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## Report

# Analysis of DRAM-related proteins reveals evolutionarily conserved and divergent roles in the control of autophagy

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**Abbreviations:** TetOn, tetracycline-responsive; TA-, transactivation-competent; Dox, doxycycline; qPCR, quantitative-PCR; DRAM, damage-regulated autophagy modulator; *DmDRAM*, *Drosophila melanogaster* DRAM-like protein

**Key words:** DRAM, DRAM2, autophagy, lysosome, human, *Drosophila*

Autophagy is a membrane-trafficking process that serves to deliver cytoplasmic proteins and organelles to the lysosome for degradation. The process is genetically defined and many of the factors involved are conserved from yeast to man. Recently, a number of new autophagy regulators have been defined, including the Damage-Regulated Autophagy Modulator (DRAM), which is a lysosomal protein that links autophagy and the tumor suppressor, p53. We describe here analysis of DRAM-related proteins which reveals evolutionary conservation and divergence of DRAM's role in autophagy. We report that humans have 5 other proteins that show significant homology to DRAM. The closest of these, which we have termed DRAM2, displays 45% identity and 67% conservation when compared to DRAM. Interestingly, although similar to DRAM in terms of homology, *DRAM2* is different from *DRAM* as it not induced by p53 or p73. *DRAM2* is also a lysosomal protein, but again unlike *DRAM* its overexpression does not modulate autophagy. In contrast to humans, the *Drosophila* genome only encodes one DRAM-like protein, which is approximately equal in similarity to human *DRAM* and *DRAM2*. This questions, therefore, whether *DRAM* function is conserved from fly to man or whether *DRAM*'s capacity to regulate autophagy has evolved in higher eukaryotes. Expression of *DmDRAM*, however, clearly revealed an ability to modulate autophagy. This points, therefore, to a conserved role of *DRAM* in this process and that additional human proteins have more recently evolved which, while potentially sharing some similarities with *DRAM*, may not be as intrinsically connected to autophagy regulation.

## Introduction

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved membrane trafficking process that effects the lysosomal degradation of cytoplasmic proteins and is the only mechanism for the degradation of organelles.<sup>1,2</sup> At induction, membrane structures termed 'isolation membranes' originate within the cytoplasm of the cell. These membranes then grow and mature to form double-membrane vesicles (autophagosomes) which encapsulate the cellular cargos destined for degradation.<sup>1</sup> Autophagosomes can then undergo fusion events with multi-vesicular bodies and endosomes, but ultimately fusion occurs with lysosomes to form 'autolysosomes'. The acidic hydrolases provided by the lysosome then effect the degradation of the contents of the autolysosome resulting in the formation of constituent parts of the cargo, e.g., amino acids and fatty acids.<sup>3</sup> These factors can then undergo further catabolic breakdown or can be recycled into biosynthetic pathways.

Although active under basal conditions as a means to monitor the integrity of long-lived proteins and organelles, the rate and cargo of autophagy can change in response to various forms of cellular stress. In response to catabolic defects or nutrient deprivation, autophagy can be activated to degrade cellular components as a self-limited cell survival mechanism which can then be used for the generation of ATP until nutrient replete conditions are regained.<sup>4-6</sup> Under other forms of cellular stress, autophagy can be activated to promote cell survival by the removal of damaged or misfolded proteins and organelles, which, if not removed, would compromise cell viability.<sup>7,8</sup>

As well as promoting cell survival, a number of other reports have also indicated that autophagy is a component of pro-death mechanisms.<sup>9-11</sup> In addition, while the cell survival aspects of autophagy could clearly be considered oncogenic, there is significant evidence that autophagy is inactivated in human cancer indicating a tumor suppressive role.<sup>12-14</sup> In line with this, mouse models where autophagic factors have been compromised have been reported to be tumor prone.<sup>15-17</sup>

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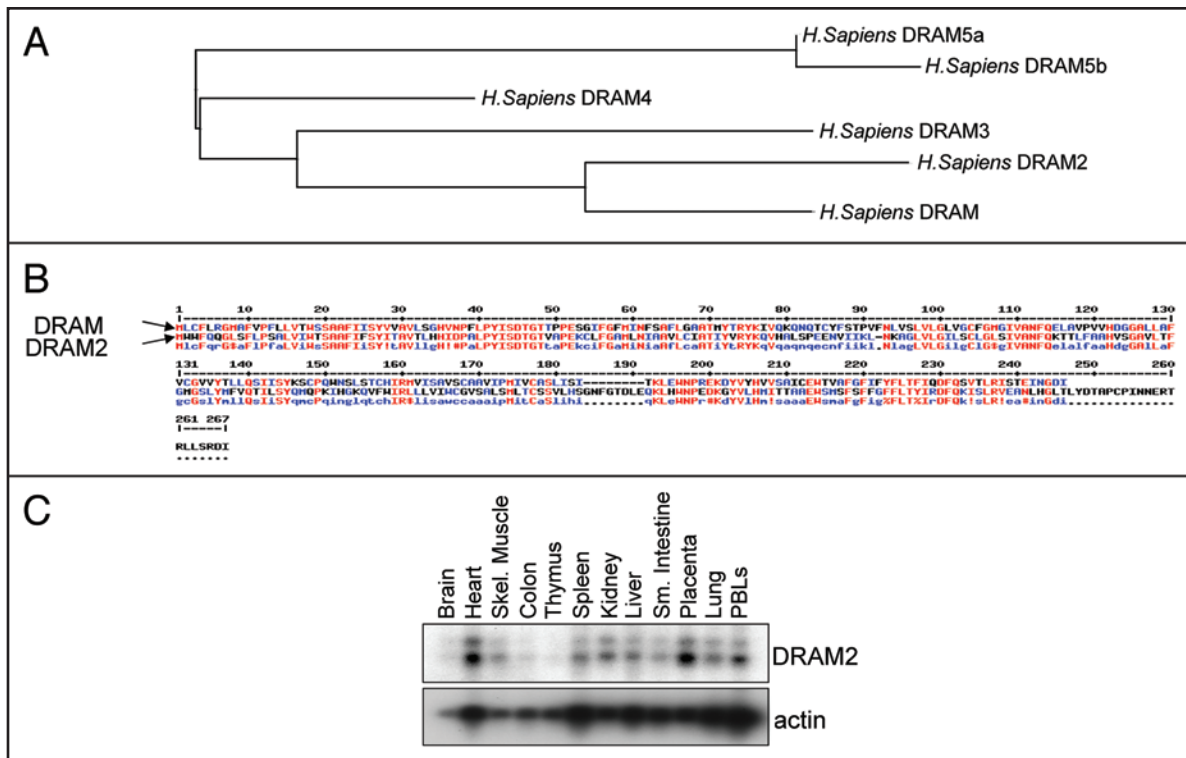


Figure 1. DRAM2/TMEM77 is the closest of a family of DRAM-related proteins. (A) A search for DRAM-related human proteins by BLAST revealed 5 proteins that have significant homology to DRAM at the protein level. (B) Line-up of the peptide sequences of DRAM and DRAM2/TMEM77. (C) Northern blot of DRAM2 expression in human tissues. Previous designations and accession numbers of DRAM family proteins are: DRAM2—TMEM77 (NP\_848549.3); DRAM3—TMEM224 (NP\_001078957.1); DRAM4—LOC441027/FLJ12993 (NP\_001073975.1); DRAM5a—TMEM150/FLJ90024 isoform 1 (NP\_001026908.1); DRAM5b—TMEM150/FLJ90024 isoform 2 (NP\_669173.1).

Despite the disparate functions of autophagy, the core process is mediated by a series of ‘*Atg*’ genes which in most cases are clearly conserved from yeast to man.<sup>1,2,18</sup> More recently, however, a number of additional autophagy regulators have been described which in some cases function in selective autophagy scenarios, but little is known of their evolutionary nature. One such factor is the Damage-Regulated Autophagy Modulator, DRAM, which was the first molecular link to be reported between autophagy regulation and the tumor suppressor p53.<sup>19,20</sup> At basal levels p53 has been shown to act as a suppressor of autophagy through protein-protein interactions at mitochondria.<sup>21,22</sup> In response to various forms of cellular stress, however, the levels of p53 become elevated and the majority of p53 translocates to the nucleus where it promotes the transactivation of a broad spectrum of target genes that mediate p53’s tumor suppressive effects.<sup>23</sup> One of the genes activated by p53 is *DRAM* which is required for the ability of p53 to induce autophagy and is also critical for the ability of p53 to induce programmed cell death.<sup>19,20</sup> Since autophagy, but not p53, is present in yeast, we questioned the nature of DRAM’s evolutionary conservation. This revealed that there are five human proteins that show significant homology to DRAM, but in simpler organisms such as *Drosophila* there is only one, *DmDRAM*. We present here the comparative analysis of DRAM, *DmDRAM* and DRAM2—the most closely related human protein to DRAM. These studies reveal insights into the conserved and yet divergent roles of DRAM protein in autophagy regulation.

## Results and Discussion

**Humans contain a family of DRAM proteins.** In order to gain insight into the evolutionary nature of DRAM, BLAST searches were undertaken for human proteins which share significant homology to the DRAM peptide sequence. This resulted in the identification five previously undescribed human proteins that were similar to DRAM, indicating that DRAM belongs to an uncharacterized protein family. Phylogenetic analysis of these proteins revealed varying degrees of relatedness to DRAM and as a result we nominally refer to these proteins as DRAM2 through DRAM4, DRAM5a and DRAM5b (the last two proteins are isoforms from the same gene) (Fig. 1A). DRAM2 is by far the most closely related human protein to DRAM and shares 45% identity and 67% conservation at the amino acid level (Fig. 1B). Different to DRAM, however, which is undetectable by northern analysis in unstressed cells, DRAM2 expression was clearly detectable in a number of tissues. Expression was highest in placenta and heart, although most tissues exhibited some level of expression, with only brain and thymus seemingly devoid of detectable mRNA expression.

**DRAM2 is biologically distinct from DRAM.** Since DRAM2 is so closely related to DRAM, we decided to investigate if DRAM2 was also like DRAM biologically. Firstly, since expression of *DRAM* is known to be induced by p53 and its related family member p73,<sup>19,24</sup> we sought to determine if *DRAM2* was

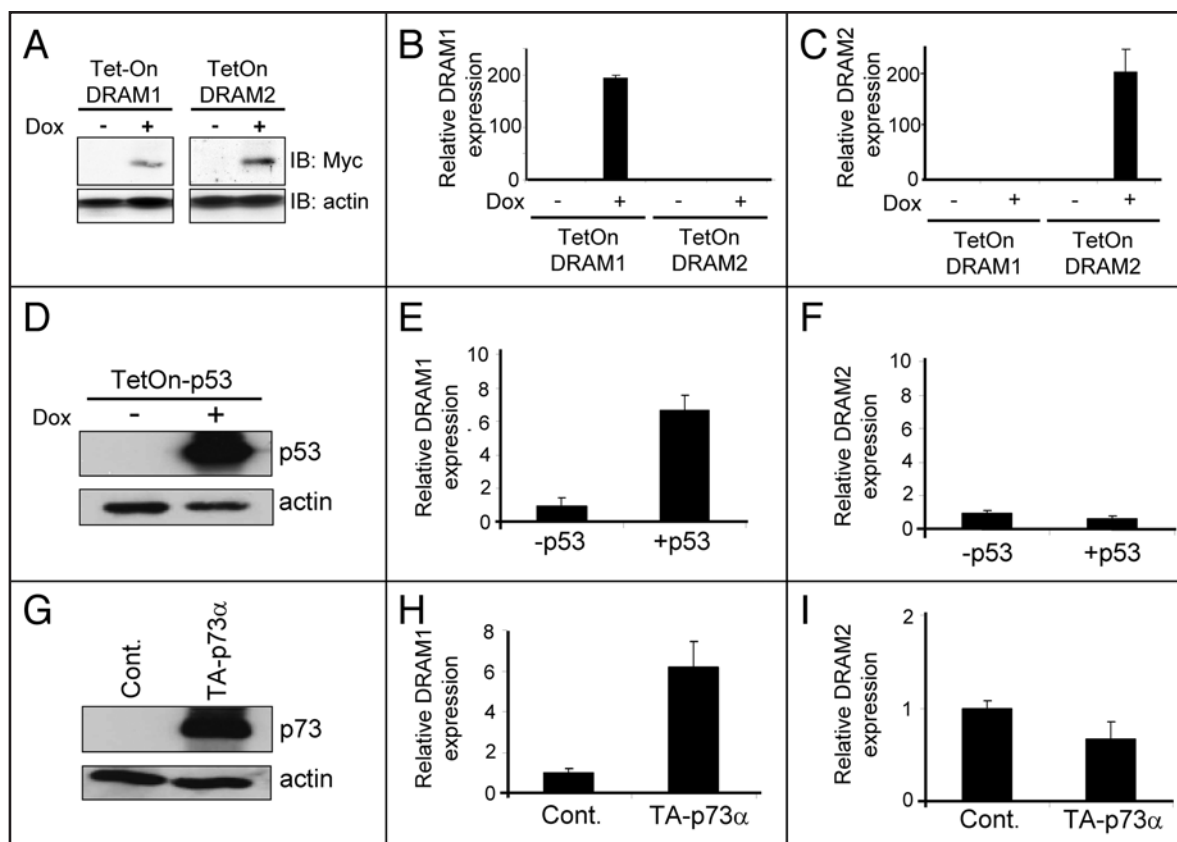


Figure 2. *DRAM2*, unlike *DRAM* is not induced by p53 or p73. (A–C) The specificity of qPCR primers for *DRAM* (B) and *DRAM2* (C) were determined by analysis of TetOn-*DRAM* and TetOn-*DRAM2* inducible cell lines (A). (D–F) p53 induces *DRAM*, but not *DRAM2*. TetOn-p53 inducible cells were treated with Dox for 24 h and the presence of p53 determined by western blotting (D). Expression levels of *DRAM* (E) and *DRAM2* (F) mRNA in these TetOn-p53 cells was determined by qPCR. (G–I) p73 induces *DRAM*, but not *DRAM2*. Saos-2 cells were infected with an adenovirus expressing TA-p73 or control “empty” adenovirus and expression of the p73 was determined by western blotting (G). Expression levels of *DRAM* (H) and *DRAM2* (I) following infection of these cells with adenovirally-delivered TA-p73 was determined by qPCR. Dox, doxycycline.

also induced by these two tumor suppressive transcription factors. Due to the similarity between the mRNA sequences of *DRAM* and *DRAM2*, qPCR primers were generated and first tested for specificity in TetOn inducible cell lines for *DRAM* and *DRAM2* (Fig. 2A–C). These primers were then used to assess the relative levels of *DRAM* and *DRAM2* mRNAs following p53 induction in TetOn-p53 cells (Fig. 2D). This revealed, in agreement with previous studies,<sup>19</sup> that *DRAM* is strongly induced by p53 in this system (Fig. 2E). In contrast, no changes in *DRAM2* mRNA were observed indicating that *DRAM2* is not a transcriptional target of p53 (Fig. 2F). To test if *DRAM2*, like *DRAM*, was induced by TA-p73,<sup>24</sup> Saos-2 cells were infected with an adenovirus expressing a transactivation-competent isoform of p73, TA-p73 $\alpha$  (Fig. 2G). Also in agreement with previous studies, TA-p73 $\alpha$  caused a marked increase in *DRAM* expression (Fig. 2H),<sup>24</sup> however, similar to what was observed in TetOn-p53 cells, no increase in *DRAM2* mRNA was seen following infection with TA-p73 $\alpha$  (Fig. 2I).

Although *DRAM2* was not responsive to p53 or p73, it remained possible that *DRAM2* may still be a modulator of autophagy, perhaps in response to other stimuli. We first assessed, therefore, whether *DRAM2*, like *DRAM*, was a lysosomal protein.<sup>19</sup> *DRAM2* was induced in TetOn-*DRAM2* cells for 24 h

as previously described. Cells were then fixed and stained with an antibody against the lysosomal protein, cathepsin D. Indeed, similar to *DRAM*,<sup>19</sup> *DRAM2* was clearly co-compartmentalized with cathepsin D (Fig. 3A) indicating that *DRAM2*, as a result of regions of hydrophobicity in its peptide sequence (Fig. 1B), is most likely a lysosomal membrane protein. No co-localization was observed with other markers of sub-cellular compartments (data not shown).

TetOn-*DRAM* and TetOn-*DRAM2* cells were then infected with an adenovirus expressing GFP-LC3 (a marker of autophagosomes) which changes from a diffuse cytoplasmic localization to a more punctate pattern as the LC3 integrates in autophagosome membranes upon their formation.<sup>25,26</sup> Following treatment of these cells with Dox for a period of 24 h, the cells were stained for *DRAM*/*DRAM2* expression and the formation of autophagosomes assessed by fluorescent microscopy. In agreement with previous reports, induction of *DRAM* caused clear accumulation of autophagosomes (Fig. 3B).<sup>19</sup> No increase in autophagosomes was observed, however, as a result of *DRAM2* induction (Fig. 3B) despite similar levels of protein expression being observed by western blotting (Fig. 2A). This result was confirmed in long-lived protein assays—which are a measure of autophagic flux—in which

Figure 3. DRAM2 is a lysosomal protein, but different from DRAM, DRAM2 does not regulate autophagy. (A) Tet-On DRAM2 cells were induced with Dox for 24 h. Cells were then stained for DRAM2 (Myc) and for cathepsin D, stained with DAPI and analysed by fluorescent microscopy. (B) Tet-On-DRAM and Tet-On-DRAM2 cells were infected with an adenovirus expressing GFP-LC3 for 16 h. Cells were then induced with Dox for 24 h stained for DRAM or DRAM2 (Myc) and with DAPI and analysed by fluorescent microscopy. Dox, doxycycline.

either DRAM2 overexpression or DRAM2 knockdown during p53-induced autophagy had no effect on the rate of degradation observed (data not shown).

**Drosophila only contain one DRAM protein which can modulate autophagy.** Sequence homology searches using BLAST for DRAM polypeptides in other organisms revealed that many simpler organisms for example, *Drosophila*, contain only one obvious DRAM protein (Fig. 4A). This raises the question, therefore, as to whether human DRAM's ability to modulate autophagy is an evolutionarily conserved process or whether DRAM has emerged later in evolution and that *Drosophila* DRAM (*DmDRAM*) is more like DRAM2. To test this, *DmDRAM* was expressed in cells and assessed for its ability to modulate autophagy (Fig. 4B). This clearly revealed that *DmDRAM*, like human DRAM, causes a marked increase in autophagosome number as judged by the appearance of GFP-LC3 puncta (Fig. 4C), indicating that the regulation of autophagy by DRAM proteins is an evolutionarily conserved process.

Although these studies provide clear insight into the evolutionary nature of DRAM-induced autophagy, perhaps the biggest question raised is what is the function of DRAM2? Despite having no role in autophagy it may be that DRAM2 shares some other function with DRAM. We have also previously shown that DRAM is critical for cell death downstream of p53, and while it is perceived that this is through DRAM's ability to modulate autophagy, it remains formally possible that these two functions of DRAM are separable. Our studies, however, into the possibility that DRAM2 may regulate cell death indicates once again that DRAM2 is different from DRAM. DRAM2 appeared to have no effects on cell death when overexpressed and knockdown of DRAM2 by RNAi had no effect on cell death induced by p53 (data not shown).

Due to the extent of sequence homology between DRAM and DRAM2 it seems unlikely that the

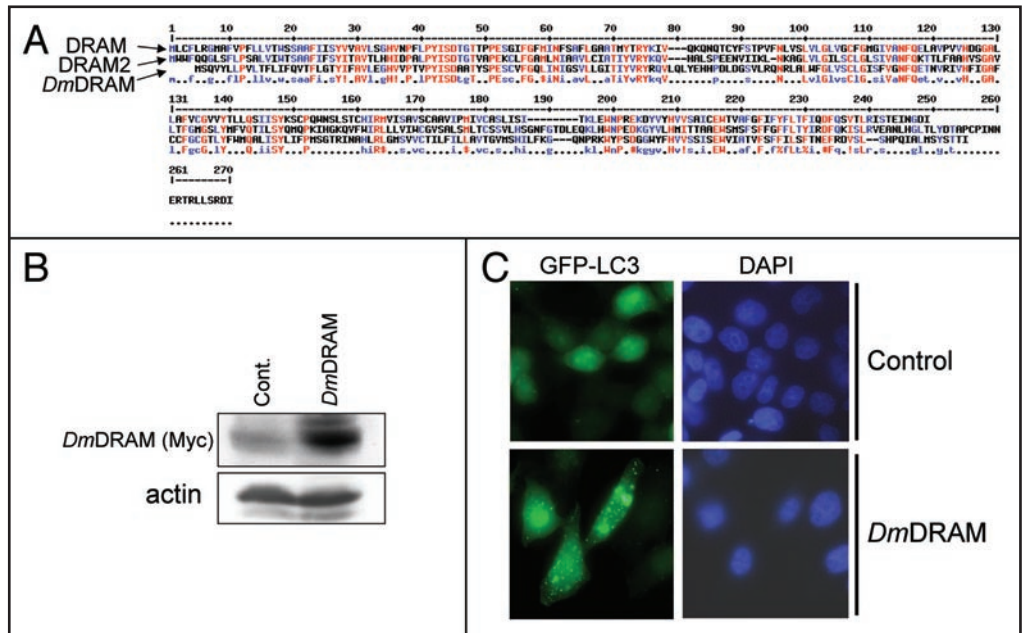
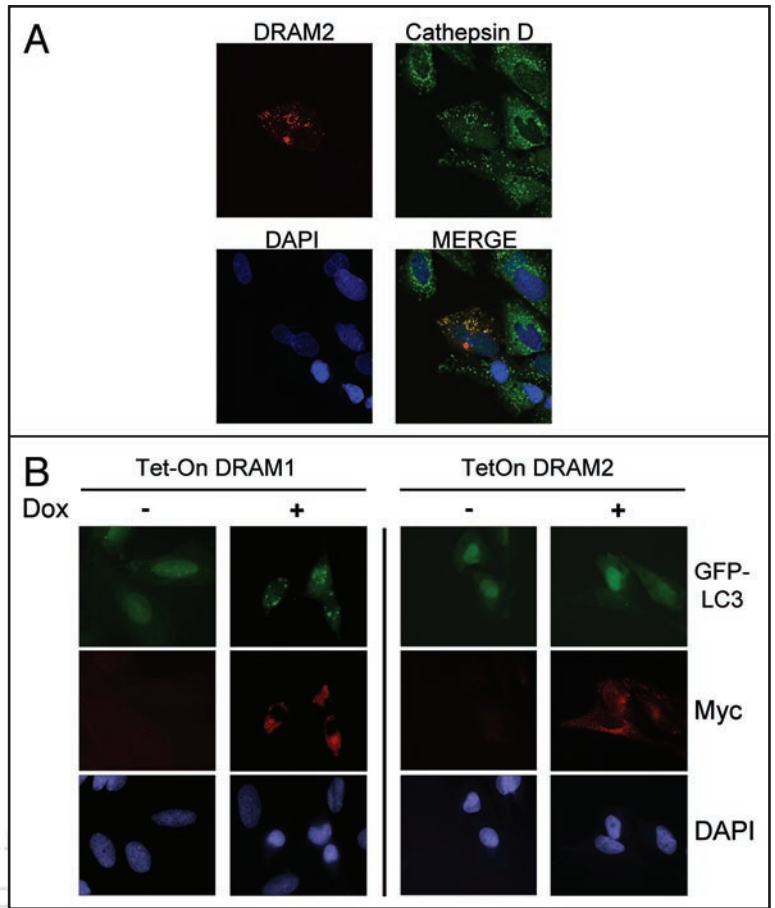


Figure 4. *Drosophila* DRAM can modulate autophagy. (A) Line-up of the peptide sequences of DRAM, DRAM2 and *DmDRAM*. (B and C) Cells were transiently transfected with *DmDRAM* or empty vector as control. 24 h later cells were infected with an adenovirus expressing GFP-LC3. Following a further 24 h cells were either harvested for western blotting (B) or stained with DAPI and analysed by fluorescent microscopy (C).

two proteins are not in some way functionally related. It remains possible that since DRAM2 is constitutively expressed in many tissues, that induction of *DRAM* by p53 somehow perturbs the function of DRAM2 and thereby induces autophagy, or that the cell responds by inducing autophagy. In this regard, it would be interesting to know whether DRAM and DRAM2 interact within the cell. It seems clear too, that to understand DRAM2 function it may be necessary first to understand the function of DRAM. Maybe the proteins do indeed have a similar function, but for example, have different substrates such that the effects they produce are markedly different. The generation of DRAM-deficient animals—both mice and flies—would indeed be very rewarding and would yield information with respect to both the functional and evolutionary nature of DRAM function. For example, although *DmDRAM* can modulate autophagy, is this a functional aspect downstream of p53 in *Drosophila*? In this regard, it is important to note that *Drosophila* p53 is considered to be more like human p73 than human p53,<sup>27</sup> and our previous studies have shown that while p73 induces *DRAM*, its ability to modulate autophagy is DRAM-independent.<sup>24</sup> Ultimately, therefore, it is clear that many questions remain and that further investigation of not only DRAM and DRAM2, but the entire DRAM family should be highly rewarding.

## Materials and Methods

**Plasmids.** pcDNA3-DRAM2-MycHis was generated by PCR from the I.M.A.G.E clone for TMEM77 Clone ID: 5491665 using the following primers: (forward) ATA AGA TCT ATG TGG TGG TTT CAG CAA GGC CTC, (reverse) ATA TCT AGA AAT ATC TCT GGA AAG TAG CCG TGT. PCR products were digested with BglII and XbaI and cloned into the BamHI and XbaI sites of pcDNA3-MycHisA (Invitrogen). Similarly, pcDNA3-*DmDRAM*-MycHis was amplified from *Drosophila* S2R<sup>+</sup> RNA by RT-PCR using the following primers (forward) ATG CGA ATT CGC CAC CAT GTC ACA GGT TTA CTT GCT GCC G (reverse) CTG ACT CGA GAT GGT GGT CGA ATA GGA CAT CAG. Products were digested with EcoRI and XhoI and cloned into pcDNA3MycHisA (Invitrogen). pcDNA3-DRAM-MycHis has been previously described.<sup>19</sup>

**Cell culture, transfections and infections.** Saos2, HeLa and S2R<sup>+</sup> cells can all be obtained from ATCC. TetOn-p53 and TetOn-DRAM cells are Saos2 derived lines and have been previously described.<sup>19,28</sup> All human cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. *Drosophila* cells were maintained in Schneider's medium (Invitrogen). Induction of transgene expression in Tet-On cell lines was achieved by addition of 1 µg/ml doxycycline (Dox) (Sigma). Where indicated, cells were transfected by CaPO<sub>4</sub> precipitation as previously described.<sup>29</sup> Adenoviruses for TA-p73 $\alpha$  and GFP-LC3 (a gift from Aviva Tolkovsky, University of Cambridge) were generated and purified as previously described<sup>24,25</sup> and were added to cell cultures where indicated at a concentration of 12 infectious units per cell.

**Western blotting.** Cells were lysed in a 2x western sample buffer and transferred to nitrocellulose membranes as

previously described.<sup>30</sup> Membranes were probed using standard immunoblotting techniques with antibodies that recognize HA-tagged p73 (HRP-conjugated HA, Roche), p53 (DO-1, Pharmingen), Myc-tagged DRAM, DRAM2 and *Dm*-DRAM (4A6, Upstate) and actin (clone 1A4, Sigma).

**Immunofluorescence.** For analysis of autophagosomes, cells were infected with an adenovirus expressing GFP-LC3 as described above. 16 h later cells were, where indicated, incubated either in the absence or presence of Dox. For transiently transfected cells, infection with adenovirally expressed GFP-LC3 where undertaken 16 h hours after transfection. 24 h after infection with GFP-LC3, cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% triton. Cells were then stained with antibodies that recognize Myc-tagged DRAM proteins (4A6, Upstate). Following incubation with a Texas red conjugated secondary antibody (Molecular Probes Inc.), cells were analyzed by confocal microscopy. For DRAM2 localization studies, cells were co-stained for Myc-tagged DRAM2 and for Cathepsin D (DAKO) as a lysosomal marker. In all immunofluorescence studies, cellular DNA was stained with 4',6-diamidino-2-phenylindole (Sigma) prior to mounting and visualization.

**Quantitative RT-PCR.** RNA was prepared using TRIzol Reagent (Invitrogen). qPCR analysis was undertaken using the DyNAmo SYBR Green 2-step qRT-PCR kit (Finnzymes). Data collection was carried out using a Chromo4 real-time PCR detector and analysed with Opticon Monitor 3. Primers for *DRAM* and *18S* have been previously described.<sup>19</sup> Primers for *DRAM2* were: (forward) AAG CAA GTT CAT GCT CTG AGT C, (reverse) CCA GAT AAC CAA CAA CAG TCT G. qPCR cycling parameters were 95°C 15 min [94°C 10 sec, 55°C 30 sec, 72°C 30 sec] 34 cycles, 72°C 10 min. Expression levels of genes analysed by qPCR were normalized relative to levels of 18S rRNA.

**Sequence alignments and phylogenetic analysis.** Alignment of peptide sequences was undertaken using MultAlin (<http://bioinfo.genotoul.fr/multalin/multalin.html>).<sup>31</sup> For phylogenetic analysis, sequences from NCBI were analyzed using Blast Tree View (<http://blast.ncbi.nlm.nih.gov>)

**Northern.** Northern analysis was undertaken as previously described.<sup>32</sup> Briefly, <sup>32</sup>P-labelled full-length cDNA probes were generated for *DRAM2* and *actin* using RediPrime (GE Healthcare) as per the manufacturer's instructions. Probes were then hybridized sequentially to a tissue Northern membrane (Clontech) as previously described<sup>33</sup> Following 24 h of hybridization, the membrane was washed 2 x 20 min in 2x SSC/0.1% SDS at room temperature, followed by 45 min in 0.1x SSC/0.1% SDS at 65°C and then subsequently exposed to X-Omat film (Kodak). Between sequential probings of the membrane, radioactive probe was stripped from the membrane by soaking in boiling 0.1% SDS followed by shaking at room temperature until ambient temperature was reached.

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