GRWD1 negatively regulates p53 via the RPL11–MDM2 pathway and promotes tumorigenesis

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Abstract

The ribosomal protein L11 (RPL11) binds and inhibits the MDM2 ubiquitin ligase, thereby promoting p53 stability. Thus, RPL11 acts as a tumor suppressor. Here, we show that GRWD1 (glutamate-rich WD40 repeat containing 1) physically and functionally interacts with RPL11. GRWD1 is localized to nucleoli and is released into the nucleoplasm upon nucleolar stress. Silencing of GRWD1 increases p53 induction by nucleolar stress, whereas overexpression of GRWD1 reduces p53 induction. Furthermore, GRWD1 overexpression competitively inhibits the RPL11–MDM2 interaction and alleviates RPL11-mediated suppression of MDM2 ubiquitin ligase activity toward p53. These effects are mediated by the N-terminal region of GRWD1, including the acidic domain. Finally, we show that GRWD1 overexpression in combination with HPV16 E7 and activated KRAS confers anchorage-independent growth and tumorigenic capacity on normal human fibroblasts. Consistent with this, GRWD1 overexpression is associated with poor prognosis in cancer patients. Taken together, our results suggest that GRWD1 is a novel negative regulator of p53 and a potential oncogene.

Keywords GRWD1; nucleolar stress; p53/RPL11; tumorigenesis

Subject Categories Cancer; Post-translational Modifications, Proteolysis & Proteomics

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Introduction

The p53, an important tumor suppressor, has been referred to as the “guardian of the genome” [1]. Under unstressed conditions, p53 levels are kept low via the activity of the ubiquitin proteasome system. The RING-type E3 ubiquitin ligase MDM2 negatively regulates p53 by promoting its degradation, thereby functioning as an oncoprotein [2]. When cells undergo various stresses, such as DNA damage, mitogenic stress, or nucleolar stress, p53 evades these ubiquitin ligases and the stabilized protein acts as a transcriptional activator to stimulate expression of multiple target genes, including the cyclin-dependent kinase inhibitor p21\textsuperscript{waf1} and several pro-apoptotic factors [3].

Nucleolar stress, also referred to as ribosomal stress, is one of the cellular stresses that evoke the p53-mediated response [4–10]. The molecular mechanisms and biological relevance of nucleolar stress are not fully understood. In one simple model, when cells encounter a stress such as nutrient deprivation, inhibition of rRNA transcription, or (more directly) functional impairment of certain ribosomal proteins (RPs) by genetic mutations (e.g., RPL5, RPL11, RPL35A, RPS10, RPS17, RPS19, RPS24, and RPS26 in Diamond–Blackfan anemia or RPS14 in 5q– syndrome), nucleolar integrity is disrupted, leading to exportation of “effector RPs” such as RPL5 and RPL11 to the nucleoplasm [11]. These factors in turn inhibit MDM2 ubiquitin ligase, ultimately activating p53 to maintain cellular and genetic homeostasis.

The term “effector RPs” was proposed recently by another group [12] to refer to proteins that bind MDM2 and inhibit MDM2-mediated ubiquitination and degradation of p53 when ectopically overexpressed. Among the effector RPs, RPL5 and RPL11 are of prime importance in the nucleolar stress response: siRNA-mediated silencing of RPL5 and RPL11, but not RPL23, RPL26, or RPS7, impairs p53 accumulation upon nucleolar stress [5,7,13]. Furthermore, basal p53 levels are decreased by silencing of RPL5 and RPL11 but increased by silencing of RPL23, RPL26, RPS7, or RPS14 [5,7,13–15]. Mutations in RPL5 and RPL11 genes cause Diamond–Blackfan anemia, a disorder associated with predisposition to cancer, consistent with the idea that they play important roles in the general p53 response. In other words, RPL5 and RPL11 may be tumor suppressors [11,16,17]. Other RPs, such as RPS14, may also play critical roles in the p53 response.

GRWD1 (glutamate-rich WD40 repeat containing 1), a WD40 protein that is highly conserved among eukaryotes, has been
functionally implicated in ribosome biogenesis [18–20]. In addition, GRWD1 is one of the DDB1-interacting WD40 proteins, which are predicted to function as substrate receptors of Cull4-DDB1 ubiquitin ligases [21,22]. Indeed, Cdt2, one of these WD40 proteins, turned out to be a receptor for Cdt1, a crucial replication licensing factor. However, no substrates of the Cull4-DDB1-GRWD1 ubiquitin ligase have yet been identified. More recently, we found that GRWD1 also functions as a histone chaperone to promote MCM loading at replication origins [23]. Taken together, these findings suggest that GRWD1 is a multifunctional protein involved in multiple cellular regulatory pathways, particularly those associated with cell growth control.

Here, we show that GRWD1 is a novel negative regulator of p53 induction by nucleolar stress and thus acts as a potential oncogene. Consistent with this, clinical data revealed that GRWD1 overexpression is associated with poor prognosis in several cancers.

Results

GRWD1 silencing enhances, and GRWD1 overexpression reduces, p53 induction and stability upon nucleolar stress

Using a combination of FLAG-GRWD1 immunoaffinity purification and mass spectrometric analysis, we identified RPL5, RPL11, and RPL23, as candidate GRWD1 interactors (Fig EV1), suggesting that GRWD1 may contribute to control of ribosome biogenesis and/or nucleolar functions. This idea is consistent with previous findings that Rrb1, the budding yeast homolog of GRWD1, is essential for growth and participates in early ribosome assembly [19,20]. GRWD1 is further implicated in ribosome biogenesis by the fact that its depletion leads to a 20% reduction in total protein synthesis [18]. By contrast, siRNA-mediated depletion of GRWD1 in HeLa cells does not significantly affect the levels of DNA replication initiation factors [23].

Given that GRWD1 is overexpressed in cancer cells [23], we hypothesized that it negatively regulates the nucleolar stress response that induces p53. To test this, we first investigated the effect of siRNA-mediated GRWD1 depletion. We transfected HCT116 cells with three different siRNAs targeting GRWD1, treated them with actinomycin D to evoke nucleolar stress, and sequentially harvested the cells. As expected, p53 and its targets p21, PUMA, and MDM2 accumulated over time in control siRNA-treated cells, and this induction was augmented in GRWD1-depleted cells (Figs 1A, left panel and EV2A). In actinomycin D-treated cells, phosphorylation of ATM kinase at Ser1981 was not induced (Fig 1A, middle panel), indicating that little DNA damage is induced by the treatment. Similar hyperinduction of p53 and p21 was also observed in U2OS cells treated with actinomycin D and expression of the siRNA-resistant HA-GRWD1 alleviated the hyperinduction (Fig 1B), supporting that the effect of siRNA-mediated GRWD1 depletion is mostly specific. In addition, hyperinduction of the p53 pathway was also observed when HCT116 cells were treated with bleomycin, a DNA-damaging agent inducing the ATM phosphorylation (Fig EV2B). Consistent with the hyperinduction of p53 and PUMA, the levels of cleaved PARP, a marker for apoptosis, were also elevated in GRWD1-depleted HCT116 cells (Fig 1A, middle panel). Such induction of PUMA and cleaved PARP was largely dependent on the presence of p53 (Figs 1A, right panel and EV2C). To determine whether GRWD1 depletion affects the stability of p53, we added cycloheximide (CHX) to the siRNA-treated HCT116 cells and monitored protein levels (Figs 1C and EV2D). The data revealed that GRWD1 depletion stabilizes p53 proteins.

We noticed that although GRWD1 depletion by siRNAs enhances activation of p53 pathway in response to nucleolar stress, it per se somewhat induces p53 without actinomycin D treatment (e.g., the data for time 0 in Fig 1A). Also in U2OS cells, GRWD1 depletion by siRNAs per se induced up-regulation of p53 and accumulation of sub-G1 cells likely representing apoptotic cells (Fig 2A and B). It has been suggested that GRWD1 may be required for ribosome biogenesis [18–20]. Therefore, we thought it possible that GRWD1 depletion per se induces nucleolar stress. To address this issue, we first revisited cellular localization of GRWD1. Although GRWD1 is present in nuclei and binds chromatin [23], it tends to accumulate in nucleoli [23,24]. We examined the localization of GRWD1 by immunostaining after non-ionic detergent extraction of cells to remove nucleoplasmic proteins. This assay revealed that GRWD1 is enriched in nucleoli and co-localizes with fibrillarin, a well-known nucleolar marker (Fig 2C). Furthermore, nucleolar GRWD1, like fibrillarin, dispersed into nuclei upon nucleolar stress induced by actinomycin D (Fig 2C). We then investigated whether nucleolar integrity is affected by GRWD1 depletion. As shown in Fig 2D, nucleolar fibrillarin dispersed when cells were treated with siRNAs targeting GRWD1, suggesting that its depletion impairs nucleolar integrity and thereby induces nucleolar stress response. Therefore, only with the data obtained with endogenous GRWD1-depleted cells, it would be difficult to clarify whether endogenous GRWD1 actively suppresses p53 pathway in addition to maintaining nucleolar integrity, although the hyperinduction of p53 pathway by GRWD1 depletion is in line with the idea.

To gain further insight into the GRWD1 function, we next investigated the effect of GRWD1 overexpression on activation of the p53 pathway by nucleolar stress. As shown in Fig 1D, GRWD1 overexpression reduced induction of p53 and MDM2 in response to nucleolar stress in untransformed human fibroblasts. Addition of the proteasome inhibitor MG132 to the cells alleviated the induction of p53 and MDM2 by GRWD1 overexpression (Fig 1D). Furthermore, the data obtained with cycloheximide treatment indicated that GRWD1 overexpression destabilizes p53 in cells undergoing actinomycin D-induced nucleolar stress (Fig 1E). Taken together, these findings strongly suggest that GRWD1 may actively counteract the nucleolar stress response by destabilizing p53. Therefore, we sought to elucidate the underlying molecular mechanisms in detail. As mentioned in the Introduction section, many RPs are implicated in the nucleolar stress response. However, a great deal of attention is now focused on RPL5 and RPL11, which play pivotal roles in p53 regulation in response to both nucleolar stress and other types of cellular stresses [11,17]. Therefore, we also focused on RPL11 and RPL5 in our subsequent experiments.

GRWD1 physically interacts with RPL11, and the interaction requires the N-terminal and acidic domains

To investigate the physical interaction between GRWD1 and RPL11 or RPL5 in more detail, we overexpressed RPL11-FLAG and
Figure 1. GRWD1 silencing increases, whereas GRWD1 overexpression reduces, p53 induction and stability upon nucleolar stress.

A Wild-type (left and middle panels) and p53+/− (right panel) HCT116 cells were transfected with control (mixture of control DS scrambledNeg, siLuci, and siGFP) or GRWD1-targeting (mixture of siGRWD1-3 and 4) siRNAs for 36 h, treated with 5 nM actinomycin D, and harvested at the indicated times. Whole-cell extracts were analyzed by immunoblotting with the indicated antibodies. Coomassie brilliant blue (CBB) staining serves as a loading control.

B Control U2OS cells infected with control retroviruses with the empty vector and U2OS cells overexpressing the siRNA-resistant HA-GRWD1 were treated and analyzed as above.

C HCT116 cells were transfected with control (mixture of control DS scrambledNeg, siLuci, and siGFP) or GRWD1-targeting (mixture of siGRWD1-3 and 4) siRNAs for 24 h, treated with 50 μg/ml cycloheximide, and harvested at the indicated times. Whole-cell extracts were analyzed by immunoblotting with the indicated antibodies.

D HFF2/T cells stably overexpressing HA-GRWD1 were established by retroviral infection. Cells were cultured in the presence or absence of actinomycin D (5 nM) for 12 h and then treated with or without MG132 (20 μM) for 6 h as indicated. Whole-cell extracts were analyzed as above.

E Control and HA-GRWD1-overexpressing HFF2/T cells were first treated with actinomycin D (5 nM) for 12 h, then further treated with 50 μg/ml cycloheximide, and harvested at the indicated times. Whole-cell extracts were analyzed by immunoblotting with the indicated antibodies. The means and SDs from two independent experiments are shown.

Source data are available online for this figure.
RPL5-FLAG along with HA-GRWD1 in 293T cells and immunoprecipitated cell lysates with an anti-FLAG antibody. As shown in Fig 3A, HA-GRWD1 was co-precipitated with RPL11-FLAG, but not with RPL5-FLAG. In addition, endogenous RPL11 co-immunoprecipitated with endogenous GRWD1 in both 293T cells and HCT116 cells (Fig 3B and C). We investigated whether the co-precipitation efficacy is changed by actinomycin D-induced nucleolar stress (Fig 3D). Using this assay, we examined the abilities of various GRWD1 truncated mutants (Fig 3F) to bind RPL11. The results revealed that an N-terminal region spanning amino acids (aa) 1–136, including the acidic domain, is required for efficient binding (Fig 3E and F). Similar results were obtained in immunoprecipitation assays performed on GRWD1-overexpressing cells (see later in Fig 6A). As described above, GRWD1 is enriched in nucleoli, co-localizing with fibrillarin, and disperses into nuclei upon nucleolar stress (Fig 2C). This behavior was very similar to that of RPL11 [7] (Fig 3C). In light of the stable interaction between GRWD1 and RPL11, we hypothesized that GRWD1 influences the nucleolar stress response via a physical interaction with RPL11.

**GRWD1 inhibits the RPL11–MDM2 interaction, alleviates RPL11-mediated suppression of MDM2 ubiquitin ligase activity, and abrogates RPL11-mediated p53 stabilization**

Overexpression of RPL11 inhibits MDM2 ubiquitin ligase activity and thereby increases the level of p53 [7,10]. Therefore, we investigated whether GRWD1 prevents stabilization of p53 by RPL11. As expected, in H1299 cells transfected with MDM2 and p53, co-expression of RPL11 increased p53 levels (Fig 4A). MDM2 is known to self-ubiquitinate [25]. Consistent with this, MDM2 levels were also increased by RPL11 co-expression (Fig 4A). Interestingly, co-expression of GRWD1 prevented the RPL11-mediated up-regulation of p53 and MDM2 (Fig 4A). These data strongly indicate that GRWD1 overexpression interferes with RPL11-mediated inactivation of MDM2 and the resultant activation of p53. When p53 and GRWD1 only was co-expressed in H1299...
Figure 3. GRWD1 interacts with RPL11 via the N-terminal domain including the acidic domain.

A 293T cells were co-transfected with HA-GRWD1 (1.6 μg) and RPL5-FLAG (2.4 μg) or RPL11-FLAG (2.4 μg) as indicated for 42 h and subjected to immunoprecipitation with anti-FLAG antibody. Immunoprecipitates (IPs) and 3.125% of inputs were immunoblotted with the indicated antibodies. *, immunoglobulin light chains.

B Lysates from untransfected 293T cells were immunoprecipitated with anti-GRWD1 antibody or control IgG. Immunoprecipitates (IPs) and 2.7% of inputs were immunoblotted with the indicated antibodies.

C HCT116 cells were treated with 5 nM actinomycin D for 12 h or left untreated and subjected to immunoprecipitation with anti-GRWD1 antibody or control IgG. Immunoprecipitates (IPs) and 3.5% of inputs were immunoblotted with the indicated antibodies.

D GST-RPL11 or GST alone was incubated with purified GRWD1 and bound proteins analyzed by immunoblotting with the indicated antibodies. I, input; P, pull-down.

E Cell lysates were prepared from 293T cells transfected with wild-type (WT) HA-GRWD1-FLAG (12 μg) or various N-terminal truncated mutants of HA-GRWD1-FLAG (12 μg) for 42 h and then subjected to GST pull-down assay with purified GST-RPL11 or GST. Bound proteins were analyzed by CBB staining (GST and GST-RPL11) and immunoblotting with the indicated antibodies. *, non-specific bands.

F Schematic representation of WT GRWD1 and N-terminal truncated mutants used in (E) and following studies.

G HCT116 cells were transfected with RPL11-FLAG expression vector (0.56 μg) or empty vector (0.56 μg) for 24 h and then treated with 5 nM actinomycin D or vehicle (PBS) for 12 h. The cells were first extracted with Triton X-100 to remove nucleoplasmic proteins, double-immunostained with anti-GRWD1 antibody (green) and anti-FLAG M2 antibody (red), and counterstained with DAPI. Scale bar, 20 μm.

Source data are available online for this figure.
cells without MDM2, GRWD1 did not decrease p53 levels (Fig EV3A). Therefore, although GRWD1 may also function as a component of several ubiquitin ligases [21,22], it may not directly control proteolysis of p53. We also examined whether overexpression of RPL5 could increase p53 levels in H1299 cells, but found that it has a smaller effect than RPL11 under our experimental conditions (Fig EV3B).

RPL11 binds to MDM2 and inhibits its ubiquitin ligase activity toward p53 [7,10]. It has been suggested that the acidic domain of MDM2 is important for the interaction [26]. We therefore investigated the possibility that GRWD1 competitively sequesters RPL11, thereby preventing its interaction with MDM2. As shown in Fig 4B, overexpression of GRWD1 competitively prevented co-precipitation of MDM2 with RPL11. We next examined whether GRWD1 overexpression affects RPL11-mediated inhibition of the MDM2 ubiquitin ligase, monitoring in vitro ubiquitination of MDM2 as a readout of its activity. As expected, in H1299 cells overexpressing His-Xpress-MDM2 along with HA-Ub, MDM2 was ubiquitinated, and co-expression of RPL11 significantly reduced the ubiquitination (Fig 4C, compare lane 3 with lane 4). Co-overexpression of GRWD1 prevented the RPL11-mediated inhibition of MDM2 ubiquitination levels (Fig 4C, lane 5). Taken together, these findings suggest that the GRWD1–RPL11 interaction prevents RPL11 from binding to MDM2 and suppressing its ubiquitin ligase activity.

We further confirmed that GRWD1 blocks RPL11-mediated suppression of MDM2 ubiquitin ligase activity using an in vitro ubiquitination assay with purified proteins. To this end, we immunopurified His-Xpress-MDM2 from 293T cells and measured its ability to ubiquitinate recombinant p53. As shown in Fig 4D, a ladder of high-molecular weight p53, representing the ubiquitinated forms, appeared when Xpress-MDM2 was present, but was hardly detectable in control immunoprecipitates from cells not expressing Xpress-MDM2. When recombinant RPL11 was added to the reaction, p53 ubiquitination was significantly inhibited (compare lane 2 with lane 3); however, addition of recombinant GRWD1 alleviated the RPL11-mediated inhibition (lanes 4–6). These data further support the model described above.

**GRWD1 overexpression in combination with HPV16 E7 and KRAS G12V induces oncogenic transformation in normal human cells**

GRWD1 is overexpressed in several cancer-derived cell lines [23]. Therefore, in light of our observation that GRWD1 down-regulates p53 function, we investigated whether GRWD1 overexpression confers tumorigenic potential on human cells. For this purpose, we utilized HFF2/T cells, normal human fibroblasts immortalized by introduction of telomerase [27]. In general, inactivation of the p53 pathway, inactivation of the Rb pathway, and activation of the Ras pathway are required for in vitro multistep carcinogenesis [28,29]. Because overexpressed GRWD1 suppresses p53, we used high-titer retrovirus vectors to serially introduce GRWD1, the E7 protein of human papilloma virus type 16 (HPV16), which inactivates Rb [30,31], and the KRAS mutant G12V. Expression of the transduced proteins was confirmed by immunoblotting (Fig 5A).

Oncogenic hypergrowth stimuli such as expression of oncogenic mutant Ras induce p53 activation [32–34]. In this regard, it has recently been suggested that expression of oncogenic Ras perturbs ribosome functions and thereby induces RPL11-mediated p53 activation [33]. In agreement with these findings, we found that steady-state p53 levels were up-regulated in HFF2/T cells expressing activated KRAS and E7 (Fig 5A, lower panel). As expected, such increase in p53 levels was abrogated by co-expression of GRWD1 (Fig 5A, lower panel).

Although activated Ras is required for cells to gain the ability to form tumors in nude mice, inactivation of the p53 and Rb pathways may be sufficient for anchorage-independent growth in certain cell types [35]. Accordingly, we first investigated whether overexpression of GRWD1 in combination with E7 induces anchorage-independent growth. As shown in Fig 5B and C, HFF2/T cells overexpressing GRWD1 and E7 formed numerous, relatively large colonies in soft agar. Cells expressing E7 alone also formed colonies, albeit smaller and fewer in number.

We then investigated the tumorigenic properties of HFF2/T cells overexpressing GRWD1, E7, and KRAS G12V. As shown in Fig 5D and E, HFF2/T cells expressing all three proteins formed large colonies in soft agar. The number and size of colonies were significantly higher than those of cells overexpressing E7 and KRAS G12V without GRWD1. To further investigate the effect of GRWD1 overexpression on oncogenic transformation, we injected the transduced cells into nude mice (Fig 5F). Cells overexpressing GRWD1, E7, and KRAS G12V exhibited greater tumorigenicity than control cells without GRWD1, although the tumors regressed late in the experimental period. From these data, we conclude that GRWD1 overexpression promotes oncogenic transformation by repressing p53 in combination with E7 and activated KRAS.

**GRWD1 overexpression is associated with poor prognosis in cancer patients**

Taken together, the data presented above demonstrate that GRWD1 overexpression can increase tumorigenic potential. Therefore, using databases that contain gene expression profiles of various clinical cancer samples in conjunction with patient outcomes [36,37], we investigated the possible association between GRWD1 overexpression and cancer prognosis. As shown in Fig EV4, high expression of GRWD1 was associated with poor prognosis in two expression profile studies from Gene Expression Omnibus (GEO) #GSE13507 (bladder cancer) and jacob-00182-MSK (lung cancer).

We further mined publicly available databases that contain information of the p53 mutation status together with gene expression profiles and prognosis of various cancer patients. As a result, we found that in a cohort of brain lower grade glioma (the cbioPortal for Cancer Genomics; http://www.cbioportal.org) [38,39], high expression of GRWD1 is highly associated with poor prognosis in patients with wild-type p53 but not those with mutated p53 (Fig 5G).

**The ability of GRWD1 to inhibit p53 and promote tumorigenesis requires an interaction with RPL11 mediated by the N-terminal and acidic domains**

As described above, GRWD1 interacts with RPL11 via the N-terminal and acidic domains (Fig 3E and F). This finding was confirmed by immunoprecipitation assay (Fig 6A). Furthermore, the GRWD1 Δ1–136 mutant could not prevent the RPL11 binding to MDM2 (Fig 6B). We next examined whether overexpression of the
Figure 4. GRWD1 inhibits the RPL11-MDM2 interaction and alleviates RPL11-mediated suppression of MDM2 ubiquitin ligase activity.

A H1299 cells were co-transfected with the indicated expression vectors (p53, 7.5 ng; HA-Ub, 0.5 μg; His-Xpress-MDM2, 2 μg; RPL11-FLAG, 1 μg; HA-GRWD1-FLAG, 1.5 μg; GFP, 0.04 μg) for 48 h and analyzed by immunoblotting with the indicated antibodies. GFP serves as a control protein to show equal transfection efficiencies.

B Lysates were prepared from 293T cells co-transfected with His-Xpress-MDM2 (1.2 μg), FLAG-RPL11 (1.8 μg), and HA-GRWD1 (1 μg) as indicated for 42 h and then immunoprecipitated with anti-FLAG antibody. Immunoprecipitates (IPs) and 1.5% of inputs were immunoblotted with the indicated antibodies.

C In vivo ubiquitination assay to detect MDM2 autoubiquitination in H1299 cells. Lysates were prepared from H1299 cells transfected with the indicated expression vectors (His-Xpress-MDM2, 2 μg; HA-Ub, 0.5 μg; RPL11-FLAG, 1 μg; HA-GRWD1-FLAG, 1.5 μg) for 48 h and treated with proteasome inhibitors for 6 h before harvest, and then immunoprecipitated with anti-MDM2 antibody. Immunoprecipitates (IPs) and inputs were immunoblotted with the indicated antibodies. SE, short exposure.

D In vitro ubiquitination of p53 by immunopurified MDM2. His-Xpress-MDM2 was immunopurified from transfected 293T cells with anti-Omni probe antibody. Recombinant p53 was incubated with E1, E2 (UbcH5a), His-ubiquitin, ATP, GST-RPL11, GRWD1-His, and immunopurified His-Xpress-MDM2 or control immunoprecipitates at 30°C for 120 min as indicated. The samples were resolved by SDS–PAGE followed by immunoblotting with the indicated antibodies.

Source data are available online for this figure.
GRWD1 Δ1–136 mutant affects RPL11-mediated suppression of MDM2 activity. As shown in Figs 6C and EV5A, wild-type GRWD1 repressed up-regulation of MDM2 levels by RPL11, whereas the Δ1–136 mutant affected MDM2 levels only slightly. Similar effects were observed for p53, although the changes were smaller in magnitude (Fig 6C). Furthermore, MDM2 self-ubiquitination assays

Figure 5.
in H1299 cells revealed that co-expression of wild-type GRWD1, but not the Δ1–136 mutant, restored ubiquitination levels inhibited by RPL11 (Fig EV5B). Finally, we examined the ability of the GRWD1 Δ1–136 mutant to transform HFF2/T cells in combination with HPV16 E7 and KRAS G12V (Fig 6D). As shown in Fig 6E and F, the capacity of HFF2/T cells expressing E7, KRAS G12V, and GRWD1 Δ1–136 to form anchorage-independent colonies was significantly smaller than that of cells expressing wild-type GRWD1. Taken together, these data strongly indicate that the ability of GRWD1 to interfere with the p53 pathway and promote tumor formation is dependent upon its interaction with RPL11.

Discussion

Our results indicate that GRWD1 is a negative regulator of p53 induction in response to nucleolar stress. GRWD1 depletion promotes p53 induction upon nucleolar stress (Figs 1A–C and EV2A and D), whereas GRWD1 overexpression suppresses it (Fig 1D and E). In addition, GRWD1 binds to RPL11 (Fig 3), an important positive regulator of p53 activation that acts as a tumor suppressor gene, and competitively inhibits the RPL11–MDM2 interaction (Fig 4B), suggesting that the inhibitory effect of GRWD1 on p53 induction by nucleolar stress is mediated by interference with the interaction between RPL11 and MDM2, which inhibits MDM2-mediated p53 degradation. In vivo and in vitro ubiquitination assays using MDM2 also strongly support this conclusion (Fig 4C and D). Moreover, the GRWD1 Δ1–136 mutant, which cannot engage in the interaction with RPL11, is less able to inhibit the RPL11–MDM2 interaction and alleviate RPL11-mediated inhibition of MDM2 ubiquitin ligase activity (Figs 6 and EV5), highlighting the importance of the interaction with RPL11 for GRWD1 functions. Given that GRWD1 could also interact with RPL5 and RPL23, it remains possible that GRWD1 interferes with the RP–MDM2–p53 pathway by binding to other RPs. However, GRWD1 overexpression only slightly diminishes the ability of RPL5 to inhibit MDM2 (Fig EV3B).

In addition to response to nucleolar stress, GRWD1 may also play a role in negative regulation of p53 induced by genotoxic stress (Fig EV2B) and hypergrowth stimuli by activated Ras plus E7 (Fig 5A). This seems consistent with the fact that RPL11 insufficiency may lead to impairment of general p53 responses rather than specific deficiency in p53 activation by nucleolar dysfunction. For example, in mouse fibroblasts, even heterozygous deletion of RPL11 impairs the activation of p53 by DNA damage as well as by nucleolar stress [17]. In addition, it has been recently reported that RPL5/11-dependent nucleolar stress pathway plays a crucial role also in activated Ras-mediated p53 induction [33]. It is also shown that in mouse harboring the RPL11 binding-deficient MDM2 C305F mutant, p53 induction by overexpressed c-myc is impaired [16]. Taken together, these findings could explain why Diamond–Blackfan anemia is associated with predisposition to cancer.

Co-immunoprecipitation assay between GRWD1 and RPL11 suggests that they interact even in unstimred cells and the binding efficacy is not enhanced by nucleolar stress (Fig 3B and C). It is generally considered that MDM2 ubiquitin ligase functions in nucleoplasm while RPL11 mainly exists in nucleoli. Therefore, even if GRWD1 binds to RPL11 in nucleoli, it may only subtly influence MDM2–p53 pathway. On the other hand, when the levels of ribosome-free RPL11 increase, for example, by nucleolar stress, GRWD1 binding may have a significant effect on the pathway. Especially when overexpressed, GRWD1 may accelerate malignant transformation, as shown in here. It is so far unclear whether the GRWD1–RPL11 interaction in nucleoli has a certain physiological role. In line with the previous findings that GRWD1 is required for ribosome biogenesis [18–20], we found that GRWD1 depletion per se induces nucleolar stress (Fig 2). Therefore, GRWD1–RPL11 interaction in unstimred cells could contribute to maintenance of ribosome biogenesis.

While it seems clear that GRWD1 counteracts p53 and this effect is likely mediated by interfering with the interaction between RPL11 and MDM2 and thereby relieving MDM2 ubiquitin ligase activity form RPL11-mediated suppression, it remains possible that GRWD1 suppresses p53 function by a different mechanism(s). Thus, it has been suggested that neddylation of RPL11 promotes its nucleolar localization and protects it from degradation and that upon nucleolar stress, RPL11 neddylation decreases, resulting in its release from nucleoli to activate p53 pathway [40]. Furthermore, it has been also suggested that de-neddylated RPL11 is recruited to p53-regulated promoters through the MDM2 interaction to relieve p53 from MDM2-mediated transcriptional suppression [41]. Therefore, it is possible that GRWD1 affects RPL11 neddylation. Considering that GRWD1 is the chromatin-bound protein with histone chaperone activity [23], it is also possible that GRWD1 is recruited to p53-regulated promoters and affects p53 transcriptional activity. For the former point, our findings that bacterially produced recombinant GRWD1 binds to bacterially
**Figure 6.** The abilities of GRWD1 to inhibit the nucleolar stress response and promote tumorigenesis require interaction with RPL11 via the N-terminal domain.

A 293T cells were co-transfected with wild-type HA-GRWD1 (WT) (0.5 µg) or HA-GRWD1 Δ1–136 (3.5 µg) and RPL11-FLAG (1.5 µg) as indicated for 42 h and then immunoprecipitated with an anti-FLAG antibody. Immunoprecipitates (IPs) and 1.6% of inputs were immunoblotted with the indicated antibodies.

B 293T cells were co-transfected with His-Xpress-MDM2 (0.6 µg), RPL11-FLAG (0.9 µg), and HA-GRWD1 WT (0.5 µg) or HA-GRWD1 Δ1–136 (4.5 µg) as indicated for 42 h and subjected to immunoprecipitation with an anti-FLAG antibody. Immunoprecipitates (IPs) and 1.6% of inputs were immunoblotted with the indicated antibodies.

C H1299 cells were co-transfected with the indicated expression vectors (p53, 7.5 ng; HA-Ub, 0.5 µg; His-Xpress-MDM2, 2 µg; RPL11-FLAG, 1 µg; HA-GRWD1-FLAG WT, 1.5 µg; HA-GRWD1-FLAG Δ1–136, 1.5 µg; GFP, 0.04 µg) for 48 h and then analyzed by immunoblotting with the indicated antibodies. GFP serves as a control protein to show equal transfection efficiencies.

D HFF2/T cells stably expressing wild-type HA-GRWD1 (WT) or Δ1–136, E7, and KRAS G12V were established using retroviral vectors and analyzed by immunoblotting with the indicated antibodies. *

E, F Anchorage-independent growth of HFF2/T cells overexpressing E7 and activated KRAS G12V with HA-GRWD1 WT or Δ1–136 (or without HA-GRWD1). At 9 days after seeding, colonies were analyzed as above. Representative images are shown in (E). Scale bars, 500 µm. Average numbers of colonies (> 200 µm in diameter) per photographed area with SDs are shown in (F). Seventy randomly selected areas were counted.

Source data are available online for this figure.
produced recombinant RPL11 in vitro (Fig 3D), that the recombinant RPL11 inhibits MDM2 ubiquitin ligase activity in vitro (Fig 4D), and that the recombinant GRWD1 alleviates the RPL11-mediated suppression (Fig 4D) all suggest that RPL11 neddylation may not be necessarily required for the GRWD1–RPL11–MDM2 regulatory pathway. However, these possibilities should be investigated in detail in future.

When expressed in combination with HPV E7 (which inhibits the RB protein) and activated mutant KRAS, GRWD1 transforms telomerase-immortalized normal human fibroblasts, enabling them to form large colonies in soft agar in an anchorage-independent manner (Fig 5D and E). Furthermore, these transformed cells can form tumors in nude mouse xenografts (Fig 5F), albeit to a limited extent. In agreement with the previous findings [33], expression of activated Ras plus E7 increases p53 levels and this increase is attenuated by co-expression of GRWD1 (Fig 5A, lower panel). In addition, a GRWD1 mutant defective in RPL11 binding lacks the transforming activity (Fig 6). Taken together, these data strongly indicate that oncogenic activity of GRWD1 is mediated by down-regulation of the RPL11–MDM2–p53 pathway. Combined expression of E7, activated Ras, and HPV E6 confers aggressive transformation on telomerase-immortalized normal human cells [28,29]. The E6 protein blocks p53 function by accelerating its ubiquitination-mediated degradation [31]. In addition, E6 facilitates cell growth and oncogenic transformation by intervening in several different signaling cascades that regulate growth [30]. Therefore, it is to be expected that GRWD1 would confer a more moderate tumor formation capability than E6. Nevertheless, our data clearly demonstrate that GRWD1 is a transformation-promoting factor. Finally, we cannot formally exclude the possibility that GRWD1 overexpression up-regulates ribosome biogenesis [18] and/or pre-replication complex formation [23], both of which could also contribute to its oncogenic activity.

Consistent with its transforming activity, overexpression of GRWD1 correlates with poor prognosis in cancer patients (Fig EV4). Especially, in a cohort of lower grade glioma, we found that high expression of GRWD1 is highly associated with poor prognosis in patients with wild-type p53 but not those with mutated p53 (Fig 5G). The data provide further support for our conclusion that GRWD1 promotes malignant transformation by inhibiting p53, although future in depth clinical studies should be required to establish significance of GRWD1 overexpression in cancer patients.

PICT1, a nucleolar protein conserved in budding yeast and metazoans, is a negative regulator of the RP–MDM2–p53 pathway [42]. PICT1 binds to RPL11 and retains it in nucleoli, thereby preventing activation of the nucleolar stress pathway. However, this cascade may not function in fission yeast; consequently, the conserved function of PICT1 remains to be determined. Nop53, a yeast homolog of PICT1, is required for biogenesis of the ribosome large subunit and is essential for cell viability [43,44], suggesting that PICT1 has an essential function in ribosome biogenesis. In mammalian cells, PICT1 may down-regulate p53 function, as described above, and overexpression of PICT1 in cancer correlates with a poor prognosis [45], indicating that it may act as an oncogene. In contrast to GRWD1, it remains unclear whether PICT1 overexpression actually promotes cellular transformation and/or tumorigenesis. Nevertheless, the overall biological relevance of PICT1 is likely to be similar to that of GRWD1. Future studies should address the relationship between GRWD1 and PICT1.

Materials and Methods

Cells

HCT116 cells (wild type and p53–/–) were obtained from Dr. Bert Vogelstein. 293T cells were obtained from Dr. Masao Seto. H1299 and U2OS cells were obtained from ATCC. HFF2/T cells (human foreskin fibroblasts immortalized with human telomerase reverse transcriptase [hTERT]) were established in our laboratory [27,46]. These cells were cryopreserved in small aliquots and were passaged in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 8% fetal calf serum (FCS) for ≤ 6 months after resuscitation.

Plasmids

The retroviral expression vectors pCMSCV-puro and pCMSCV-puro-16E7 were described previously [46]. pCMSCV-EM7-bsd and pCMSCV-EM7-bsd-Kras*V12 were described previously [29]. pCLH’CX was constructed by replacing the neomycin resistance gene of pCLNCl [47] with the hygromycin resistance gene. pHCMV-VSV-G and pCL-GagPol were described previously [48,49]. pcDNA3.1-HA-Ub was obtained from Dr. Chiba (Tsukuba University). pCMV-p53 and pAcGFP1-Mem were purchased from Clontech.

pGEX-6P-1-p53 was constructed by inserting full-length p53 into pGEX-6P-1 (GE Healthcare). pcDNA4/Hismax-mdm2-WT was constructed by inserting wild-type MDM2 into pcDNA4/HismaxC (Promega). The retroviral vector pCLH’CX-HA-GRWD1 was constructed by inserting HA-GRWD1 [50] into pCLH’CX. pFLAG-CMV-5b-HA-GRWD1 and pFLAG-CMV-5b-HA-GRWD1 Δacid were constructed by inserting HA-GRWD1 and HA-GRWD1 Δacid from the pGEX vectors [23] into pFLAG-CMV-5b (Sigma). Detailed procedures for constructing the other GRWD1 truncation mutants (Δ81–136, Δ40–136, Δ1–40, and Δ1–136) in pET28b vectors will be provided upon request. These GRWD1 mutants were inserted into pFLAG-CMV-5a (Sigma). To construct the HA-GRWD1Δ1–136 expression vector, oligonucleotides encoding HA were synthesized and inserted into pET28b-GRWD1Δ1–136, and then HA-GRWD1Δ1–136 was inserted into pCLH’CX.

pFLAG-CMV-5a-RPL5 and pFLAG-CMV-5a-RPL11 were generated by inserting RPL5 or RPL11 cDNA (from pCR4-TOPO-RPL5 and pOT7-RPL11, respectively; Open Biosystems) into pFLAG-CMV-5a (Sigma). The following primers were used: for RPL5, 5’-GAACCGTCAGAATTCCACCATGG GGTTGGTTAAGTTGTGAAAG-3’ (RPL5 forward) and 5’-ACCGAGATCC GTGACGCTTCACGCACCCGT-3’ (RPL5 reverse); for RPL11, 5’-G ACGGTCAAGAATCATTCCACGTGGGTAAGGTG-3’ (RPL11 forward) and 5’-ACCGGATCCGTCGACCTTGGCAGAAGGATCC-3’ (RPL11 reverse). pGEX6P-1-RPL11 was constructed by inserting RPL11 into pGEX6P-1 (GE Healthcare).

Transfection

For Figs 3A, 4B, and 6A and B, 293T cells in 60-mm dishes (1 × 10⁶ cells/dish) were transiently transfected with expression vectors (4–6 μg total) using PEImax (Polysciences) as described previously.
[23]. At 42 h after transfection, cells were subjected to immunoprecipitation. For Figs 3E and 4D, 293T cells in 100-mm dishes (2 × 10^6 cells/dish) were transiently transfected with expression vectors (12 μg total) using PEImax. At 42 h after transfection, cells were subjected to pull-down or immunopurification of His-Xpress-MDM2. For Figs 4A and C, and 6C, H1299 cells in 60-mm dishes (2 × 10^5 cells/dish) were transiently transfected with expression vectors (5 μg total) using Lipofectamine 2000 (Invitrogen). At 48 h after transfection, cells were subjected to immunoblotting or in vivo ubiquitination assay.

**Cycloheximide treatment**

Cells were treated with 50 μg/ml cycloheximide (Wako) and harvested at the indicated time points.

**Immunopurification of FLAG-GRWD1**

293T cells were transfected with either HA-GRWD1-FLAG or HA-GRWD1 as a control, lysed in NP-40 buffer (150 mM NaCl, 1% NP-40, 50 mM Tris–HCl [pH 7.4]) containing multiple protease inhibitors, and then subjected to immunoprecipitation with anti-FLAG antibody (FLAG M2 Affinity Gel, Sigma). After washing three times with 150 mM NET gel buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.4], 0.1% Triton X-100, 1 mM EDTA), the immunoprecipitates were eluted with elution buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 10% glycerol) containing 3×FLAG peptidase at 150 μg/ml.

**Mass spectrometry**

The bound proteins were concentrated by trichloroacetic acid precipitation and separated by 10% SDS–PAGE followed by silver staining. The gel was cut into 33 slices mainly according to the visible bands, numbered as shown in Fig EV1A. Proteins in each gel slice were digested with trypsin. The obtained peptides were dried, dissolved in a solution containing 0.1% trifluoroacetic acid and 2% acetonitrile, and then subjected to nanoscale liquid chromatography (nanoLC)–MS/MS analysis with a system consisting of an LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with a nanoLC instrument (Paradigm MS4 Michrom BioResources, Auburn, CA) and HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). Peptide separation was performed with an in-house pulled fused silica capillary packed with 3-μm C18 L-column (Chemicals Evaluation and Research Institute, Japan). The mobile phases consisted of 0.1% formic acid/2% acetonitrile and 0.1% formic acid/90% acetonitrile, and peptides were eluted with the linear gradient. Collision-induced dissociation spectra were acquired automatically in the data-dependent scan mode with the dynamic exclusion option. The peak lists were generated by MsN.exe (Thermo Fisher Scientific) with a minimum scan/group value of 1 and were compared with ipi_HUM_NEW database using the Mascot algorithm (ver. 2.4.1).

**siRNA experiments**

siRNA oligonucleotides (IDT) with the following sequences (sense strand) were synthesized: siGRWD1-1 (5′-GGGAUGAGCGGCCCCAA AUGAAGCdC-3′), siGRWD1-3 (5′-GCGUGUGGGCCCAUAAAGG AdCdc-3′), siGRWD1-4 (5′-GACGUAAUGUUCUCUGAAGGCCdTd G-3′), siRPL11-1 (5′-AUAUGACCCCAAGCAUUUGGUAUCdAdC-3′), siRPL11-2 (5′-CAGAUAUAUUUCGUUCAUCdAdC-3′), control DS scrambledNeg (5′-CUUCUCUCCUUUCUCUCCUUGdGda-3′), control siLuci (5′-GGUUCCUGGAACAAUGGUUUUAdCda-3′), and control siGFP (5′-ACCUGAAGUUCACUGCAACCGAc-dG-3′). HCT116 or U2OS cells (1 × 10^5) in 12-well plates were transfected with 18 pmol siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions.

**Immunoprecipitation**

For Figs 3A, 293T cells were transiently transfected with the indicated expression vectors, and cell extracts were prepared with mCSK buffer (10 mM Pipes [pH 6.8], 100 mM NaCl, 300 mM sucrose, 1 mM EGTA, 1 mM MgCl2) containing 0.1% Triton X-100, 1 mM DTT, and multiple protease inhibitors. Aliquots of the extracts were then immunoprecipitated with anti-FLAG antibody and protein G-Sepharose beads (Amersham Bioscience). For Fig 3B and C, 293T or HCT116 cells were lysed in NP-40 buffer (150 mM NaCl, 1% NP-40, 50 mM Tris–HCl [pH 7.4]) containing multiple protease inhibitors. Aliquots of the lysates were immunoprecipitated with anti-GRWD1 antibody and protein G-Sepharose beads. For Figs 4B, and 6A and B, 293T cells were transiently transfected with the indicated expression vectors, and cell extracts were prepared with NP-40 buffer containing multiple protease inhibitors. Aliquots of the extracts were immunoprecipitated with anti-FLAG antibody and protein G-Sepharose beads.

**GST pull-down assay**

GST-RPL11 or GST was bacterially expressed and purified on glutathione beads as described previously [51]. For Fig 3D, GST-RPL11 or GST was incubated with purified GRWD1-His [23] and then bound to glutathione beads. After washing four times with buffer A (20 mM Tris–HCl [pH 7.4], 200 mM NaCl, 1 mM DTT, 0.1% Triton X-100), the bound proteins were eluted and analyzed by immunoblotting.

For Figs 3E, 293T cells were transfected with the indicated GRWD1 truncation mutant and lysed in NP-40 buffer supplemented with phosphatase inhibitors and protease inhibitors. The lysates were then mixed with GST-RPL11 or GST pre-bound to glutathione beads. After washing four times with 150 mM NET gel buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.4], 0.1% Triton X-100, 1 mM EDTA), bound proteins were eluted.

**Immunoblotting and antibodies**

Immunoblotting was performed as described previously [23,50]. Preparation and specificity of polyclonal rabbit antibodies against human GRWD1 was described previously [23,50]. The anti-human RPL11 antibody was confirmed by the data that RPL11 fusion protein containing full-length human RPL11. Specificity of the anti-RPL11 antibody was confirmed by the data that RPL11 depletion by siRNAs diminished the signals detected by the antibody in immunoblotting (Fig EVSC). Other antibodies were purchased from different companies: MDM2 (OP46, Calbiochem), p53 (OP43,
Calbiochem), p21 (#2947, Cell Signaling Technology), FLAG (PAI-94B, Thermo Scientific), FLAG M2 (F3165, SIGMA), GST (G7781, SIGMA), fibrillarin (ab4566, Abcam), Omni probe (sc-499, Santa Cruz), HPV16-E7 (28-0006, Invitrogen), PUMA (#12450, Cell Signaling Technology), and pan-Ras (sc-166691, Santa Cruz).

**Immunofluorescent staining**

After brief rinse with cold phosphate-buffered saline (PBS), cells were permeabilized with 0.1% Triton X-100 in mCSK buffer for 1 min and then fixed with chilled 100% methanol for 10 min. Cells were additionally fixed with 3.7% formaldehyde in PBS for 20 min, washed with PBS, incubated with primary antibodies in PBS for 1 h at room temperature, incubated with secondary antibodies for 1 h at room temperature, and counterstained with DAPI. Cells were finally mounted in Fluoro-KEEPER Antifade Reagent (Nacalai Tesque) and analyzed using a KEYENCE BZ-9000 microscope.

**In vivo ubiquitination assay**

H1299 cells were transiently transfected with vectors encoding His-Xpress-MDM2, HA-Ubiquitin, FLAG-RPL11, HA-GRWD1-FLAG, and/or empty vectors in various combinations for 48 h. Cells were further treated with 10 μM MG132 (PEPTIDE Institute) and 0.5 μM epoxomicin (PEPTIDE Institute) for 6 h before harvest and then lysed in RIPA buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) containing multiple protease inhibitors. Aliquots of lysates were immunoprecipitated with anti-MDM2 and protein G-Sepharose beads. Samples were adjusted to equalize the level of immunoprecipitated MDM2.

**In vitro ubiquitination assay**

293T cells were transfected with His-Xpress-MDM2 for 42 h and lysed in 1 ml of mCSK buffer. Aliquots of the extracts were reacted with anti- Omni antibody probe for 4 h at 4°C. Immunoprecipitates were collected with protein G-Sepharose beads, washed twice with mCSK buffer containing 0.2% Triton X-100 and 0.1 mM ATP, twice with mCSK buffer containing 0.1 mM ATP, and twice with UBAB buffer (25 mM Tris–HCl [pH 7.4], 50 mM NaCl, 10 mM MgCl2) containing 0.1 mM ATP and 1 mM DTT. The beads were resuspended in 23 μl of UBAB buffer containing 50 ng recombinant p53, 6 μg/ml E1 (BIOMOL), 15 μg/ml E2 (UbcH5a, Enzo), 0.4 mg/ml His-tagged ubiquitin (SIGMA), 2 mM ATP, and 1 mM DTT. GST-RPL11 (5 μg) and GRWD1-His (1–3 μg) were also added as indicated. The samples were incubated at 30°C for 120 min and subjected to SDS–PAGE followed by immunoblotting. Recombinant p53 was prepared from bacterially produced and purified GST-p53 by digestion with PreScission Protease (Amersham).

**Establishment of HFF2/T cells stably expressing HA-GRWD1, E7, and KRAS G12V and U2OS cells stably expressing HA-GRWD1**

HFF2/T, non-transformed human fibroblasts immortalized with hTERT [27], were serially infected with recombinant retroviruses encoding GRWD1, HPV16 E7, and KRAS G12V or the corresponding control retroviruses. Recombinant retroviruses were produced as described previously [27,46]. Infected cells were selected with hygromycin B (100 μg/ml) for GRWD1, puromycin (0.25 μg/ml) for E7, and blasticidin (1 μg/ml) for RAS. U2OS cells stably overexpressing HA-GRWD1 were similarly established using retroviral vectors. Because our GRWD1 cDNA was codon-optimized [50], expression of the exogenous HA-GRWD1 proteins was expected to be resistant to siRNAs targeting endogenous GRWD1.

**Soft agar colony formation assay**

Cells were seeded in 6-well plates (2.5 × 10⁴ cells/well) in DMEM containing 20% FCS and 0.4% agarose with a 0.7% agarose underlay (SeaPlaque). Duplicate wells were prepared for each sample. Media were replaced once per week. For Fig 5B and C, colonies were analyzed after 3 weeks with a CKX41 microscope equipped with a DP21 digital camera (OLYMPUS). For Figs 5D and E, and 6E and F, colonies were analyzed after 9 days. To count the colonies, photographs of at least six randomly selected microscope fields for each sample were acquired at a magnification of 100× and colonies over the indicated sizes were counted.

**Tumorigenesis in nude mice**

Protocols for animal experiments were approved by the Kyushu University Animal Care and Use Committee (permit numbers: A23-022 and A27-001). Four-week-old female BALB/c nu/nu nude mice were obtained from Kyudo Co., Ltd. Mice were inoculated by subcutaneous injection on both sides of the backs with 1 × 10⁶ HFF2/T cells expressing E7 and KRAS G12V, with or without GRWD1, mixed with Matrigel (50 μl of cell suspension + 50 μl of Matrigel, 356234, BD Biosciences). The size of the xenografted tumor was measured using digital calipers, and volume was calculated based on the following formula: tumor volume (mm³) = length × (width)² × π/6. Body weight and tumor size were measured three times per week.

**Analysis for association of GRWD1 overexpression with cancer patient prognosis**

To evaluate the relationship between GRWD1 expression levels and cancer patient prognosis, we used the PrognoScan database [36,37]. In the database, association of gene expression with patient survival was evaluated by the minimum P-value approach. Patients were arranged by expression levels of a gene of interest and then classified into high- and low-expression groups at all possible cutoff points; the risk differences of any two groups were estimated by the log-rank test. Finally, the cutoff point that gave the most pronounced P-value was selected.

We also used the TCGA databases (the cBioPortal for Cancer Genomics; http://www.cbioportal.org) that contain gene expression profiles of various cancers, patient outcomes, and mutation status of p53 [38,39]. In these analyses, the average of expression levels of GRWD1 was calculated for each cohort and patients were then classified into high- and low-expression groups using the average as a cutoff point. The risk differences of any two groups were estimated by the log-rank test.
Data presentation and statistical analysis

Unless otherwise stated, quantitative data are represented as the mean ± SD of three or more independent experiments. For qualitative data and semi-quantitative data, a representative image from multiple independent experiments is shown; for all such figures, essentially the same results were obtained in the multiple independent experiments. Unless otherwise stated, statistical analyses were performed with a two-tailed Student’s t-test. P-values of < 0.05 were considered statistically significant.

Expanded View for this article is available online.

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Author contributions

KK, NS, and MF designed the experiments and wrote the manuscript. KK conducted most of the experiments regarding the novel functions of GRWD1. BS performed the immunopurification of FLAG-GRWD1 and identification of novel GRWD1-binding proteins. TTA, TTs, and KH supported some of the experiments. MM and KIN conducted mass spectrometric analysis of FLAG-GRWD1 immunoprecipitates. ME, TKo, and KS contributed to analysis of cancer databases. KY, ME, and TKi contributed to the experimental design and provided scientific advice.

Conflict of interest

The authors declare that they have no conflict of interest.

References


