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Research Article

RNF138/NARF is a Cell Cycle Regulated E3 Ligase that Poly-ubiquitinates G2E3

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Abstract

G2E3 is a G_2/M-specific gene that is down-regulated in response to DNA damage. It is highly regulated at both the transcriptional and post-translational levels. It plays a critical role in early embryonic development, as murine G2E3-deficient blastula undergoes a massive apoptosis. To identify proteins interacting with G2E3 a yeast two-hybrid was conducted, which revealed eight putative G2E3-binding proteins including two ubiquitin ligases (RNF138/NARF, DZIP3/hRUL138), a proteasomal subunit (PSMB4), and one cell cycle regulatory molecule (INCA1). We examined a possible functional interaction between G2E3 and the putative oncogene RNF138/NARF, demonstrating that RNF138/NARF has a subcellular localization and cell cycle regulated expression pattern similar to that of G2E3. The regions of each protein necessary for interaction were mapped to the C-terminus of RNF138/NARF and the N-terminus of G2E3. Furthermore, we demonstrated that RNF138/NARF catalyzes the poly-ubiquitination of G2E3 *in vitro* and *in vivo*. Ubiquitination of G2E3 by RNF138/NARF provides another mechanism for the regulation of this protein. Because depletion of either G2E3 or RNF138/NARF causes cellular apoptosis, this functional interaction may be important in the regulation of cell death.

INTRODUCTION

Covalent modification of proteins by ubiquitin regulates numerous cellular processes by altering protein function, subcellular localization, or stability. The selection of proteins for ubiquitination is a specific and highly regulated process that is possible because there are a large number of distinct E3 enzymes, each with a limited number of potential target proteins. At least four distinct domains can catalyze ubiquitination of target proteins, including HECT domains [1] and the structurally similar RING [2], PHD [3], and U-box [4] domains. Not surprisingly, proteins that are involved in the process of ubiquitination such as ubiquitin conjugating enzymes [5], ubiquitin ligases [6], components of multi-protein ubiquitin ligases [7], and molecules that negatively regulate ubiquitination [8,9] may also be targeted for degradation by other ubiquitin ligases.

G2E3 is an ubiquitin ligase originally identified in a screen for cell cycle regulated molecules with DNA damage responsive expression [10]. Subsequent studies have shown that G2E3 localization is altered in response to genotoxic stress, suggesting a role for the protein in DNA damage signaling or repair [11]. Two distinct domains in G2E3 catalyze the ubiquitination of target proteins; one RING domain and one PHD domain [12]. A catalytically inactive HECT domain in the carboxy-terminus of G2E3 regulates intracellular trafficking of the protein. As with many other E3s, inactivation of G2E3 has a pronounced effect on development. G2E3 deficiency leads to pre-implantation embryonic lethality as a result of massive apoptosis [12]. While G2E3 is a very important protein for mammalian development, it is not yet clear how it exerts its anti-apoptotic effect.

RNF125/TRAC-1 was the first characterized member of a family of four ubiquitin ligases (RNF125/TRAC-1, RNF166, RNF114/ZFP313, and RNF138/NARF) with a RING domain, zinc fingers, and an ubiquitin interacting motif (UIM) [13]. These proteins likely play distinct roles in eukaryotes since they have limited homology to one another and are expected to interact with and ubiquitinate distinct target proteins. At this time little is known about the function of RNF166. RNF114/ZFP313 [14,15] and RNF125/TRAC-1 [13,16,17] are involved in immune and anti-viral signaling. The fourth member of this protein family, RNF138/NARF (NEMO-like kinase associated RING finger protein), was initially shown to physically interact with NEMO-

like kinase and to catalyze the ubiquitination of TCF/LEF [18]. Through this activity, RNF138/NARF suppresses β-catenin signaling in *Xenopus* embryos. More recently, RNF138/NARF has been shown to play a role in cell cycle progression and apoptosis. siRNA-mediated knockdown of RNF138/NARF slows cell proliferation through the G_2/M phase and induces apoptosis [19]. Moreover, RNF138/NARF is overexpressed in glioma cell lines and human tissues [19,20], implicating it as a putative oncogene.

In this study we employed a yeast two-hybrid screen to identify eight putative G2E3-interacting proteins. Using GST-pulldown assays, interactions with G2E3 were confirmed for a cell cycle regulatory protein (INCA1), a component of the proteasome (PSMB4), and two RING finger ubiquitin ligases (DZIP3/hRUL138 and RNF138/NARF). Using deletion mutants of G2E3 and RNF138/NARF, we demonstrate that their interaction is mediated by the N-terminal region of G2E3 and the C-terminus of RNF138/NARF. We further demonstrate that G2E3 is a substrate for the E3 activity of RNF138/NARF both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Construct preparation

A full length cDNA for RNF138/NARF was obtained from OpenBiosystems. Deletion mutagenesis of G2E3 and RNF138/NARF, point mutagenesis of RNF138/NARF, and cloning into the indicated vectors was performed using standard methodology. Point mutations (cysteine34→alanine/histidine36→alanine) in RNF138/NARF were introduced by PCR using mutant primers RNF-CHA-F (5'- GGCCGCTCAGGCCGTTTTCTGTAG) and RNFCHA-R (5'- AACGGCCTGAGCGGCCGTGGTC). All constructs were sequenced to confirm that no unexpected mutations were present.

Yeast two hybrid screening

Yeast two-hybrid screening was performed using the Matchmaker system and reagents (BD Biosciences). Full length G2E3 was cloned in-frame with the Gal4 DNA binding domain in the pAS2-1 plasmid to generate G2E3/pAS2-1. A human testis cDNA library (500μg) in the pACT2 vector was co-transformed with 1 mg of G2E3/pAS2-1into the yeast strain AH109. The transformants were grown in SD-Leu-Trp media (SD without leucine and tryptophan) to exponential phase and then plated on SD agar-Leu-Trp-His-Ade (SD agar without leucine, tryptophan, histidine and adenine) with 3AT (3-amino-1,2,4-triazole). Plates were incubated at 30ºC for 3-5 days. Large colonies were re-streaked on fresh plates and a colony lift assay was performed to test for β-galactosidase activity. Plasmid DNA was isolated from each blue colony and transformed into *E.coli* for sequence analysis. Plasmids with cDNAs cloned in-frame with the Gal4 activation domain were identified as putative interacting proteins. To confirm these interactions using yeast, each was subcloned in-frame with Gal4 activation domain of GAD10 plasmid and then co-transformed with G2E3/pAS2-1 into the yeast strain Y187.

Recombinant Protein Purification and GST Pull Down

cDNAs for four of the putative G2E3-interacting proteins were subcloned into pGEX-2T then transformed into a competent BL21(DE3) strain of *E. coli*. Following induction with IPTG, each recombinant GST-tagged protein was purified using glutathione sepharose 4B according to manufacturer's instructions.

For GST pull down assays, full length G2E3 and deletion mutants were subcloned into pCMV10-3XFLAG. Each was transiently transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen). Twenty-four hours following transfection, cells were lysed in mammalian cell lysis buffer [10] and cleared by centrifugation. One mg of each lysate was incubated with 50 μg of GST-tagged protein and 100 μL of glutathione-sepharose 4B slurry for 1 hour at 4°C. Sepharose beads were washed extensively with lysis buffer then boiled 5 min in protein sample buffer to elute co-purifying proteins. Proteins were separated by SDS-PAGE, transferred overnight to nitrocellulose and immunoblotted with the indicated antibody.

Fluorescent microscopy

RNF138/NARF cDNA was subcloned into pEGFP-C3 (Clontech) to generate RNF138/NARF-GFP. RNF138/NARF-GFP and G2E3-GFP [11] were transiently transfected into Cos-7 cells using Lipofectamine 2000 (Invitrogen). Protein localization was assayed for EGFP fluorescence using an Olympus AX70 fluorescent microscope as previously described [11]. Images were assembled in Adobe Photoshop.

Cell Synchronization and northern Blotting

HeLa cells synchronization, analysis by flow cytometry, and Northern blotting were performed as previously described [10]. For Northern blotting, a ^{32}P-labeled RNF138/NARF probe was generated using the Megaprime Labeling Kit according to manufacturer's recommendation. Multiple tissue Northern blots were obtained from Clontech. A ^{32}P-labeled GAPDH probe was used as an internal control for loading.

Ubiquitin ligase assays

For G2E3 ubiquitination assays, 750 ng GST, G2E3$^{(2-373)}$-GST, or G2E3$^{(334-706)}$-GST HECT domain were used as substrate with 1 μg GST-tagged wild-type or RING mutant (C34A/H36A) RNF138/NARF, 1 μg UbcH5a, 100 ng human E1, 6XHis-ubiquitin and assay buffer (50mM Tris-7.5, 2.5mM $MgCl_2$, 0.5mM dithiothreitol, 300 μM ATP, and 1X Energy Regeneration System). Reactions were incubated for 1.5 hours at 30°C then terminated by boiling in an equal volume of 2X protein sample buffer. SDS-PAGE and immunoblotting were performed as described above. Ubiquitinated proteins were detected with an anti-His antibody.

To analyze *in vivo* ubiquitination, HEK293T cells were transfected with G2E3-GFP, 6XHis-myc-ubiquitin, and either empty FLAG vector or the same vector directing expression of wild-type or RING mutant RNF138/NARF. Twenty-four hours later, G2E3 was immunoprecipitated using an anti-GFP antibody and protein A-sepharose. Following washing, proteins were separated by SDS-PAGE and Western blotting was performed with the indicated antibodies.

RESULTS AND DISCUSSION

G2E3 physically interacts with several proteins from human testis

G2E3 has cell cycle regulated and DNA damage responsive

expression [10] and has been shown to re-localize from the nucleolus to the nucleoplasm in response to genotoxic stress [11] suggesting that it may play a role in the control of cell division or the cellular response to DNA damage. In order to gain a better understanding of the role that G2E3 may play in these processes, we conducted a yeast two-hybrid screen using full length G2E3 fused with the Gal4 activation domain. Since G2E3 mRNA is most highly expressed in human testis (unpublished data), we screened a human testis cDNA library fused with a Gal4 DNA binding domain (pACT2) for potential interacting partners in the yeast strain AH109. We screened approximately $2x10^6$ clones using Trp/Leu/His/Ade selection. Colonies surviving this selection were screened for β-galactosidase activity, a final screen to increase the stringency of the selection process. Eight of these clones were not analyzed further because the cDNA insert was not in the correct reading frame (6) or the insert was genomic DNA rather than cDNA (2). The remaining eight cDNAs are listed in Table 1 along with their reported function. The putative G2E3-interacting proteins identified in this screen are also shown schematically in Figure 1. The portion of each cDNA that was isolated in our screen is shown as a bold line with the remaining full-length cDNA shown as dotted lines. Recognized domains in each protein are shown diagrammatically.

Four of the eight cDNAs identified in this screen were selected for additional analysis because of their role in cell cycle regulation (INCA1) [21,22], protein ubiquitination (DZIP3/hRUL138 and RNF138/NARF) [18-20,23,24], or proteasome-mediated protein degradation (PSMB4) [25]. These cDNA fragments were cloned into a bacterial expression vector to allow expression and purification of GST-tagged recombinant proteins. Each of these GST-tagged proteins was incubated with cell lysates from HEK293T cells expressing FLAG-tagged G2E3. The interaction between each of these four cDNAs was confirmed in this experiment (Figure 2) using GST alone as a negative control. We have not attempted to confirm the interaction between G2E3 and the four remaining putative interacting proteins, but at least two of these may represent artifactual interactions since their mRNAs are very highly expressed (ribosomal protein L18 and eIF3 subunit 4) and therefore disproportionately represented in the cDNA library.

The interaction of G2E3 with other proteins involved in the ubiquitin-proteasome system could indicate that G2E3 may be part of a multi-protein complex involved in protein degradation. Alternatively, G3E3 could serve as either the substrate of or ligase for these other E3 proteins. The interactions of G2E3 with

Table 1:

Putative G2E3-Interacting Protein	Abbreviation	Reported Function
Ribosomal Protein L18	RPL18	Translation
Inhibitor of CDK interacting with cyclin A1	INCA1	Cyclin/ CDK inhibitor
RING finger protein 138/NLK associated RING finger	RNF 138/ NARF	Ubiquitin ligase
Proteasome subunit beta 4	PSMB4	Proteasome mediated proteolysis
MGC72075	MGC72075	Unknown
Eukaryotic initiation factor 3 subunit 4	EIF3S4	Translational Initiation
Chromosome 2 open reading frame 29	C2orf29	Unknown
DAZ-interacting protein 3	DZIP3/hRL138	Ubiquitin ligase

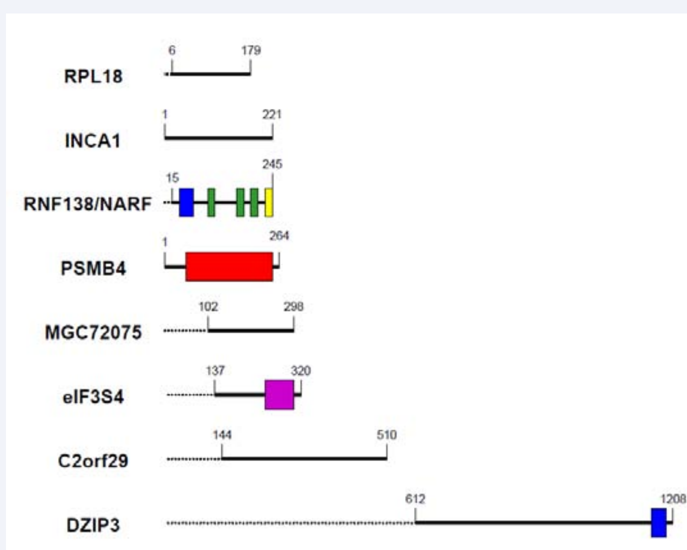

Figure 1 Putative G2E3 interacting proteins. Eight putative G2E3 interacting proteins were identified in a yeast two-hybrid screen of approximately 2 X 10^6 clones from a testis cDNA library. Each of these was confirmed using the yeast strain Y187. The portion of each protein encoded by the cDNA fragment isolated in our screen is numbered and indicated as a bold line. In most cases, the truncated cDNA encoded a protein with a deletion of a portion of the N-terminus, as shown with a dotted line. Several domains are present in one or more proteins including RING domains (blue), C2H2 and C2HC zinc fingers (green), a ubiquitin interacting motif (UIM, yellow), and a RNA recognition motif (RRM, purple).

RNF138/NARF and INCA1, both of which play roles in cell cycle regulation, were especially interesting as G2E3 appears to also be involved in the cell cycle. INCA1 is a cyclin-dependent kinase inhibitor that interacts with several cyclin proteins [22]. It is down-regulated in some human leukemias. We chose to focus our additional studies, however, on RNF138/NARF since its depletion, like G2E3, increases cellular apoptosis [19].

The N-terminus of G2E3 interacts with the C-terminus of RNF138/NARF

We prepared full-length GST-tagged RNF138/NARF as well as similar GST-tagged C-terminal and N-terminal deletion mutants (residues 2-122 and 123-245) using a bacterial expression system. These constructs are shown in a schematic in Figure 3A. As expected the negative control (GST alone) failed to interact with G2E3-FLAG while the positive control (RNF138/NARF-GST) pulled down the G2E3-FLAG very effectively (Figure 3A, lower panel). No interaction between G2E3 and the N-terminus of RNF138/NARF was detected, but this mutant was expressed very poorly. The C-terminus of RNF138 retained full capacity for interaction with G2E3. This region of the protein includes 2 zinc finger domains and the UIM. From this experiment, it is not possible to determine if the RNF138/NARF N-terminus can bind to G2E3, but it clearly shows that the C-terminus is sufficient for the proteins to form a stable physical interaction.

We next performed the reciprocal experiment to identify the region of G2E3 responsible for interaction with RNF138/NARF. Three G2E3 deletion mutants (shown schematically in Figure 3B, upper panel) were expressed as FLAG-tagged proteins in HEK293T cells and assayed for binding to full-length RNF138/NARF-GST. An immunoblot demonstrates that each G2E3 deletion mutant is expressed at similar levels in HEK293T cells (Figure 3B, lower panel). GST-pulldown was performed to identify which mutant(s) are capable of binding to RNF138/NARF-GST. As shown in the lower right panel of Figure 3B, only the N-terminal portion of G2E3 ($G2E3^{(2-235)}$) is co-purified in this assay suggesting that the RNF138/NARF-interacting domain resides entirely within this region of G2E3, which includes the first 2 PHD/RING domains of the protein.

Finally, we sought to confirm the results of these two deletion mutant analyses using only the two interacting regions. As shown in Figure 3C, the interacting region from RNF138/NARF (residues 123-245) was sufficient to pull-down the interacting domain from G2E3 (residues 2-235). This experiment confirms that these respective regions of G2E3 and RNF138/NARF are sufficient for physical interaction.

RNF138/NARF and G2E3 are each nuclear proteins

If the interaction between G2E3 and RNF138/NARF is physiologically relevant, the two proteins should be found in similar locations within mammalian cells. Therefore, we expressed G2E3-GFP and RNF138/NARF-GFP to determine if they have an overlapping pattern of intracellular localization. We previously demonstrated that G2E3 is primarily localized to the nucleus in cultured cells [19]. As shown in Figure 4, both G2E3-GFP and RNF138/NARF-GFP are localized to the nucleoplasm in Cos-7 cells, supporting the legitimacy of the G2E3-RNF138/NARF interaction. As previously shown, G2E3 has at least two distinct nuclear localization signals (NLSs) [19]. Although RNF138/NARF does not have a prototypic NLS, there are regions rich in basic residues that may direct its nuclear import. The localization of RNF138/NARF to the nucleoplasm differs from the related RNF125/TRAC-1, which is membrane bound [13] suggesting that these proteins are unlikely to have overlapping functions.

RNF138/NARF is cell cycle regulated and expressed in cultured cell lines

Because RNF138/NARF and G2E3 physically interact and the latter protein is expressed in a cell cycle phase-specific pattern, we hypothesized that RNF138/NARF would also have G_2/M-specific expression. To test this, Northern blotting was performed using RNA harvested from synchronized HeLa cells. Flow cytometric analysis demonstrated synchronous cell cycle progression of cells after release from a double thymidine block (Figure 5A). Northern blotting demonstrated that multiple splice forms of RNF138/NARF mRNA are specifically expressed as the cells near completion of mitosis (Figure 5B), similar to that previously seen for G2E3 [10]. Four different transcripts were detected, as expected based on the existence of two known splice variants that affect protein sequence (examples are NM_016271 and NM_198128) and two that alter the poly-adenylation site (examples are ESTs AA079189.1 and BU536063.1). This indicates that like G2E3, RNF138/NARF mRNA expression is regulated in a cell cycle-phase specific manner.

To determine tissues and cell lines in which RNF138/NARF is

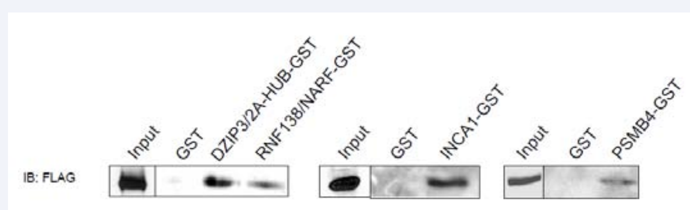

Figure 2 Confirmation of the interaction between G2E3 and proteins identified in the yeast two-hybrid screen. One milligram of a lysate prepared from HEK293T cells overexpressing FLAG-tagged G2E3 was incubated with each recombinant GST-tagged protein or GST control protein (50 µg each). GST-tagged proteins and associated proteins were purified using glutathione sepharose and extensive washing. Precipitated proteins were immunoblotted using a FLAG monoclonal antibody. Each of the four GST-tagged proteins (DZIP3/2A-HUB, RNF138/NARF, INCA1, and PSMB4) formed a stable interaction with G2E3-FLAG as compared with the GST control. 100 µg of the HEK293T cell lysates was also subjected to FLAG immunoblotting (labeled as Input) to determine protein interaction efficiency.

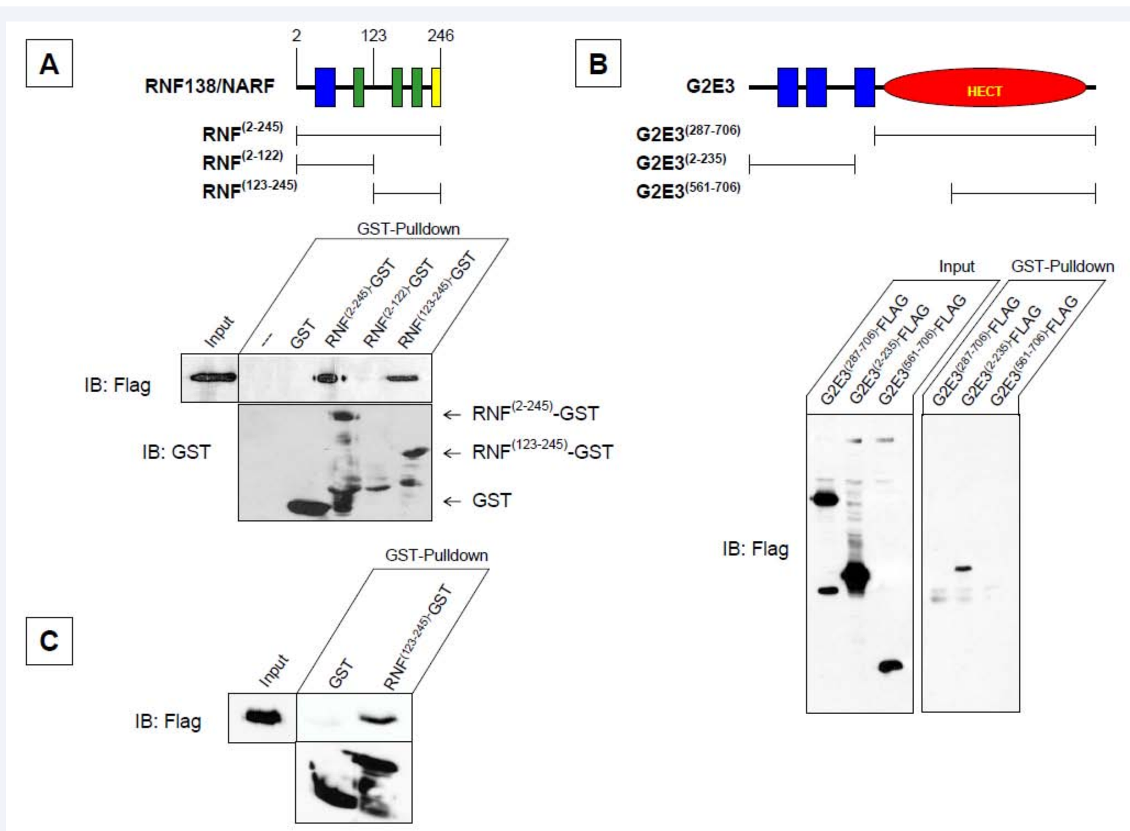

Figure 3 The N-terminus of G2E3 binds to the C-terminus of RNF138/NARF. Full-length and N- and C-terminal deletion mutants of RNF138/NARF were expressed and purified using glutathione beads. Purified proteins were incubated with HEK293T cell lysates over-expressing FLAG-tagged G2E3 or G2E3 deletion mutants. A. Full-length RNF138/NARF-GST (amino acids 2-245) and two deletion mutants (amino acids 2-122 and 123-245) are shown schematically in the upper panel. The purified proteins were incubated with HEK293T cell lysates expressing G2E3-FLAG. In the lower panel, efficient co-purification of G2E3-FLAG with both full-length RNF138/NARF (labeled $RNF^{(2-245)}$) and the C-terminus of RNF138/NARF (labeled $RNF^{(123-245)}$) is observed. No co-purification is observed with the negative control (GST) or the N-terminus of RNF138/NARF (labeled $RNF^{(2-122)}$. GST immunoblotting demonstrates that the RNF138/NARF N-terminus (residues 2-122) is very poorly expressed but similar amounts of all other proteins were used in this pulldown assay. B. Three G2E3 deletion mutants used in these assays are shown schematically in the upper panel. These were expressed as FLAG-tagged proteins in HEK293T cells. In the lower left panel, expression levels of the FLAG-tagged proteins are shown to be similar. Lysates expressing each mutant were tested for binding to RNF138/NARF-GST by GST pulldown (lower right blot). Efficient co-purification is observed only with the G2E3 N-terminus. C. The G2E3-interacting portion of RNF138/NARF (residues 123-245) was used to assess interaction with the RNF138/NARF-interacting portion of G2E3 (residues 2-235). $RNF138/NARF^{(123-245)}$-GST was incubated with a lysates from HEK293T cells expressing $G2E3^{(2-235)}$–FLAG. An efficient interaction between the N-terminus of G2E3 and the C-terminus of RNF138/NARF is observed in this assay.

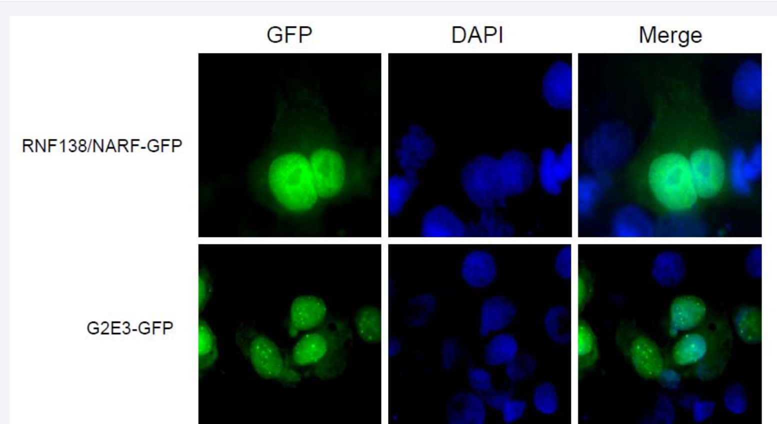

Figure 4 RNF138/NARF and G2E3 are localized to the nucleus. RNF138/NARF and G2E3 were expressed separately as GFP fusion proteins to define the subcellular localization of the proteins. Cells were stained with DAPI and analyzed by fluorescence microscopy.

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expressed, we probed multiple tissue northern blots (Clontech) with an RNF138/NARF probe (Figure 5C). As was seen in synchronized HeLa cells, four mRNA transcripts were detected. RNF138/NARF showed very low expression in organs other than testis and thymus where high and moderate expression was detected, respectively. We also examined transcript levels in a variety of malignant cell lines. Unlike normal tissue, numerous cell lines expressed moderate levels of RNF138/NARF mRNA and several (K562 chronic myelogenous leukemia, SW480 colon carcinoma, and A549 lung carcinoma cells) expressed high levels of the mRNA. These data correlate with that which has indicated RNF138/NARF is overexpressed in malignant gliomas [19,20]. The heightened expression of RNF138/NARF in malignant tissues indicates that it likely plays a positive role in cell cycle progression or the prevention of apoptosis.

G2E3 is a substrate for the RNF138/NARF ubiquitin ligase

Since G2E3 and RNF138/NARF physically interact and are expressed in a similar temporal pattern and subcellular location, we hypothesized that RNF138/NARF would serve as an ubiquitin ligase for G2E3. We first tested the ability of RNF138/NARF to poly-ubiquitinate G2E3 *in vitro*. For these experiments, we used G2E3 deletion mutants expressed in bacteria as GST fusion proteins. RNF138/NARF catalyzes extensive polyubiquitination of the N-terminus of G2E3 (G2E3$^{(2-373)}$) *in vitro* (Figure 6A) but does not ubiquitinate the G2E3 C-terminus (G2E3$^{(334-706)}$) or the GST negative control. This correlates with the data indicating that RNF138/NARF interacts with the N-terminus of G2E3 (Figure 3B). Although G2E3 can auto-ubiquitinate when expressed in Sf9 cells [12], this activity is very weak or absent when the protein is expressed in bacteria (unpublished data). However, to formally rule out the possibility that the RNF138/NARF-mediated ubiquitination of G2E3 is actually auto-ubiquitination of G2E3 or alternatively that G2E3 is ubiquitinating RNF138/NARF in this reaction, we performed an additional experiment comparing *in vitro* ubiquitination of G2E3$^{(2-373)}$ by wild-type and RING mutant RNF138/NARF. As expected, RNF138/NARF catalyzes extensive poly-ubiquitination of G2E3$^{(2-373)}$ while RNF138/NARF(C34A/H36A) has no such activity (Figure 6B), confirming that RNF138/NARF ubiquitinates G2E3 *in vitro* in a RING-dependent manner.

To determine if RNF138/NARF ubiquitinates G2E3 *in vivo*, we co-expressed myc-tagged ubiquitin and GFP-tagged G2E3 along with an empty FLAG-tagged expression vector or the same expression vector directing expression of either wild-type or RING-mutant RNF138/NARF. In each case, the GFP-tagged

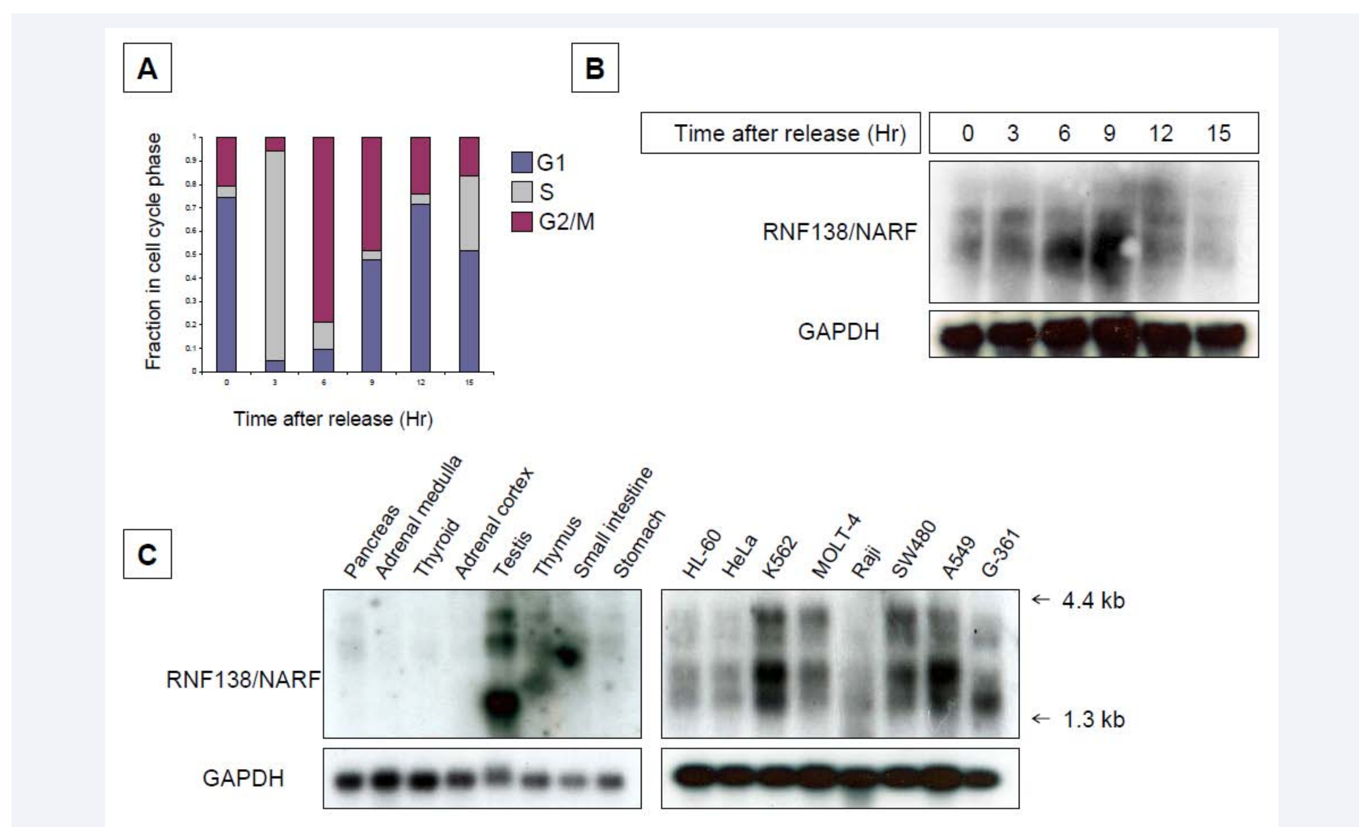

Figure 5 RNF138/NARF is expressed in a cell cycle dependent manner and expressed in cancer cell lines. HeLa cells were synchronized using a double-thymidine block and release and then harvested for analysis by flow cytometry and Northern blotting. A. Synchronous progression through the cell cycle is observed with most cells completing mitosis and entering G1-phase between 6 and 12 hours after release from the second thymidine block. B. Northern blotting reveals maximal accumulation of several RNF138/NARF transcripts at 9 hours after release. C. Multiple tissue Northern blots were probed with RNF138/NARF and GAPDH probes. There is no detectable RNF138/NARF mRNA in most tissues examined other than testis and thymus. Numerous cancer cell lines express easily detectable levels of RNF138/NARF mRNA. GAPDH blotting demonstrates similar loading in these blots.

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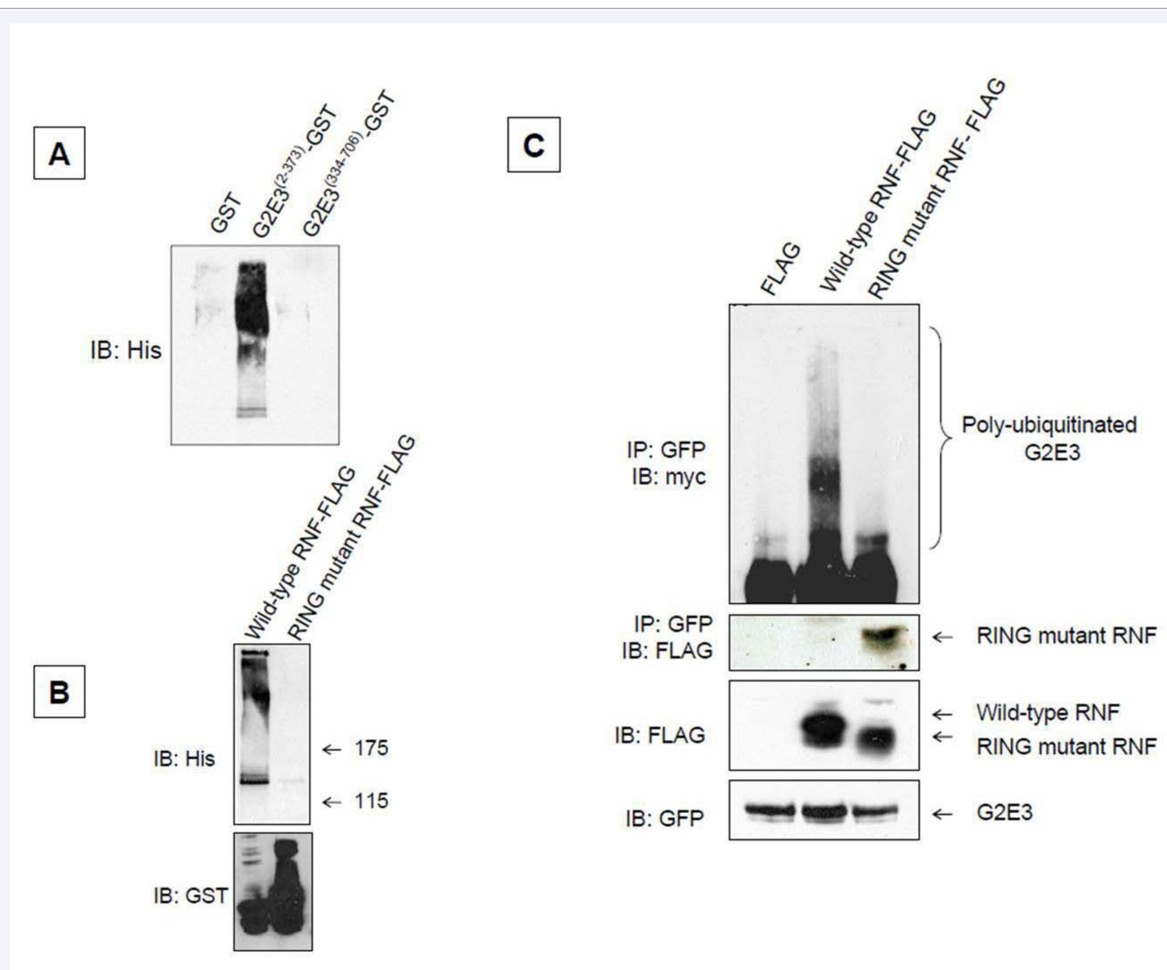

Figure 6 RNF138/NARF is an ubiquitin ligase for G2E3 *in vivo* and *in vitro*. A. Ubiquitination reactions were performed using E1, 6XHis-ubiquitin, UbcH5a, and RNF138/NARF-FLAG. GST and GST-tagged G2E3 deletion mutants were used as substrates in the ubiquitination reaction performed *in vitro*. After the reaction was performed, the reaction was terminated by boiling in SDS and then diluted. Poly-ubiquitinated proteins were detected using anti-His immunoblotting. RNF138/NARF-FLAG catalyzes extensive poly-ubiquitination of the N-terminus of G2E3 but does not ubiquitinate the G2E3 C-terminus or the GST negative control. B. Wild-type and RING-mutant (C34A/H36A) RNF138/NARF were tested for their ability to polyubiquitinate G2E3 *in vitro* using the same reaction conditions as before. Inactivation of the RING completely abrogates poly-ubiquitination of G2E3 by RNF138/NARF. C. Expression plasmids for G2E3-GFP and myc-ubiquitin were co-transfected into HEK293T cells along with either empty FLAG expression vector, wild-type RNF138/NARF-FLAG, or RING-mutant RNF138/NARF. Cells were lysed 36 hours later and GFP tagged proteins were immunoprecipitated from 1 mg of cell lysate. The immunoprecipitated proteins were immunoblotted for myc to detect poly-ubiquitinated FLAG-tagged proteins (upper panel). After stripping, the membranes were re-probed for FLAG-tagged proteins that co-immunoprecipitate with G2E3-GFP (second panel from top). Whole cell lysates (100 µg) were also probed with FLAG (third panel from top) and GFP (bottom panel) to confirm expression levels of RNF138/NARF and G2E3 proteins.

G2E3 was immunoprecipitated and ubiquitinated proteins were detected by immunoblotting for myc-tagged ubiquitin. As shown in the top panel of Figure 6C, expression of RNF138/NARF catalyzed poly-ubiquitination of G2E3, while the negative control and RING mutant RNF138/NARF did not. The same blot was re-probed with an anti-FLAG antibody to identify proteins that co-immunoprecipitate with G2E3-GFP. Since the interaction between RNF138/NARF and G2E3 results in the ubiquitination and degradation of the latter protein, the physical interaction between the two proteins (as represented by their co-immunoprecipitation) appears to be greatly enhanced by inactivation of the RNF138/NARF RING domain. As a control, total lysates were probed for RNF138/NARF-FLAG and G2E3-GFP to confirm that differences in co-immunoprecipitation and G2E3 ubiquitination were a result of inactivation of the RING domain rather than differences in protein expression. Note that the RNF138/NARF RING mutant protein consistently migrates slightly faster than the wild-type protein on SDS-PAGE.

CONCLUSIONS

In this study, we have identified and confirmed by yeast two-hybrid and GST-pulldown assay four proteins physically interacting with G2E3: RNF138/NARF, INCA1, PSMB4, and DZIP3/hRUL138. We mapped the interaction domains of G2E3 and RNF138/NARF to the N- and C-terminus, respectively. We have shown that both G2E3 and RNF138/NARF primarily localize to the nucleus and that RNF138/NARF like G2E3 is cell cycle regulated with maximal expression during the G_2/M cell cycle

phase. Furthermore, our data indicates that G2E3 is a substrate for the E3 ubiquitin ligase function of RNF138/NARF.

We hypothesize that the ubiquitination of G2E3 by RNF138/NARF serves as a signal for its proteasome-dependent degradation. While we have made attempts to demonstrate a decline in G2E3 stability in the presence of RNF138/NARF, we have failed to observe such a decline due to the absence of a suitable antibody to detect endogenous G2E3. It is possible; however, that RNF138/NARF-mediated ubiquitination of G2E3 may play a different role in the protein's regulation such as altering its own enzymatic activity or localization. It appears that G2E3 and RNF138/NARF may both promote cell survival since knockdown of RNF138/NARF leads to cell cycle arrest and apoptosis [19] and G2E3 insufficiency results in apoptosis of the embryonic inner cell mass [12]. This commonality in function of the proteins supports a role for G2E3 ubiquitination by RNF138/NARF that is distinct from degradation. The proteins could work cooperatively to promote cell survival and/or cell cycle progression.

In this work, we have focused on the role of RNF138/NARF in the regulation of G2E3. It is also possible, however, that the converse may also be true. Since G2E3 is an ubiquitin ligase with 2 distinct E3 domains, it is possible that one or both may catalyze the poly-ubiquitination of RNF138/NARF, thereby counteracting the negative regulation of β-catenin signaling imposed by RNF138/NARF. Future experiments will evaluate the possibility that G2E3 positively regulates Wnt signaling. Inactivation of G2E3 causes early embryonic lethality [12], a different phenotype than is observed for knockout of Wnt genes [26]. Therefore, even if G2E3 does modulate Wnt signaling, it likely influences other distinct pathways as well.

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REFERENCES

1. Huibregtse JM, Scheffner M, Beaudenon S,Howley PM. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc Natl Acad Sci U S A. 1995; 92: 5249.

2. Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, Weissman AM. RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. Proc Natl Acad Sci U S A. 1999; 96: 11364-11369.

3. Dul BE, Walworth NC. The plant homeodomain fingers of fission yeast Msc1 exhibit E3 ubiquitin ligase activity. J Biol Chem. 2007; 282: 18397-18406.

4. Aravind L, Koonin EV. The U box is a modified RING finger - a common domain in ubiquitination. Curr Biol. 2000; 10: R132-134.

5. Yamanaka A, Hatakeyama S, Kominami K, Kitagawa M, Matsumoto M, Nakayama K. Cell cycle-dependent expression of mammalian E2-C regulated by the anaphase-promoting complex/cyclosome. Mol Biol Cell. 2000; 11: 2821-2831.

6. Linares LK, Kiernan R, Triboulet R, Chable-Bessia C, Latreille D, Cuvier O, et al. Intrinsic ubiquitination activity of PCAF controls the stability of the oncoprotein Hdm2. Nat Cell Biol. 2007; 9: 331-338.

7. einstein J. Cell cycle-regulated expression, phosphorylation, and degradation of p55Cdc. A mammalian homolog of CDC20/Fizzy/slp1. J Biol Chem. 1997; 272: 28501-28511.

8. Margottin-Goguet F, Hsu JY, Loktev A, Hsieh HM, Reimann JD, Jackson PK. Prophase destruction of Emi1 by the SCF(betaTrCP/Slimb) ubiquitin ligase activates the anaphase promoting complex to allow progression beyond prometaphase. Dev Cell. 2003; 4: 813-826.

9. Guardavaccaro D, Kudo Y, Boulaire J, Barchi M, Busino L, Donzelli M, et al. Control of meiotic and mitotic progression by the F box protein beta-Trcp1 in vivo. Dev Cell. 2003; 4: 799-812.

10. Crawford DF, Piwnica-Worms H. The G(2) DNA damage checkpoint delays expression of genes encoding mitotic regulators. J Biol Chem. 2001; 276: 37166-37177.

11. Brooks WS, Banerjee S, Crawford DF. G2E3 is a nucleo-cytoplasmic shuttling protein with DNA damage responsive localization. Exp Cell Res. 2007; 313: 665-676.

12. Brooks WS, Helton ES, Banerjee S, Venable M, Johnson L, Schoeb TR, et al. G2E3 is a dual function ubiquitin ligase required for early embryonic development. J Biol Chem. 2008; 283: 22304-22315.

13. Giannini AL, Gao Y,Bijlmakers MJ. T-cell regulator RNF125/TRAC-1 belongs to a novel family of ubiquitin ligases with zinc fingers and a ubiquitin-binding domain. Biochem J. 2008; 410: 101-111.

14. Capon F, Bijlmakers MJ, Wolf N, Quaranta M, Huffmeier U, Allen M, et al. Identification of ZNF313/RNF114 as a novel psoriasis susceptibility gene. Hum Mol Genet. 2008; 17: 1938-1945.

15. Bijlmakers MJ, Kanneganti SK, Barker JN, Trembath RC, Capon F. Functional analysis of the RNF114 psoriasis susceptibility gene implicates innate immune responses to double-stranded RNA in disease pathogenesis. Hum Mol Genet. 2011; 20: 3129-3137.

16. Arimoto K, Takahashi H, Hishiki T, Konishi H, Fujita T, Shimotohno K. Negative regulation of the RIG-I signaling by the ubiquitin ligase RNF125. Proc Natl Acad Sci U S A. 2007; 104: 7500-7505.

17. Shoji-Kawata S, Zhong Q, Kameoka M, Iwabu Y, Sapsutthipas S, Luftig RB, et al. The RING finger ubiquitin ligase RNF125/TRAC-1 down-modulates HIV-1 replication in primary human peripheral blood mononuclear cells. Virology. 2007; 368: 191-204.

18. Yamada M, Ohnishi J, Ohkawara B, Iemura S, Satoh K, Hyodo-Miura J, et al. NARF, an nemo-like kinase (NLK)-associated ring finger protein regulates the ubiquitylation and degradation of T cell factor/lymphoid enhancer factor (TCF/LEF). J Biol Chem. 2006; 281: 20749-20760.

19. Zhou YX, Chen SS, Wu TF, Ding DD, Chen XH, Chen JM, et al. A novel gene RNF138 expressed in human gliomas and its function in the glioma cell line U251. Anal Cell Pathol (Amst). 2012; 35: 167-178.

20. Anderson TW, Wright C,Brooks WS. The E3 Ubiquitin Ligase NARF Promotes Colony Formation in vitro and Exhibits Enhanced Expression Levels in Glioblastoma Multiforme in vivo. Am J Undergrad Res. 2010; 9: 23-30.

21. Diederichs S, Bäumer N, Ji P, Metzelder SK, Idos GE, Cauvet T, et al. Identification of interaction partners and substrates of the cyclin A1-CDK2 complex. J Biol Chem. 2004; 279: 33727-33741.

22. Bäumer N, Tickenbrock L, Tschanter P, Lohmeyer L, Diederichs S, Bäumer S, et al. Inhibitor of cyclin-dependent kinase (CDK) interacting with cyclin A1 (INCA1) regulates proliferation and is repressed by oncogenic signaling. J Biol Chem. 2011; 286: 28210-28222.

23. Zhou W, Zhu P, Wang J, Pascual G, Ohgi KA, Lozach J, et al. Histone H2A monoubiquitination represses transcription by inhibiting RNA polymerase II transcriptional elongation. Mol Cell. 2008; 29: 69-80.

24. Kreft SG, Nassal M. hRUL138, a novel human RNA-binding RING-H2 ubiquitin-protein ligase. J Cell Sci. 2003; 116: 605-616.

25. Kopp F, Kristensen P, Hendil KB, Johnsen A, Sobek A, Dahlmann B. The human proteasome subunit HsN3 is located in the inner rings of the complex dimer. J Mol Biol. 1995; 248: 264-272.

26. Wodarz A, Nusse R. Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol. 1998; 14: 59-88.

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