

Depletion of Neuroguidin/CANu1 sensitizes human osteosarcoma U2OS cells to doxorubicin

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Osteosarcoma is a primary bone cancer which occurs mainly in children. Neuroguidin/CANu1 is a nucleolar protein involved in the maintenance of ribosomal structure. In this study, we investigated the effect of Neuroguidin/CANu1 depletion on the response of osteosarcoma cells to doxorubicin. In normal circumstances, Neuroguidin/CANu1 is localized at nucleoli, which translocates to nuclear foci in the presence of doxorubicin. shRNA knockdown of Neuroguidin/CANu1 did not affect cell viability in the absence of doxorubicin, but led to enhanced cytotoxicity in doxorubicin-treated cells. Doxorubicin increased the population of apoptotic cells by 3-fold in Neuroguidin/CANu1-depleted cells compared to that in control cells. Depletion of Neuroguidin/CANu1 mRNA induced the expression of p21 and the cleavage of PARP, leading to increased caspase-3/7 activity. Together, these results suggest that Neuroguidin/CANu1 is required for maintaining cellular homeostasis and may contribute to the improved efficiency of chemotherapy. [BMB reports 2011; 44(1): 46-51]

INTRODUCTION

Osteosarcoma is primary bone cancer which particularly affects pediatric patients. Osteosarcoma is an aggressive tumor with frequent occurrence of pulmonary metastasis (1). Since the introduction of multi-agent chemotherapy for osteosarcoma over 30 years ago, overall survival has exceeded 70% (2, 3). Current treatment regimens usually involve neoadjuvant and adjuvant chemotherapy with high-dose methotrexate, doxorubicin, cisplatin, and ifosfamide. However, the degree of success achieved with current drugs with known activity in osteosarcoma has reached a plateau. Moreover, for osteosarcoma patients treated with the current highly aggressive chemotherapy regimens, acute and long-term toxicity is a significant problem. In addition, a large portion of patients do not re-

spond to chemotherapy protocols and go on to develop metastasis (4). It is crucial to understand the mechanisms of chemotherapy resistance and to identify new targets to improve the management of osteosarcoma.

Recently, we identified a novel 315-aa nucleolar protein, human Neuroguidin/CANu1, which is encoded by a gene on chromosome 14q11.2. Neuroguidin/CANu1 is strongly expressed in tumor cells and localizes to the nucleolus (5). Neuroguidin (Ngd), a mouse homolog of CANu1, localizes to the axons of neurons and forms punta at dendrites. The N-terminus of Ngd comprises the binding motif of eukaryotic initiation factor 4E (eIF4E). Ngd associates with cytoplasmic polyadenylation element binding protein (CPEB), stimulating elongation of the mRNA polyadenine tail to regulate the translation of CPE-containing mRNA during neurogenesis (6).

Various nucleolar proteins diffuse into the nucleoplasm in response to UV, radiation, and cytotoxic agents. These proteins are Werner syndrome protein (WRN), ARF, Ki-67, nucleolin, fibrillarin, topoisomerase 1 (Topo 1), and PARP-1/2 (7, 8). Previous studies have shown that nucleolin induces p53-dependent movement outside the nucleolus to block the initiation of DNA replication and stimulate DNA repair (9). Under stressful conditions, acetylated WRN moves outside the nucleolus to form repair foci, where it mediates DNA recombination and repair (9, 10). According to our previous study, Neuroguidin/CANu1 translocates from the nucleolus to the nucleoplasm in response to UV irradiation (5). These results suggest that Neuroguidin/CANu1 may be involved in sensing stress in the nucleolus and may, like other nucleolar proteins, be involved in damage response activity. In order to overcome the dose-limiting side effects of conventional chemotherapeutic agents, we explored the effectiveness of doxorubicin in a Neuroguidin/CANu1-shRNA model system. We determined the cell viability of the Neuroguidin/CANu1-knocked down osteosarcoma U2OS cells in response to doxorubicin.

RESULTS

The translocation of Neuroguidin/CANu1 in U2OS cells in response to doxorubicin

In a previous study, we observed that the Neuroguidin/CANu1 protein in U2OS cells translocates to the nucleoplasm from the nucleolus after UV irradiation. In present study, we inves-

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tigated the cellular distribution of Neuroguidin/CANu1 protein in U2OS cells treated with doxorubicin. As expected, doxorubicin prompted the localization of Neuroguidin/CANu1 to the nucleoplasm and the formation of nuclear foci (Fig. 1). We also determined which region of CANu1 is critical for this translocation by testing C- and N-terminal deletions. As shown in Fig. 1, the N-terminal deleted (1-107 a.a.) Neuroguidin/CANu1 protein (CANu1ΔN) did not form foci after doxorubicin treatment, but exhibited normal distribution in the absence of doxorubicin. The C-terminal deleted mutant (296-315 a.a.) of Neuroguidin/CANu1 (CANu1ΔC20) did not localize to the nucleolus even in the absence of doxorubicin, indicating that the nucleolar localization signal may exist in the C-terminal region of Neuroguidin/CANu1. In addition, our data suggest that the N-terminal region of Neuroguidin/CANu1 is critical for its translocation after exogenous stress.

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Neuroguidin/CANu1 depletion and cell viability in response to doxorubicin

Previous studies have shown that Neuroguidin/CANu1 is a stress response protein, although its role remains unclear. Since the pattern of Neuroguidin/CANu1 translocation under exogenous stresses is similar to those of other nucleolar stress-sensing proteins such as PML, p53, and hsp70 (11-13), we conducted a loss of function study using a shRNA system. First, we examined whether knockdown of Neuroguidin/CANu1 affects cell viability. In the absence of exogenous stress, viability in Neuroguidin/CANu1-depleted cells was not different from control cells (Fig. 2A). However, the viability of Neuroguidin/CANu1-depleted cells changed in response to

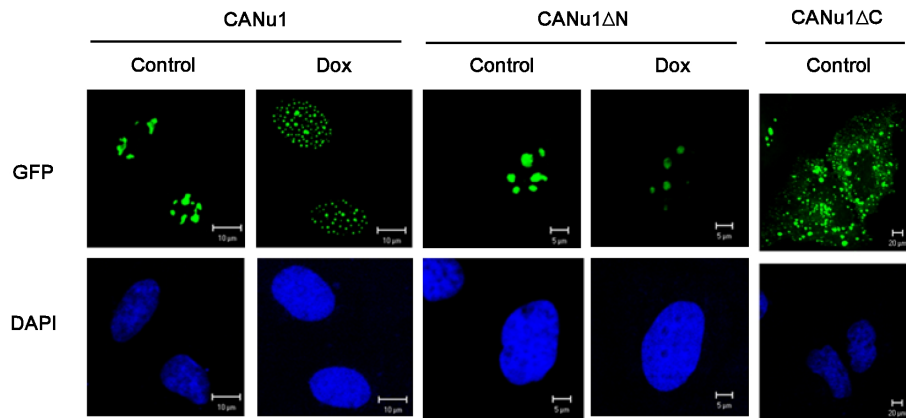


Fig. 1. The cellular localization pattern of GFP-Neuroguidin/CANu1 fusion protein in U2OS cells exposed to doxorubicin. GFP-CANu1-, GFP-CANu1ΔN-, or GFP-CANu1ΔC- expressing cells were treated with doxorubicin (1 μM). After 36 hr, these samples were fixed and stained with DAPI as a nuclear marker. Localization patterns were observed by fluorescence microscopy. Dox = doxorubicin.

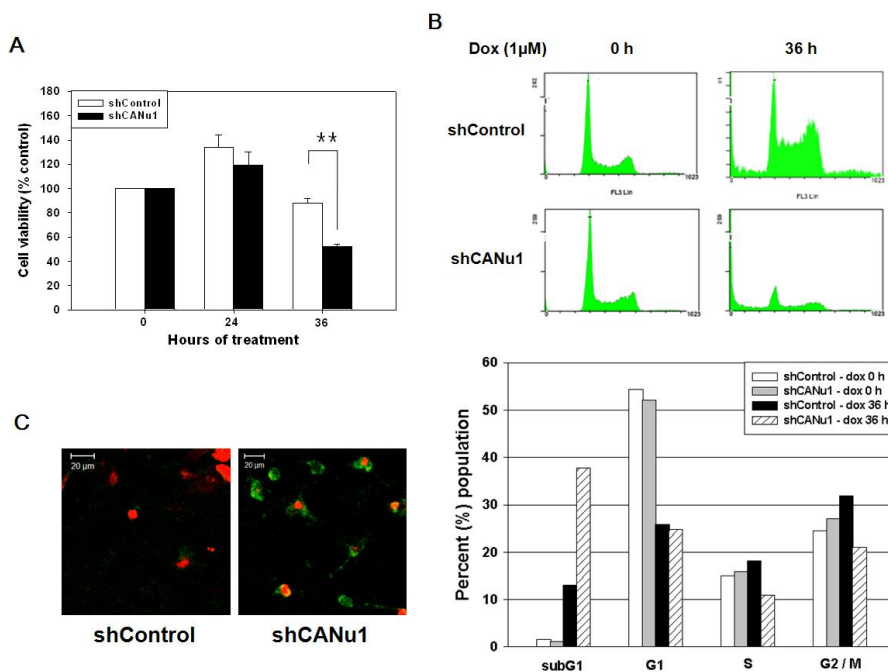


Fig. 2. Neuroguidin/CANu1-depleted U2OS cells reduce cell viability in response to doxorubicin. (A) Neuroguidin/CANu1-depleted U2OS cells were exposed to 1 μM doxorubicin. At the indicated time points, cell viability was determined by the MTS assay. Data represent the average of three independent experiments. Error bars represent the standard error. **P < 0.01. (B) The cells were infected with the Neuroguidin/CANu1 shRNA vector and treated with doxorubicin for 36 hr. The DNA contents in Neuroguidin/CANu1-depleted cells were analyzed by flow cytometry. The proportions of cells in the subG1, G1, S, and G2/M phases are indicated below each gate. Experiments were repeated three times with similar results. (C) Apoptotic cells induced by 1 μM doxorubicin were observed by annexin V-FITC/PI staining under fluorescence microscopy.

doxorubicin. After 36 h doxorubicin treatment, the population of Neuroguidin/CANu1 -depleted cells was reduced by 53% compared to that of control cells. We also analyzed the cell cycle profile in Neuroguidin/CANu1-depleted cells by flow cytometry. In the absence of exogenous stress we did not observe any differences between the cell cycle profiles of Neuroguidin/CANu1-deficient and control cells (Fig. 2B). However, at 36 h post-doxorubicin treatment, the depletion of Neuroguidin/CANu1 expression in all cells reduced the number of cells in the S and G2/M phases and was accompanied by the accumulation of cells in the sub G1 phase, whereas the population of S and G2/M phase was increased in control cells. Following treatment with 1 μ M doxorubicin, the apoptosis rate in the control group increased from 1.3% to 13%, whereas that in the Neuroguidin/CANu1-depleted cells dramatically increased from 1.2% to 37.8% (Fig. 2B). The enhanced rate of apoptosis induced by the depletion of Neuroguidin/CANu1 was confirmed by the annexin-V staining assay. We observed increased numbers of pre-apoptotic cells (annexin-positive) and post-apoptotic cells (both annexin- and PI-positive) among the Neuroguidin/CANu1-deficient cells, whereas few dead cells (PI-positive) were found in the control cells (Fig. 2C). These results indicate that Neuroguidin/CANu1 depletion attenuates cellular stability to induce apoptosis in osteosarcoma cells in response to doxorubicin, suggesting that Neuroguidin/CANu1 could serve as a stress response protein.

The effect of Neuroguidin/CANu1 depletion on the apoptotic pathway in response to doxorubicin

Next, we determined the expression of apoptosis-related proteins in Neuroguidin/CANu1-depleted cells after anti-tumor drug treatment. As shown in Fig. 3, the cleavage of poly (ADP-ribose) polymerase (PARP), an apoptosis marker, was observed in both the depleted and control strains. In the control cells the pattern of PARP cleavage appeared 12 h post-doxorubicin treatment, and was induced 6 h earlier in the Neuroguidin/CANu1-depleted cells, indicating that Neuroguidin/CANu1-deficient cells have increased chemosensitivity to doxorubicin. In addition, in the absence of doxorubicin, the expression of p21 and p53 in the Neuroguidin/CANu1-depleted cells was enhanced as same as our previous data (5). However, in the presence of doxorubicin, the induction of p21 protein was accelerated in Neuroguidin/CANu1-depleted cells by synergistic effect of doxorubicin, although the enhanced level of p53 in Neuroguidin/CANu1-depleted cells was not increased as much as that of control cells (Fig. 3). Its induction was delayed in control cells after doxorubicin treatment. These results suggest that the expression of p21 induced by doxorubicin in Neuroguidin/CANu1-depleted cells may be dependent of transcriptional activation of p53. Consistent with the p21 level and PARP cleavage, the activity of caspase 3/7 in the Neuroguidin/CANu1-knocked down cells was also increased by 3-fold compared to the control cells in the presence of doxorubicin

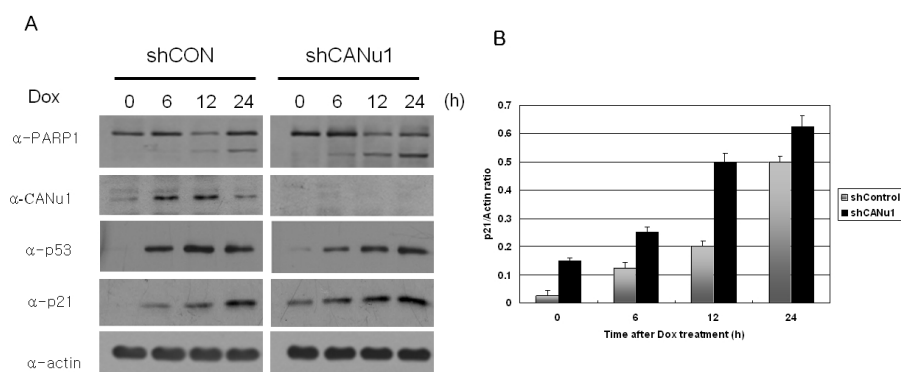


Fig. 3. Neuroguidin/CANu1 depletion affects apoptosis-related proteins in the presence of doxorubicin. (A and B) The expression of Neuroguidin/CANu1 in U2OS cells was knocked down by the shRNA approach. After treatment with doxorubicin, proteins related to apoptosis in U2OS cells were assayed by Western blotting. The ratio of p21 to actin was calculated at each time point. The bar graphs in (B) shows mean optical density and standard error of Western blot bands derived from three independent experiments.

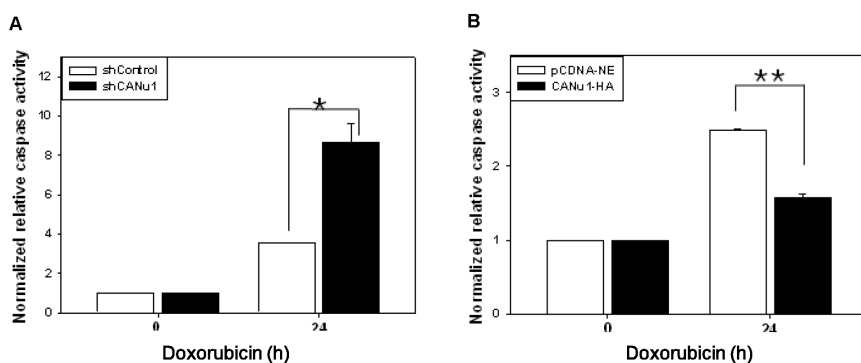


Fig. 4 Depletion of Neuroguidin/CANu1 stimulates caspase activity in U2OS cells in response to doxorubicin. (A) Neuroguidin/CANu1-depleted U2OS cells were treated with 1 μ M doxorubicin. Caspase-3/7 activity in Neuroguidin/CANu1-depleted cells was determined by caspase-Glo 3/7 luminescent assay. (B) shRNA Neuroguidin/CANu1-depleted cells were transiently transfected with an expression plasmid encoding Neuroguidin/CANu1 cDNA. After 24 h the cells were treated with 1 μ M doxorubicin. Caspase-3/7 activity was measured by caspase-Glo 3/7 luminescent assay. Data represent the average of three independent experiments performed in triplicate. Error bars represent the standard error. *P < 0.05, **P < 0.01.

(Fig. 4A). In order to confirm whether the increased activity of caspase 3/7 was induced by the depletion of Neuroguidin/CANu1, plasmids containing Neuroguidin/CANu1 cDNA were transfected into Neuroguidin/CANu1-depleted cells for the recovery of Neuroguidin/CANu1 protein. In the presence of doxorubicin, the activity of caspase 3/7 in Neuroguidin/CANu1 shRNA-treated cells was reduced after transfection with the Neuroguidin/CANu1 gene (Fig. 4B). Thus, doxorubicin-induced apoptosis in Neuroguidin/CANu1-depleted U2OS cells is directly mediated by the loss of function of Neuroguidin/CANu1. These results indicate that the depletion of Neuroguidin/CANu1 enhances chemosensitivity of human osteosarcoma cells to doxorubicin.

DISCUSSION

Although neoadjuvant chemotherapy is efficient in curing osteosarcoma, chemotherapy resistance and drug-induced side-effects remain serious problems (14, 15). Doxorubicin is commonly used as an anticancer drug with proven pharmacological effects, but it is limited by serious side effects, particularly cardiac toxicity (16-18). For these reasons, efforts to improve the efficacy of doxorubicin are essential and could benefit many patients suffering from a variety of cancers. In this study, we show synergistic cytotoxicity between Neuroguidin/CANu1 depletion and doxorubicin in osteosarcoma cells. Since Neuroguidin/CANu1 is strongly expressed in cancer cells (5), targeting its reduction may be a rational approach. In our study, we found that cell death induced by doxorubicin in the absence of Neuroguidin/CANu1 induces p21 expression. The p21 protein is stimulated by various therapeutic agents and is known to involve apoptotic processes (19). For example, the enhanced expression of p21 improves the sensitivity of human tumor cells to drugs including retinoid and oxysterol agents (20, 21). In addition, p21 increases the cytotoxic effects of cisplatin in human hepatoma and ovarian carcinoma (22, 23).

Since nucleolar proteins are known to regulate genomic stability, nucleoli are critical sites for maintaining cellular homeostasis (24-27). According to recent studies, nucleoli in cancer cells alter their morphology and the rate of ribosome biogenesis by neoplastic transformation (27, 28). In addition, the overexpression of nucleolar proteins is often found in tumor cells. The nucleolar protein nucleostemin regulates the proliferation of cancer cells and is highly expressed in many tumors (29, 30). Knockdown of nucleostemin expression induces a severe decline in cell proliferation (31, 32). In our study, Neuroguidin/CANu1 is also highly expressed in tumor cells (5) and the expression of Neuroguidin/CANu1 knocked down by shRNA decreased cellular viability in the presence of doxorubicin. When cells were exposed to DNA damage agents, the localization of Neuroguidin/CANu1 changed into nucleolar foci in nucleoplasm. These foci are similar to those of the DNA repair genes such as RECQL4 and helicase, which

are known to translocate in nuclei in response to exogenous stress (33). In addition, nucleolin is known to increase cellular sensitivity via stabilization of bcl-2 in leukemia cells (34). However, the mechanism by which nucleolar proteins improve the chemosensitivity in tumor cells is still unclear.

Recently, studies of synthetic lethality are very active in the field of cancer therapeutics. The concept of synthetic lethality is used to describe the phenomenon wherein mutation of a single gene is compatible with viability, but simultaneous mutation of both genes results in cell death (35, 36). This strategy is applied to kill tumor cells without affecting normal cells. Suppression of TBK1 or NF- κ B by RNAi killed cancer cells that harbored oncogenic KRAS, but not normal KRAS (37). This strategy suggests that a loss-of-function of additional genes in tumor cells is critical to increase cellular sensitivity. Similar approaches used in cancer drug discovery have also been developed in cancer therapeutics (38). Yuan *et al.* (11) report that IFN α alone did not affect cell growth but enhanced doxorubicin-induced cytotoxicity in osteosarcoma U2OS cells. Akt inhibitors also enhance the effect of doxorubicin in breast cancer T47D cells (39). We have also shown that the depletion of Neuroguidin/CANu1 alone did not alter cell viability but affected doxorubicin-induced cytotoxicity in U2OS cells. These results suggest that the low level of Neuroguidin/CANu1 may activate a default pathway induced by doxorubicin resulting in apoptosis in osteosarcoma cells. This work also supports the view that the proper combination of Neuroguidin/CANu1 and conventional chemotherapeutic agents may be a rational strategy for improving the treatment of osteosarcoma. Combinatorial approaches with Neuroguidin/CANu1 and doxorubicin exhibit a synergistic antitumor activity. Currently, we are investigating synthetic lethal screening for putative target genes of Neuroguidin/CANu1 in tumors. Targeting of the Neuroguidin/CANu1 pathway could represent a promising therapeutic approach to control the cytotoxicity of tumor cells. In conclusion, combined regimens of genotoxin-based chemotherapy concurrent with Neuroguidin/CANu1 may hold promise for a more effective treatment of osteosarcoma.

MATERIALS AND METHODS

Cell culture and reagents

Human osteosarcoma U2OS cells were cultured in McCoy 5A medium containing 10% fetal bovine serum and antibiotics and maintained in a humidified incubator at 37°C with 5% CO₂. Doxorubicin was purchased from Sigma (St. Louis, USA).

Lentiviral shNeuroguidin/CANu1 production

Lentiviral shCANu1 was produced as described previously (5). A short-hairpin sequence complementary to CANu1 was cloned into the lentiviral vector pLKO.1-TRC (Addgene, Boston, USA). Control cells were transduced with virus carrying a scrambled shRNA sequence. U2OS cells were transduced with the shRNA lentivirus and selected by incubation with 1.5

µg/ml puromycin for 2 days. The expression Neuroguidin/CANu1 protein was determined by western blotting.

Caspase-3/7 activity

Cells were seeded in 96-well plates (10,000 cells/well). After 24 h cells were treated with 1 µM doxorubicin. Caspase 3/7 activity was measured in accordance with the manufacturer's instructions. In brief, 100 µl of caspase-Glo 3/7 reagent (Promega, Madison, USA) was added to each well. After 1 h incubation at room temperature, luminescence was measured using a GloMax[®]20/20 luminometer (Turner Biosystem, Sunnyvale, USA).

Western blotting

Cells were washed with cold phosphate buffered saline (PBS) and lysed in ice-cold buffer [50 mM Tris-HCl, 150 mM NaCl, 1% NP40, 0.1% SDS, protease inhibitor cocktail (Roche, Mannheim, Germany), 50 mM NaF, and 0.2 M Na₃VO₄]. The protein concentration was determined by Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, USA). Protein extracts were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in 5% non-fat milk and incubated with p53 antibodies (Santa Cruz Biotechnologies, Santa Cruz, USA), Ngd, a mouse homolog of CANu1 (Sigma, St. Louis, USA), actin (Sigma, St. Louis, USA), and PARP (Becton-Dickinson Biosciences, Franklin lakes, USA) at room temperature. After washing with PBS, the membranes were incubated with horseradish-peroxidase conjugated secondary antibodies. The protein signals were visualized by an enhanced chemiluminescence reaction system (Santa Cruz Biotechnologies, Santa Cruz, USA). Actin was used as an equal loading control.

Cell viability assay

Cells in 96-well plates (10,000 cells/well) were incubated with 1 µM doxorubicin. Cell viability was measured using CellTiter 96 Aqueous One Solution (Promega, Madison, USA), which assesses the capacity of cells to reduce formazan and thus is an indicator of metabolic activity. The absorbance was measured using a Versa Max microplate reader (Molecular Devices, Sunnyvale, USA) at 490 nm. The absorbances were averaged and expressed as a percentage of the control vehicle. Statistical significance was determined using a two-way ANOVA.

Flow cytometry analysis

Cells were suspended in 70% ethanol for 30 min at -20°C. Fixed cells were washed twice and rehydrated in PBS. Prior to fluorescence-activated cell sorting (FACS) analysis, the cells were incubated with DNA staining solution (PBS with 0.1 mg/ml propidium iodide, 1 mg/ml RNase and 0.1% triton X-100) for 30 min. DNA content was analyzed using Cell Quest software on a flow cytometer (Becton-Dickinson, Franklin Lakes, USA).

Immunofluorescence microscopy

U2OS cells expressing GFP-CANu1, GFP-CANu1ΔN, or GFP-CANu1ΔC20 fusion proteins were cultured on coverslips for 24 h and exposed to doxorubicin. The construction of these expression plasmids was performed as described previously (5). After 36 h, cells were fixed with 4% paraformaldehyde for 15 min. After washing, cells were DAPI-stained for 5 min. DAPI (0.1 µg/ml) was used to detect nuclei. The samples were mounted and visualized on an LSM510 microscope (Carl Zeiss, Oberkochen, Germany).

Annexin V-FITC labeling

For the detection of apoptotic cell death, labeling tests involving both propidium iodide (PI) and annexin-V were performed using an Annexin-V-FLUOS staining kit (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions. Briefly, after treatment with 1 µM doxorubicin for 36 h, the cells were fixed in 4% (w/v) paraformaldehyde for 15 min and incubated with both FITC-labeled annexin-V and PI stock solution (50 µg/ml) for 10 min at room temperature. The samples were mounted and visualized using an LSM510 microscope (Carl Zeiss, Oberkochen, Germany).

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