responsible for directing its dynamic localization pattern. Although the HECT domain is predicted to function as an ubiquitin ligase, we have demonstrated that it plays an important role in regulating the subcellular location of G2E3.

A schematic showing important regions of G2E3 summarizes many of the findings of this study (Figure 7). We have demonstrated that residues 18-35 are responsible for nucleolar targeting of the protein. A CRM1-independent nuclear export domain has been localized to residues 364-544 of the HECT domain. C-terminal to the NES is a region (including residues 654-674 of the HECT domain) that inhibits the function of the NES in the full-length protein. We speculate that this NES inhibition might be regulated under physiologic conditions, allowing export of the protein from the nucleus in response to a particular stimulus. The dynamic accumulation of G2E3 in the nucleolus is altered after DNA damage, leading to relocalization of the protein to the nucleoplasm.

The nuclear export of many proteins is dependent on CRM1, but a small, diverse group of proteins exported from the nucleus in a CRM1-independent manner have been identified (summarized in [40]), though many more likely exist. Most of the known proteins are either viral proteins or cellular proteins involved in cell signaling, transcriptional regulation, or intracellular transport of other molecules. Since G2E3 has a very large, divisible nuclear export domain, it is possible that multiple proteins may be involved in this process. Identification of

the proteins that regulate this process will provide important insights into the basic process of nuclear export.

G2E3 localizes to the nucleoplasm of most cultured cells under normal growth conditions, but in HeLa cells, it accumulates within nucleoli. We have identified a region of the protein that is necessary for nucleolar localization and rich in the positively charged amino acids lysine and arginine. Some nucleolar targeting sequences rich in positively charged amino acids have been found to mediate interactions with the RNA-rich environment of the nucleolus [41]. It is possible that G2E3 may interact with RNA within the nucleolus and that this is what mediates its nucleolar localization. The nucleolus has traditionally been defined as the sight of ribosome biogenesis, but it has become increasingly apparent that many other cellular functions including cell cycle regulation are carried out in conjunction with this structure. For example, the nucleolus houses several cell cycle regulators including $p14^{ARF}$ which sequesters Mdm2, an E3 ligase for the tumor suppressor p53, to prevent degradation of p53 during cellular stress. In response to a variety of DNA damaging agents, G2E3 delocalizes from nucleoli, a process that could be a result of either inhibition of nucleolar import or increased nucleolar export or a combination of these two processes. We showed by FRAP that in unstimulated cells the protein rapidly shuttles through the domain. Nucleolar delocalization in response to treatment with topoisomerase I inhibitors or other DNA damaging agents has been described for several other proteins including topoisomerase I [42], WRN helicase [43], nucleolin [44], and $p14^{ARF}$ [45]. Each of these proteins plays a role in the cellular response to DNA damage by regulating DNA repair, cell cycle arrest, or apoptosis. We hypothesize that G2E3 will also be involved in the cellular response to DNA damage. We have not yet defined the function of G2E3, but have demonstrated that it is essential early in development since G2E3 knockout mice die *in utero* prior to E8.5 (manuscript under review). Early embryonic lethality is also observed when several other genes involved in the DNA damage response are inactivated [46-53].

When G2E3's localization was analyzed within primary WI-38 fibroblasts we noted that in many cells the protein failed to concentrate in the nucleus. This differs from all transformed cell lines examined in which G2E3 was almost exclusively nuclear. Reasons for this difference are not clear, although, the rapid proliferation of transformed cell lines as compared with primary cells is a possible explanation. We have shown that G2E3 is both imported and exported from the nucleus. Compartmentalization of the protein within either the nucleus or cytoplasm would restrict its access to substrate proteins and could limit or promote activity which would affect cell cycle progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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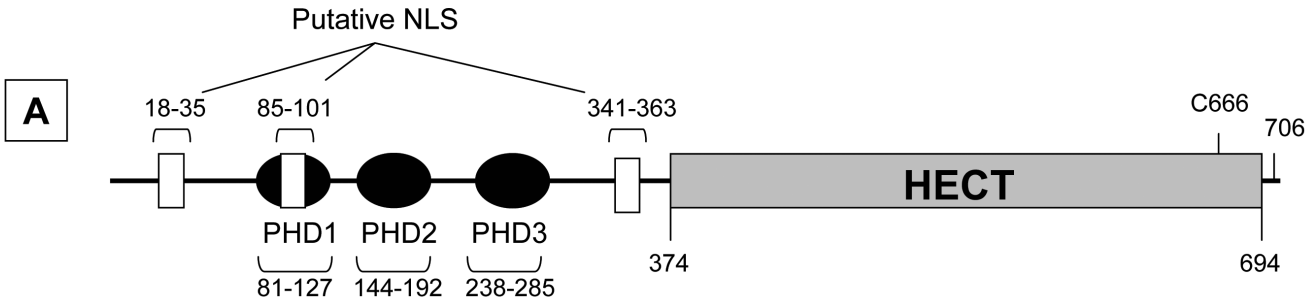

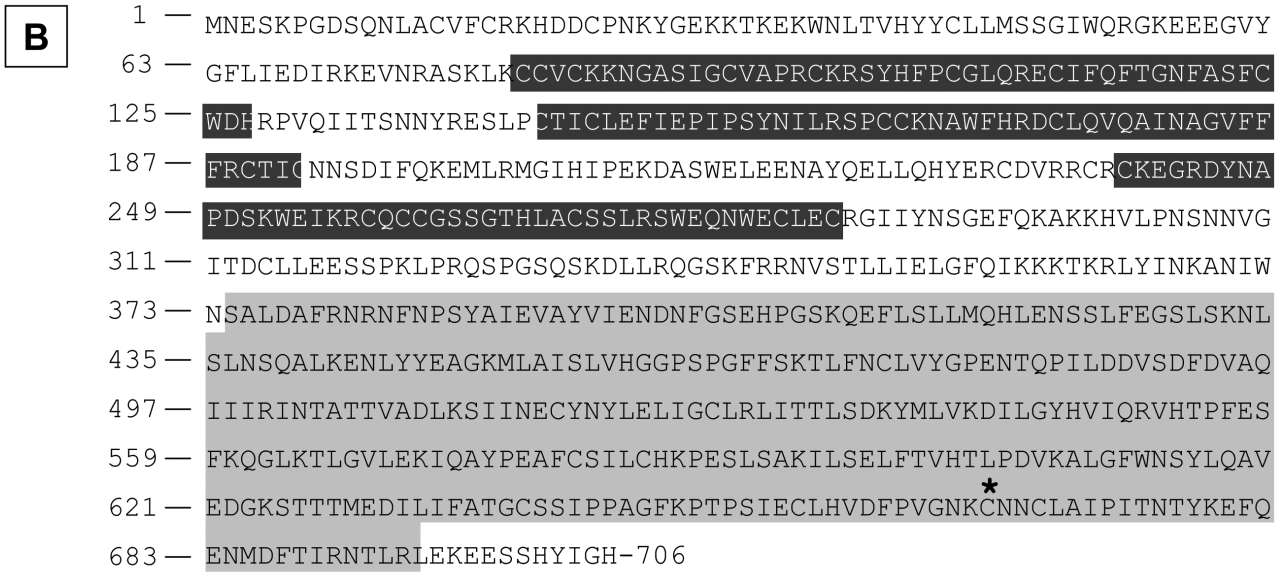

Figure 1.

G2E3 domain stucture and amino acid sequence. (A) G2E3 is a 706 amino acid protein with three zinc binding domains in the n-terminus that most closely match the consensus for PHD domains. Also within the n-terminal half of the protein are three putative nuclear localization signals (NLSs). A HECT domain is found in the c-terminus. (B) The amino acid sequence for human G2E3 is shown. The three cysteine-rich PHD domains are highlighted in black. The HECT domain is highlighted in gray. An asterisk marks the conserved cysteine residue required for HECT domain function.

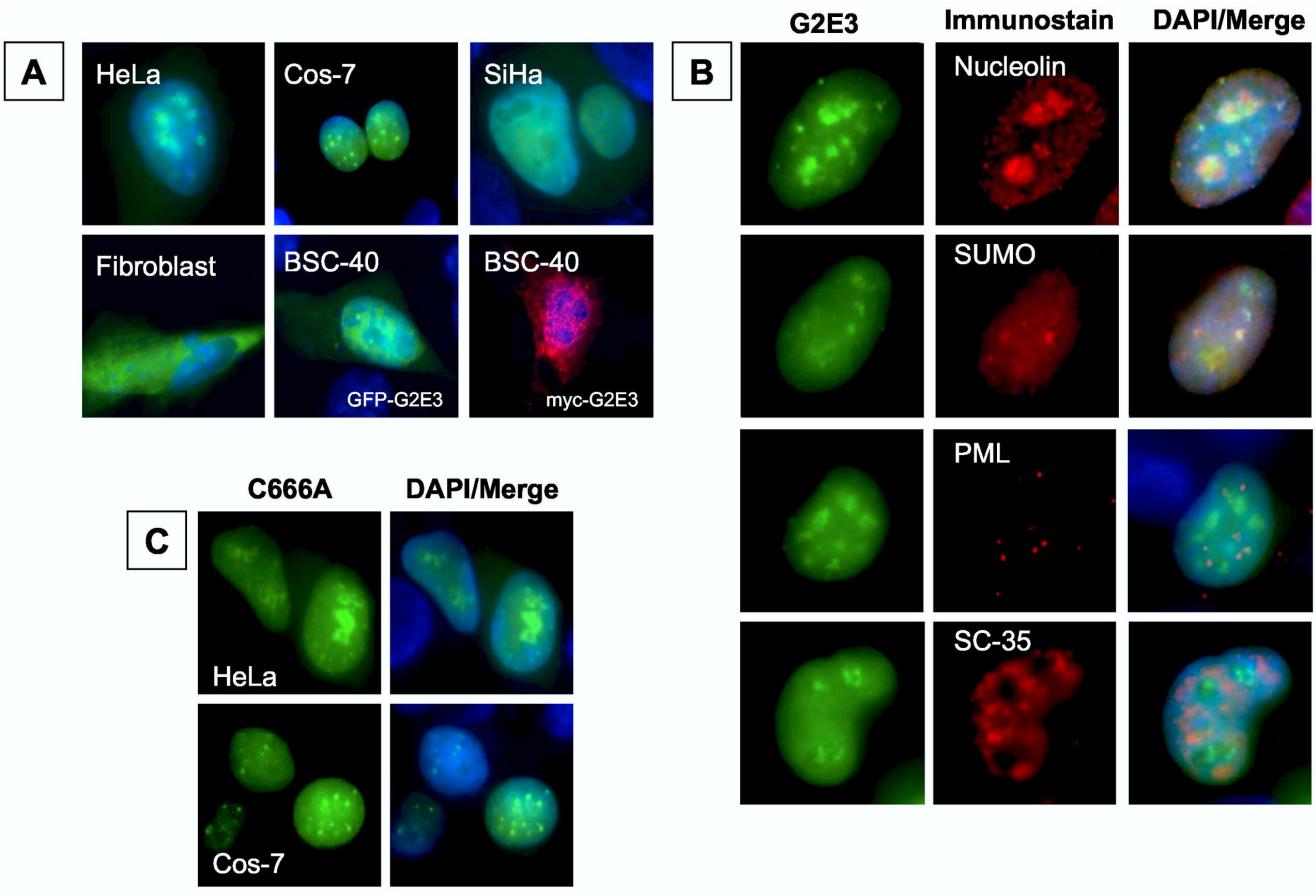

Figure 2.

G2E3 localizes to the nucleus of cultured cells and to nucleoli of HeLa cells. (A) HeLa, Cos-7, SiHa, WI-38 fibroblast, and BSC-40 cells were transiently transfected with GFP-G2E3. DAPI staining is in blue and GFP staining is in green. BSC-40 cells were transiently transfected with G2E3-myc and immunostained for myc. GFP-G2E3 is primarily nuclear in all transformed lines with prominent enrichment in a subdomain of the nucleus in HeLa cells. (B) HeLa cells were transiently transfected with GFP-G2E3 and immunostained with the indicated antibody to detect sites of co-localization. G2E3 primarily co-localizes with nucleolin in the nucleus of HeLa cells. (C) GFP-C666A was transiently expressed in HeLa or Cos-7 cells and its localization was examined.

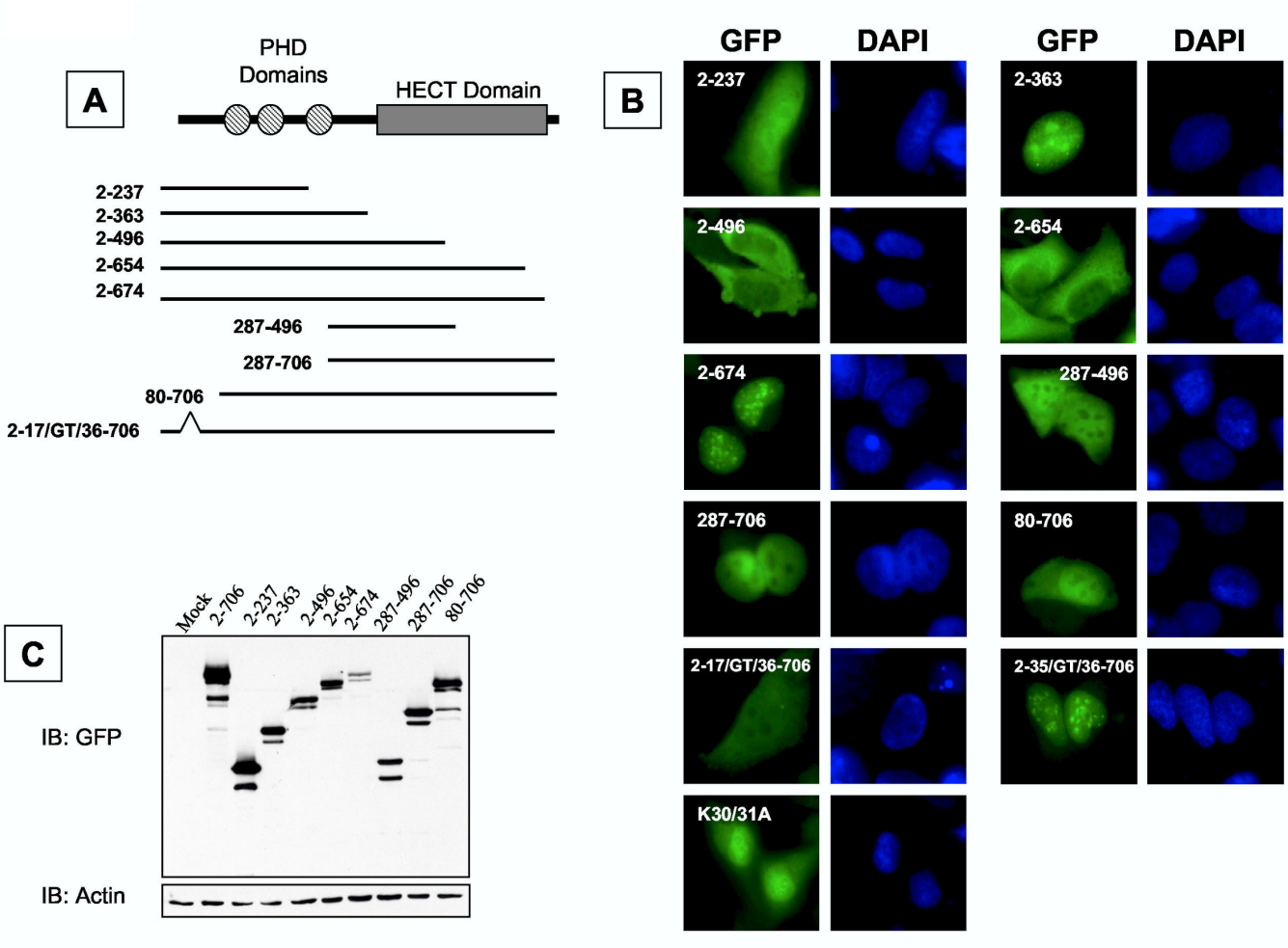

Figure 3.

G2E3 contains multiple signals directing its nuclear and nucleolar localization. (A). A domain schematic of G2E3 is shown along with each deletion mutant analyzed. (B) Each deletion mutant construct was transfected into HeLa cells and analyzed for localization by fluorescence microscopy as before. The amino acids included in each fusion construct are indicated on each photomicrograph. DAPI staining indicates the nuclei. Several regions alter nuclear and nucleolar accumulation of GFP-G2E3. Magnification 100X. (C) GFP-G2E3 deletion mutant constructs were transfected into 293T cells and expression was detected by immunoblotting for GFP. Expression of most deletion mutants is comparable to full length GFP-G2E3.

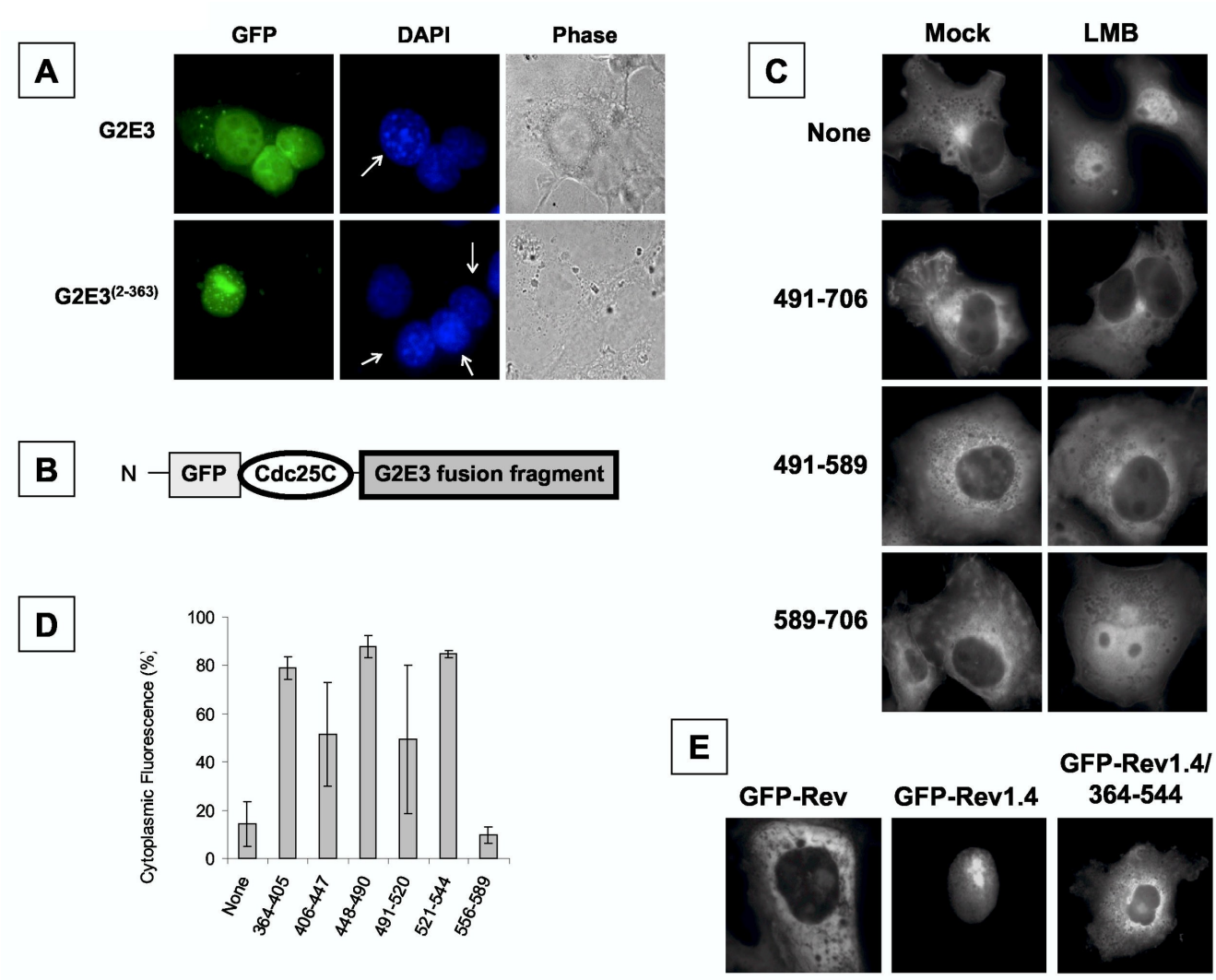

Figure 4.

G2E3 is a nucleo-cytoplasmic shuttling protein. (A) An interspecies heterokaryon assay was conducted. GFP-G2E3 or GFP-G2E3$^{(2-363)}$ expressing Cos-7 cells were co-cultured with NIH-3T3 cells in the presence of CHX. Arrows indicate NIH-3T3 cells as determined by the presence of mouse satellite DNA foci. (B) Schematic showing encoded fusion protein containing GFP in the n-terminus and Cdc25C immediately downstream and a portion of G2E3 fused in-frame at the c-terminus. (C) Constructs with the indicated amino acids from G2E3 were transfected into Cos-7 cells and either mock-treated (mock) or LMB-treated (LMB) for 3 hours prior to analysis by fluorescence microscopy. Representative cells show that all fusion proteins are primarily cytoplasmic in untreated cells but only constructs containing amino acids 491-589 and 491-706 are cytoplasmic after LMB treatment. (D) Fusion constructs were prepared as before with portions of the G2E3 NES from amino acids 364-544. Fusion proteins containing the indicated amino acids were expressed in Cos-7 cells and cells were treated with LMB. After 3 hours, they were analyzed by fluorescence microscopy for predominant localization in either the cytoplasm or nucleus. A small fraction of cells expressing GFP-Cdc25C or GFP-Cdc25C-G2E3$^{(556-589)}$ have primarily cytoplasmic protein, but all constructs bearing fragments of the NES have a significant increase in cells with predominantly cytoplasmic protein. (E) Constructs were prepared as in Figure 4B with the exception of HIV

Rev or HIV Rev1.4 in place of Cdc25C. Each was transfected into Cos-7 cells and predominant localization of the GFP-tagged protein was detected by fluorescence microscopy. The NES from Rev is inactivated in Rev1.4 so the protein is retained in the nucleus. Nuclear export is restored if the G2E3 export domain is included in the fusion protein.

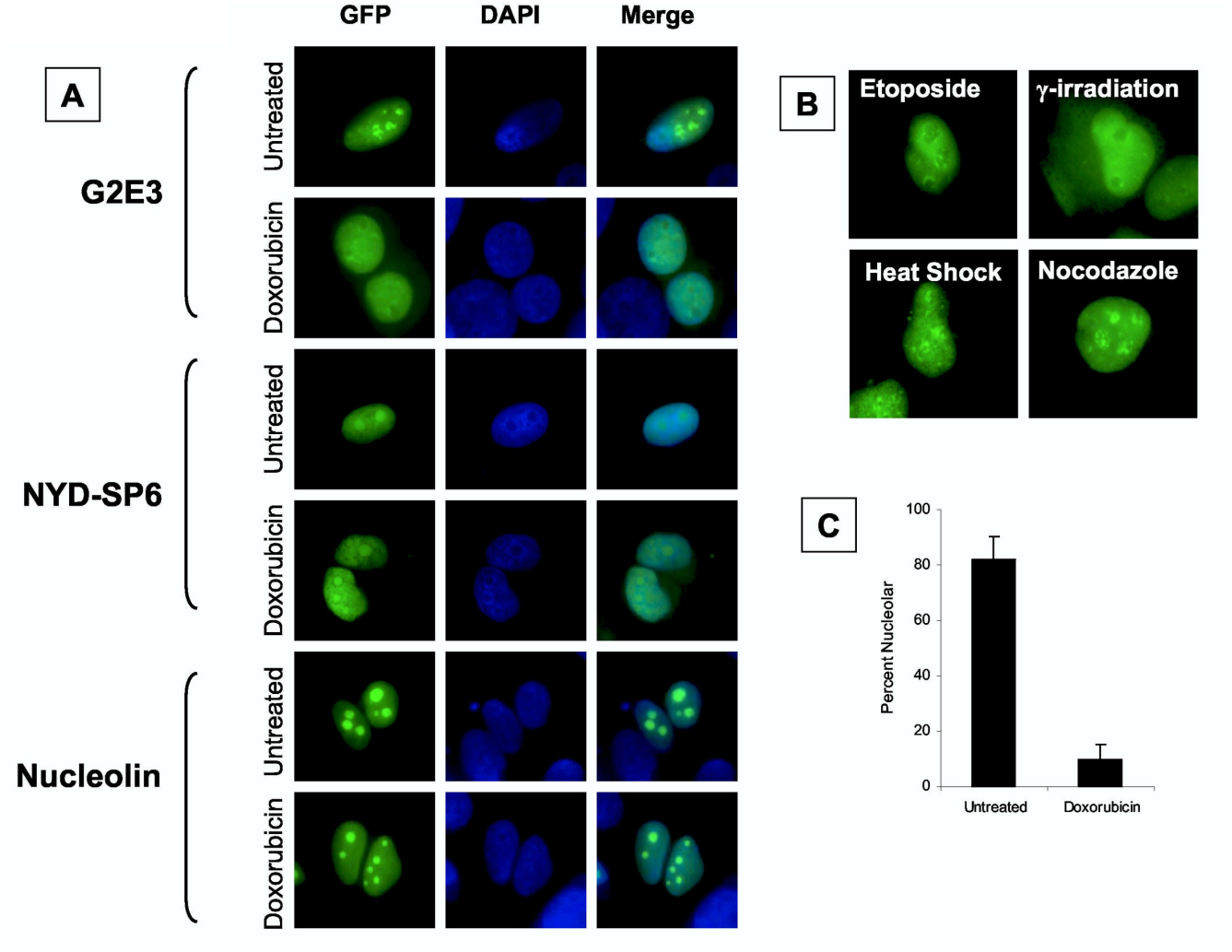

Figure 5.

G2E3 delocalizes from HeLa nucleoli in response to DNA damage. (A) HeLa cells were transiently transfected with GFP-G2E3, GFP-NYD-SP6, or GFP-nucleolin. Twenty-four hours later, cells were mock treated or treated with 1 μM doxorubicin (GFP-G2E3) or 10 μM doxorubicin (GFP-NYD-SP6, GFP-nucleolin) for 2 hours followed by fixation and DAPI staining. GFP-G2E3 is primarily nucleolar prior to drug treatment and more diffusely localized to the nucleoplasm after doxorubicin treatment. GFP-NYD-SP6 is diffusely localized throughout the nucleus with accumulation in the nucleolus prior to treatment and unchanged in localization after doxorubicin treatment. GFP-Nucleolin is unchanged following DNA damage. A larger dose of doxorubicin was used for NYD-SP6 and Nucleolin to ensure that adequate DNA damage was incurred. (B) HeLa cells expressing GFP-G2E3 were treated with the indicated stimuli and analyzed by fluorescence microscopy. γ-irradiation and etoposide both cause relocalization out of the nucleolus. Heat shock and nocodazole treatment do not alter subcellular localization. (C) HeLa cells treated with or without doxorubicin for 2 hours were scored for presence or absence of nucleolar GFP-G2E3 in triplicate by a blinded observer. The fraction of cells with prominent nucleolar accumulation of the protein is graphed.

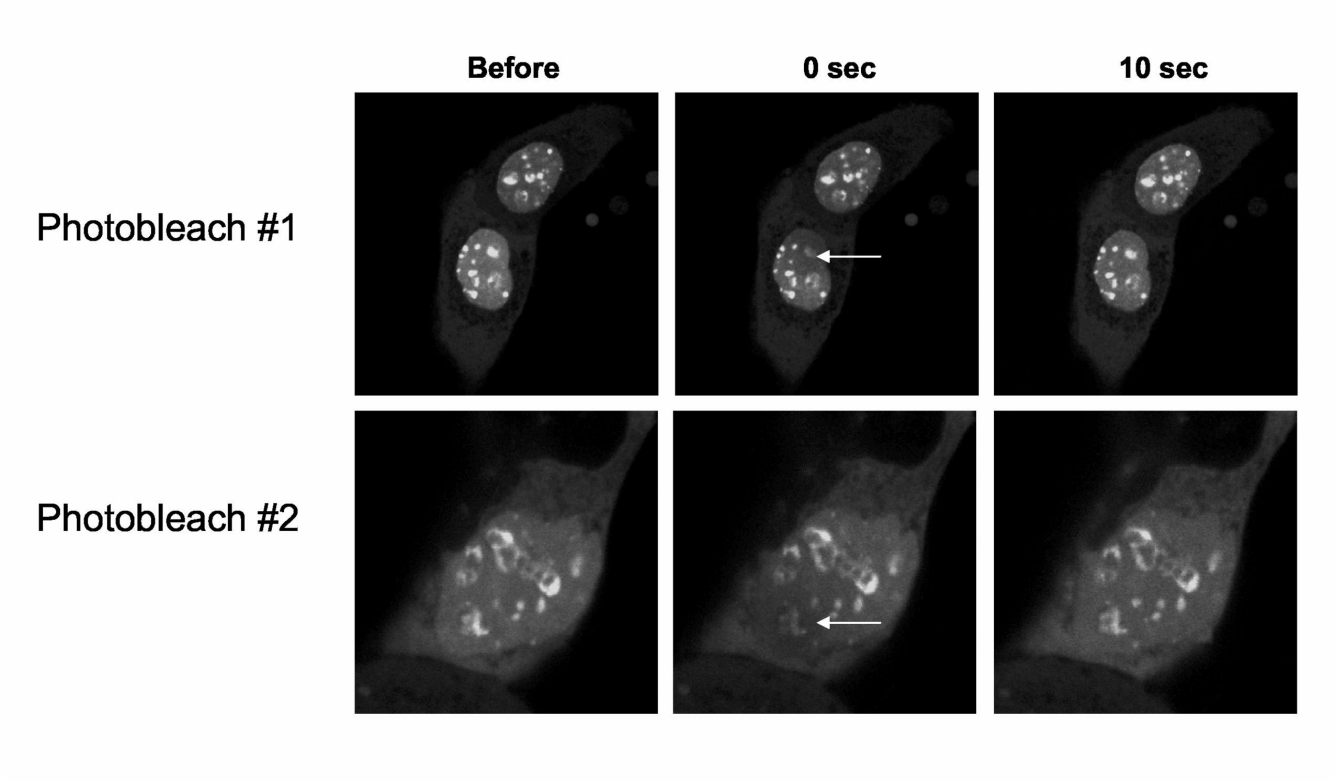

Figure 6.

G2E3 shuttles through the nucleolus of HeLa cells. Live HeLa cells expressing GFP-G2E3 were incubated at room temperature in the presence of cell culture medium supplemented with 25 mM HEPES buffer. Selected nucleoli were photobleached and imaged before, immediately after (0 sec), and at indicated intervals following photobleaching. Images were normalized to prebleach. The arrow indicates photobleached nucleolus.

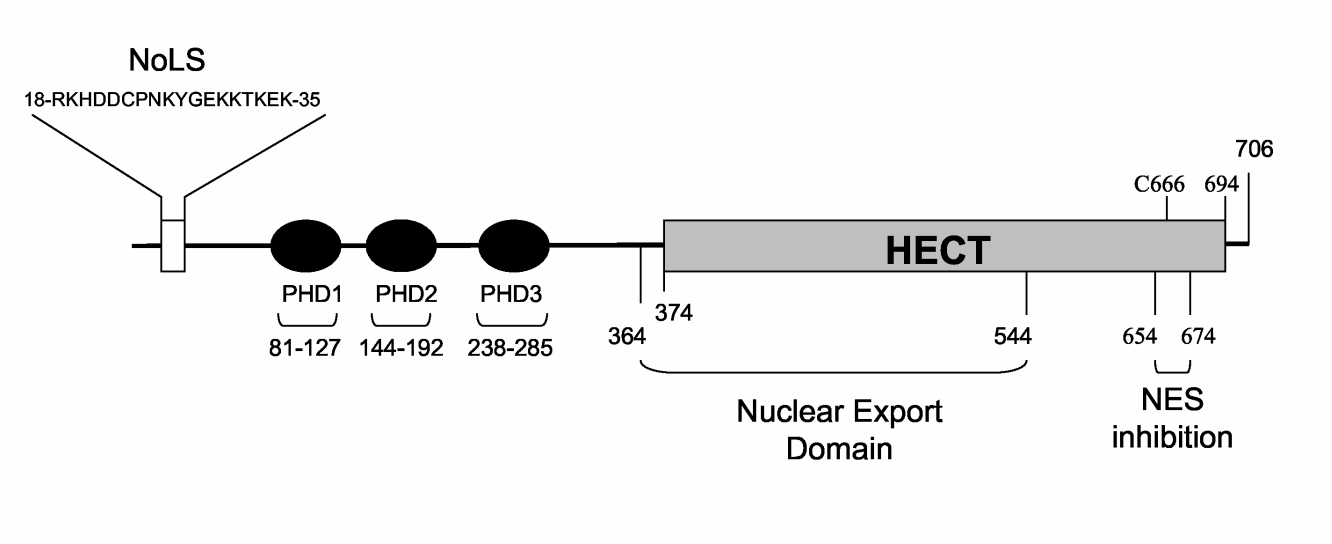

Figure 7.

G2E3 domains and sequences directing its subcellular localization. As noted previously, three PHD domains are observed in the G2E3 n-terminus and a HECT domain is found in the c-terminus. A sequence that directs accumulation within HeLa nucleoli is found in amino acids 18-35. Within the HECT domain, at least two distinct regions regulate localization of the protein. A CRM1-independent nuclear export domain is found in amino acids 364-544. Finally, a sequence within the c-terminus that includes amino acids 654-674 function to antagonize the NES.