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Human hAtg2A protein expressed in yeast is recruited to preautophagosomal structure but does not complement autophagy defects of *atg2Δ* strain

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Yeast Atg2, an autophagy-related protein, is highly conserved in other fungi and has two homologues in humans, one of which is hAtg2A encoded by the *hATG2A/KIAA0404* gene. Region of homology between Atg2 and hAtg2A proteins comprises the C-terminal domain. We used yeast *atg2Δ* strain to express the *GFP-KIAA0404* gene, its fragment or fusions with yeast *ATG2,* and study their effects on autophagy. The GFP-hAtg2A protein localized to punctate structures, some of which colocalized with Ape1-RFP-marked preautophagosomal structure (PAS), but it did not restore autophagy in *atg2Δ* cells. N-terminal fragment of Atg2 and N-terminal fragment of hAtg2A were sufficient for PAS recruitment but were not sufficient to function in autophagy. Neither a fusion of the N-terminal fragment of hAtg2A with C-terminal domain of Atg2 nor a reciprocal fusion were functional in autophagy. hAtg2A, in contrast to yeast Atg2, did not show interaction with the yeast autophagy protein Atg9 but both Atg2 proteins showed interaction with Atg18, a phospholipid-binding protein, in two-hybrid system. Moreover, deletion of *ATG18* abrogated PAS recruitment of hAtg2A. Our results show that human hAtg2A can not function in autophagy in yeast, however, it is recruited to the PAS, possibly due to the interaction with Atg18.

Keywords: autophagy, human hAtg2A protein, yeast Atg2, nitrogen starvation

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INTRODUCTION

Cell growth and death are determined by the presence or absence of specific proteins. Depending on the extracellular conditions, specific balance is maintained between protein synthesis and degradation. Proteins are degraded by two major pathways: the ubiquitin-proteasome pathway (Hershko & Ciechanover, 1998; Goldberg, 2003) and autophagy (Klionsky & Ohsumi, 1999; Xie & Klionsky, 2007). During authophagy cytoplasmic components are enwrapped into an autophagosome and delivered to the vacuole for degradation. In yeast, at least 35 autophagy-related genes (*ATG*) have been found to be essential for autophagy (Klionsky *et al.*, 2003; Kabeya *et al.*, 2007). Many Atg proteins, including Atg2, localize to the pre-autophagosomal structure or phagophore assembly site (PAS; Xie & Klionsky, 2007), the center of autophagosome formation (Suzuki *et al.*, 2001; Suzuki & Ohsumi, 2010). In the yeast *Saccharomyces cerevisiae* autophagy overlaps the cytoplasm-to-vacuole targeting (Cvt) pathway which is responsible for the biosynthetic delivery of aminopeptidase I (Ape1) from the cytoplasm to the vacuole (Klionsky *et al.*, 1992; Lynch-Day & Klionsky, 2010). The Cvt pathway operates in growth conditions and autophagy is induced by starvation. Many Atg proteins are common to both the Cvt and autophagy pathways.

Atg2 is a peripheral membrane protein of 1592 amino acids (aa) and a predicted molecular mass of 178 kDa (Shintani *et al.*, 2001; Wang *et al.*, 2001) which is localized in the perivacuolar PAS and is also found in the cytosol. Efficient localization of Atg2 to the PAS is dependent on a membrane protein, Atg9, which binds Atg2 (Wang *et al.*, 2001; Suzuki *et al.*, 2001; 2007). Atg2 forms a complex with Atg18 (Obara *et al.*, 2008b) and together they are required for Atg9 recycling between the PAS and non-PAS membraneous compartments (Reggiori *et al.*, 2004; Suzuki *et al.*, 2007). Moreover, Atg2 and Atg18 localize to the PAS interdependently in autophagy-inducing conditions (Suzuki *et al.*, 2007). The domain structure of Atg2 is unknown. A point mutation causing the G83E substitution results in a severe defect in Atg2 function (Shintani *et al.*, 2001).

In higher eukaryotic cells autophagy is mechanistically identical to the process that occurs in yeast (Reggiori & Klionsky, 2002). In mammalian cells autophagy has multiple physiological functions: it is involved in cell death, tumor suppression, aging, age-associated diseases and neurodegeneration (reviewed in: Bergamini *et al.*, 2004; Gozuacik & Kimchi, 2004; van der Vaart *et al.*, 2008). Numerous genes for components of the human autophagic machinery have been cloned thanks to their homology to yeast *ATG* genes and shown to complement autophagy defects of respective yeast mutants (Reggiori & Klionsky, 2002). The homology of yeast Atg2 and the human autophagy-related protein 2 homolog A encoded by the *hATG2A/KIAA0404* gene and further referred to as hAtg2A was pointed out previously (Shintani *et al.*, 2001). The *hATG2A/KIAA0404* gene further referred to as *KIAA0404* was upregulated in the etoposide-induced apoptosis in HeLa cells and by doxorubicin, an inhibitor of topoisomerase II (Kusama *et al.*, 2009). However, hAtg2A has not been characterized until very

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Abbreviations: aa, amino acid; Cvt, cytoplasm-to-vacuole targeting; GFP, green fluorescent protein; orf, open reading frame; PAS, preautophagosomal structure; RFP, red fluorescent protein; S.D., standard deviation

recently when several hAtg2A-interacting proteins were identified (Behrends *et al.*, 2010).

Yeast is widely used as a model organism to study the function of human proteins and better to understand many human diseases (reviewed in: Foury, 1997; Petranovic & Nielsen, 2008). Here we studied *in silico* the similarity of yeast Atg2 protein to proteins from other organisms and investigated whether the human hAtg2A protein expressed in yeast could function in autophagy. We also asked if the C-terminus of Atg2 is required for Atg2 function and/or could be replaced by the C-terminus of hAtg2A, and, conversely, if hAtg2A with the corresponding Atg2 part is functional in autophagy. Moreover, we studied the interaction of Atg2 and hAtg2A with Atg9 and Atg18 by using the two-hybrid system.

MATERIALS AND METHODS

Strains, media and growth conditions. The *S. cerevisiae* strains used were TN125 MAT α *ade2 ura3 leu2 his3 trp1 lys2 PHO8::pho8⊿60*, YTS24 *atg2::LEU2 PHO8::pho8⊿60* (isogenic to TN125) (Shintani *et al.*, 2001) and PJ69-4A *MAT a trp1-901 leu2-3, 122 his3-200 ura3-52 gal4⊿ gal80⊿ LYS2::GAL1-HIS3, GAL2-ADE2 met2::GAL7-lacZ* (James *et al.*, 1996). Yeast growth and transformation followed standard procedures (Sherman, 2002). YPD (1% yeast extract, 1% peptone, 2% glucose), SC-leu or SC-leu-his (containing 0.68% yeast nitrogen base without amino acids, and 20 mg/l of adenine, uracil and required amino acids minus leucine and histidine) with 2% glucose as a carbon source were used. For nitrogen starvation cells were shifted to YB-N (0.17% yeast nitrogen base without ammonium sulphate and amino acids) containing glucose as a carbon source. For two-hybrid interaction transformants were grown on SC-leu-trp-his with addition of 5–10 mM 3-aminotriazole at 28 °C for 3–7 days.

In strains TN125 and YTS24 the *LEU2* gene was deleted to anable use of plasmids with the *LEU2* marker in further studies and were substituted with a *KanMX* cassette by genomic integration of *Bam*HI-digested pLEU2::kanMX (Voth *et al.*, 2003) to produce strains with a *leu2::kanMX* marker, YPJ2 and YPJ1, respectively.

The ability of cells to form colonies was used to measure their survival under nitrogen starvation conditions. Cells were grown to OD_{600} about 1 in YPD or SC-leu, washed once and then resuspended in YB-N to an OD_{600} about 0.3. At the indicated times, OD_{600} was measured, aliquots were removed, serially diluted and plated on YPD plates in triplicate. Results shown are representative of two independent experiments.

Plasmids and plasmid construction. The plasmids used in this study were: BS-KIAA0404 (Kazusa DNA Research Institute, Japan), pUG36 (Niedenthal *et al.*, 1996), $p415_{GPD1}$ [*LEU2* marker (Mumberg *et al.*, 1995)], pAPE1-RFP (based on pRS313, kind gift of M. Thumm, University of Gottingen, Germany), YEp-ATG2 (Shintani *et al.*, 2001), pGBDUAtg9 (Reggiori *et al.*, 2005), PS-150 bearing the *AD-ATG18* fusion (kind gift of D. J. Klionsky, University of Michigan, USA (Nair *et al.*, 2010)), pGEM (pGEM-TEasy, Promega), pGBT-9 (Clontech) and pACT2 (Clontech).

To express human *KIAA0404* gene in yeast, plasmid p415-GFP-KIAA0404 bearing *GFP* fused with *KIAA0404* gene under the constitutive *GPD1* promoter was constructed. First the *Xba*I fragment of pUG36 bearing the *GFP* gene was transferred to *Xba*I sites of $p415_{GPD1}$ plasmid to obtain p415-GFP. Then *Bam*HI-*Eco*RV 6221 bp fragment of a BS-KIAA0404 derivative, in which a *Bam*HI restriction site had been introduced between the *Mlu*I and *Nsi*I sites by PCR site-directed mutagenesis of pGEM-MluI-NsiI, was ligated with *Bam*HI and *Sma*I-digested p415-GFP. The primers used for *in vitro* site specific mutagenesis and other PCR reactions are shown in Table S1. To obtain plasmid p415-GFP-KIAA0404-N expressing fragment of hAtg2A protein containing amino acids 1–1561, plasmid p415-GFP-KIAA0404 was digested with *Eco*47III and religated.

Plasmid p415-GFP-ATG2 bearing *GFP* fused to the yeast *ATG2* gene was also constructed for control. To this end *Sph*I–*Sac*II DNA fragment containing *ATG2* was transferred from YEp-ATG2 to pGEM and pGEM-ATG2 was used for two rounds of *in vitro* PCR mutagenesis to insert *Xma*I and *Sal*I restriction sites flanking *ATG2* open reading frame (orf). Resulting pGEM-ATG2-XS was sequenced and was digested with *Xma*I, ends were filled in with Klenow polymerase, and after *Sal*I digestion the fragment bearing *ATG2* was isolated and ligated with *Sma*I and *Sal*I-digested p415-GFP. To obtain p415-GFP-ATG2-N encoding Atg2 protein devoid of C-terminal domain (aa 1–1184), *Blp*I–*Stu*I fragment of p415-GFP-ATG2 was replaced with similar fragment in which *Sal*I site had been introduced by *in vitro* PCR mutagenesis and the 1235-bp *Sal*I fragment was removed by *Sal*I digestion and religation.

To analyze the possible function of hAtg2A N-terminal and C-terminal fragments, plasmids expressing appropriate gene fusions, p415-GFP-ATG2-N-KIAA0404-C and p415-GFP-KIAA0404-N-ATG2-C, were constructed by fusion-PCR. Fragment *Blp*I-*Stu*I of p415-GFP-ATG2 was transferred to pGEM to obtain pGAF. pGAF was used as a template for PCR to amplify *ATG2* fragment and to add 18 bp overhang of *KIAA0404* sequence. This fragment together with PCR-amplified fragment of *KIAA0404* encoding C-terminal part of the hAtg2A protein, with 18 bp overhang of *ATG2* sequence (Table S1), was used as a template for fusion PCR. Product of fusion PCR was digested with *Blp*I and *Sal*I and was used to replace *Blp*I–*Sal*I fragment of p415-GFP-ATG2 and obtain p415-GFP-ATG2-N-KIAA0404-C which encodes a fusion of N-terminal part of Atg2 (aa 1–1184) with C-terminal part of hAtg2A (aa 1540–1938). Plasmid p415-GFP-KIAA0404-N-ATG2-C encoding fusion protein of N-terminal part of hAtg2A (aa 1–1539) fused with C-terminal part of Atg2 (aa 1185-1592) was obtained by replacing *Sac*II–*Sal*I fragment of p415-GFP- KIAA0404 with *Sac*II and *Sal*I-digested product of fusion PCR. In this fusion-PCR two PCR products were used as templates: one DNA product of 1277 bp amplified by using p415-GFP-ATG2 as a template and *ATG2*-specific oligonucleotides, one with 18-nucleotide *KIAA0404* overhang added, and the other DNA product of 1238 bp amplified by using p415-GFP-KIAA0404 as a template and *KIAA0404*-specific oligonucleotides, one with 18-nucleotide *ATG2* overhang added.

To analyze the physical interaction of proteins by two-hybrid system, plasmids expressing fusions of *GAL4* domains with full lenght *ATG2*, *ATG9* or *KIAA0404* were constructed. To obtain pGBT9-ATG2, pGEM-ATG2-XS (see above) was digested with *Xma*I and *Sal*I, and 4794 bp fragment was transferred to pGBT9. To obtain pGBT9-KIAA0404, 7.2-

Table S1. Oligonucleotides used

Name	Sequence	Nucleotide position in *ATG2*, *ATG9* or *KIAA0404* orf	Description
Xma11	TTTTTTTTGATTT**CCCGGG**AATGGCATTTTGG	ATG2 1–12	introduce *Xma*I site
Xma11rev	CCAAAATGCCATT**CCCGGG**AAATCAAAAAAAA		
Sal11	CGTAAAAATTTTT**GTCGAC**ATTTTTGATATAT	*ATG2* 4779–4775	introduces *Sal*I site
Sal11rev	ATATATCAAAAAT**GTCGAC**AAAAATTTTTACG		
BlpI_F	**GCTCAGC**CAAAAGAAGAAGAC	*ATG2* 3016–3036	amplification of *ATG2* fragment
StuI_R	GACAGGAGCC**AGGCCT**TCCAA	*ATG2* 4047–4027	
M13F	TGTAAAACGACGGCCAGT		universal primer complementary to pGEM T-Easy, used for fusion PCR
YK_R	CGTGTGTAGGTACAGGAATTTATTCCAGGTGG	*ATG2* 3552–3539	5′ end contains 18 nucleotide overhang complementary to *KIAA0404* (4716–4699), used for fusion PCR
YK_F	TCC CAG ATC AAC AAG TTT GTA ACT TTG CTA AAG CA	*ATG2* 3553–3572	5′ end contains overhang complementary to *KIAA0404* (4684–4698), used for fusion PCR
SalATG_R	TAACTAATTACATGACTCGAG**GTC GAC**A		complementary to terminator sequence of *ATG2*, introduces *Sal*I site
Sal3_F	CCTGGAATAAA**GTCGAC**ACTTTG	*ATG2* 3542–3564	introduces *Sal*I site
Sal3_R	GCAAAGT**GTCGAC**TTTATTCC	*ATG2* 3565–3545	
0404LBam	ACA T**GG ATC C**CT CGG AGA CCGCCG GGC CTG	*KIAA0404* 4–1	introduces *Bam*HI site
0404UBam	CGA G**GG ATC C**AT GTC ACG ATG GCT GTG GCC A	*KIAA0404* 1–21	
KY_L	CCACCTGGAATAAATTCCTGTACCTACACACG	*KIAA0404* 4699–4716	5′ end contains 14 nt overhang complementary to *ATG2* (3539–3552)
SalKIA_R	ACTCGAG**GTCGAC**TCAGTCTTGGGCAC	*KIAA0404* 5899–5885	introduces *Sal*I site to *KIAA0404*
KY_R	TTAGCAAAGTTACAAACTTGTTGA TCTGGGAGGA	*KIAA0404* 4698–4681	5′ end contains overhang to *ATG2* (3568–3553)
SacII_F	CCTCATCA**CCGCGG**AGACC	*KIAA0404* 3477–3495	complementary to *KIAA0404*
Atg94	GAAATTGAGATGGTGCA**GGATCC**ACAGTTGAAGTG		introduces *Bam*HI site after *ATG9* orf
Atg93	GGAATTCCCGATCC**CCATGG**AGAGAGATGAATAC	*ATG9* 1–18	introduces *Nco*I site in start codon of *ATG9* orf

kb *Eco*RI–*Eco*RV fragment was transferred from the KS-KIAA0404-EcoRI plasmid (BS-KIAA0404 derivative), in which an *Eco*RI restriction site had been introduced by PCR before start codon, to pGBT9 *Eco*RI and *Sma*I restriction sites. Similarly, to construct pGBT9-KIAA0404-N the *Eco*47III fragment of plasmid KS-KIAA0404-*Eco*RI was deleted and than *Eco*RI-*Eco*RV fragment was transferred to pGBT9. To obtain pACT2-ATG9, the *ATG9* orf was amplified by PCR by using primers containing *Nco*I and *Bam*HI restriction sites, and pGBDUAtg9 as a template, and was cloned to pACT2 *Nco*I and *Bam*HI restriction sites. pACT2-ATG9 sequence was confirmed.

Total protein extracts and Western blot analysis. Protein extracts were prepared as described (Baggett *et al.*, 2003). Samples were analyzed by Western blotting using anti-GFP (Roche), anti-aminopeptidase I (anti-Ape1, kindly provided by D. Klionsky, University of Michigan, Ann Arbor, USA) and secondary anti-mouse or anti-rabbit HRP-conjugated antibodies (DACO), followed by enhanced chemiluminescence (Millipore). A representative result of two experiments is shown.

Fluorescence microscopy. For GFP fluorescence yeast were grown to the logarithmic phase in SD-leu-his medium at 30 °C. After that cells were shifted to YB-N starvation medium for 4 hours. At least 100 cells of each strain were viewed with an Eclipse (Nikon) fluorescence microscope equipped with an ORCA (Nikon) camera. Images were collected using Lucia General 5.1 software (Laboratory Imaging Ltd.).

Monitoring of authophagy by Pho8Δ60 alkaline phosphatase test. For measurements of autophagic activity, the alkaline phosphatase test was performed as described (Noda *et al.*, 1995). Five independent experiments were performed and means ±S.D. (standard deviation) are shown.

Bioinformatic analyses. The non-redundant database at NCBI was scanned for sequences similar to the Atg2 protein using the PSI-Blast program (Altschul *et al.*, 1997). Fifty fungal Atg2 orthologues were aligned using the MAFFT program (Katoh *et al.*, 2002) and manually inspected for differences. Among human proteins, Atg2 homologues were found using the PSI-Blast program (Altschul *et al.*, 1997) with the first hits detected in the first iteration with e-values of 5e-11 and 4e-15 for hAtg2A and Atg2B, respectively. Multiple sequence alignment of yeast Atg2 with the *Aspergillus nidulans* AnAtg2 and human hAtg2A homologues was

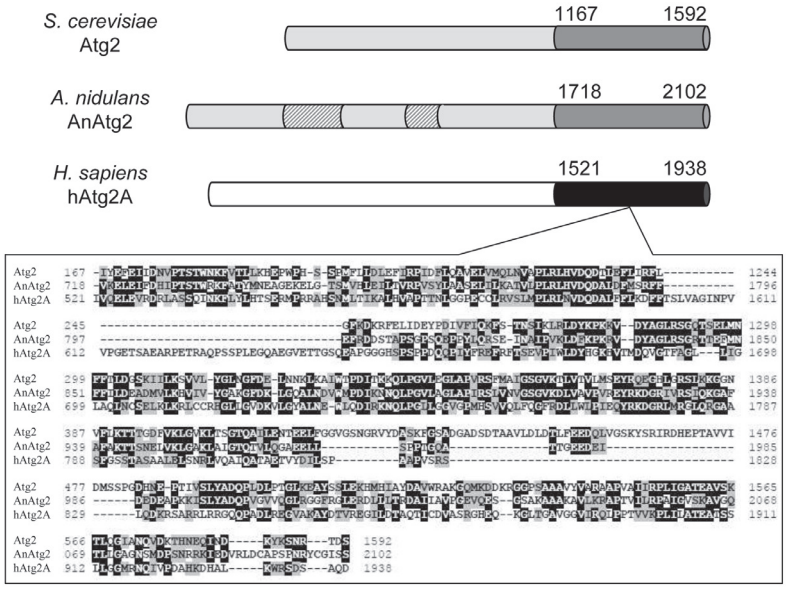

Figure 1. Atg2 domain structure and homology to other Atg2 proteins
Schematic representation of domain structure of Atg2, AnAtg2 and hAtg2A. N-terminal and C-terminal fragments of fungal AnAtg2 and Atg2 are in *light grey* and *dark grey*, respectively. N-terminal fragment of hAtg2A is in *white* and C-terminal domain of hAtg2A in *black*. Regions specific to proteins of filamentous fungi are shown *striped*.

done using MAFFT with 1000 iterations and then formatted in BioEdit (Hall, 1999).

RESULTS

Homologues of yeast Atg2 protein

Autophagy is a process conserved in evolution from yeast to humans, and several yeast Atg proteins involved in autophagy have orthologues in human cells (Reggiori & Klionsky, 2002). Specifically, the yeast Atg2 protein was reported to be related to the protein encoded by the human *KIAA0404* gene (Shintani *et al*., 2001), hAtg2A. We performed *in silico* analysis using PSI-Blast to find homologues of yeast Atg2 in other organisms and to predict the Atg2 domain structure.

Our analysis revealed that *S. cerevisiae* Atg2 is highly homologous to Atg2 proteins from other fungi forming a fifty-strong Atg2 family with 24 members belonging to the *Pezizomycotina* subphylum, 22 to *Saccharomycotina* and 4 — to *Basidiomycotina*. All three subphyla seem to have quite similar Atg2s, except for large insertions in the filamentous fungi. The largest insertions are located in regions 260–480 and 720–850 of the filamentous fungi polypeptide sequences. The provenience and function of these regions have not been studied yet. The C-terminal region of Atg2 (aa 1167–1592) is also homologous to the C-terminal region of human hAtg2A protein (aa 1521–1938) (25% identity, e-value 3e-12, score 72.4. using BLAST) and the corresponding region of the hAtg2B protein (aa 1644–2078) encoded by the *C14orf103* gene (Fig. 1 and data not shown).

KIAA0404 gene can be expressed in yeast

The data about the cellular function of the hAtg2A protein encoded by the *KIAA0404* gene was lacking, thus we used yeast to express the *KIAA0404* gene and to study its potential function in autophagy. Plasmid p415-GFP-KIAA0404 encoding hAtg2A protein N-terminally fused to GFP was constructed. The fusion gene was expressed from a strong constitutive *GPD1* promoter. A plasmid encoding yeast Atg2 fused to GFP was also constructed to serve as a control. *atg2*⊿ yeast cells were transformed with p415-GFP-KIAA0404 and control plasmids (p415-GFP, p415-GFP-ATG2), grown in medium containing glucose, shifted or not to nitrogen starvation medium for 4 hours, and protein extracts were prepared and analyzed by Western blotting using anti-GFP antibody. As expected, when *GFP-ATG2* was expressed an abundant protein product of about 220 kDa was observed. When the *GFP-KIAA0404* gene was expressed the GFP-hAtg2A fusion protein of about 315 kDa was present but its level was low and two smaller proteins of 130 kDa and about 30 kDa were observed, probably representing partial degradation products, the smaller one resembling GFP (Fig. 2a and data not shown). The *atg2*⊿ transformants expressing *GFP-ATG2* or *GFP-KIAA0404* shifted to nitrogen starvation medium showed protein products of similar size as in growth conditions, but less abundant (not shown).

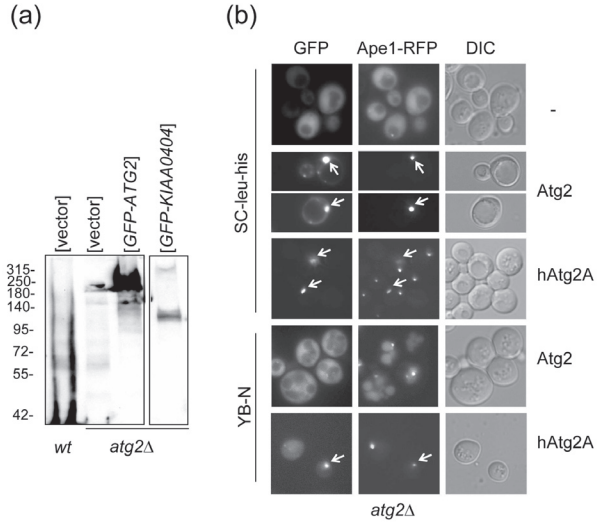

Figure 2. Expression of *KIAA0404* in yeast
(a) Western blot analysis. *atg2Δ* strain (YPJ1) was transformed with plasmids expressing *GFP*, *GFP-ATG2* or *GFP-KIAA0404* genes and grown in SD-leu medium to OD_{600} of 0.4–0.6. Extracts were prepared and Western blot analysis was performed using anti-GFP antibody. **(b)** Localization of human hAtg2A in yeast cells. *atg2Δ* strain (YPJ1) was transformed with plasmids expressing *GFP*, *GFP-ATG2* or *GFP-KIAA0404* and with plasmid expressing *APE1-RFP*. Transformants were grown in SC-leu-his medium to OD_{600} of 0.4–0.6 and were shifted to nitrogen starvation medium for 4 hours. Cells were viewed by epifluorescence or by DIC optics. Arrows indicate punctate structures where GFP-hAtg2A and Ape1-RFP colocalize.

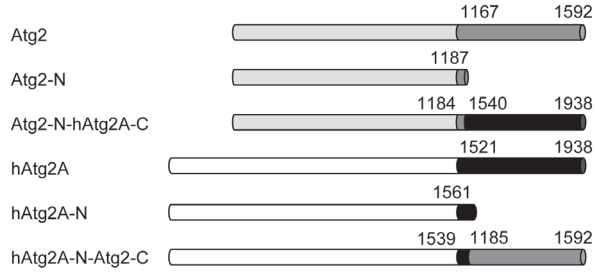

Figure 3. Schematic representation of Atg2 and hAtg2A domain structure and their constructs expressed with N-terminally fused GFP
Fragments and domains are as in Fig. 1.

We also transformed wild type cells with the above plasmids and tested for growth on YPD, and SD-leu at three temperatures: 28 °C, 34 °C and 37 °C. Cells expressing *GFP-ATG2*, *GFP-KIAA0404* grew similarly to control cells in these conditions (not shown). Thus, the *GFP-KIAA0404* gene can be expressed in yeast cells and hAtg2A is not toxic to yeast cells in growth conditions.

hAtg2A is localized in a few cytoplasmic dots and one of these dots colocalizes with Ape1-RFP, so it is recruited to the PAS

Atg2 localizes in yeast cells to one or two punctate cytoplasmic structures in growth conditions and to one such structure adjacent to a vacuole called PAS in starvation condition (Shintani *et al.*, 2001; Suzuki *et al.*, 2007). PAS is the site of recruitment of membranes and cargo proteins, such as Ape1, to the growing autophagosome. So, Ape1 fused to a fluorescent tag such as red fluorescent protein (Ape1-RFP) can be used as a marker for PAS in growing cells (Kawamata *et al.*, 2005; Suzuki *et al.*, 2007). Ape1-RFP is localized to the vacuole in starved cells. Similarly as published, in our experiments in *atg2⊿* cells in growth conditions we observed GFP-Atg2 in one to three cytoplasmic dots, one of which colocalized with Ape1-RFP, the PAS marker. Also slight diffused cytoplasmic staining was observed probably due to overproduction of the protein (Fig. 2b). In starvation conditions GFP-Atg2 was cytoplasmic in most cells and some cells showed also a single dot while Ape1-RFP was vacuolar and in some cells also localized to the PAS (Fig. 2b).

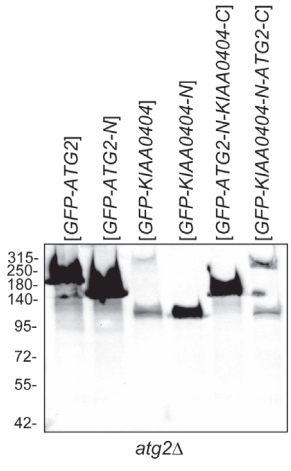

Figure 4. Cellular levels of GFP-Atg2-N, GFP-hAtg2A-N, and fusions of Atg2 with hAtg2A.
atg2Δ strain was transformed with plasmids expressing *GFP-ATG2-N, GFP-KIAA0404-N, GFP-ATG2-N-KIAA0404-C* or *GFP-KIAA0404-N-ATG2-C* and grown in SD-leu medium to OD_{600} about 1. Extracts were prepared and Western blot analysis was performed using anti-GFP antibody. Extracts from cells expressing *GFP-ATG2* or *GFP-KIAA0404* were used as a control.

Cellular localization of the human GFP-hAtg2A protein was observed in *atg2⊿* cells, and was compared with the localization of Ape1-RFP, GFP-Atg2 and of GFP alone. In growth conditions GFP was diffused in the cytoplasm while the GFP-hAtg2A protein was localized to 1–3 punctate structures (Fig. 2b). One of them colocalized with the Ape1-RFP punctum in about 60% of *atg2⊿* cells in which both GFP and RFP signals were visible, suggesting that GFP-hAtg2A could be recruited to the PAS. In control experiments employing *atg1⊿* cells in which PAS does not form we did not find colocalization of GFP-Atg2A and Ape1-RFP, which supports this conclusion (not shown). The identity of the punctate structures other than PAS containing GFP-Atg2A is unknown. In starvation conditions GFP-hAtg2A was cytoplasmic in 95% of cells and 5% of cells showed it in one punctate structure (Fig. 2b). The cytoplasmic staining may also derive from the partial degradation product, GFP. In these cells Ape1-RFP was found in the PAS, not in the vacuole, indicating a lack of complementation of the autophagy defects of *atg2⊿* by *GFP-KIAA0404*.

N-terminal fragments of Atg2 and hAtg2A are sufficient for PAS recruitment but do not support Cvt transport of Ape1-RFP to the vacuole

Since hAtg2A protein, despite being partially recruited to the PAS, did not complement the Cvt defect of *atg2⊿* cells, in which it differed from the yeast Atg2, several questions arose. Which part of Atg2 is required for PAS recruitment and for functioning in the Cvt pathway? Is the function of the C-terminus of Atg2 conserved? Can the C-terminal part of Atg2 be replaced with a homologous fragment of hAtg2A? To answer these questions several plasmids were constructed and the cellular level and localization of protein products encoded was analyzed. These were plasmids expressing: *GFP-ATG2-N* (aa 1–1184) devoid of a fragment encoding the C-terminus of Atg2, *GFP-KIAA0404-N* (aa 1–1561) devoid of a fragment encoding the C-terminus of hAtg2A, *GFP-ATG2-N-KIAA0404-C* fusion in which the fragment encoding the C-terminus of Atg2 (aa 1185–1592) was replaced by a fragment encoding the C-terminus of hAtg2A (aa 1540–1938), and *GFP-KIAA0404-N-ATG2-C* encoding a fusion protein of the N-terminal part of hAtg2A (aa 1–1539) fused to the C-terminal part of Atg2 (aa 1185–1592) (Fig. 3). The site of fusion of the Atg2 and hAtg2A proteins was chosen between amino acids KR which are identical in both proteins and are located in the first most conserved block of amino acids (see Fig. 1). Expression of these fusion genes in *atg2⊿* strain was analyzed by Western blotting and compared with the expression level of *GFP-ATG2* and *GFP-KIAA0404* in growth and starvation conditions (Fig. 4 and data not shown). The expression of *GFP-ATG2-N* was very efficient and comparable to that of *GFP-ATG2*, and Atg2-N protein of expected size of about 170 kDa was observed. The expression of *GFP-KIAA0404-N* was less efficient and hAtg2A-N migrated faster than expected, of about 130 kDa instead of 220 kDa. This size was similar to the major

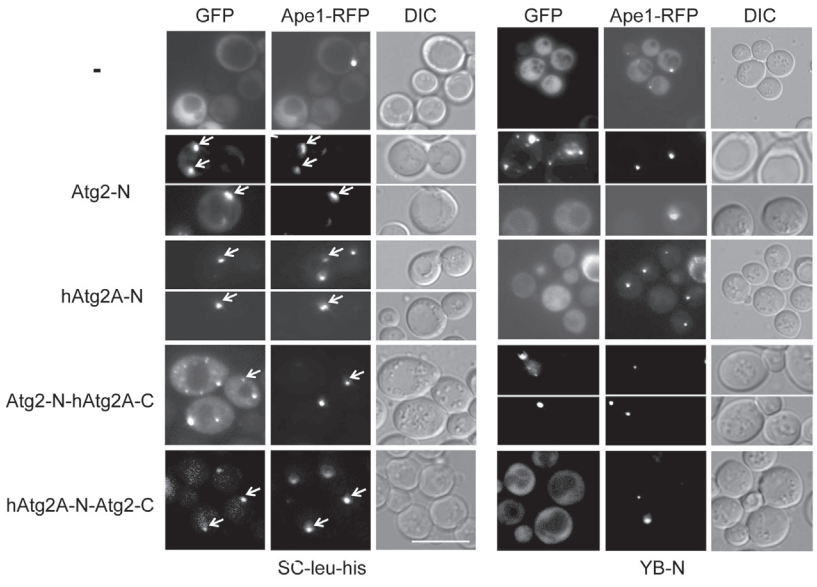

Figure 5. Localization of GFP-Atg2-N and GFP-hAtg2A-N fragments, and GFP-Atg2-N-hAtg2A-C and GFP-hAtg2A-N-Atg2-C fusions.
atg2Δ strain was transformed with plasmids expressing *GFP*, *GFP-ATG2-N, GFP-KIAA0404-N, GFP-ATG2-N-KIAA0404-C* or *GFP-KIAA0404-N-ATG2-C* and with plasmid expressing *APE1-RFP*. Transformants were grown in SC-leu-his medium to OD_{600} of 0.4–0.6 and were shifted to nitrogen starvation medium for 4 hours. Cells were viewed by epifluorescence or by DIC optics. Arrows indicate punctate structures where GFP and RFP signal colocalize. Scale bar, 10 μm.

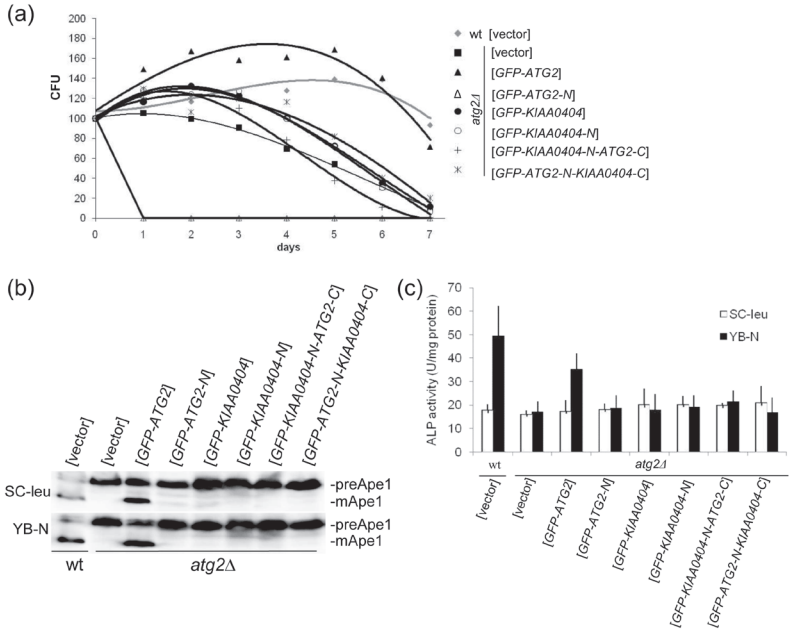

Figure 6. Lack of complementation of *atg2Δ* autophagy defects by *GFP-ATG2-N*, *GFP-KIAA0404*, *GFP-KIAA0404-N* and fusions of *KIAA0404* with *ATG2*.
(a) Survival of *atg2Δ* (YPJ1) trasformants during starvation. Wild type strain transformed with empty vector for control and *atg2Δ* cells transformed with plasmids expressing *GFP*, *GFP-ATG2, GFP-ATG2-N, GFP-KIAA0404, GFP-KIAA0404-N, GFP-ATG2-N-KIAA0404-C* or *GFP-KIAA0404-N-ATG2-C* were grown in SC-leu, shifted to YB-N, and tested for viability after 1 to 7 days of incubation. Colony forming units (CFU) were scored. **(b)** Complementation of *atg2Δ* (YPJ1) defect of Cvt pathway. Same set of transformants as in **(a)** was grown in SD-leu, shifted to YB-N for 4 hours, then extracts were prepared and analyzed by Western blotting using anti-Ape1 antibody. **(c)** Complementation of *atg2Δ* (YPJ1) defect of nonselective autophagy. Wild type cells transformed with empty vector and *atg2Δ* cells expressing *GFP*, *GFP-ATG2, GFP-ATG2-N, GFP-KIAA0404, GFP-KIAA0404-N, GFP-ATG2-N-KIAA0404-C* or *GFP-KIAA0404-N-ATG2-C* were grown in SD-leu, shifted or not to YB-N for 4 hours, then extracts were prepared and Pho8Δ60 test was performed.

band of GFP-hAtg2A representing partial degradation product, indicating that the C-terminal part of hAtg2A could be unstable in yeast. The apparent molecular mass of the protein product of *GFP-ATG2-N-KIAA0404-C* was about 180 kDa and was lower than expected (220 kDa), again indicating an instability of the C-terminus of hAtg2A. The full length product of *GFP-KIAA0404-N-ATG2-C* showed the expected size of about 260 kDa but a partial degradation product of about 130 kDa was also observed. We cannot exclude the possibility that some of these proteins migrate aberrantly and in fact are not even partially degraded. After a shift to starvation conditions all these proteins were present but much less abundant (not shown). The localization of the N-terminal parts of the proteins, Atg2-N and hAtg2A-N, expressed from plasmids described above was observed in *atg2Δ* strain in growth and nitrogen starvation conditions and compared with the localization of GFP-Atg2, GFP-hAtg2A and Ape1-RFP. GFP-Atg2-N was present in 1–3 dots and slightly diffused in the cytoplasm, similarly as GFP-Atg2. One of these dots colocalized with Ape1-RFP in about 50% of cells indicating that the N-terminal fragment of Atg2 is sufficient for PAS recruitment. Interestingly, about 20% of cells showed more that one Ape1-RFP dot indicating that more than one PAS could be present in some cells or Ape2-RFP could be located also in other punctate structure in this condition (Fig. 5). Observation of GFP-Atg2-N in starvation conditions showed cytoplasmic localization or in 1–5 dots, and Ape1-RFP was localized in the PAS. On the other hand, GFP-hAtg2A-N was localized in one or two dots and slightly diffused in the cytoplasm and colocalized with Ape1-RFP in 60% of cells indicating that the N-terminal part of hAtg2A is also sufficient for PAS recruitment. Ape1-RFP was localized in two dots in some of these cells.

Fusion proteins GFP-Atg2-N-hAtg2A-C and GFP-hAtg2A-N-Atg2-C were localized in few dots or one, respectively, and slightly diffused in the cytoplasm in *atg2Δ* cells in growth conditions (Fig. 5). One of these dots colocalized with Ape1-RFP, and the recruitment to PAS was more efficient for the

hAtg2A-N-Atg2-C fusion. In starvation conditions GFP-Atg2-N-hAtg2A-C was found in few cytoplasmic dots while GFP-hAtg2A-N-Atg2-C was in the dots and the cytoplasm. Ape1-RFP localized exclusively to the PAS, not to the vacuole, in cells expressing both fusion proteins showing a lack of complementation of Cvt defects of *atg2⊿* (Fig. 5).

Intact *KIAA0404* and fusions containing fragments of *KIAA0404* do not complement defects of atg2Δ cells in survival upon starvation, preApe1 maturation and nonselective autophagy

The involvement of hAtg2A protein in yeast authophagy could be assessed by investigating complementation of individual phenotypes of *atg2⊿* cells by *GFP-KIAA0404* or its derivatives. The *atg2⊿* cells do not survive for longer than five days under nitrogen starvation conditions when glucose is present in the medium, they show a block in preApe1 maturation and a defect in nonselective autophagy measured by Pho8Δ60 test (Shintani *et al.*, 2001; Wang *et al.*, 2001). First we analyzed the complementation of the *atg2⊿* viability defects by *GFP-KIAA0404,* its N-terminal fragment *GFP-KIAA-0404-N*, and the fusions *GFP-KIAA0404-N-ATG2-C* and *GFP-ATG2-N-KIAA0404-C* relative to the complementation by *GFP-ATG2* and *GFP-ATG2-N*. The respective transformants were grown in SC-leu, transferred to nitrogen starvation medium for seven days and tested for viability by scoring colony forming units after each day. The wild type control strain remained viable throughout the experiment while the *atg2⊿* cells bearing the empty plasmid died after six days, in good agreement with published data. Plasmids expressing *GFP-ATG2* fully complemented the *atg2⊿* defect, cells remained viable, similarly as wild type strain, after 7 days of starvation. Survival of transformants bearing plasmids expressing *GFP-KIAA0404*, *GFP-KIAA0404-N* or the fusion genes declined during starvation and was similar to the *atg2⊿* control cells after 7 days of starvation (Fig. 6a). This result indicates that expressed *KIAA0404* gene, its 5' fragment or fusions do not complement the *atg2⊿* defects in survival. Surprisingly, *GFP-ATG2-N* was very toxic to *atg2⊿* cells, they did not survive even the first day of starvation. However, the *KIAA0404* fragment encoding homologous C-terminal part of hAtg2A fused with *GFP-ATG2-N* (resulting in *GFP-ATG2-N-KIAA0404-C*) prevented this toxicity, similarly as the respective native fragment of *ATG2*. Although we do not observe full length fusion protein expressed from *GFP-ATG2-N-KIAA0404-C* in our Western blot analysis (Fig. 4), this protein may be present and function in the cells and be partially degraded during protein extraction.

Another test performed for complementation of *atg2⊿* by *GFP-KIAA0404* was the Western blot test for preApe1 maturation. In growth conditions in wild type cells preApe1 is transported *via* the Cvt pathway to the vacuole where it is proteolytically processed. This pathway is blocked in *atg2⊿* strains (Shintani *et al.*, 2001; Wang *et al.*, 2001). The maturation of preApe1 was analyzed by Western blotting in the set of transformants described above grown in SD-leu medium and shifted to nitrogen starvation conditions. Neither *GFP-KIAA0404,* its fragment *GFP-KIAA0404-N* nor the *GFP-KIAA0404-N-ATG2-C* or *GFP-ATG2-N-KIAA0404-C* fusions restored preApe1 maturation in *atg2⊿*, in conditions when *GFP-ATG2* expressed from the plasmid was fully functional (Fig. 6b). These results indicate that the human hAtg2A protein or its fragments cannot take over the yeast Atg2 function in the Cvt pathway. This is in agreement with our Ape1-RFP localization results in the *atg2⊿* strain bearing *KIAA0404*-containing plasmids, described above (see Fig. 5). Similarly, preApe1 maturation was not restored in *atg2⊿* cells expressing *GFP-ATG2-N,* showing that the Atg2-N fragment is not sufficient for function.

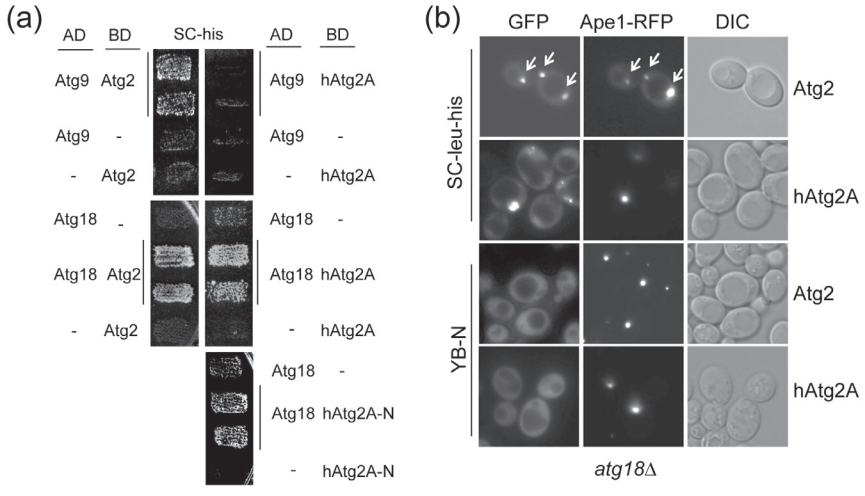

Figure 7. hAtg2A interacts with Atg18 and requires Atg18 for recruitment to the PAS.
(a) Interaction of Atg2 and hAtg2A with Atg9 and Atg18. Two-hybrid tester strain (PJ69-4A) was co-transformed with plasmids expressing activating domain (AD)-fused Atg9 or Atg18 and Atg2, hAtg2A or hAtg2A-N fused with the DNA binding domain (BD) or with empty vectors (-). Interactions were monitored by the ability of cells to grow on plates lacking histidine and containing 5–10 mM 3-aminotriazol (SC-his). **(b)** Localization of Atg2 and hAtg2A in *atg18Δ* strain. *atg18Δ* strain was transformed with plasmids expressing *GFP-ATG2* or *GFP-KIAA0404* and with plasmid bearing *APE1-RFP*. Transformants were grown in SC-leu-his medium to OD_{600} of 0.4–0.6 and were shifted to nitrogen starvation medium for 4 hours. Cells were viewed by epifluorescence or by DIC optics. Arrows indicate punctate structures where GFP and RFP signals colocalize.

We also tested Pho8Δ60 activity, a measure of nonselective autophagy, in the set of transformants described above. Wild type cells transformed with empty vector responded to nitrogen starvation by increasing the activity of Pho8Δ60 more than 2-fold while in *atg2⊿* cells this activity was similar as in growth conditions (Fig. 6c), in agreement with previously published data (Shintani *et al.*, 2001). Expression of *GFP-ATG2* complemented this phenotype, the Pho8Δ60 activity was significantly increased upon starvation. This complementation was lost when *GFP-ATG2-N* was expressed, indicating that the N-terminal fragment of Atg2 is not sufficient to function in nonselective autophagy. Complementation was also not observed in *atg2⊿* cells expressing *GFP-KIAA0404,* its fragment *GFP-KIAA0404-N* or fusions with fragments of

ATG2, showing that hAtg2A does not function in non-selective autophagy in yeast.

hAtg2A interacts with Atg18 but does not interact with Atg9 in two-hybrid system

Atg2 was shown to interact with the membrane protein Atg9 (Wang *et al.*, 2001) and lipid binding protein Atg18 (Obara *et al.*, 2008b; Nair *et al.*, 2010) and to be required for Atg9 cycling between PAS and non-PAS peripheral compartment (Reggiori *et al.*, 2004). Therefore, we tested if these interactions of Atg2 could be reproduced in yeast two-hybrid system and if hAtg2A also interacted with the same proteins. Transformation of a tester strain with plasmids bearing gene fusions of $GAL4_{AD}$ with *ATG9* or *ATG18* and plasmids bearing $GAL4_{BD}$ fused with *ATG2* or *KIAA0404* followed by the analysis of transformants for growth on SC-his plates showed that Atg2 interacted with both Atg9 and Atg18 in this system (Fig. 7a). hAtg2A did not interact with Atg9 but an interaction with Atg18 was observed (Fig. 7a). The interaction of hAtg2A with Atg18 suggests that Atg18 is probably one of the proteins mediating recruitment of hAtg2A to PAS. Consistent with the fluorescence microscopy studies showing hAtg2A-N recruitment to the PAS, the N-terminal fragment of hAtg2A was also able to interact with Atg18 in two-hybrid system (Fig. 7a).

To analyze the involvement of Atg18 in hAtg2A recruitment to PAS we observed the cellular localization of GFP-hAtg2A in the *atg18*⊿ strain in growth and nitrogen starvation conditions and compared it with localization of Atg2. We found that in growth conditions Atg2 was present in one or two cytoplasmic dots and colocalized with Ape1-RFP1 in most cells. In some cells Ape1-RFP was found in two dots, as before. In contrast, hAtg2A was found in one to five dots, but none of those dots colocalized with Ape1-RFP. Upon nitrogen starvation Atg2 and hAtg2A diffused in the cytoplasm while Ape1-RFP was in PAS and not in the vacuole (Fig. 7b). These results indicate that *ATG18* is important for PAS localization of hAtg2A in both growth and starvation conditions. It was previously reported that *ATG18* is required for PAS recruitment of Atg2 in autophagy-inducing conditions (rapamycin treatment) (Suzuki *et al.*, 2007) and our results are consistent with those findings. We also indicated that some factors may help to recruit Atg2 to Ape1-RFP-containing dots in growing *atg18*⊿ cells.

DISCUSSION

The function of human hAtg2A protein encoded by *KIAA0404* has not been reported to date. When this work was in progress several proteins interacting with hAtg2A were identified, including some proteins involved in autophagy in humans (Behrends *et al.*, 2010). Here we studied the homology of Atg2 and hAtg2A and used yeast as a model organism to test the possible involvement of hAtg2A in autophagy. We showed that *GFP-KIAA0404* could be expressed in yeast and its protein product, GFP-hAtg2A, was recruited to the PAS. This recruitment is probably mediated by Atg18 which interacted with hAtg2A in the two hybrid system. Human hAtg2A did not support growth of *atg2*⊿ cells in starvation conditions and could not substitute for the yeast Atg2 protein in the Cvt pathway or non-selective autophagy. Moreover, we showed that the N-terminal fragments of Atg2 or of hAtg2A were sufficient for the recruitment to the PAS but they did not function in autophagy. Also, the C-terminal fragment of Atg2 could not be functionally substituted for homologous C-terminal fragment of hAtg2A and *vice versa*. Surprisingly, we also found that Atg2-N was toxic to yeast cells in starvation conditions and this toxicity was prevented by fusion with the hAtg2A C-terminus.

Human hAtg2A and yeast Atg2 show only limited homology, restricted to the C-terminal domain. However, the hAtg2A protein did localize to cytoplasmic punctate structures, some of which colocalized with Ape1-RFP-marked PAS, similarly as did yeast Atg2. One of the proteins mediating PAS recruitment of hAtg2A could be Atg18 since these proteins interacted in the two-hybrid assay in our experiments and deletion of *ATG18* abolished PAS localization of hAtg2A. Atg18 binds Atg2 independently of its phosphatidylinositol-3-phosphate (PI3P)-binding ability (Obara *et al.*, 2008b), and this complex is recruited to PAS by PI3P, in which PAS is enriched (Obara *et al.*, 2008a) and by other factors (Suzuki *et al.*, 2007). One such factor is Atg9 (Wang *et al.*, 2001). However, in contrast to Atg2, hAtg2A did not bind Atg9 in two-hybrid system suggesting that other proteins must also contribute to the hAtg2A recruitment to PAS. Very recently, studies of proteomic network organization of the human autophagy system have identified nine proteins interacting with hAtg2A (Behrends *et al.*, 2010). Among them are hAtg2B, and WIPI1 and WDR45, two WD40 domain-containing proteins homologous to Atg18. This supports our finding and indicates that the Atg2-Atg18 interaction is conserved in evolution from yeast to humans. Moreover, hAtg2A was found among interacting proteins when any of the Atg8 human homologues (GABARAP, GABARAPL1, GABARAPL2, MAP1LC3B, MAP1LC3B) was used as a bait in these proteomic studies (Behrends *et al.*, 2010). This suggests that Atg8 may contribute to the PAS recruitment of hAtg2A in yeast. However, PAS assembly studies showed that Atg8 is not required for Atg2 PAS localization (Suzuki *et al.*, 2007) and rather the Atg18-Atg2 complex facilitates PAS recruitment of lipidated Atg8 (Nair *et al.*, 2010), not the opposite. We therefore hypothesize that Atg18 could be the only protein required for the PAS localization of hAtg2A.

The N-terminal part of hAtg2A, also similarly as the N-terminal part of Atg2, was sufficient for the punctate structure-localization and PAS recruitment, indicating that this sequence, although not showing homology, has retained its PAS-addressing function. However, this PAS localization was not sufficient to support hAtg2A, hAtg2A-N or Atg2-N function in selective and nonselective autophagy. Therefore, the C-terminal domain of Atg2 must be important for the function in autophagy but not necessary for Atg2 localization. The C-terminal domain of hAtg2A seems rather unstable in yeast cells since it was removed in some pool of hAtg2A and a shorter form, similar in size to hAtg2A-N, predominated on Western blots. This could be one of the reasons of the lack of complementation of *atg2*⊿ defects by *GFP-KIAA0404*. On the other hand, the amount of full length hAtg2A could be nominally sufficient to function but the C-terminal domain of hAtg2A could lack some important protein interactions. However, the presence of hAtg2A-C in fusion with Atg2-N prevented its toxicity and kept Atg2-N-hAtg2A-C in the punctate structures in starvation conditions. We tested if replacing the C-terminal domain of hAtg2A affected the function. Although the fusion protein hAtg2A-N-Atg2-C was more

stable than hAtg2A and was recruited to the PAS, it still was not functional. So, the C-terminal Atg2 domain can properly function only when attached to the N-terminus of Atg2. Similarly, the C-terminal domain of hAtg2A can not functionally substitute for the C-terminal domain of Atg2 even though these domains can regulate each other's function. Some human proteins, GAS41 for example, are unable to complement phenotypes of the loss of a yeast homologue but can provide a conserved domain which is able to functionally substitute for the yeast domain (Wang *et al.*, 2009). In our case only the combination of the Atg2-N and Atg2-C domains provides the essential functions necessary for autophagy and cell survival upon starvation.

Our results on the survival of *atg2⊥* expressing *GFP-ATG2-N* pointed to previously unreported effects of this overproduced Atg2 fragment on cells upon starvation. These cells did not survive even one day of starvation, they also grew slower in growth conditions in liquid medium compared to empty plasmid-bearing cells (not shown). The mechanism of this toxicity is unknown at present but is an interesting subject for further investigation.

In our localization studies we used Ape1-RFP as a marker for PAS. However, Ape1-RFP is found in two cytoplasmic dot structures in some cells in our and other studies (Suzuki *et al.*, 2007). Considering that there is only one PAS in the yeast cell (Xie & Klionsky, 2007) the second Ape1-RFP complex must be located in a non-PAS compartment. Since PAS is characterized by the presence of Atg proteins we assumed that sites where Atg2 or hAtg2A colocalized with Ape1-RFP is the PAS. mCherry-Atg8, another PAS marker, also localized in more that one dot in our experimental conditions, in cells expressing *GFP-ATG2* from *GPD1* promoter (not shown), rising the possibility that overproduction of GFP-Atg2, or ectopic production of GFP-hAtg2A, may somehow affect PAS formation or Ape1-RFP/mCherry-Atg8 recruitment to PAS.

Recycling between PAS and an Atg9-containing compartment adjacent to mitochondria has been observed for transmembrane domain-containing Atg9 protein which interacts with Atg2, and Atg2 is required for this recycling (Reggiori *et al.*, 2005; Mari & Reggiori, 2007; Mari *et al.*, 2010). We observed the Atg2-Atg9 interaction by using the two-hybrid system. However, we were unable to map the interaction site in Atg2 since both C- and N-terminal truncations abolished the binding (not shown). Considering our finding that hAtg2A did not interact with Atg9 in yeast two-hybrid system it is possible that hAtg2A, even recruited to PAS, cannot perform the Atg9-recycling function. This could be a possible reason for the lack of complementation of *atg2⊥* by *KIAA0404*.

To conclude, our results showed that human hAtg2A is recruited to the PAS in yeast, possibly by interaction of its N-terminal fragment with Atg18 protein. Thus, even though hAtg2A did not function in autophagy in yeast, it is probably involved in autophagy in mammalian cells, but further work will be required to find out if indeed hAtg2A has such a role in human cells.

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