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New Phenomenon

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The fourth transmembrane helix is important for DRAM function

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Damage-regulated autophagy modulator gene (*DRAM*) was first identified by Crighton *et al.* in 2006 when they treated Saos-2 cells with doxycycline and adriamycin after p53 induction and analyzed gene expression by using microarray technology [1]. The *DRAM* gene locates on the human chromosomal 12q23.2 [2] and encodes a 238 amino acid lysosomal protein whose role in autophagy is phylogenetically conserved. As a direct target of P53, DRAM is a critical modulator in p53-induced autophagy and programed cell death [3]. Nevertheless, the exact mechanism through which DRAM modulates autophagy has not been clarified. Sequence analysis predicted that the DRAM protein contains six transmembrane domains and several other highly conversed domains [1], therefore understanding the exact role of each domain of DRAM in autophagy may help to clarify the exact mechanism.

To get a preliminary understanding of the functions of DRAM and its different transmembrane domains, we constructed eukaryotic expression plasmids of DRAM, pEGFP-DRAM and of its deletants, including pEGFP-DN1, pEGFP-DN2-3, pEGFP-DN4-5, pEGFP-DN4, pEGFP-DN5, and pEGFP-DN6 according to the prediction results [1] (**Fig. 1A**), and transfected them into HepG2 cells. The empty vector pEGFP-N1 was used as the negative control. The mRNA levels of *DRAM* or its deletants were identified by real-time PCR at 24 h after transfection (**Fig. 1B**). The protein levels for the two deletants (pEGFP-DN4, pEGFP-DN5) were further determined by western blot analysis (**Supplementary Fig. S1**). Primers used are listed in **Supplementary Table S1**. As DRAM is an autophagy modulator and autophagy is involved in apoptosis as well as cell proliferation under different conditions [4–6], we detected apoptosis, cell proliferation, and colony formation ability of these transfected cells.

The results showed that expression of DRAM alone was not sufficient to affect apoptosis (**Supplementary Fig. S2**), but it was able to promote cell proliferation (**Fig. 2A**) and colony formation (**Fig. 2B**) of HepG2 cells, which are consistent with the observation of Kerley-Hamilton *et al.* [2] in Saos cell lines stably expressing DRAM. By comparing the results in the cell proliferation and colony formation assays (**Fig. 2A,B**), we found that the deletion of the fourth–fifth transmembrane domain significantly impaired DRAM's ability in promoting proliferation and colony formation of HepG2 cells, indicating that this region is crucial for the function of DRAM.

To determine which of the fourth and the fifth transmembrane domains is more important than the other, the deletants of pEGFP-DN4 and pEGFP-DN5 were further analyzed. It was found that both regions were important in DRAM's ability of promoting cell proliferation (**Fig. 2C**), but the effect of the fourth transmembrane domain was more obvious than the fifth transmembrane domain. The soft agar colony formation assay revealed that DRAM could still cause a significant increase in the clonogenic potential of HepG2 cells when the fifth transmembrane domain was deleted. However, the deletion of the fourth transmembrane domain significantly impaired DRAM-induced cell colony formation (**Fig. 2D**). That is, the fourth transmembrane domain might play a major role in DRAM-mediated cell proliferation and colony formation.

Based on these results, we further investigated the role of the fourth transmembrane domain in DRAM-induced autophagy. Results showed that deletion of the fourth transmembrane domain impaired the expression of LC3 (**Supplementary Fig. S3A**), a well-characterized autophagy marker [7]. Detection of subcellular localization of DRAM revealed that pEGFP-DRAM exhibited diffuse fluorescence within nucleus and cytoplasm, and it showed DRAM protein accumulation in the internal side of cytoplasmic membrane. The result of transfection cells with pEGFP-DN4 was that fluorescence distributed exclusively in the cytoplasm (**Supplementary Fig. S3B**), which implied that the fourth transmembrane is closely associated with DRAM's location in nucleus. Lysosome stain revealed that there was a marked reduction in the lysosome amount in HepG2 cells that ectopically expressing DRAM. Based on previous studies, it is highly likely that full-length DRAM was able to promote lysosomes fusing with autophagosomes to form autolysosomes, leading

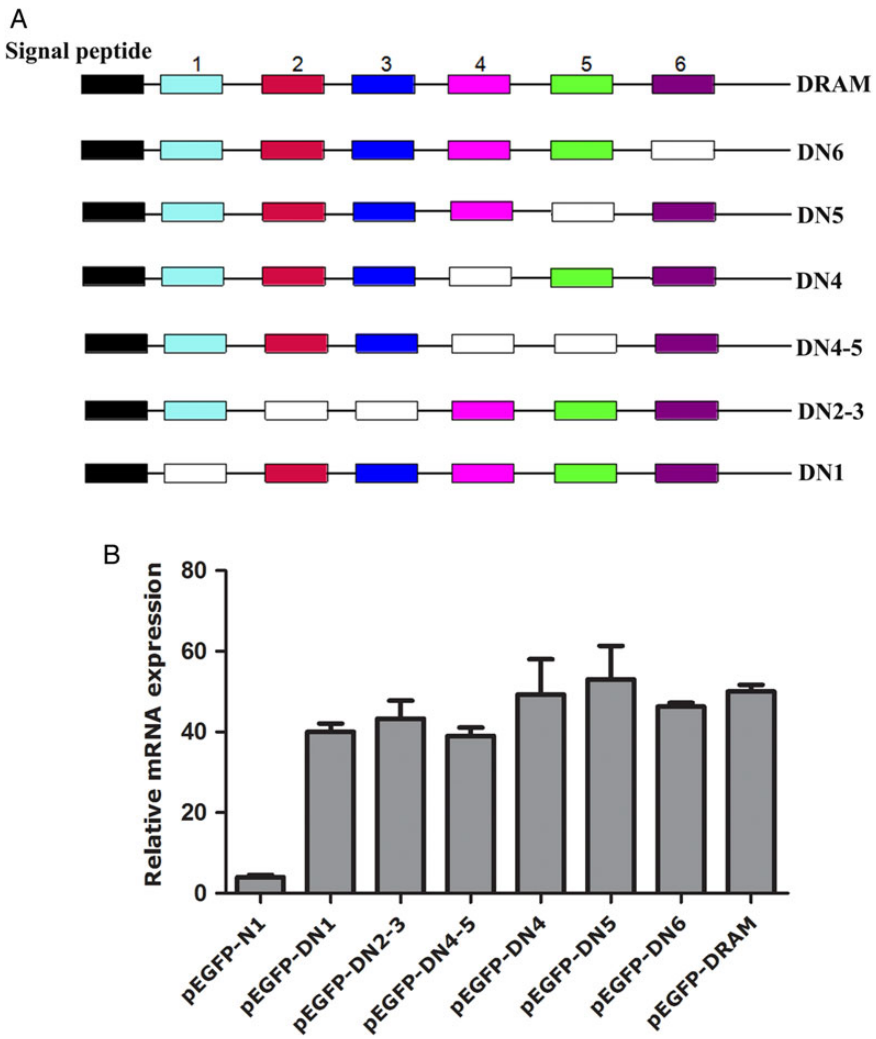

Figure 1. Construction of eukaryotic expression plasmids of DRAM and its deletants (A) Schematic diagram of different deletants of DRAM, white boxes indicate the transmembrane domains that were deleted. (B) Relative mRNA levels of *DRAM* or its deletants were determined by real-time PCR at 24 h after transfection HepG2 cells with the constructed plasmids. Data were normalized to that of GAPDH.

to the failure of recognizing lysosomes by an acid probe. However, no obvious change of lysosomes was observed in HepG2 cells transfected with pEGFP-DN4 (**Supplementary Fig. S3C**). That is to say, the fourth transmembrane domain was critical for DRAM-induced autophagosomes formation. From this perspective, we further examined autophagosomes by TEM. It turned out that cells transfected with pEGFP-DN4 exhibited the formation of vacuolated lysosomes instead of autophagosomes (**Supplementary Fig. S3D**).

All these results revealed that the fourth transmembrane domain may be the key region to the functions of DRAM in promoting cell proliferation, colony formation, and autophagy. However, this is just a preliminary study, and more detailed investigation should be carried out in other cell lines. In addition, as DRAM is a big transmembrane protein with multiple domains, the deletions of one or two domains may affect the entire structure of DRAM, especially when the domain deleted has structural contact with other domains. Therefore, the clarification of the exact structure of DRAM is urgently needed. Because crystallization of membrane proteins is extremely difficult, new technology such as cryoelectron microscopy may be useful to gain structural information of DRAM. All these information will provide researchers with new ideas to design future prospective studies on DRAM and to develop new anticancer therapeutics.

Supplementary Data

Supplementary data are available at *ABBS* online.

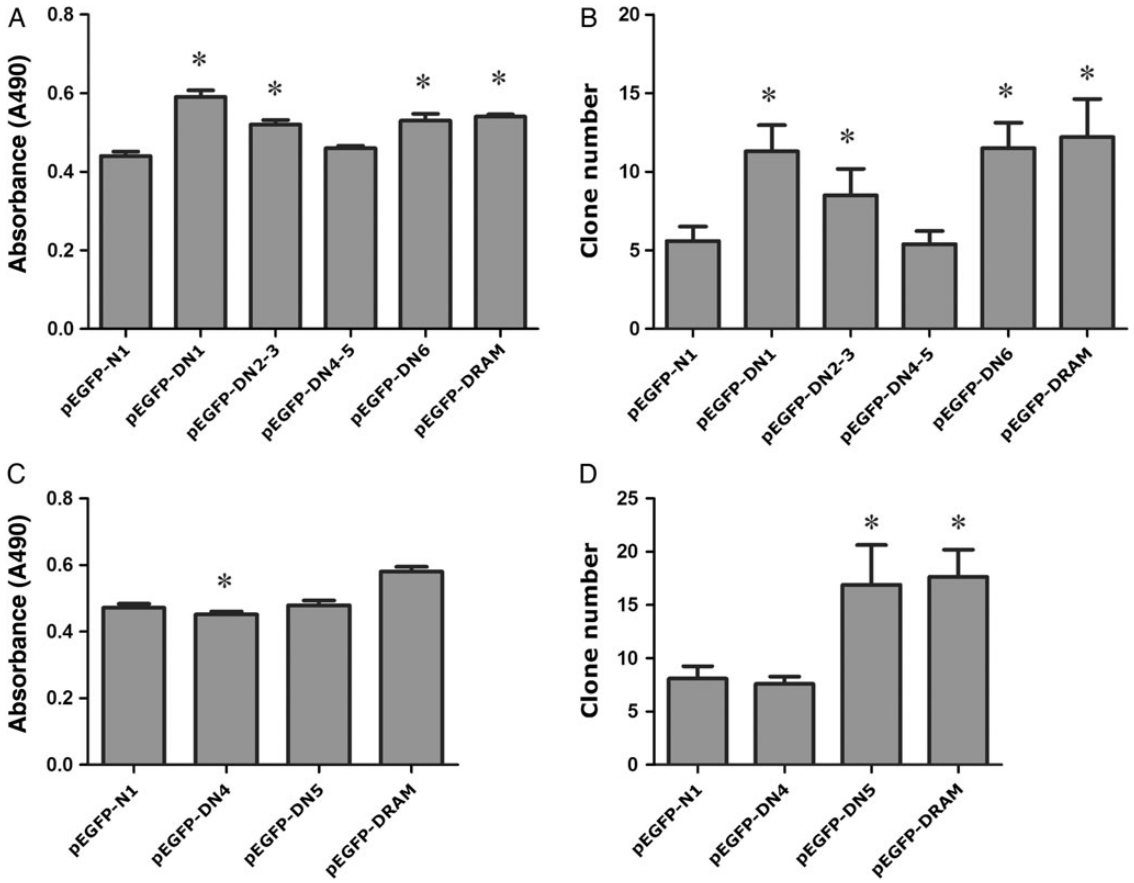

Figure 2. Effect of DRAM and its deletants on cell apoptosis and colony formation in HepG2 cells HepG2 were transfected with pEGFP-DRAM, pEGFP-DN1, pEGFP-DN2-3, pEGFP-DN4-5, pEGFP-DN4, pEGFP-DN5, pEGFP-DN6, or empty vector pEGFP-N1 (negative control). (A,C) Cell proliferation was measured by the MTS cell proliferation assay. (B,D) Colony formation was determined by the soft agar assay. *$P < 0.05$ vs. pEGFP-N1.

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