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Functional conservation between mammalian MGRN1 and plant LOG2 ubiquitin ligases

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Abstract

Plant LOSS OF GDU 2 (LOG2) and mammalian MAHOGUNIN RING FINGER 1 (MGRN1) proteins are RING-type E3 ligases sharing similarity N-terminal to the RING domain. Deletion of this region disrupts the interaction of LOG2 with the plant membrane protein GLUTAMINE DUMPER 1 (GDU1). Phylogenetic analysis identified two clades of LOG2/MGRN1-like proteins in vertebrates and plants. The ability of MGRN1 to functionally replace LOG2 was tested. MGRN1 ubiquitylates GDU1 *in vitro* and can partially substitute for LOG2 in the plant, partially restoring amino acid resistance to a *GDU1-myc over-expression, log2-1 background*. Altogether, these results suggest a conserved function for the N-terminal domain in evolution.

Structured summary of protein interactions

GDU1 physically interacts	with **rnMGRN1**	by two hybrid (1, 2)
rnMGRN1 binds to **GDU1**	by pull down	(View interaction)
GDU6 physically interacts	with **rnMGRN1**	by two hybrid (1, 2)
GDU3 physically interacts	with **hsMGRN1**	by two hybrid (1, 2)
rnMGRN1 physically interacts	with **GDU7**	by two hybrid (1, 2)
GDU5 physically interacts	with **hsMGRN1**	by two hybrid (1, 2)

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hsMGRN1 physically interacts	with **GDU1**	by two hybrid (1, 2)
rnMGRN1 physically interacts	with **GDU3**	by two hybrid (1, 2)
rnMGRN1 physically interacts	with **GDU5**	by two hybrid (1, 2)
hsMGRN1 physically interacts	with **GDU7**	by two hybrid (1, 2)
GDU6 physically interacts	with **hsMGRN1**	by two hybrid (1, 2)
LOG2 binds to **GDU1**	by pull down (1, 2)	
hsMGRN1 physically interacts	with **GDU4**	by two hybrid (1, 2)
rnMGRN1 physically interacts	with **GDU2**	by two hybrid (1, 2)
GDU4 physically interacts	with **rnMGRN1**	by two hybrid (1, 2)
LOG2 physically interacts	with **GDU1**	by two hybrid (1, 2, 3, 4)
GDU2 physically interacts	with **hsMGRN1**	by two hybrid (1, 2)

Keywords

ubiquitin ligase; membrane trafficking; Mahogunin; GDU1; LOG2

Introduction

Ubiquitylation, the post-translational ligation of ubiquitin (Ub) to intracellular proteins, is a modification by which robust and specific changes in protein longevity, activity, and localization can be attained in eukaryotes. After thioesterification by an activating enzyme (E1) and transfer to a conjugating enzyme (E2), Ub ligases (E3s) bind protein substrates and E2-Ub thioesters in a manner conducive to substrate ubiquitylation [1]. Both 26S proteasome- and lysosome/vacuole-dependent protein degradation, as well as non-proteolytic protein inhibition and activation, are facilitated by specific E3s [2]. RING (Really Interesting New Gene) proteins, characterized by a two-zinc cysteine/histidine-coordinated cross-brace motif (the RING domain) that interacts with E2s, are a diverse E3 family instrumental in numerous biological processes, ranging from protein quality control to development of the central nervous system [3,4].

MAHOGUNIN RING FINGER 1 (MGRN1) is a RING-type E3 in mammals that suppresses signaling through the G protein-coupled melanocortin receptors-1 (MC1R), -2 (MC2R) and -4 (MC4R) through ubiquitylation-dependent and -independent processes [5-7]. In mice, a MGRN1 loss-of-function allele (*mahoganoid, md*^{nc}) fails to suppress MC1R and MCR3,4 signaling in the follicle and hypothalamus, respectively, resulting in leaner mice with a darker coat color [8,9]. MGRN1 also plays a role in membrane trafficking: TSG101, a subunit of Endosomal Sorting Complex Required for Transport-1 (ESCRT-1) that drives endosome maturation in eukaryotes [10], interacts with and is ubiquitylated by MGRN1 [3,11]. While MGRN1 ligase activity is necessary for signaling through MCRs for pigment-type switching, interaction with TSG101 is not [7]. As the function of MGRN1 in pigment switching becomes clearer, additional substrates of MGRN1 may be identified [5,7].

We previously characterized LOSS OF GDU1 2 (LOG2), an *Arabidopsis thaliana* RING E3 ligase, and noted a region of amino acid similarity to MGRN1 [12]. In plants, LOG2

physically interacts with and ubiquitylates the plant protein GLUTAMINE DUMPER1 (GDU1). Characterized by a membrane domain and the family signature amino acid motif Val-Ile-Met-Ala-Gly (VIMAG), GDU1 belongs to a family of proteins whose over-expression promotes amino acid export from plant cells [13,14]. Mutations in LOG2 suppress all phenotypes associated with GDU1 over-expression, suggesting that LOG2 and GDU1 are involved in the same process [12]. In addition, more roles have been linked to LOG2. *log2* loss-of-function plants are hyposensitive to exogenous application of the stress hormone abscisic acid [15], although neither the mechanism nor LOG2's or potentially GDU1's roles in this process are known. Reasoning that sequence similarity between MGRN1 and LOG2 might beget similar function, we compared LOG2 and MGRN1 protein properties. Our results underscored a shared functionality between the two proteins, likely conferred by regions common to LOG2/MGRN1 family proteins.

Materials and Methods

DNA constructs

Rat MGRN1 coding sequence was amplified by RT-PCR from IMAGE clone 7134018, cloned into pDONR201 (Life Technologies) using the Gateway technology, and recombined into pCDNA3.2/V5DEST (Life Technologies) and pCDNA3.2/mCherry [16]. Myristoylation-inhibited $MGRN1^{G2A}$ was procured via site-directed mutagenesis with the QuikChange kit (Stratagene). HsMGRN1 was cloned from cDNA from HEK cells and recombined into pDONRZeo. Both human and rat MGRN1 clones were recombined into Gateway pGBT9 and pACT2 yeast-two-hybrid vectors [12]. Deletion variants of LOG2 were created by PCR and transferred to yeast or *E. coli* expression vector by Gateway cloning. For plant expression, RnMGRN1 cDNA in pDONR201 was recombined into pGWUBQ10, a modified pGWB14 plasmid [17] with 1003 bp upstream of *UBQ10* coding region replacing the 35S promoter, and the resulting plasmid introduced into *Agrobacterium tumefaciens* strain AGL1 and then into *Arabidopsis th*aliana ecotype Col GDU-myc over-expressing *log2-2* double homozygous plants by vacuum infiltration [18].

Sequence analyses

Protein sequences were retrieved from Genbank by PSI-BLAST [19] using the DAR2 of LOG2 and MGRN1 as queries. Protein domains and motif were identified by MEME (meme.nbcr.net/) [20]. Sequences were aligned and the phylogenic tree created by MEGA5 [21] (see Supp. Tables 1 and 2 for protein sequences).

Protein-protein interaction assays

Yeast-two-hybrid, GST pull-down, and ubiquitylation assays were performed as described in [12].

Cell Culture and imaging

BHK21 cells (ATCC-CCL10) cells were purchased from ATCC. Cells were cultured in Dulbecco's Modified Eagles Medium supplemented with 5% cosmic calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were cultured at 37°C under 5% CO_2. For expression assays, cells were transfected with 12 μg of each construct per flask (25 cm^2).

For imaging, cells were plated on an 8-well, glass-bottom chamber coated with poly-L-lysine, and transfection was performed with 400 ng MGRN1- and MGRN1^{G2A}-mCherry construct/well (100 mm^2). Cells were imaged 72 h post-transfection by confocal microscopy using the same settings as in [12].

Protein purification and western blotting

Cells were harvested 72 hours after transfection. Total proteins were extracted according to [5]. Membrane fraction purification was based on [22]. Differences in membrane association of MGRN1 and MGRN1^{G2A} were investigated as described in [12].

Plant analysis

Transgenic plants were isolated and the transgene made homozygous in subsequent generations by antibiotic selection. Independent lines were generated and tested for the ability to grow on germination plates supplemented with leucine as previously described using 20 seeds of each line per plate (5 plates total) [14]. The number of arrested seedlings was counted after 10 days of growth. Seedlings were scored as “arrested” if seedlings failed to turn green, failed to produce expanded cotyledons nor had visible true leaves, as previously observed [14]. Results were analyzed by ANOVA in jmp (http://www.jmp.com/).

Results

LOG2 and its 4 paralogs (LUL1-4) from *Arabidopsis thaliana* have been reported to share amino acid similarity with MGRN1 outside of the RING domain, a region referred to as DAR2 (for Domain Associated with RING 2) [12]. To further characterize the similarities, and to define the distribution of LOG2/MGRN proteins among species, sequences from LOG2-like and MGRN1-like proteins were retrieved from Genbank. Overall, 267 proteins from Metazoa and plants were identified (Supp. Table 1), verifying a wide distribution of these proteins in diverse species. Phylogenic reconstruction, performed on DAR2 and RING domain sequences, identified multiple clades among plant and metazoan proteins. While one MGRN1-like gene was found in arthropods and nematodes, two clades, corresponding to MGRN1 and uncharacterized RING FINGER157 (RNF157) proteins, were identified in vertebrates (Supp. Fig. 1). Only MGRN1 vertebrate proteins (but not RNF157 or non-vertebrate homologs) have a conserved PSAP sequence (Fig. 1), previously found necessary for interaction between MGRN1 and TSG101 [11]. Plant genomes also contained LOG2-like encoding genes grouping in 5 clades, two for dicots, and three for monocots. Bootstrap values suggested that one clade was monocot-specific while the two other monocot caldes were related to the two dicot clades.

Conservation of the DAR2 domain in LOG2 and MGRN1 (Supp. Fig. 2) prompted the study of the interaction of this domain between LOG2 and GDU1. Recombinant full-length LOG2 was previously found to associate with the cytosolic domain of GDU1 (cGDU1) in GST pull-down assays and yeast-two-hybrid, and this association required an intact VIMAG motif in GDU1 [12]. To test whether the conserved DAR2 region is required for interaction with GDU1, we examined the affinity of LOG2 truncations for cGDU1 in yeast-two-hybrid and GST pull-down assays. VIMAG motif mutants known to impair association with LOG2

(cGDU1^{m} = G100R and cGDU1$^{\Delta}$= ΔVIMAG; see Fig. 2 and [12]) were included as negative controls. The RING domain and variable LOG2 N-terminal region were dispensable for interaction in yeast (Fig. 2A). Truncations of the DAR2 abolished all detectable interaction in yeast with cGDU1 (Fig. 2A). A LOG2 fragment comprising the DAR2 alone (Δ4) interacted with wild-type cGDU1 but not the VIMAG mutants. In GST pull-down assays, the LOG2-cGDU1 interaction was also tolerant of, but sensitive to, RING finger and DAR2 truncations, respectively (Fig. 2B). Importantly, the LOG2 DAR2 alone could pull down cGDU1. These results indicate the LOG2-GDU1 association is primarily conferred by intact DAR2 of LOG2 and VIMAG region of GDU1.

The question whether DAR2 from MGRN1 could interact with GDU1 was tested as above by yeast two hybrid and GST-pull down assays. Rat (RnMGRN1) and human (HsMGRN1) MGRN1 interacted with Arabidopsis cGDU1-3,5 in yeast in both plasmid conformations (Fig. 3A), as previously observed for Arabidopsis LOG2 [12]. Consistent with the yeast-two-hybrid result, GST-RnMGRN1 interacted with cGDU1 *in vitro*, albeit to a smaller extent than LOG2 (Fig. 3B, left panels). Moreover, the G100R mutation in cGDU1 yielded undetectable interactions (Fig. 3B, right panels). These data demonstrate that multiple mammalian MGRN1 can directly associate with multiple plant cGDU paralogs in a manner dependent on an intact VIMAG motif in GDU1.

Because MGRN1 is an E3 ligase, we next examined whether cGDU1 could serve as a ubiquitylation substrate for RnMGRN1. While a single, unmodified form of cGDU1 was detected in the presence of all ubiquitylation components without an E3, multiple higher molecular mass cGDU1 species were observed in the presence of RnMGRN1 (Fig. 3C). These bands were detected with antibodies against both substrate epitopes and ubiquitin itself, and differences in electrophoretic mobility were consistent with ubiquitin addition (Fig. 3C). Notably, both flag- and HA-cGDU1 were ubiquitylated, indicating the modification is not peculiar to the substrate tag. Given that cGDU1 is also an *in vitro* substrate of LOG2 [12], these data suggest that the mode of cGDU1 binding by RnMGRN1 is similar to that of LOG2.

Both LOG2 and HsMGRN1 have been shown to be myristoylated [12,23]. Myristoylation prediction algorithms suggest that rat MGRN1 could also be myristoylated. While the N-terminal amino acids of myristoylated proteins are somewhat variable, an N-terminal glycine (typically following removal of the initiator methionine) is required for recognition by the myristoylation enzyme N-myristoyl transferase [24]. Therefore, RnMGRN1 and a non-myristoylatable RnMGRN1^{G2A} mutant were expressed in rabbit reticulocyte lysates in the presence of tritiated myristic acid, and protein expression was assessed by fluorographic imaging. While both proteins expressed at similar levels (evidenced by leucine incorporation), only wild-type RnMGRN1 was myristoylated (Supp. Fig. 3). MGRN1 localizes to endosomes and associates with plasma membrane-resident MCRs [3,5], phenomena that may be facilitated by N-myristoylation (reviewed in [25,26]). LOG2^{G2A} mutant was deficient in *in vitro* myristoylation and exhibited weaker membrane anchorage than wild-type LOG2 [12]. To assess the importance of myristoylation in membrane localization, RnMGRN1 and RnMGRN1^{G2A} were fused at their C-termini to mCherry and expressed in hamster cells. Confocal observation of RnMGRN1-mCherry is in agreement

with previous investigations [5,11]. Interestingly, the G2A mutation did not yield dramatic changes in sub-cellular localization, as both proteins were present around the nucleus in punctate structures (Fig. 4A). In a second approach, RnMGRN1 and RnMGRN1^{G2A} were C-terminally V5-tagged and expressed in hamster cells. Microsomes were purified, and the strength of RnMGRN1's membrane affinity was tested by incubation with NaCl, Na_2CO_3, and Triton X-100 detergent, treatments that extract peripheral, luminal, and integral membrane proteins, respectively [27]. RnMGRN1 was partially solubilized only by detergent (Fig. 4B), whereas the G2A mutation exhibited weaker membrane association, being additionally solubilized by NaCl (Fig. 4B). Overall, these data suggest that while dispensable for RnMGRN1 membrane localization *per se*, myristoylation contributes to the affinity of the protein for membranes.

Sequence comparison, interaction with GDU1, and myristoylation suggested that RnMGRN1 could have similar *in vivo* functional properties to LOG2. To test this hypothesis, an *in vivo* assay for MGRN1 function in plants was performed. *gdu1-1D* plants are tolerant to high concentrations of amino acids, while *gdu1-1D log2-2* double mutant plants exhibit growth arrest, i.e. plants germinate but fail to develop expanded, green cotyledons and emergent true leaves after radicle emergence [12]. Similarly, over-expression of a myc epitope-tagged version of GDU1, GDU1-myc, recapitulates the Gdu1D phenotype [12] and confers resistance to exogenous amino acids in a *LOG2*-dependent manner (unpublished). RnMGRN1 was expressed in the *GDU1-myc log2-2* double mutant, and triply homozygous progeny (*GDU1-myc log2-2 RnMGRN1*) were grown on synthetic growth media containing 2.5 mM leucine. Two independent transgenic lines had fewer growth arrested seedlings than the progenitor line, GDU1-myc *log2-2*, but not as many as *GDU1-myc* alone, which had no arrested seedlings (Fig. 5B), suggesting that RnMGRN1 is active in plants and can partially substitute for LOG2 function in plants.

Discussion

We show here that the mammalian MGRN1 and the plant LOG2 share the strongest sequence similarity in the RING domain and DAR2 region, the latter uniquely defining this subfamily of RING proteins. It is important to note that the C-terminal extension of the animal MGRN proteins is very variable within Metazoa: the sequence of the C-terminal domain of the vertebrates is not similar to the C-terminal domain of the arthropods or nematodes, suggesting that this domain has evolved differently within lineages to be endowed with functions distinct from the DAR2. In addition plant and vertebrate genomes contain multiple MGRN1-like genes (Supp. Fig. 2). The RNF157 gene, encoding an uncharacterized protein present in the vertebrate lineage, displays similarity to MGRN1 and LOG2, but its C-terminal domain is not similar to the MGRN1 domain. The sequence similarity of RFN157 with LOG2 and MGRN1 suggests that RNF157 may also be involved in the regulation of membrane protein trafficking.

In addition to sequence similarity, MGRN1 and LOG2 are functionally similar. Both proteins associate with the plasma membrane and are endomembrane-resident [3,5,12] and proper localization requires myristoylation. In addition, MGRN1 and LOG2 play important roles in their native contexts as regulators of membrane protein function. MGRN1 can

control the turnover of melanocortin receptors [6,28], compete with G proteins for binding MCR [5] and ubiquitylate TSG101, a regulator of endosomal trafficking [3]. While LOG2's role is not understood, together with GDU1 it appears to be a necessary activator of a pathway leading to increased amino acid export in plants, possibly by controlling membrane protein turnover [12].

Rat MGRN1 and plant LOG2 share biochemical properties: RnMGRN1 interacts with and ubiquitylates plant GDU1 *in vitro*. The G100R mutation in GDU1, which markedly reduces association with LOG2 [12], inhibits interaction with MGRN1 in yeast-two-hybrid and pull-down assays (Fig. 2), suggesting that the interaction of GDU1 and MGRN1 is similarly mediated by the VIMAG domain and the DAR2. RnMGRN1, like LOG2 and HsMGRN1 [12,23], is myristoylated *in vitro*. Suppression of myristoylation has different effects on the two proteins: the myristoylation-deficient G2A mutant of MGRN1 is anchored to the membrane with only slightly less affinity than the wild type protein, while $LOG2^{G2A}$ membrane affinity is significantly weakened [12]. Such differences could hint at stronger interaction of MGRN1 with membrane proteins than LOG2, which seems to depend mainly on myristoylation for membrane localization.

Finally, we show that the *log2* null mutation can be complemented by expression of mammalian RnMGRN1, showing that MGRN1 bears most of the functions of LOG2 with respect to the GDU1 over-expression phenotype. This extreme functional conservation suggests a conserved function for the DAR2 and RING domains. While the ubiquitin ligase activity of the two proteins is known, it is worth noting that no *in vivo* function has yet been assigned to the DAR2 of MGRN1 proteins. The conservation of this domain in mammals, nematodes and arthropods suggests it is biologically relevant. The GDU1 VIMAG domain is predicted to have a beta-hairpin fold (data not shown, Robetta *ab initio* prediction [29]), a relatively common supersecondary structural motif. Thus, while no protein similar to GDU1 has been identified outside the plant kingdom, it is possible that proteins with divergent sequences but a similar fold exist in metazoans. Recent work indicates that although the E3 ligase activity of MGRN1 is critical for the regulation of MCR-dependent pigment switching, it is independent of the interaction with TSG101 [7], suggesting that MGRN1 has additional *in vivo* interactors. This as-yet unidentified interactor could bind to sequences within the DAR2 of MGRN1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

cGDU1	cytosolic domain of GDU1
DAR2	DOMAIN ASSOCIATED WITH RING 2
ESCRT	Endosomal sorting complex required for transport
GDU	GLUTAMINE DUMPER
GST	Glutathione-S-Transferase
LOG2	LOSS OF GDU 2
MCR	Melancortin receptors
MGRN1	Mahogunin Ring Finger 1
RING	Really Interesting New Gene
RNF	Ring Finger
VIMAG	Val-Ile-Met-Ala-Gly

Highlights

- Arabidopsis E3 ligase LOG2 interacts with cGDU1 through DAR2, a conserved domain.
- There are two clades of DAR2-containing proteins in both vertebrates and plants.
- Mammalian MGRN1, homologous to plant LOG2, binds to and ubiquitylates GDU1 in vitro.
- MGRN1 functionally complements log2 mutant plants indicating functional conservation.
- MGRN1 may have *in vivo* binding partners with GDU1-like VIMAG motifs.

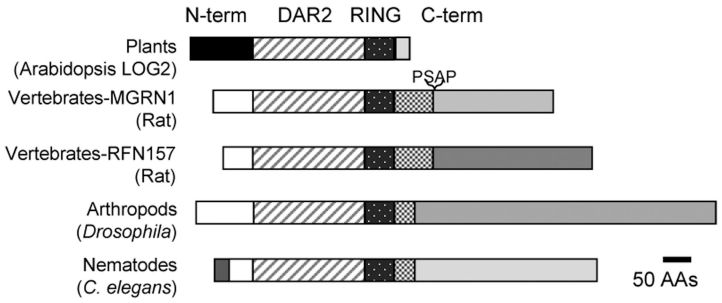

Figure 1. Structure of LOG2/MGRN1-like proteins from selected plants and metazoans (Arabidopsis, rat, *Drosophila* and *Caenorhabditis elegans*)

N-termini of animal proteins are conserved, averaging 70 amino acids (AAs), while the corresponding plant sequences are proline-rich and highly variable in composition and length (47-187 AA). The ∼260 AA DAR2-RING region is conserved between plants and animals and forms the signature of LOG2/MGRN1 proteins. Vertebrate MGRN1 C-termini contain a conserved ∼25 AA region located just before the PSAP motif. Domains are drawn to scale. Domain were identified using MEME; proteins represented are Arabidopsis LOG2 (NP_566356), *C. elegans* (NP_510385.1), Drosophila (AAO42645), rat MGRN1 and RNF157 (NP_001013986 and XP_001081716, respectively).

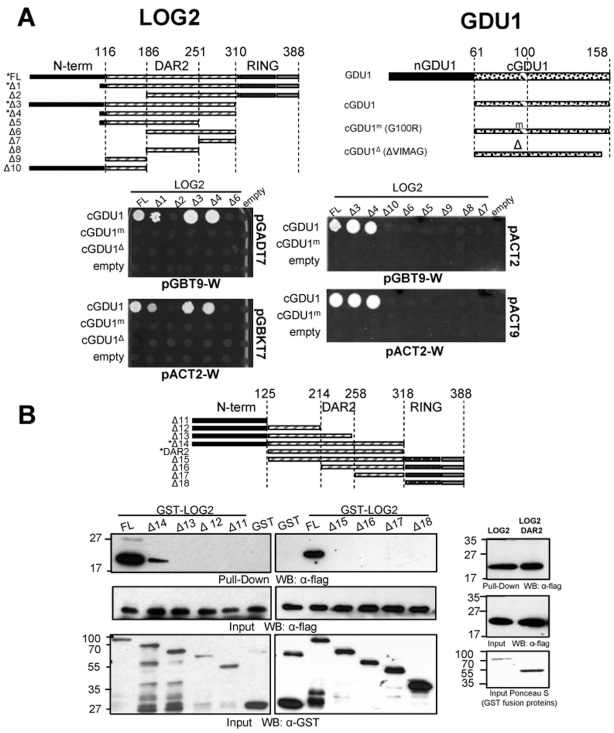

Figure 2. The DAR2 of LOG2 is necessary and sufficient for interaction with cGDU1

A. Yeast-two-hybrid assay of the interaction of cGDU1 and cGDU1 mutants with LOG2 deletions. Yeast cells carrying the various constructs were spotted on selective medium lacking adenine and histidine. Panels show swapping of inserts between bait (pGBT9 and pGBKT7) and prey (pACT2 and pGADT7) plasmids. LOG2 RING finger and N-terminal deletions interact with cGDU1, while truncation of the DAR2 abolishes the interaction. cGDU1: cytosolic GDU1. cGDU1^{m}: cGDU1^{G100R}. cGDU1$^{\Delta}$: cGDU1$^{\Delta VIMAG}$ (amino acids 93-111 replaced with Phe).

B. GST-pull down assays of the interaction of flag-cGDU1 with GST-LOG2 deletion and GST-LOG2^{DAR2}.

LOG2 and GDU1 deletion constructs are diagrammed with positions in amino acids from the beginning of the protein. Numbers on the left indicate molecular mass in kD. WB: Western blot. Asterisks indicate the constructs interacting with cGDU1.

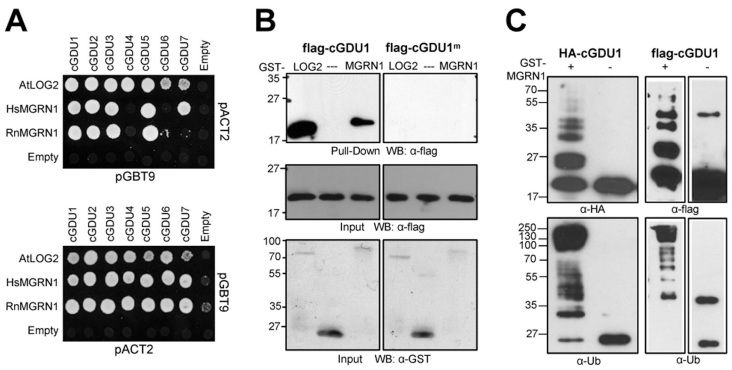

Figure 3. MGRN1 interacts with cytosolic regions of GDU proteins and ubiquitylates cGDU1

A. Multiple Arabidopsis cGDUs (GDU1 61-158; GDU2 59-129; GDU3 59-148; GDU4 64-156; GDU5 59-131; GDU6 41-111; GDU7 49-97) interact with human and rat MGRN1 in both bait-prey orientations in yeast. Yeast cells carrying the various constructs were spotted on selective medium lacking adenine and histidine. Panels show swapping of inserts between bait (pGBT9) and prey (pACT2) plasmids.

B. GST-LOG2 and GST-RnMGRN1 pull down flag-cGDU1 (but not flag-cGDU1^{m}) *in vitro*.

C. Flag-cGDU1 and HA-cGDU1 are ubiquitinated in vitro by GST-RnMGRN1. Numbers on the left indicate molecular mass in kD.

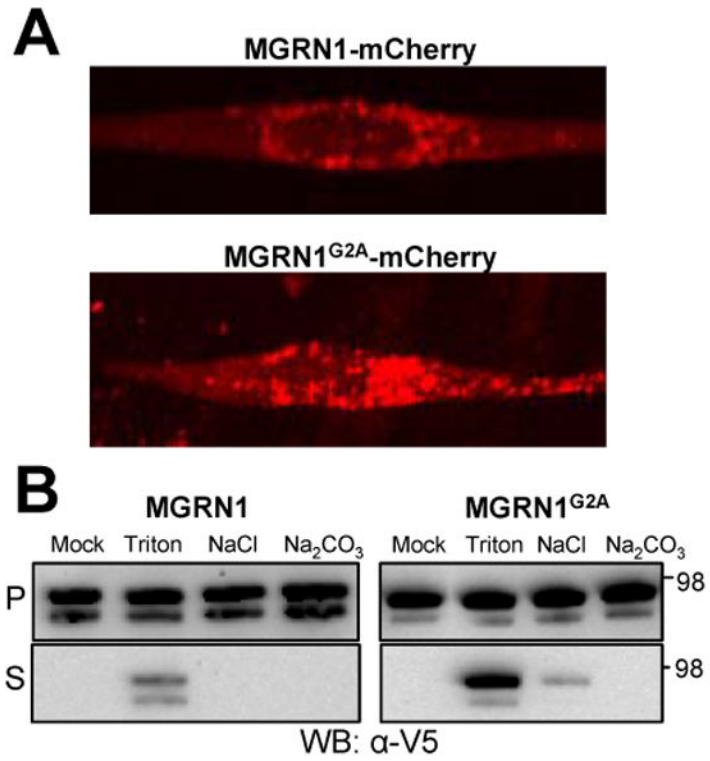

Figure 4. Effect of the G2A mutation on RnMGRN1 membrane localization in BHK21 cells

A. Subcellular localization of RnMGRN1 and RnMGRN1^{G2A} fused to the mCherry protein by confocal microscopy. Both proteins were concentrated in punctate, perinuclear structures. B. Sensitivity of microsomal RnMGRN1 and RnMGRN1^{G2A} to 1 M NaCl, 0.1 M Na_2CO_3 pH 11.5, or 1% (v/v) Triton X-100 washes. The myristoylation mutant is somewhat more susceptible than wild-type RnMGRN1 to extraction with detergent and salt. P: Pellet. S: supernatant.

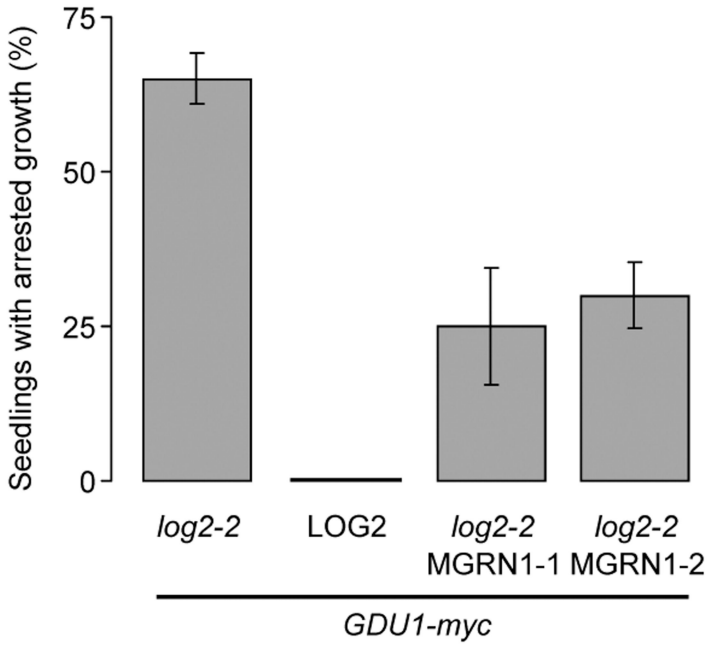

Figure 5. Expression of RnMGRN1 in *Arabidopsis thaliana* partially rescues the ability of *GDU1-myc log2-2* plants to grow on exogenous leucine

Plants over-expressing GDU1-myc and homozygous for the *log2-2* loss-of-function allele (*log2-2*) were transformed with an *RnMGRN1* plant expression construct. Growth of two independent triply homozygous RnMGRN1-expressing plant lines were compared to progenitor line *GDU1-myc log2-2* and *GDU1-myc LOG2* plants grown on 2.5 mM leucine (20 seeds per repeat for each line). ANOVA analysis with Tukey post-hoc tests for multiple comparisons indicates that the two *RnMGRN1* lines are significantly different from progenitor line GDU1-myc *log2-2* ($p < 0.01$) lacking LOG2, and not different from each other. Mean ± SE (n=5).