Guanine nucleotide binding protein like-1 (GNL1) promotes cancer cell proliferation and survival through AKT/p21^{CIP1} signaling cascade

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ABSTRACT **Human guanine nucleotide binding protein like 1 (GNL1) is an evolutionary conserved putative nucleolar GTPase belonging to the HSR1_MMR1 subfamily of GTPases. GNL1 was found to be highly up-regulated in various cancers. Here, we report for the first time that GNL1 inhibits apoptosis by modulating the expression of Bcl2 family of proteins and the cleavage of caspases 7 and 8. Furthermore, GNL1 protects colon cancer cells from chemodrug-induced apoptosis. Interestingly, GNL1 up-regulates the expression of p53 and its transcriptional target, p21 but the up-regulation of p21 was found to be p53 dependent as well as independent mechanisms. Our results further demonstrate that GNL1 promotes cell growth and survival by inducing cytoplasmic retention and stabilization of p21 through AKT-mediated phosphorylation. In addition, GNL1 failed to inhibit apoptosis under p21 knockdown conditions which suggests the critical role of p21 in GNL1-mediated cell survival. Finally, an inverse correlation of GNL1, p21, and AKT expression in primary colon and breast cancer with patient survival suggests their critical role in tumorigenesis. Collectively, our study reveals that GNL1 executes its antiapoptotic function by a novel mechanism and suggests that it may function as a regulatory component of the PI3K/AKT/p21 signaling network to promote cell proliferation and survival in cancers.**

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INTRODUCTION

Tumors utilize a wide variety of mechanisms for survival under the attack of radiation and chemotherapy. Despite recent advances in cancer treatment, chemoresistance in metastatic disease remains a hurdle. Hence, there is a need to identify new biomarkers and to elucidate the underlying molecular mechanisms that regulate chemoresistance. Guanine nucleotide binding proteins are emerging as crucial coordinators of signaling cascades during cell proliferation. The YawG/YlqF/HSR1-MMR1 GTP-binding protein subfamily of GTPases is evolutionarily conserved from prokaryotes to mammals. The members of this family are characterized by the presence of circular permutated guanine nucleotide binding motifs (Essers *et al.*, 2014). Human guanine nucleotide binding protein like 1 (GNL1), GNL2, GNL3, and GNL3L are the four well-known members of this family in humans and are found to be highly expressed in cancers (Liu *et al.*, 2004; Kafienah *et al.*, 2006; Chennupati *et al.*, 2011; Thoompumkal *et al.*, 2015).

GNL1 encodes 607 amino acids with a molecular mass of 65 kDa, contains basic amino acids rich N-terminus, acidic amino acid-rich C-terminus, a proline-rich domain, and five GTP-binding motifs. GNL1 encodes a novel arginine/lysine-rich nuclear/nucleolar localization signal at the N-terminus and is found to be localized to different subcellular compartments in cell cycle-dependent manner (Boddapati *et al.*, 2012). GNL1 was reported to have a critical role in

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Abbreviations used: ASK1, apoptosis signaling kinase 1; ERp29, endoplasmic reticulum protein 29; FBS, fetal bovine serum; GNL1, guanine nucleotide binding protein-like 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly-ADP ribose polymerase; PEI, polyethyleneimine; PMSF, phenylmethylsulfonyl fluoride; RP, ribosomal protein.

liver cell proliferation (Gao *et al.*, 2008). Expression of GNL1 was up-regulated in bladder and ovarian cancers and in a panel of squamous cell carcinoma cell lines (Jensen *et al.*, 2008; Fang *et al.*, 2013; Delcourt *et al.*, 2017). A recent report from our laboratory demonstrates that GNL1 interacts with ribosomal protein S20 (RPS20) and promotes cell proliferation by modulating G1/S and G2/M phase transition (Krishnan *et al.*, 2018). GNL1 promotes hyperphosphorylation of pRb and results in faster cell cycle progression (Krishnan *et al.*, 2018). Together, these reports suggest that GNL1 might regulate a network of pathways to promote cell growth and survival, but the mechanism(s) remains poorly understood.

The p53/p21 pathway has been widely implicated in various cellular processes including cell cycle regulation, apoptosis, and tumorigenesis. DNA damage often activates the p53/p21 pathway and causes G1/S phase arrest in mammalian cells; p21 is a well-known target of p53 and interacts with various cyclin-CDK complexes to control cell cycle progression. A recent report suggests that p21 could also function as a positive regulator of the cell cycle (Cheng *et al.*, 1999). Interestingly, p21 induces the assembly and activation of the cyclin D1-CDK4/6 complex and promotes faster G1/S phase transition (Cheng *et al.*, 1999). Expression of p21 positively correlated with cell survival (Asada *et al.*, 1999). Recent studies suggested that p21 may act as a tumor suppressor and as an oncogene based on its cellular localization (Asada *et al.*, 1999; Blagosklonny, 2002; Koster *et al.*, 2010). The growth inhibitory functions of p21 are associated with its nuclear localization, whereas cytoplasmic localization was associated with its oncogenic or antiapoptotic activities (Asada *et al.*, 1999; Blagosklonny, 2002; Koster *et al.*, 2010). Furthermore, cytoplasmically localized p21 interacts with apoptosis signaling kinase 1 (ASK1) and inactivates its activity to inhibit cellular apoptosis (Zhan *et al.*, 2007).

Recent reports have suggested that AKT/PKB promotes cell growth and survival by modulating p21 activity in response to extracellular signals (Li *et al.*, 2002). Phosphorylation of p21 at Thr145 and Ser146 residues by AKT resulted in cytoplasmic localization and stabilization of p21 protein (Li *et al.*, 2002). Results from the present investigation provided evidence that GNL1 promotes AKT-mediated p21 phosphorylation, which is critical for the cytoplasmic localization and stabilization of p21. Finally, our results suggest that AKT/p21 signaling pathway is essential for the antiapoptotic function of GNL1 to promote cell proliferation and survival during tumorigenesis.

RESULTS

GNL1 inhibits apoptosis

GNL1 was initially identified as a putative nucleolar GTPase. Recent reports provided evidence that GNL1 and other of members of GNL family are significantly up-regulated in several cancers and promote cell proliferation (Liu *et al.*, 2004; Meng *et al.*, 2008; Boddapati *et al.*, 2012; Datta *et al.*, 2015; Thoompumkal *et al.*, 2015, 2016; Krishnan *et al.*, 2018), but the mechanisms remain poorly understood. Toward this, we have selected GNL1 to define the mechanism by which GNLs regulate cell proliferation. GNL1-expressing cells were stained with annexin-V/APC and the apoptotic cell population was measured by flow cytometry as described in *Materials and Methods*. Results indicate that the ectopic expression of GNL1 in HCT116$^{p53+/+}$ (Figure 1A) and SKBR3 (Supplemental Figure S1A) cells significantly reduced the apoptotic cell numbers with corresponding increase of live cell population. Toward understanding the mechanism by which GNL1s modulate cell survival, expression levels of key molecular players involved in apoptotic pathway were analyzed in GNL1-expressing cells. Western blot analysis indicates that GNL1 up-regulates the expression of antiapoptotic Bcl-xL, Bcl-2, and survivin; in contrast, the expression of proapoptotic BAX and BID was significantly reduced in both HCT116$^{p53+/+}$ (Figure 1B) and SKBR3 (Supplemental Figure S1B) cells. Furthermore, significant reduction in the levels of cleaved caspases 7 and 8 were observed on GNL1 expression in HCT116$^{p53+/+}$ cells (Figure 1C). To further define the mechanism of GNL1-mediated inhibition of apoptosis, endogenous GNL1 was depleted in HCT116$^{p53+/+}$ cells and measured cell apoptosis by flow cytometry. Interestingly, knockdown of GNL1 resulted in a significant increase of apoptotic cell population with a corresponding decrease of live cell population (Figure 1D). Results from the Western blot analysis indicate that knockdown of GNL1 resulted in significant down-regulation of antiapoptotic Bcl-xL, Bcl-2, and survivin, with increased levels of proapoptotic proteins BAX and BID (Figure 1E). Furthermore, increased levels of cleaved caspases 7 and 8 were observed on GNL1 knockdown in HCT116$^{p53+/+}$ cells (Figure 1F). The knockdown efficiency of GNL1 was determined by Western blot analysis using anti-GNL1 antibodies. Together, these data suggest the possibility that GNL1 promotes cell proliferation and survival by inhibiting caspase-dependent cell apoptosis via modulating the expression of Bcl-2 family proteins in cancers.

GNL1 promotes chemoresistance in cancer cells

Different chemotherapy drugs are usually offered to cancer patients, but the response to chemotherapy reduces subsequently with each episode and resulted in disease progression (Acharyya *et al.*, 2012). Chemoresistance and metastasis are the key problems in cancer treatment. The observed high-level expression of GNL1 in various cancers (Liu *et al.*, 2004; Boddapati *et al.*, 2012; Thoompumkal *et al.*, 2015; Krishnan *et al.*, 2018) together with its antiapoptotic function observed in the present investigation led to the hypothesis that GNL1 expression in cancers may induce chemoresistance. We therefore next tested whether GNL1 expression modulates chemo–drug-induced apoptosis in cancer cells. Toward this, GNL1-expressing HCT116$^{p53+/+}$ cells were treated with different chemotherapeutic drugs (camptothecin, oxaliplatin, and 5-Flurouracil) and we analyzed the cell viability by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described in *Materials and Methods*. Results indicate that GNL1 expression provides growth advantage to cells in the presence of indicated chemo-drugs compared with GPF expression (Figure 2A). In contrast, significant reduction of cell viability was noticed under GNL1 knockdown condition with indicated drug treatment (Figure 2B). These data suggest that the observed increased cell viability in GNL1-expressing cells in the presence of chemo-drugs might be due to apoptosis inhibition. Camptothecin and oxaliplatin were selected for further experiments to understand the antiapoptotic function of GNL1. Toward this, GNL1-expressing HCT116$^{p53+/+}$ cells were treated with indicated drugs for 24 h and stained with annexin-V/APC followed by flow cytometry analysis to determine the status of cell apoptosis. Results indicate that the number of apoptotic cell population was significantly reduced with a corresponding increase in live cell population in GNL1-expressing cells treated with camptothecin (Figure 2C) or oxaliplatin (Supplemental Figure S2A) compared with DMSO-treated cells. Camptothecin is a topoisomerase I inhibitor (Liu *et al.*, 2006) shown to induce apoptotic death of cultured embryonic cortical neurons by BAX-dependent and mitochondrial pathways (Enokido *et al.*, 1996; Keramaris *et al.*, 2000; Morris *et al.*, 2001). Oxaliplatin forms both inter- and intrastrand cross-links with DNA and prevents DNA replication/transcription to induce cell death (Graham *et al.*, 2004). Drug treatments resulted in single- or double-

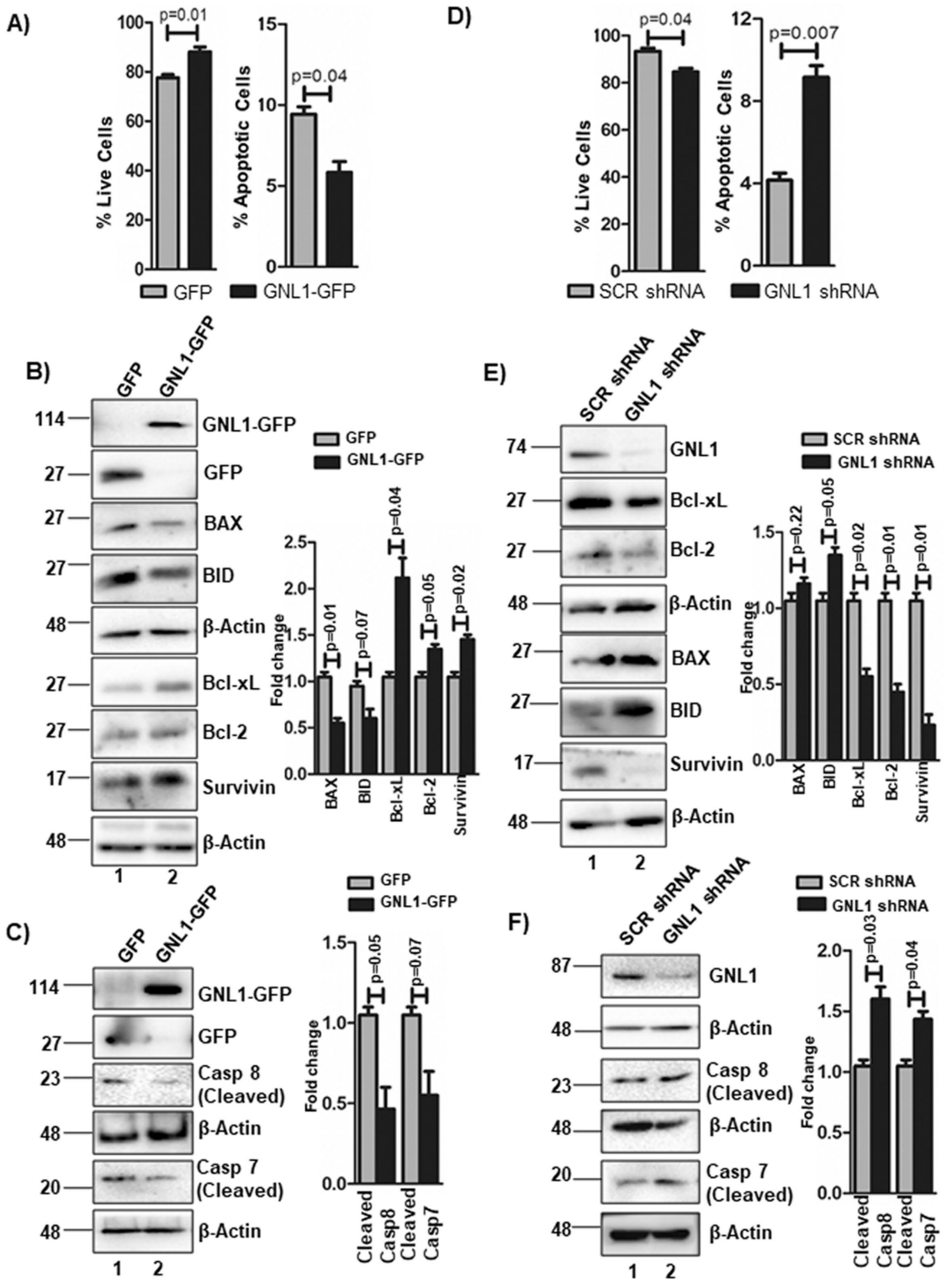

FIGURE 1: GNL1 inhibits apoptosis. (A) GNL1expressed HCT116 $^{p53+/+}$ cells were stained with Annexin-V/APC to determine the status of apoptosis by flowcytometry analysis. (B) Expression status of indicated proapoptotic and antiapoptotic proteins were determined by Western blot analysis using respective antibodies. (C) GNL1 alters the cleavage of caspase 7 and 8 in HCT116$^{p53+/+}$ cells. (D) GNL1 depletion by specific shRNA promotes apoptosis in HCT116 $^{p53+/+}$ cells. (E) Knockdown of GNL1 enhances the expression of proapoptotic BAX and BID proteins and reduces the expression of antiapoptotic proteins. (F) Depletion of endogenous GNL1 by shRNA promotes the cleavage of caspase 7 and 8. The densitometry analyses of the corresponding Western blots were carried out by normalizing the expression levels of the indicated endogenous proteins to β-actin.

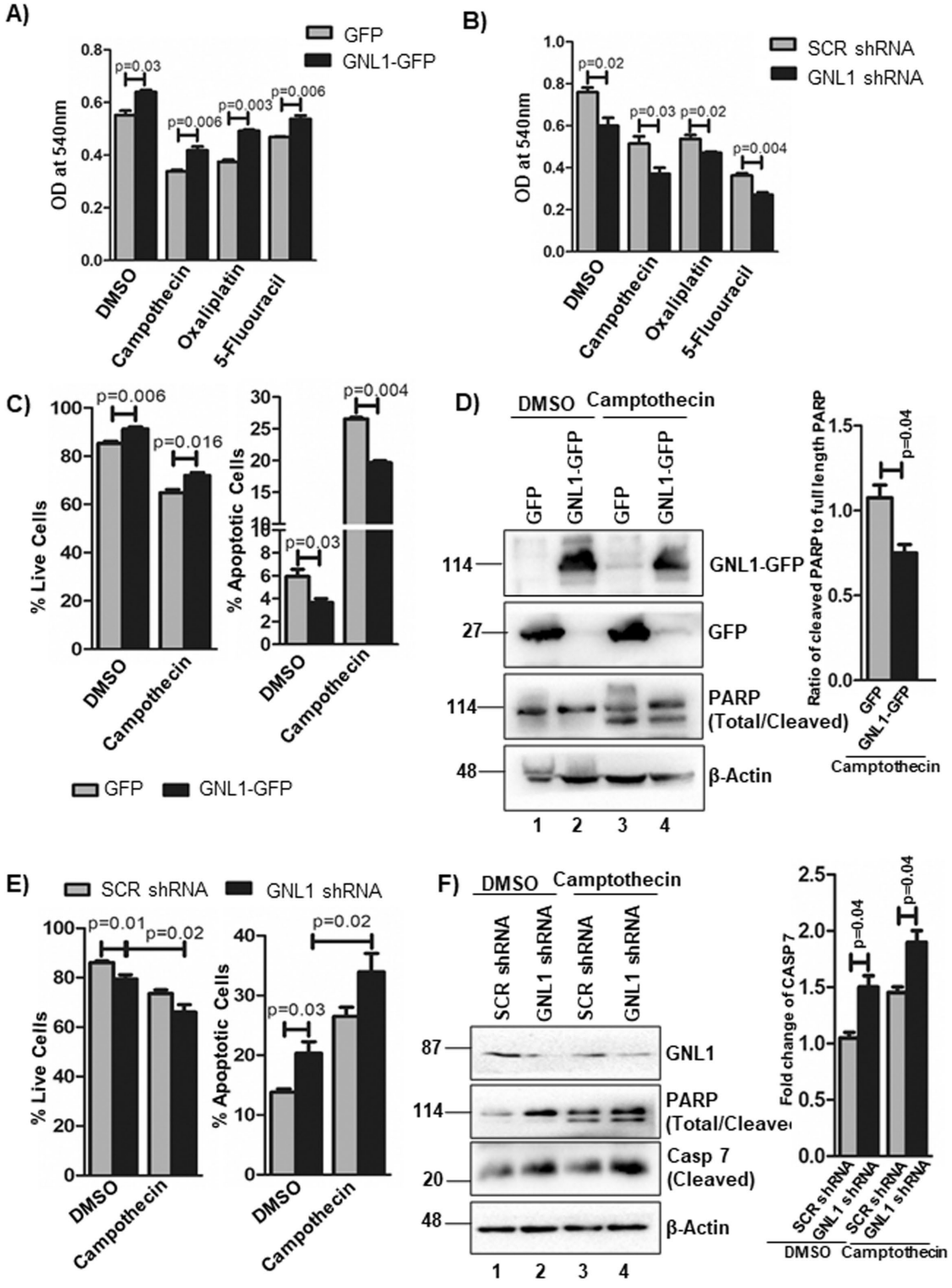

FIGURE 2: GNL1 protects colon cancer cells from chemo-drug-induced apoptosis. HCT116$^{p53+/+}$ cell viability was enhanced in the presence of indicated chemotherapeutic drugs with GNL1 expression (A) and was reversed under a GNL1-depleted condition (B). The cell viability was determined by MTT assay as described in *Materials and Methods*. (C) GNL1 inhibits camptothecin-induced cell apoptosis in HCT116 $^{p53+/+}$ cells. Status of apoptosis was determined by Annexin-V/APC staining followed by flowcytometry analysis. (D) Camptothecin-induced PARP cleavage was reduced in the presence of GNL1expression. (E) Depletion of GNL1 sensitizes HCT116 $^{p53+/+}$ cells to camptothecin-induced cell apoptosis. (F) GNL1 knockdown resulted in increased PARP and caspase 7 cleavages in HCT116 $^{p53+/+}$ cells in the presence of camptothecin. The densitometry analyses of the corresponding Western blots were carried out by normalizing the expression levels of the indicated endogenous proteins to β-actin.

strand DNA breaks and activate poly-ADP ribose polymerase (PARP) to promote DNA repair (Herceg and Wang, 2001). If damage is irreparable, PARP is inactivated by caspase-mediated cleavage, leading to programmed cell death (Kaufmann *et al.,* 1993; Nicholson *et al.,* 1995; Tewari *et al.,* 1995). To further understand the mechanism of antiapoptotic function of GNL1, we next analyzed the effect of GNL1 expression on PARP cleavage during drug treatment. Western blot analysis showed that the level of cleaved PARP was significantly decreased in both camptothecin- (Figure 2D, lane 4) and oxaliplatin- (Supplemental Figure S2B, lane 4) treated GNL1-expressing cells compared with GFP-expressing cells (Figure 2D, lane 3; Supplemental Figure S2B, lane 3). These data suggest that GNL1 blocks PARP cleavage to inhibit drug-induced cell apoptosis.

To further define the status of drug-induced cell apoptosis under GNL1 knockdown condition, GNL1-depleted HCT116$^{p53+/+}$ cells were treated with indicated drugs and stained with annexin-V/APC followed by flow cytometry analysis. Results indicate that a significant increase in apoptotic cell population with a corresponding decrease in live cell population in camptothecin-treated cells under GNL1 knockdown condition compared with DMSO treatment (Figure 2E). This was further supported with an increased cleavage of caspase 7 and PARP (Figure 2F, lane 4) under GNL1 knockdown conditions. Caspase 7 is one of the executioner caspases and inactivates PARP by cleavage (Germain *et al.,* 1999). Knockdown efficiency of GNL1 was determined by Western blot analysis using anti-GNL1 antibodies (Figure 2F). Together, these results provide evidence that GNL1 modulates caspase 7 activity to regulate cell apoptosis.

GNL1 modulates p53 and p21 expression in colon and breast cancer cell lines

It is well known that the p53/p21 pathway is activated during DNA damage. The p53 protects the genome by regulating various DNA damage response mechanisms (Williams and Schumacher, 2016). When DNA damage occurs before the cells enter into S phase, p53 arrests the cell at G1 phase by activating the expression of cyclin-dependent kinase inhibitor CDKN1A/p21^{CIP1} (El-Deiry *et al.,* 1993). If the damage is irreparable, the cell undergoes programmed cell death. It is known that camptothecin and oxaliplatin treatment promotes cell death by p53 and mitochondria-dependent mechanisms (Toscano *et al.,* 2007; Rudolf *et al.,* 2011). These data, together with results from the present study, led to the hypothesis that GNL1 may inhibit the drug-induced cell apoptosis by modulating the p53/p21 pathway. In support of this, recent studies suggest that members of YawG/YIqF/HSR1-MMR1 GTP-binding protein subfamily of GTPases such as GNL2 (NGP-1), GNL3, and GNL3L have been shown to induce cell proliferation by modulating p53 pathway (Datta *et al.,* 2015; Meng *et al.,* 2008, 2011). We therefore immediately tested whether GNL1 expression alters p53 function to promote cell proliferation. Toward this, GNL1-GFP was ectopically expressed in HCT116$^{p53+/+}$ cells and we analyzed the expression levels of p53 and p21 by Western blot analysis. To our surprise, results in Figure 3A indicate that the p53 and p21 protein levels were up-regulated by GNL1 (lane 2). To check whether this regulation is at a transcriptional or translational level, GNL1 was expressed in HCT116$^{p53+/+}$ cells and the levels of p53 and p21 mRNA were measured. Surprisingly, RT-qPCR analysis clearly indicates that GNL1 up-regulates the transcript levels of both p53 and p21 (Figure 3B). It is interesting to note that GNL1 inhibits apoptosis and promotes cell proliferation despite up-regulating the expression of well-known tumor suppressor proteins p53 and p21, which are known to inhibit cell growth.

It is well documented that p21 expression was activated in a p53-dependent as well as an -independent manner (Rey *et al.,* 1998). To understand whether GNL1-mediated p21 up-regulation is p53 dependent, the mRNA and protein levels of p21 were measured on GNL1 expression in p53-deficient HCT116$^{p53-/-}$ cells. Results in Figure 3C clearly indicate that GNL1 up-regulates p21 protein expression (lane 2) without altering its mRNA levels (Figure 3D). To further confirm, the mRNA and protein levels of p21 were determined on GNL1 expression under p53 knockdown condition. Results in Supplemental Figure S3A indicate that the protein level of p21 was significantly higher in GNL1-expressing cells under p53 knockdown conditions (lane 4) despite that the mRNA level of p21 was significantly down-regulated under similar conditions (Figure 3E). These data clearly suggest that GNL1 modulates the p21 level in a p53-dependent as well as an -independent manner. In support of this, the higher expression level of p21 was reported in various cancers, especially breast cancers (Wakasugi *et al.,* 1997; Weiss, 2003). It is likely that p21 may play an important role in cell viability after DNA damage during tumorigenesis. Collectively, these data led to the hypothesis that GNL1 up-regulates p21 to promote cell proliferation by an unknown mechanism. To test this possibility, we next analyzed whether GNL1 expression alters the p21 protein levels in various breast cancer cell lines such as T47D, SKBR3, and MDAMB231. Interestingly, there is significant up-regulation of p21 protein levels on GNL1 expression in all cell lines tested (Supplemental Figure S3B, lanes 2, 4, and 6). Interestingly, GNL1 knockdown by two independent shRNAs in HCT116$^{p53+/+}$ cells resulted in a significant reduction of p21 protein levels (Figure 3F, lanes 2 and 3). Since knockdown efficiencies were similar for both shRNAs, we used only shRNA1 for all other experiments. These results suggest the possibility that GNL1 alters p21 protein stability to induce cell proliferation. It is known that p21 protein has a short half-life and is degraded by proteasome through ubiquitin-dependent and -independent pathways (Touitou *et al.,* 2001; Zhang *et al.,* 2004; Kim et al., 2008). To understand the mechanism(s) by which GNL1 regulates the stability of p21, the polyubiquitylation status of p21 in HCT116$^{p53+/+}$ cells was measured in the presence of GNL1 expression. Results in Supplemental Figure S3C indicate that expression of GNL1 reduced the levels of polyubiquitinated p21 (lane 2), whereas depletion of endogenous GNL1 by shRNA-mediated knockdown resulted in a significant increase of p21 polyubiquitination (Figure 3G, lane 2). Taken together, these results suggest that GNL1 stabilizes p21 by inhibiting ubiquitination.

GNL1 induces cytoplasmic retention of p21

CDKN1A/p21 is a critical regulator of cell cycle and cell survival. Elevated levels of p21 protein in various aggressive tumors are reported to be associated with chemoresistance (Vincent *et al.,* 2012). It is well known that the nuclear retention of p21 is critical to regulate S phase of the cell division cycle by blocking DNA synthesis through interaction with PCNA as well as by inhibiting CDK activity. In contrast, p21 functions as pro-proliferative and -survival factors when it localized to the cytoplasmic compartment (Zhou *et al.,* 2001; Child and Mann, 2006). Since GNL1 up-regulates p21 and promotes cell proliferation, we immediately analyzed the subcellular distribution pattern of p21 on GNL1 expression and knockdown conditions. Toward this, GNL1 was ectopically expressed in HCT116$^{p53+/+}$ and SKBR3 cells and analyzed the distribution of p21 in nuclear and cytoplasmic compartments. Interestingly, results from the Western blot analysis indicate that p21 was predominantly accumulated with cytoplasmic fractions in GNL1-expressing cells (Figure 4, A and B, lane 2). In contrast, reduction of p21 accumulation in the cytoplasmic fraction was observed in a GNL1-depleted condition compared with scrambled shRNA-transfected cells (Figure 4C, lane 4). It is worth

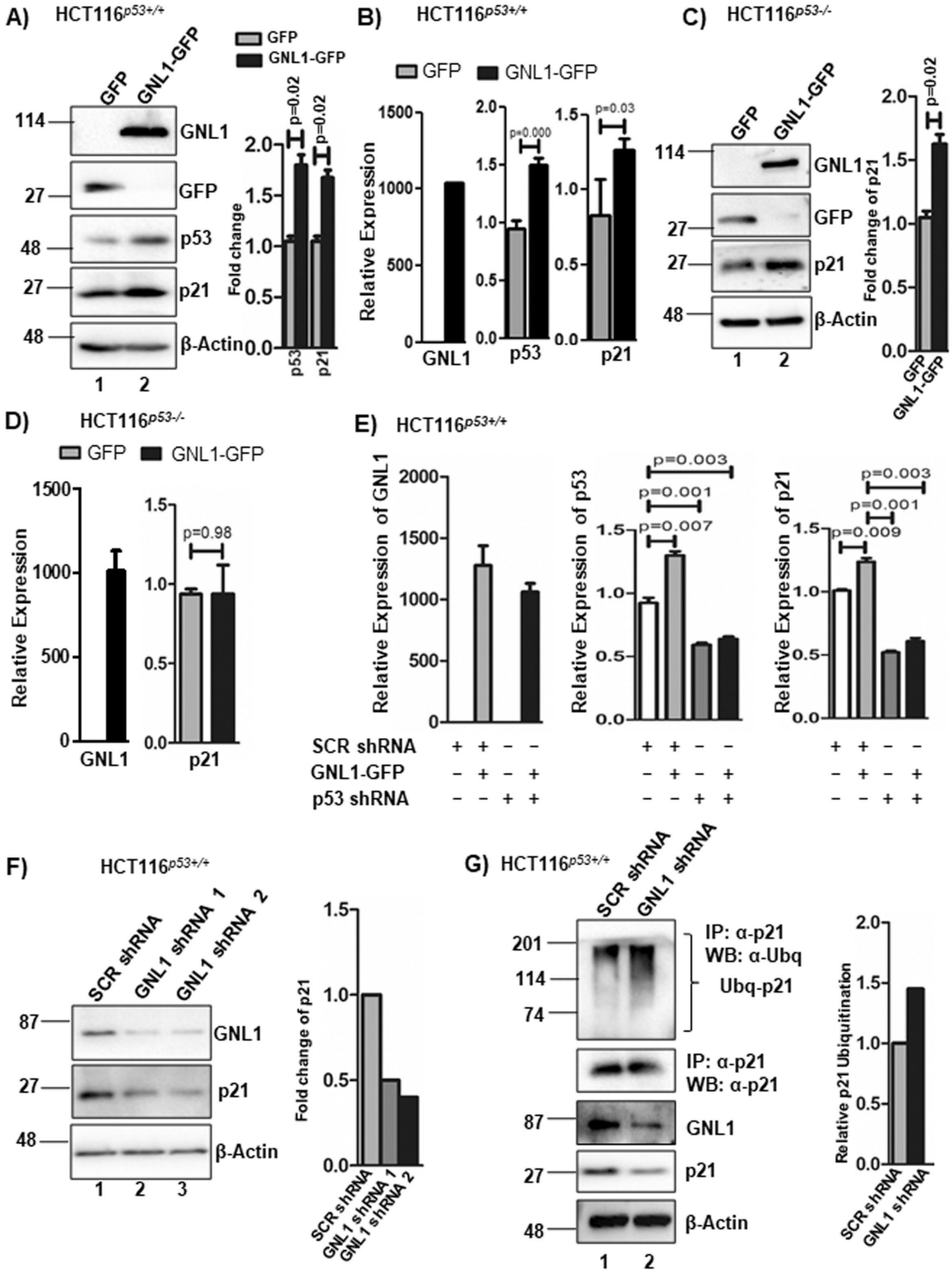

FIGURE 3: GNL1 modulates p53/p21 pathway. (A) The status of p53 and p21 proteins (A) and mRNA (B) levels were measured in HCT116$^{p53+/+}$ and HCT116$^{p53-/-}$ (C) cells in the presence of GNL1 expression. Indicated protein expression was determined by Western blot analysis using respective antibodies. mRNA levels of *GNL1*, *p53*, *and p21* were determined by qPCR analysis using specific primers. (D) RT-qPCR analysis suggests that mRNA levels of *p21* were not altered by GNL1 in HCT116$^{p53-/-}$ cells. (E) GNL1 failed to promote mRNA levels of *p21* in HCT116$^{p53+/+}$ cells under a p53-depleted condition. (F) Expression of p21 protein was reduced in HCT116$^{p53+/+}$ cells under a GNL1-depleted condition. Expression of endogenous p21 and knockdown status of GNL1 were confirmed by Western blotting using anti-p21 anti-GNL1 antibodies, respectively. (G) GNL1 depletion in HCT116$^{p53+/+}$ cells promoted p21 ubiquitination. The densitometry analyses of the corresponding Western blots were carried out by normalizing the expression levels of the indicated endogenous proteins to β-actin.

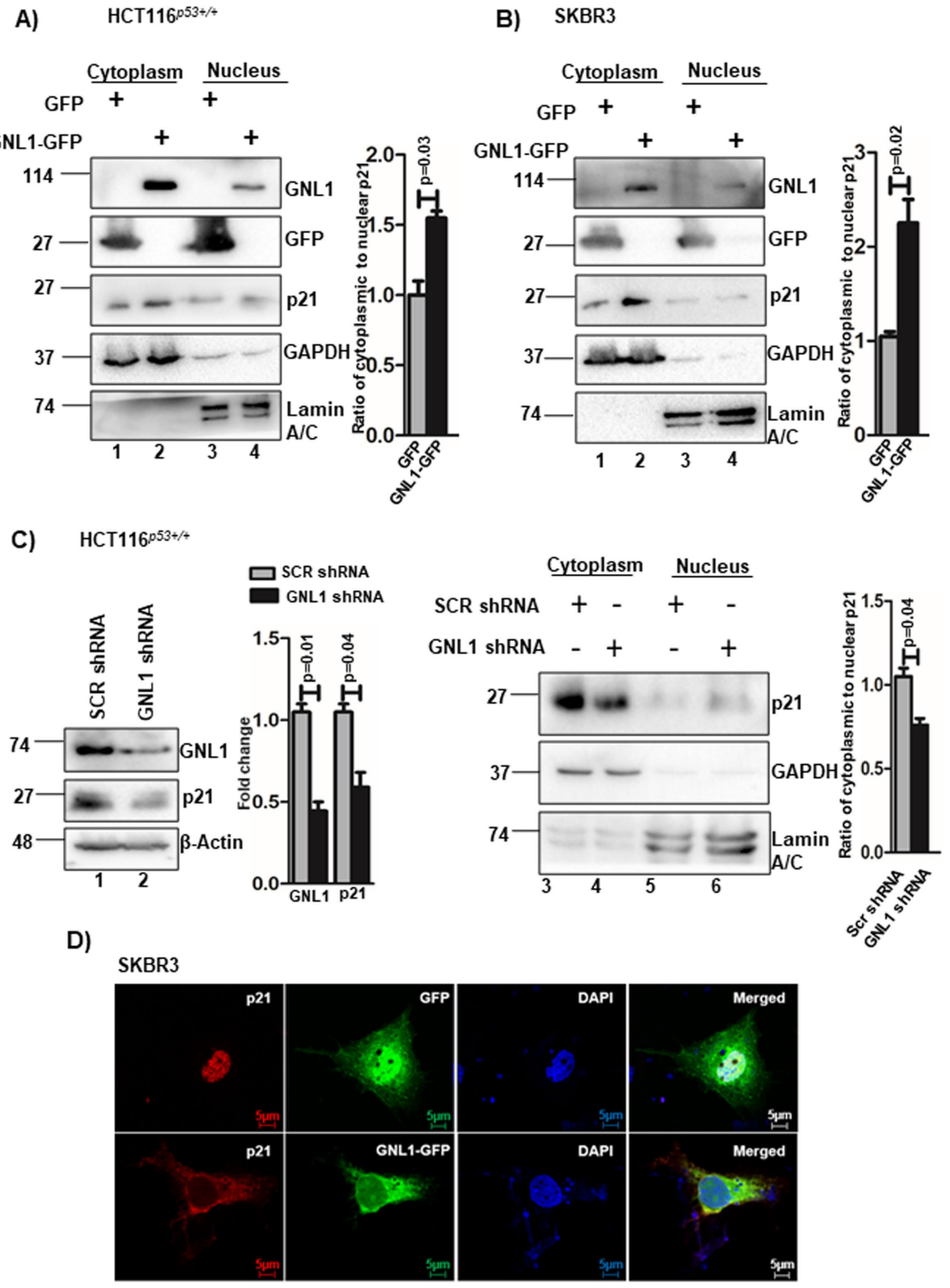

FIGURE 4: GNL1 induces cytoplasmic retention of p21. The cytoplasmic and nuclear fractions of HCT116$^{p53+/+}$ (A) and SKBR3 (B) cells expressing GNL1 were isolated and accumulation of p21 was analyzed by Western blot using anti-p21 antibody. GAPDH and Lamin A/C were used as cytoplasmic and nuclear markers, respectively. (C) Cytoplasmic and nuclear fractions were isolated from GNL1-depleted HCT116$^{p53+/+}$ cells and p21 accumulation was determined by immunoblot analysis with anti-p21 antibodies. Efficiency of GNL1 knockdown was tested by Western blot analysis using anti-GNL1 antibody. The densitometry analyses of the corresponding Western blots were carried out by normalizing the expression levels of GAPDH and Lamin A/C. (D) Immunofluorescence analysis suggests that p21was localized predominantly in the cytoplasmic compartment in the presence of GNL1 expression in SKBR3 cells. DAPI was used for nuclear staining.

noting that the total p21 expression was reduced in GNL1-depleted cells (Figure 4C, lane 2) compared with scrambled shRNA-expressing cells. Lamin A/C and GAPDH were used as nuclear and cytoplasmic markers, respectively. To define further, subcellular localization of p21 was analyzed in the presence of GNL1 by immunofluorescence. Interestingly, predominant cytoplasmic localization of p21 was observed in GNL1-expressing HCT116$^{p53+/+}$ (Supplemental Figure S4) and SKBR3 cells (Figure 4D). Collectively, these data suggest that GNL1 promotes cytoplasmic retention of p21 by a novel unknown mechanism to induce cell proliferation.

GNL1 promotes AKT-mediated p21 phosphorylation to induce cell proliferation and survival

Recent reports suggest that the nuclear localized p21 functions as a tumor suppressor, whereas the cytoplasmic p21 functions as an antiapoptotic factor and enhances cell survival (Zhou *et al.,* 2001; Child and Mann, 2006). Phosphorylation at Thr145 and/or Ser146 residues within the nuclear localization signal of p21 modulates the subcellular distribution of p21 (Xia *et al.,* 2004; Yuste *et al.,* 2005; Pérez-Tenorio *et al.,* 2006). To understand the mechanistic insights on GNL1-mediated p21 cytoplasmic localization, the status of p21 phosphorylation was determined under GNL1 expression and knockdown conditions. Toward this, GNL1 was expressed in both HCT116$^{p53+/+}$ and SKBR3 cells and the levels of p21 phosphorylation were determined by Western blot analysis. Results showed that GNL1 expression significantly up-regulated the p21 phosphorylation in both tested cell lines (Figure 5A and Supplemental Figure S5A, lane 2). In contrast, knockdown GNL1 in HCT116$^{p53+/+}$ cells significantly reduced the phospho p21 level (Figure 5B, lane 2). Total p21 protein level was normalized to measure the phosphorylation status, and the knockdown of GNL1 was determined by Western blot using anti-GNL1 antibodies. Evidence from the literature suggests that the protein kinase B/AKT is one of the main kinases attributed to p21 phosphorylation and its subsequent cytoplasmic localization (Zhou *et al.,* 2001; Héliez *et al.,* 2003; Park *et al.,* 2006). We therefore first analyzed the levels of phosphorylated AKT (p-Ser-473-AKT) on GNL1 expression by Western blot using phospho-specific AKT antibodies. Results indicate that GNL1 expression increased the phospho-AKT levels without altering the total AKT levels in both HCT116$^{p53+/+}$ and SKBR3 cells (Figure 5C and Supplemental Figure S5B, lane 2). Interestingly, GNL1 knockdown with two independent shRNAs resulted in a significant reduction of phospho-AKT levels without altering its expression of HCT116$^{p53+/+}$ cells (Figure 5D, lanes 2 and 3). Together, these data suggest that GNL1 may function as a regulatory component of the network, to activate AKT/p21 signaling pathways to promote cell growth and survival during tumorigenesis.

To further define the importance of the AKT signaling pathway in the antiapoptotic function of GNL1, GNL1-expressing HCT116$^{p53+/+}$ and SKBR3 cells were treated with AKT inhibitor and we measured the cellular apoptosis using annexin-V/APC staining followed by flow cytometry. As expected, we observed a significant decrease in apoptotic cell population in GNL1-expressing HCT116$^{p53+/+}$ (Figure 5E) and SKBR3 cells (Supplemental Figure S5C). In contrast, GNL1 was not able to block apoptotic cell death in cells treated with AKT inhibitor (Figure 5E and Supplemental Figure S5C). Collectively, these results suggest that the AKT signaling pathway plays an important role in the antiapoptotic function of GNL1. We next tested the status of p21 phosphorylation in GNL1-expressing cells in the presence of AKT inhibitor. Western blot analysis suggests that there is a significant reduction of total as well as phospho-p21 levels in GNL1-expressing HCT116$^{p53+/+}$ (Figure 5F, lanes 2 and 4) cells in the presence of AKT inhibitor. Significant reduction of phospho-AKT levels was observed in GNL1-expressing cells treated with AKT inhibitor without altering total AKT protein levels in HCT116$^{p53+/+}$ (Figure 5F, lanes 2 and 4) and SKBR3 cells (Supplemental Figure S5D). Taken together, these results suggest that GNL1 modulates AKT-mediated p21 phosphorylation to execute its antiapoptotic function.

A recent report suggested that endoplasmic reticulum protein 29 (ERp29) performs its tumor-suppressive function by repressing the activity of the AKT signaling pathway (Ye *et al.,* 2017). To understand the mechanism by which GNL1 modulates AKT signaling to promote cell growth and survival, we determined whether GNL1 alters ERp29 expression. To verify these possibilities, expression of ERp29 was analyzed under GNL1 expression and knockdown conditions. As shown in Supplemental Figure S6A, expression of GNL1 in HCT116$^{p53+/+}$ cells significantly down-regulated the ERp29 mRNA levels; in contrast, shRNA-mediated depletion of GNL1 significantly up-regulated the ERp29 expression (Supplemental Figure S6B). Interestingly, results in Supplemental Figure S6C indicate that GNL1 expression repressess the ERp29 protein levels but observed increased expression of p21 and AKT protein levels (lane 2). Interestingly, this effect was reversed under GNL1 knockdown condition (Supplemental Figure S6D, lane 2). Collectively, these data suggest that GNL1 modulates the ERp29/AKT/p21 signaling pathway to regulate the cell growth and survival during tumorigenesis.

GNL1 promotes p21 protein stabilization

Evidence from the literature suggests that AKT-mediated p21 phosphorylation plays an essential role in stabilizing p21 protein levels in cells (Child and Mann 2006; Park *et al.,* 2006). Interestingly, interference of AKT signaling resulted in destabilization of p21 by proteasomal degradation pathway (Child and Mann, 2006). The dependency of AKT activity on GNL1-mediated p21 stabilization was determined with or without AKT1 inhibitor. Interestingly, we observed rapid destabilization of p21 in cells expressing GFP or GNL1-GFP with AKT1 inhibitor (Figure 6A, lanes 3 and 4) compared with DMSO-treated cells (Figure 6A, lanes 1 and 2). To understand whether the inhibition AKT activity resulted in faster degradation p21 by proteasomal pathway, GNL1-expressing cells were treated with AKT inhibitor in the presence of proteasome inhibitor MG132 and we measured the levels of p21. Western blot analysis showed that the MG132 treatment stabilized p21 protein levels in GNL1–GFP-expressing cells despite the inhibition of AKT activity (Figure 6A, lane 6). These data support the notion that AKT activity is critical for GNL1-mediated p21 stabilization. To further confirm, ubiquitination status of p21 was determined in the presence of GNL1. Results from the ubiquitination assay reveal that GNL1 expression reduced the levels of polyubiquitinated p21, whereas we observed increased polyubiquitination of p21 in GNL1-expressing cells treated with AKT inhibitor (Figure 6B, lanes 2 and 4). Taken together, our results provided evidence that GNL1 requires functional AKT signaling pathway to stabilize p21 in cells.

Antiapoptotic function of GNL1 is p21 dependent

To further understand whether the observed antiapoptotic function of GNL1 is p21 dependent, the status of cell apoptosis was determined in cells with GNL1expression under p21 knockdown conditions. Interestingly, results presented in Figure 7A clearly suggest that GNL1 was not able to block cellular apoptosis under p21 knockdown, whereas a significant decrease in apoptotic cell population was observed in GNL1-expressing cells transfected with scrambled shRNA (Figure 7A). Furthermore, our results reveal that a significant increase in cell viability was observed in GNL1-expressing cells, and

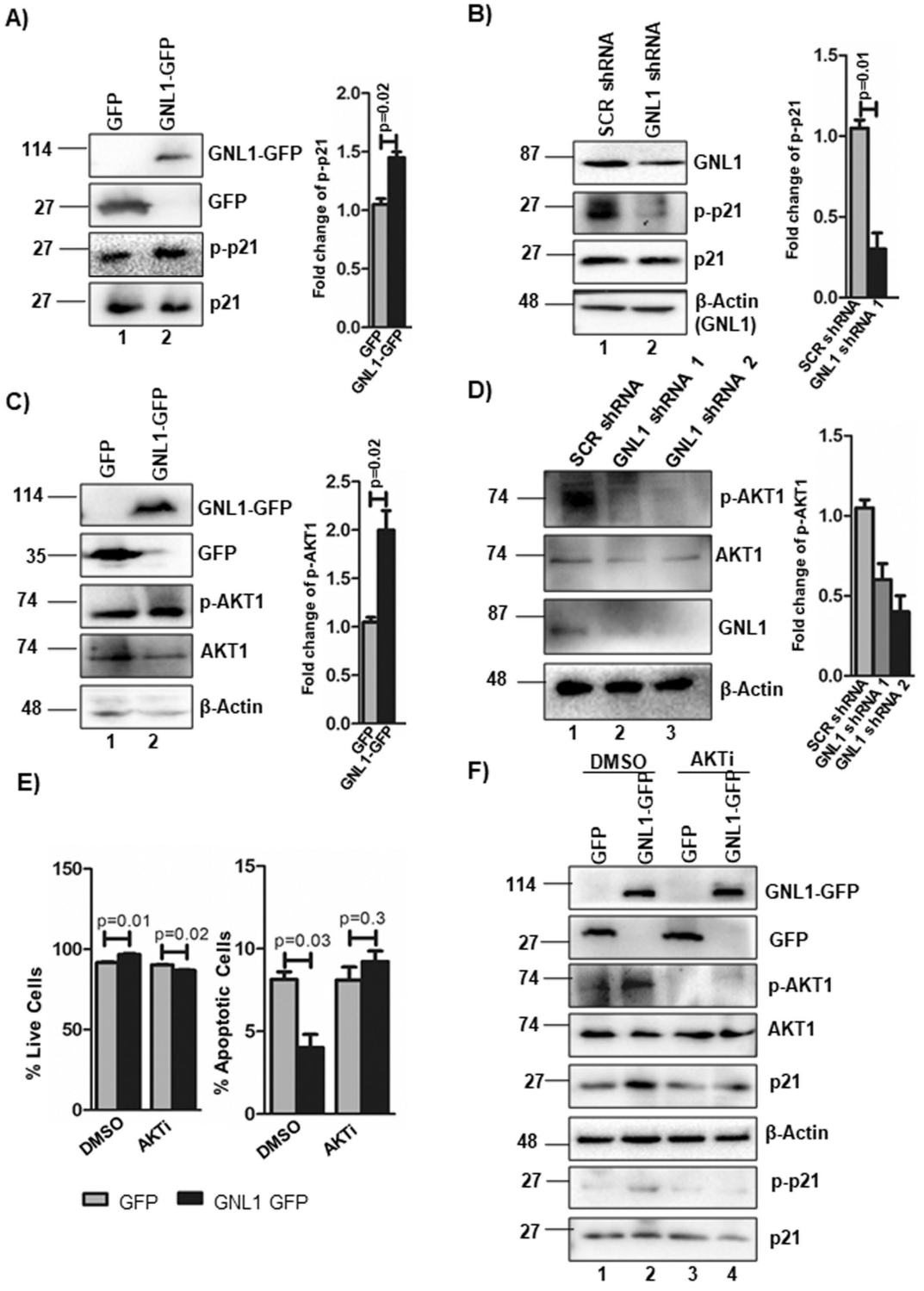

FIGURE 5: GNL1-dependent AKT-mediated p21 phosphorylation is critical to promote cell proliferation and survival. (A) GNL1 expression in HCT116$^{p53+/+}$ cells promotes p21 phosphorylation without altering total cellular p21 protein levels. Phospho-p21 and total p21 levels were measured by Western blot using anti-phospho-p21 and anti-p21 antibodies, respectively. (B) Knockdown of GNL1 by specific shRNA in HCT116$^{p53+/+}$ cells reduced the levels of p21 phosphorylation. (C) GNL1 expression in HCT116$^{p53+/+}$ cells promote AKT phosphorylation and this effect was reversed by GNL1 knockdown using specific shRNAs (D). Phosphorylation of AKT was analyzed by Western blot using anti-phospho-AKT (Ser473) antibodies. Two independent shRNAs were used for GNL1 knockdown and knockdown efficiency was determined by Western blot using anti-GNL1 antibodies. The densitometry analyses of the corresponding Western blots were carried out by normalizing the expression levels of the indicated phospho-proteins to the total endogenous protein. (E) GNL1 failed to block apoptosis in HCT116$^{p53+/+}$ cells in the presence of AKT inhibitor (AKTi). Annexin-V/APC staining was used to determine the status of apoptosis. (F) GNL1 was not able to activate AKT phosphorylation in the presence of AKTi in HCT116$^{p53+/+}$ cells. Beta actin was used as a loading control.

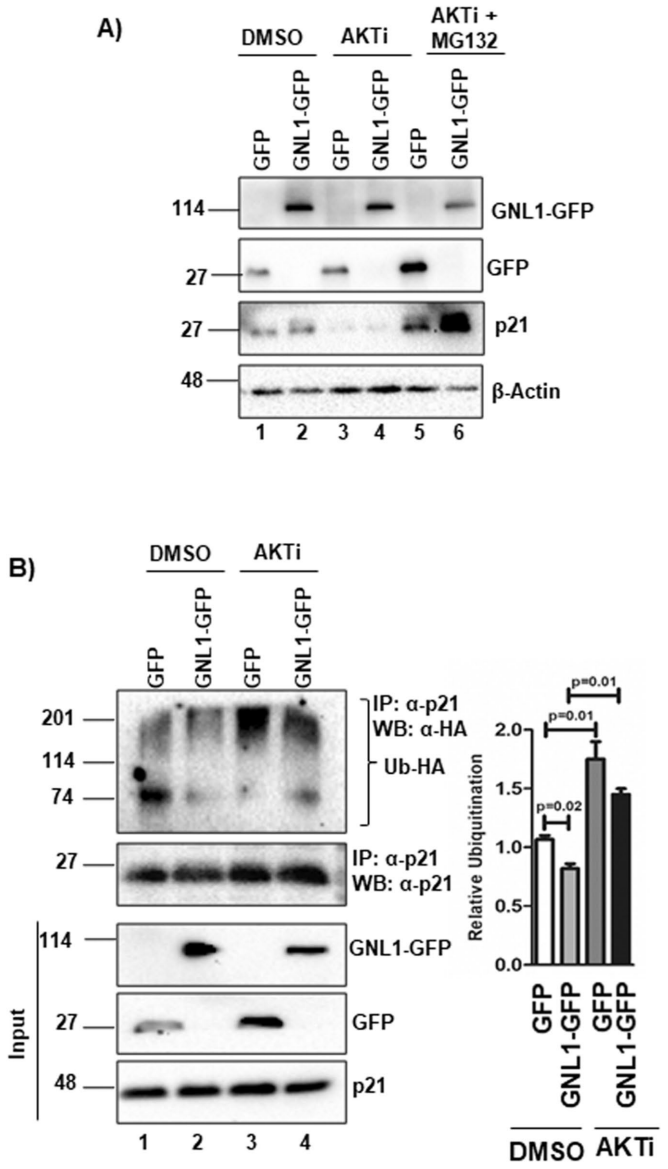

FIGURE 6: GNL1 stabilizes p21 by promoting AKT-mediated phosphorylation. (A) HCT116$^{p53+/+}$ cells transfected GNL1-GFP expression or control vector were treated with AKTi with or without MG132. GNL1 and p21protein levels were determined by Western blot using anti-GFP and anti-p21 antibodies, respectively. (B) HCT116$^{p53+/+}$ cells expressing GFP or GNL1-GFP were treated with AKTi along with MG132 and ubiquitination assay was carried out as described in *Materials and Methods*.

this effect was reversed under p21 knockdown conditions despite efficient GNL1 expression (Figure 7B). The knockdown efficiency of p21 was checked by Western blotting using anti-p21 antibodies (Figure 7C). To further understand the importance of p21 in antiapoptotic function of GNL1, the levels of antiapoptotic proteins, survivin and Bcl-xL, and the status of caspase 8 cleavage were analyzed in cells expressing GNL1 with or without p21 depletion. Results in Figure 7C suggest that GNL1 expression up-regulates the survivin and Bcl-xL protein levels, but the cleaved caspase 8 level was reduced significantly under the same conditions (Figure 7C, lane 2). Interestingly, under p21 knockdown conditions, GNL1 failed to promote the expression of antiapoptotic proteins, survivin and Bcl-xL, and was not able to inhibit caspase 8 cleavage (Figure 7C, lane 4). Collectively, these data suggest that stabilization of p21 protein is critical for GNL1 to execute its antiapoptotic function.

Evidence for an inverse correlation of GNL1, p21, and AKT expression with survival of colon and breast cancer patients

We next evaluated the prognostic effect of GNL1, p21, and AKT gene expression in overall survival of colon (Figure 8A) and breast cancer (Figure 8B) patients from the tissue data available in the PrognoScan database (Mizuno *et al.*, 2009). The results from this analysis indicate the existence of a significant negative correlation between expression status of GNL1, p21, and AKT and survival of colon and breast cancer patients. The dotted lines in the KM plot represent 95% confidence intervals for each group. Collectively, these results suggest that the interplay among GNL1, p21, and AKT may be critical to promote tumorigenesis.

DISCUSSION

GNL1 is a putative nucleolar GTPase localized in different cellular compartments in a cell cycle-dependent manner to regulate cell proliferation (Boddapati *et al.*, 2012; Krishnan *et al.*, 2018). In the present study, we have attempted to gain novel insights into the antiapoptotic function of GNL1. GNL1 was shown to modulate the expression levels of pro- and antiapoptotic proteins and inhibit cell apoptosis. Interestingly, in HCT116$^{p53-/-}$ cells, GNL1 up-regulates p21 protein, not mRNA levels, which suggest that GNL1 modulates cellular p21 stability at posttranslational levels independent of p53. In addition, GNL1 promotes the accumulation of p21 in the cytoplasmic compartment by promoting AKT-mediated p21 phosphorylation. Furthermore, our results suggest that GNL1 activates AKT signaling by suppressing the expression of ERp29, a known inhibitor of AKT. Finally, analysis using the PrognoScan database suggests that the higher-level expression of GNL1, p21, and AKT in primary colon and breast cancers was associated with poor patient survival. Collectively, data from the present investigation provided evidence that cross-talk between GNL1 and the ERp29/AKT/p21 signaling pathway plays a critical role in promoting cell proliferation and survival during tumorigenesis.

Human GNL1 was initially identified as a putative nucleolar GTPase, encodes 607 amino acids, and is highly conserved from prokaryotes to human (Vernet *et al.*, 1994). A recent report from our laboratory demonstrated that GNL1 encompasses a novel arginine/lysine-rich nucleolar localization signal and shuttles between different subcellular compartments in a cell cycle-dependent manner (Boddapati *et al.*, 2012). GNL1 exported from the nucleus via the CRM1-independent pathway (Boddapati *et al.*, 2012), but the other member of Yaw/YlqF family of GTPases, GNL3L shuttles via the CRM1-dependent pathway (Thoompumkal *et al.*, 2015). Previous reports suggest that GNL1 promotes the E2F1 release from Rb-E2F1 inhibitory complex by altering Rb phosphorylation, which is critical to induce G1/S and G2/M phase transition for faster mammalian cell proliferation (Krishnan *et al.*, 2018). Gene expression analysis revealed that increased expression of GNL1 in human cancers was associated with poor patient survival. Together, these data suggest that GNL1 plays an important role in cell cycle regulation, but the mechanism remains poorly understood.

Results from the current investigation clearly indicate that GNL1 inhibits apoptosis by modulating the expression levels of pro- and antiapoptotic proteins and the cleavage of caspases. Furthermore, depletion of GNL1 sensitizes cells to chemo-drug (camptothecin and oxaliplatin)-induced apoptosis. Both chemo-drugs are known to induce DNA damage and activate the p53/p21 pathway. Interestingly, our results suggest that GNL1 up-regulates both p53 and p21

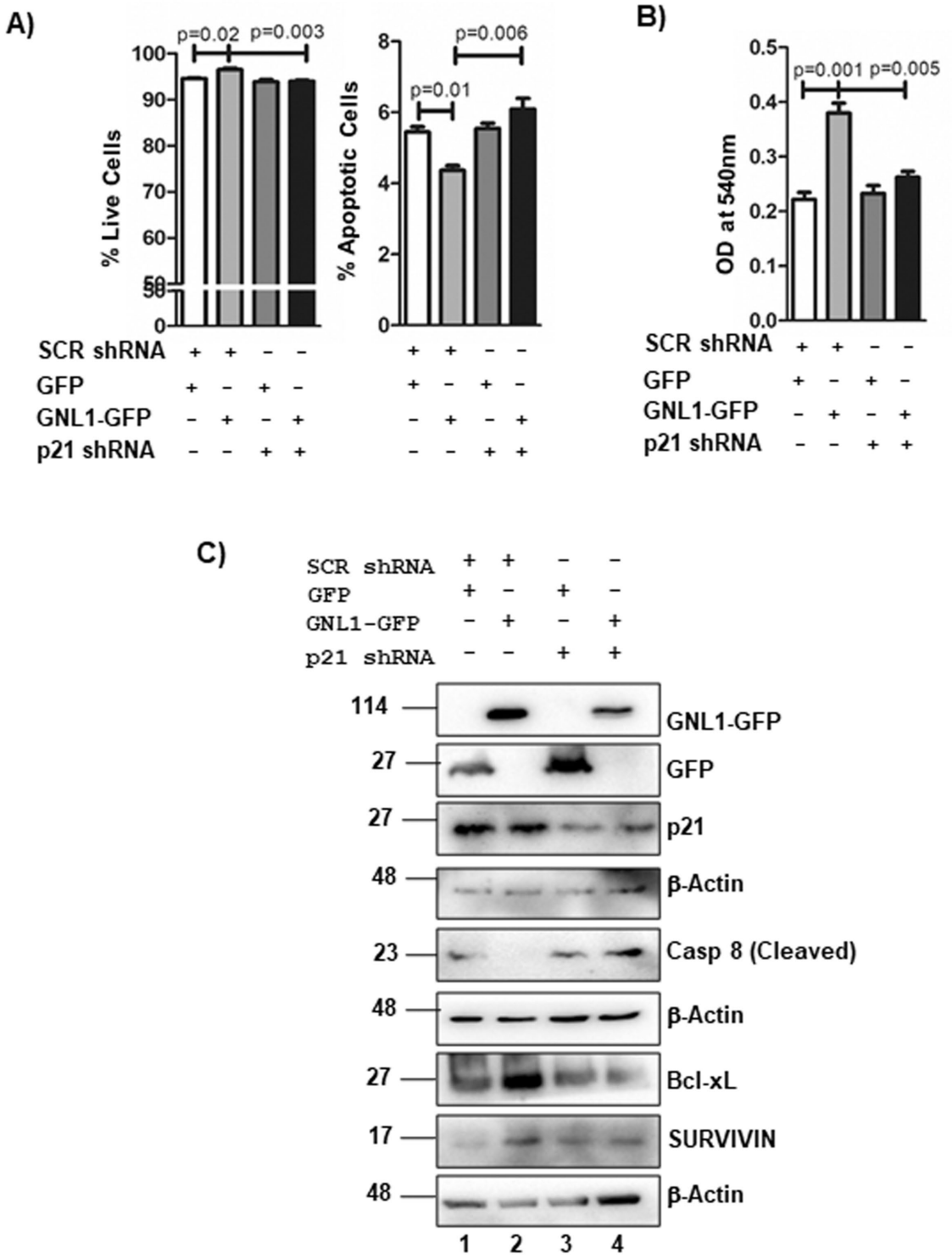

FIGURE 7: Antiapoptotic function of GNL1 is p21 dependent. (A) HCT116$^{p53+/+}$ cells were cotransfected with GNL1-GFP or control vector along with specific p21 shRNA or scrambled shRNA constructs and the status of apoptosis was measured by Annexin-V binding assay. (B) The cell viability was determined by MTT assay. (C) Expression GNL1-GFP, Bcl-xL, and survivin; cleavage of caspase 8; and the extent of p21 knockdown were determined by Western blot analysis using respective antibodies. Beta actin was used as loading control.

mRNA and protein levels in cells. It is worth mentioning that expression of GNL1 in HCT116$^{p53-/-}$ cells increased p21 protein without altering its mRNA levels. In addition, knockdown of GNL1 decreased p21 protein level in p53 null cells, suggesting that GNL1 regulates cellular p21 protein accumulation at posttranslational level via a novel p53-independent mechanism. This observation was unforeseen because p21 is a target of p53 and known to inhibit cell proliferation. Several reports suggest that p21 up-regulates G1/S progression by modulating the complex formation between cyclin D1 and CDK4 and further showed that it is a positive regulator of

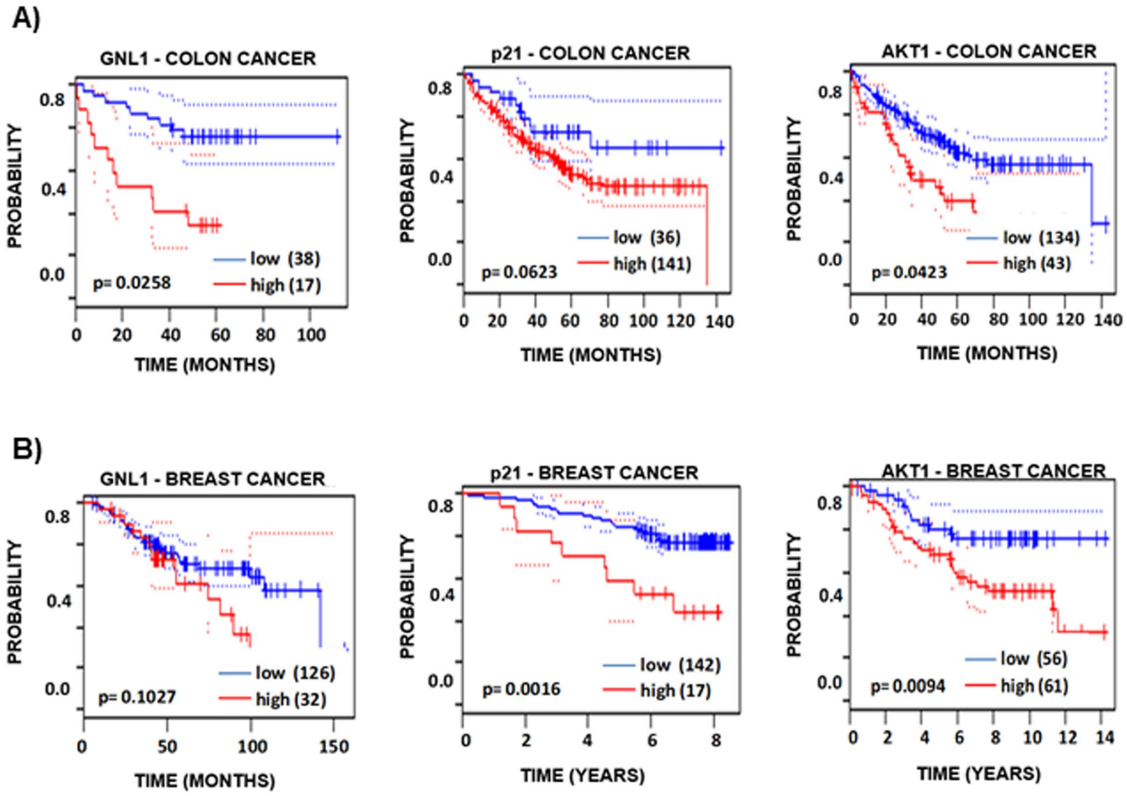

FIGURE 8: Expression of GNL1, p21 and AKT was inversely correlated with breast and colon cancer patient survival. Kaplan–Meier plots indicate the differential expression levels of GNL1, p21, and AKT in (A) colon and (B) breast cancers. The survival curves for high (red) and low (blue) expression groups indicate poor survival of patients associated with high levels of GNL1, p21, and AKT1 expression. Confidence intervals (95%) for each group are also indicated by dotted lines.

cell survival (Labaer *et al.*, 1997; Ghannam-Shahbari *et al.*, 2018). This was supported by the fact that p21 was found to be overexpressed in many human cancers and positively correlated with resistance to chemo- and radiation therapy (Shiohara *et al.*, 1994; Jung *et al.*, 1995; Elledge and Allred 1998; Aaltomaa *et al.*, 1999; Ferrandina *et al.*, 2000; Biankin *et al.*, 2001; Cheung *et al.*, 2001; Abbas and Dutta, 2009). Increased p21 levels in late-stage gliomas do not correlate with p53 levels (Jung *et al.*, 1995) and supports the notion that p21 levels may be up-regulated by p53-independent mechanism(s). Reduced levels of p21 in terminally differentiated cells such as muscle cells, hematopoietic stem cells, and macrophages resulted in apoptotic cell death (Asada *et al.*, 1999; Cheng *et al.*, 2000; Lawlor and Rotwein, 2000). In addition, the reduction of p21 expression sensitizes cells to Adriamycin, camptothecin, etoposide, gamma radiation, or prostaglandin treatment (Polyak *et al.*, 1996; Waldman *et al.*, 1996, 1997; Tian *et al.*, 2000). It is well documented that nuclear localization of p21 is critical to inhibit CDK and PCNA activity for arresting cell division cycle during DNA damage response (Harper *et al.*, 1995). On the contrary, the cytoplasmically localized p21 binds to pro-caspase 3 and promotes resistance to Fas-mediated cell apoptosis (Suzuki *et al.*, 1998, 1999; Xia *et al.*, 2004). Furthermore, p21 interacts with proapoptotic kinase ASK1 and inhibits apoptosis (Zhan *et al.*, 2007). Increased expression of cytoplasmic p21 in human primary breast carcinomas may be considered as a factor of poor prognosis (Winters *et al.*, 2001; Xia *et al.*, 2004). Together, these data suggest the possibility that the p21 subcellular localization plays an important role in determining its function. Interestingly, the observed cytoplasmic retention of p21 in the presence of GNL1 expression in the present investigation supports the notion that p21 may play an important role in regulating the antiapoptotic function of GNL1 during tumorigenesis.

Recent study suggests that p21 levels may be regulated by p53-independent mechanisms (Johannessen *et al.*, 1999). For example, epidermal growth factor up-regulates the p21 level independent of p53 by activating the PI3K/AKT pathway (Johannessen *et al.* 1999). Activated AKT phosphorylates p21 at Thr 145/Ser146 and promotes its accumulation in the cytoplasmic compartment, which is critical for p21 stability (Zhou *et al.*, 2001). Results from the present study provided evidence that GNL1 induces AKT phosphorylation and the activated AKT phosphorylates p21 at Thr145, which is critical for p21 stabilization. Immunofluorescence experiments clearly indicated that the phosphorylated p21 predominantly localized in cytoplasm (Figure 4D and Supplemental Figure S4). Interestingly, results from the present study indicate that pharmacological inhibition of AKT activity impaired antiapoptotic function of GNL1 (Figure 5E and Supplemental Figure S5C). In addition, GNL1 was unable to inhibit the apoptosis under p21 knockdown condition in colon cancer cells (Figure 7A). A recent report demonstrates that ERp29 executes its tumor suppression function by blocking AKT signaling in gastric cancers (Ye *et al.*, 2017). Interestingly, our data suggest that GNL1-mediated suppression of ERp29 expression may be critical for the activation of AKT signaling to stabilize p21 protein levels to promote cell proliferation. These results are in agreement with the AKT role in promoting cell survival (Shiohara *et al.*, 1994). Taken together, data from the present study suggest that AKT-dependent p21 phosphorylation plays an important role in GNL1-mediated cell survival during tumorigenesis.

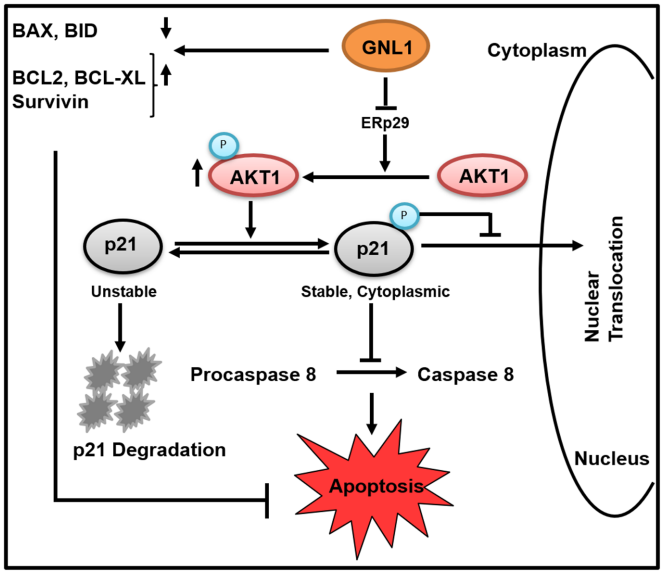

FIGURE 9: Proposed schematic model summarizing the role of GNL1 as an antiapoptotic protein. Results from the present investigation clearly illustrate that: 1) GNL1 alters the expression of pro- and antiapoptotic BCL2 family proteins; 2) suppression of ERp29 is required for the activation of AKT signaling pathway; 3) GNL1 promotes cytoplasmic localization and stabilization of p21 in AKT-mediated phosphorylation-dependent manner; and 4) cytoplasmic p21 inhibits caspase 8 cleavage, which is essential for the antiapoptotic function of GNL1.

Based on the results from the existing reports and from the current investigation, we proposed a functional model for GNL1 (Figure 9). The proposed model suggests that GNL1 promotes cell survival by regulating cellular apoptosis via the following: 1) alters the expression of pro- and antiapoptotic Bcl2 family proteins, 2) activates the AKT/p21 signaling pathway, 3) promotes cytoplasmic localization and stabilization of p21 in AKT-mediated phosphorylation dependent manner, and 4) promotes cell survival by inhibiting caspase 8 cleavage and cell apoptosis. An important function of activated PI3K/AKT pathway in cells is to modulate programmed cell death (Yao and Cooper, 1995). The activated AKT pathway regulates the translocation of Forkhead Box O transcription factors, thereby suppressing the expression of proapoptotic proteins (Datta *et al.*, 1997; Kops *et al.*, 1999). Activation of AKT pathway might be one of the possible mechanisms by which GNL1 alters the expression levels of pro- and antiapoptotic proteins. Results obtained from a current study provide evidence that GNL1 negatively regulates the expression of ERp29, and the recent report suggests that its expression was inversely associated with breast tumor development (Bambang *et al.*, 2009). Interestingly, loss of ERp29 expression in gastric cancer tissues resulted the activation of the PI3K/AKT pathway, thereby inducing epithelial-to-mesenchymal transition (Wu *et al.*, 2017). Together, the data from the present study suggest that GNL1-mediated down-regulation of ERp29 might result in the activation of the PI3K/AKT pathway. Further studies are warranted to understand the other key signaling molecules modulated by GNL1 to execute its antiapoptotic function.

The underlying mechanism(s) for cancer cells to develop resistance to chemotherapy is more complex. It is well documented that there is an association of the PI3K/AKT pathway with the acquired resistance of cancers to a variety of chemo-drugs, such as gemcitabine, irinotecan, etoposide, and Taxol (Campbell *et al.*, 2001; Pérez-Tenorio *et al.*, 2002; Shah *et al.*, 2005; Bleau *et al.*, 2009; Sinnberg *et al.*, 2009). Hyperphosphorylation of AKT has been observed in many cancers (Pérez-Tenorio *et al.*, 2002; Shah *et al.*, 2005). The results from the current investigation for the first time provided evidence that GNL1 promotes the cytoplasmic retention of p21 through AKT activation by suppressing ERp29 expression, which in turn is critical in promoting cell proliferation and survival. Collectively, these data elucidate a novel GNL1/ERp29/AKT/p21 signaling cascade to provide a growth benefit to cancer cells. It is worth mentioning that PI3K/AKT inhibitors may provide a benefit to patients with tumors expressing high levels of GNL1. The observed expression pattern of GNL1 and p21 in various cancers suggests the possibility that the synergy between GNL1 and p21 may enhance chemoresistance and this may be an ideal target for cancer therapeutic intervention.

MATERIALS AND METHODS

Request a protocol through Bio-protocol.

Plasmid construction

GNL1 open reading frame was amplified from HeLa cDNA using appropriate primers as described elsewhere (Boddapati *et al.*, 2012). The amplified product was digested with *Hin*dIII and *Bgl*II and cloned into a modified pcDNA3 vector (Invitrogen, USA) as fusion with GFP (Essers *et al.*, 2014). DNA sequencing was performed to validate the integrity of all plasmids.

Chemicals, antibodies, and reagents

DMEM, RPMI 1640, and fetal bovine serum (FBS) were from Invitrogen Life Technologies. Annexin V–APC was procured from BD Biosciences. Camptothecin and oxaliplatin were from Sigma and AKT inhibitor IV from Millipore. All other reagents used in this study are of molecular biology grade. The antibodies used in this study are detailed in Supplemental Table S2.

Cell culture, transfection, immunoprecipitation, and Western blot analysis

HCT116$^{p53+/+}$, HCT116$^{p53-/-}$, SKBR3, T47D, and MDAMB231 cells were cultured in DMEM with 10% FBS and 1% antibiotics-antimycotics (Thermo Fisher Scientific, USA). Cells were grown to 60% confluency and transfected with polyethyleneimine (PEI) or lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol. Transfected cells were lysed using 1× cell lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM KCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 0.4 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM NaF, 1 mM Na3VO4, and 1 µg/ml each of aprotinin, leupeptin, and pepstatin). For detection of phosphoprotein, cell pellets were sonicated in 1× cell lysis buffer (50 mM Tris,150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1× protease inhibitor cocktail, 1× phosphatase inhibitor cocktail, 1 mM PMSF, 2 mM Na3VO4,100 nM okadoic acid, 0.5% sodium deoxycholate, and 0.1% SDS). Proteins were separated on SDS–12% PAGE, transferred onto PVDF membrane (PerkinElmer, USA), and probed with indicated antibodies. HRP–conjugated specific secondary antibodies (Bio-Rad Laboratories, USA) were incubated with protein–antibody complexes and detected using the enhanced chemiluminescence prime detection system (GE Healthcare, Sweden). For immunoprecipitation, equal amounts of cell lysates were incubated with anti-p21 antibodies for 6 h at 4°C, and the antibody–protein complexes were eluted and resolved on SDS–12% PAGE followed by Western blotting with anti-GNL1 antibody.

Densitometry analysis of Western blots was carried out using ImageJ software.

RNA interference

GNL1 shRNA1 (TRCN0000189397: CCGGCCCGATACTTTCAGAC CTACTCTCGAGAGTAGGTCTGAAAGTATCGGGTTTTTTG), GNL1 shRNA2 (TRCN0000193121: CCGGCGATACTTTCAGACCTACTTT CTCGAGAAAGTAGGTCTGAAAGTATCGTTTTTT), and p21 shRNA (TRCN0000040127: CCGGGTCACTGTCTTGTACCCTTGTCTCGA-GACAAGGGTACAAGACAGTGACTTTTTG) were used for knockdown of GNL1 and p21, respectively, in HCT116$^{p53+/+}$ and SKBR3 cells. The universal control shRNA (SHC016: CCGGGCGCGATAGC-GCTAATAATTTCTCGAGAAATTATTAGCGCTATCGCGCTTTTT, Sigma-Aldrich, USA) was used as scrambled negative control in all the knockdown experiments. All shRNAs were purchased from Sigma-Aldrich (USA).

Fluorescence microscopy

HCT116$^{p53+/+}$ or SKBR3 cells grown on coverslips (Thermo Fisher Scientific, USA) were transfected with pCDNA3/GFP or pCDNA3/GNL1-GFP expression plasmids using PEI. For determining the subcellular distribution of proteins, transfected cells were fixed using 3% (wt/vol) paraformaldehyde and permeabilized with Triton X-100. Endogenous proteins were stained with specific antibodies for 1 h at room temperature or overnight at 4°C. Hoechst 33342 was used to stain the nuclei at a final concentration of 1 mg/ml. Samples were then viewed with LSM710 laser scanning confocal microscope (Carl Zeiss, Germany), and image acquisition was performed using Zen 2009 software (Carl Zeiss, Germany).

Ubiquitination assay

HCT116$^{p53+/+}$ cells were transiently transfected with plasmid expressing HA-ubiquitin and GNL1-GFP or GFP. After 40 h of transfection, cells were treated with 20 μM MG132 for 8 h before being harvested. Cell lysates were subjected to coimmunoprecipitation using anti-p21 antibodies and the ubiquitin conjugates were detected by Western blot analysis using anti-HA or anti-ubiquitin antibodies.

Annexin-V binding assay

HCT116$^{p53+/+}$ or SKBR3 cells were transfected with indicated expression or shRNA plasmids. Cells were trypsinized to obtain a single cell suspension after 48 h of transfection and incubated with binding buffer (0.1 M HEPES/1.4 M NaCl, 25 Mm $CaCl_2$) containing Annexin-APC and propidium iodide for 15 min at room temperature. The samples were then analyzed using a flow cytometer (FACS Canto II, BD Biosciences, USA), and the results were processed using FACS Diva software.

MTT assay

HCT116$^{p53+/+}$ were cotransfected with GNL1-GFP with or without p21-specific shRNA plasmid. MTT solution (12 mM; Thermo Fisher Scientific, USA) was added to cells and incubated for 4 h at 37°C, and the absorbance was measured at 540 nm using Enspire Multimode Reader (PerkinElmer, USA).

qPCR analysis

Total RNA from HCT116$^{p53+/+}$ cells transfected with different expression plasmids was isolated using TRIzol reagent (TaKaRa, Japan). Total RNA (2 μg) was reverse transcribed using PrimeScript RT reagent Kit (TaKaRa, Japan) according to the manufacturer's instructions. Quantitative PCR analysis was performed using SYBR-Green mix (TaKaRa, Japan) on Realplex cycler (Eppendorf, Germany). The expression levels of various genes relative to beta-actin were analyzed using $\Delta C\tau$ values according to the manufacturer's directions. The primers used for the qPCR analyzes are described in Supplemental Table S1.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5.0 software. The experiments were repeated thrice with three biological replicates. Error bars representing mean ± SD were drawn from a representative experiment with biological triplicates. The *p* values indicative of statistical significance were obtained by performing a Student's *t* test (unpaired). ImageJ software was used for densitometry analysis of Western blots.

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ETOC:

GNL1 inhibits cell apoptosis and promotes cell growth by inducing cytoplasmic retention and stabilization of p21 through AKT-mediated phosphorylation. Our data elucidate a novel GNL1/AKT/p21 signaling cascade to provide a growth benefit to cancer cells and this may be an ideal target for cancer therapeutic intervention.