

1 **Human hAtg2A protein expressed in yeast is recruited to preautophagosomal structure**
2 **but does not complement autophagy defects of *atg2* Δ strain**

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17

18 Abbreviations: aa, amino acid; GFP, green fluorescent protein; orf, open reading frame; PAS,
19 preautophagosomal structure; RFP, red fluorescent protein; SD, standard deviation

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1 **ABSTRACT**

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

18

19 **1. INTRODUCTION**

20

21 Cell growth and death are determined by the presence or absence of specific proteins.
22 Depending on the extracellular conditions, specific balance is maintained between protein
23 synthesis and degradation. Proteins are degraded by two major pathways: the ubiquitin-
24 proteosome pathway (Hershko & Ciechanover, 1998; Goldberg, 2003) and autophagy
25 (Klionsky & Ohsumi, 1999; Xie & Klionsky, 2007). During authophagy cytoplasmic

1 components are enwrapped into an autophagosome and delivered to the vacuole for
2 degradation. In yeast, at least 35 autophagy-related genes (*ATG*) have been found to be
3 essential for autophagy (Klionsky *et al.*, 2003; Kabeya *et al.*, 2007). Many Atg proteins,
4 including Atg2, localize to the pre-autophagosomal structure or phagophore assembly site
5 (PAS, (Xie & Klionsky, 2007)), the center of autophagosome formation (Suzuki & Ohsumi;
6 Suzuki *et al.*, 2001; Suzuki & Ohsumi, 2010). In the yeast *Saccharomyces cerevisiae*
7 autophagy overlaps the cytoplasm-to-vacuole targeting (Cvt) pathway which is responsible
8 for the biosynthetic delivery of aminopeptidase I (Ape1) from the cytoplasm to the vacuole
9 (Klionsky *et al.*, 1992; Lynch-Day & Klionsky, 2010). The Cvt pathway operates in growth
10 conditions and autophagy is induced by starvation. Many Atg proteins are common to both
11 the Cvt and autophagy pathways.

12 Atg2 is a peripheral membrane protein of 1592 amino acids (aa) and a predicted
13 molecular mass of 178 kDa (Shintani *et al.*, 2001; Wang *et al.*, 2001) which is localized in the
14 perivacuolar PAS and is also found in the cytosol. Efficient localization of Atg2 to the PAS is
15 dependent on a membrane protein, Atg9, which binds Atg2 (Suzuki *et al.*, 2001; Wang *et al.*,
16 2001; Suzuki *et al.*, 2007). Atg2 forms a complex with Atg18 (Obara *et al.*, 2008b) and
17 together they are required for Atg9 recycling between the PAS and non-PAS membranous
18 compartments (Reggiori *et al.*, 2004; Suzuki *et al.*, 2007). Moreover, Atg2 and Atg18 localize
19 to the PAS interdependently in autophagy-inducing conditions (Suzuki *et al.*, 2007). The
20 domain structure of Atg2 is unknown. A point mutation causing the G83E substitution results
21 in a severe defect in Atg2 function (Shintani *et al.*, 2001).

22 In higher eukaryotic cells autophagy is mechanistically identical to the process that
23 occurs in yeast (Reggiori & Klionsky, 2002). In mammalian cells autophagy has multiple
24 physiological functions: it is involved in cell death, tumor suppression, aging, age-associated
25 diseases and neurodegeneration (reviewed in (Bergamini *et al.*, 2004; Gozuacik & Kimchi,

1 2004; van der Vaart *et al.*, 2008)). Numerous genes for components of the human autophagic
2 machinery have been cloned thanks to their homology to yeast *ATG* genes and shown to
3 complement autophagy defects of respective yeast mutants (Reggiori & Klionsky, 2002). The
4 homology of yeast Atg2 and the human autophagy-related protein 2 homolog A encoded by
5 the *hATG2A/KIAA0404* gene and further referred to as hAtg2A was pointed out previously
6 (Shintani *et al.*, 2001). The *hATG2A/KIAA0404* gene further referred to as *KIAA0404* was
7 upregulated in the etoposide-induced apoptosis in HeLa cells and by doxorubicin, an inhibitor
8 of topoisomerase II (Kusama *et al.*, 2009). However, hAtg2A has not been characterized until
9 very recently when several hAtg2A-interacting proteins were identified (Behrends *et al.*,
10 2010).

11 Yeast is widely used as a model organism to study the function of human proteins and
12 better to understand many human diseases (reviewed in (Foury, 1997; Petranovic & Nielsen,
13 2008). Here we studied *in silico* the similarity of yeast Atg2 protein to proteins from other
14 organisms and investigated whether the human hAtg2A protein expressed in yeast could
15 function in autophagy. We also asked if the C-terminus of Atg2 is required for Atg2 function
16 and/or could be replaced by the C-terminus of hAtg2A, and, conversely, if hAtg2A with the
17 corresponding Atg2 part is functional in autophagy. Moreover, we studied the interaction of
18 Atg2 and hAtg2A with Atg9 and Atg18 by using the two-hybrid system.
19

20 **2. MATERIALS AND METHODS**

21
22 **2.1. Strains, media and growth conditions**
23 The *S. cerevisiae* strains used were TN125 MAT $\alpha ade2 ura3 leu2 his3 trp1 lys2$
24 *PHO8::pho8Δ60*, YTS24 *atg2::LEU2 PHO8::pho8Δ60* (isogenic to TN125)(Shintani *et al.*,
25 2001) and PJ69-4A MAT $\alpha trp1-901 leu2-3, 122 his3-200 ura3-52 gal4Δ gal80Δ$

1 *LYS2::GAL1-HIS3, GAL2-ADE2 met2::GAL7-lacZ* (James *et al.*, 1996). Yeast growth and
2 transformation followed standard procedures (Sherman, 2002). YPD (1% yeast extract, 1%
3 peptone, 2% glucose), SC-leu or SC-leu-his (containing 0.68% yeast nitrogen base without
4 amino acids, and 20 mg/l of adenine, uracil and required amino acids minus leucine and
5 histidine) with 2% glucose as a carbon source were used. For nitrogen starvation cells were
6 shifted to YB-N (0.17% yeast nitrogen base without ammonium sulphate and amino acids)
7 containing glucose as a carbon source. For two-hybrid interaction transformants were grown
8 on SC-leu-trp-his with addition of 5-10 mM 3-aminotriazole at 28°C for 3-7 days.

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

13 The ability of cells to form colonies was used to measure their survival under nitrogen
14 starvation conditions. Cells were grown to OD₆₀₀~1 in YPD or SC-leu, washed once and then
15 resuspended in YB-N to an OD₆₀₀~0.3. At the indicated times, OD₆₀₀ was measured, aliquots
16 were removed, serially diluted and plated on YPD plates in triplicate. Results shown are
17 representative of two independent experiments.

18

19 **2.2. Plasmids and plasmid construction**

20 The plasmids used in this study were: BS-KIAA0404 (Kazusa DNA Research Institute,
21 Japan), pUG36 (Niedenthal *et al.*, 1996), p415_{GPD1} [*LEU2* marker (Mumberg *et al.*, 1995)],
22 pAPE1-RFP (based on pRS313, kind gift of M. Thumm, University of Gottingen, Germany),
23 YEpl-ATG2 (Shintani *et al.*, 2001), pGBDUAtg9 (Reggiori *et al.*, 2005), PS-150 bearing the
24 *AD-ATG18* fusion (kind gift of D. J. Klionsky, University of Michigan, USA (Nair *et al.*,
25 2010)), pGEM (pGEM-TEasy, Promega), pGBT-9 (Clontech) and pACT2 (Clontech).

1 To express human *KIAA0404* gene in yeast, plasmid p415-GFP-KIAA0404 bearing
2 *GFP* fused with *KIAA0404* gene under the constitutive *GPD1* promoter was constructed. First
3 the XbaI fragment of pUG36 bearing the *GFP* gene was transferred to XbaI sites of p415_{GPD1}
4 plasmid to obtain p415-GFP. Then BamHI-EcoRV 6221 bp fragment of a BS-KIAA0404
5 derivative, in which a BamHI restriction site had been introduced between the MluI and NsiI
6 sites by PCR site-directed mutagenesis of pGEM-MluI-NsiI, was ligated with BamHI and
7 SmaI-digested p415-GFP. The primers used for *in vitro* site specific mutagenesis and other
8 PCR reactions are shown in Table S1. To obtain plasmid p415-GFP-KIAA0404-N expressing
9 fragment of hAtg2A protein containing amino acids 1-1561, plasmid p415-GFP-KIAA0404
10 was digested with Eco47III and religated.

11 Plasmid p415-GFP-ATG2 bearing *GFP* fused to the yeast *ATG2* gene was also
12 constructed for control. To this end SphI-SacII DNA fragment containing *ATG2* was
13 transferred from YEpl-ATG2 to pGEM and pGEM-ATG2 was used for two rounds of *in vitro*
14 PCR mutagenesis to insert XmaI and SalI restriction sites flanking *ATG2* open reading frame
15 (orf). Resulting pGEM-ATG2-XS was sequenced and was digested with XmaI, ends were
16 filled in with Klenow polymerase, and after SalI digestion the fragment bearing *ATG2* was
17 isolated and ligated with SmaI and SalI-digested p415-GFP. To obtain p415-GFP-ATG2-N
18 encoding Atg2 protein devoid of C-terminal domain (aa 1-1184), BlpI-StuI fragment of p415-
19 GFP-ATG2 was replaced with similar fragment in which SalI site had been introduced by *in*
20 *vitro* PCR mutagenesis and the 1235-bp SalI fragment was removed by SalI digestion and
21 religation.

22 To analyze the possible function of hAtg2A N-terminal and C-terminal fragments,
23 plasmids expressing appropriate gene fusions, p415-GFP-ATG2-N-KIAA0404-C and p415-
24 GFP- KIAA0404-N-ATG2-C, were constructed by fusion-PCR. Fragment BlpI-StuI of p415-
25 GFP-ATG2 was transferred to pGEM to obtain pGAF. pGAF was used as a template for PCR

1 to amplify *ATG2* fragment and to add 18 bp overhang of *KIAA0404* sequence. This fragment
2 together with PCR-amplified fragment of *KIAA0404* encoding C-terminal part of the hAtg2A
3 protein, with 18 bp overhang of *ATG2* sequence (Table S1), was used as a template for fusion
4 PCR. Product of fusion PCR was digested with BlpI and SalI and was used to replace BlpI-
5 SalI fragment of p415-GFP-ATG2 and obtain p415-GFP-ATG2-N-KIAA0404-C which
6 encodes a fusion of N-terminal part of Atg2 (aa 1-1184) with C-terminal part of hAtg2A (aa
7 1540-1938). Plasmid p415-GFP-KIAA0404-N-ATG2-C encoding fusion protein of N-
8 terminal part of hAtg2A (aa 1-1539) fused with C-terminal part of Atg2 (aa 1185-1592) was
9 obtained by replacing SacII-SalI fragment of p415-GFP- KIAA0404 with SacII and SalI-
10 digested product of fusion PCR. In this fusion-PCR two PCR products were used as
11 templates: one DNA product of 1277 bp amplified by using p415-GFP-ATG2 as a template
12 and *ATG2*-specific oligonucleotides, one with 18-nucleotide *KIAA0404* overhang added, and
13 the other DNA product of 1238 bp amplified by using p415-GFP-KIAA0404 as a template
14 and *KIAA0404*-specific oligonucleotides, one with 18-nucleotide *ATG2* overhang added.

15 To analyze the physical interaction of proteins by two-hybrid system, plasmids
16 expressing fusions of *GAL4* domains with full lenght *ATG2*, *ATG9* or *KIAA0404* were
17 constructed. To obtain pGBT9-ATG2, pGEM-ATG2-XS (see above) was digested with XmaI
18 and SalI, and 4794 bp fragment was transferred to pGBT9. To obtain pGBT9-KIAA0404,
19 7.2-kb EcoRI-EcoRV fragment was transferred from the KS-KIAA0404-EcoRI plasmid (BS-
20 KIAA0404 derivative), in which an EcoRI restriction site had been introduced by PCR before
21 start codon, to pGBT9 EcoRI and SmaI restriction sites. Similarly, to construct pGBT9-
22 KIAA0404-N the Eco47III fragment of plasmid KS-KIAA0404-EcoRI was deleted and than
23 EcoRI-EcoRV fragment was transferred to pGBT9. To obtain pACT2-ATG9, the ATG9 orf
24 was amplified by PCR by using primers containing NcoI and BamHI restriction sites, and

1 pGDUAtg9 as a template, and was cloned to pACT2 NcoI and BamHI restriction sites.

2 pACT2-ATG9 sequence was confirmed.

3

4 **2.3. Total protein extracts and Western blot analysis**

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

10

11 **2.4. Fluorescence microscopy**

12 For GFP fluorescence yeast were grown to the logarithmic phase in SD-leu-his medium at
13 30°C. After that cells were shifted to YB-N starvation medium for 4 hours. At least 100 cells
14 of each strain were viewed with an Eclipse (Nikon) fluorescence microscope equipped with
15 an ORCA (Nikon) camera. Images were collected using Lucia General 5.1 software
16 (Laboratory Imaging Ltd.).

17

18 **2.5. Monitoring of autophagy by Pho8Δ60 alkaline phosphatase test**

19 For measurements of autophagic activity, the alkaline phosphatase test was performed as
20 described (Noda *et al.*, 1995). Five independent experiments were performed and means ±SD
21 (standard deviation) are shown.

22

23 **2.6. Bioinformatic analyses**

24 The non-redundant database at NCBI was scanned for sequences similar to the Atg2 protein
25 using the PSI-Blast program (Altschul *et al.*, 1997). Fifty fungal Atg2 orthologues were

1 aligned using the MAFFT program (Katoh *et al.*, 2002) and manually inspected for
2 differences. Among human proteins, Atg2 homologues were found using the PSI-Blast
3 program (Altschul *et al.*, 1997) with the first hits detected in the first iteration with e-values of
4 5e-11 and 4e-15 for hAtg2A and Atg2B, respectively. Multiple sequence alignment of yeast
5 Atg2 with the *Aspergillus nidulans* AnAtg2 and human hAtg2A homologues was done using
6 MAFFT with 1000 iterations and then formatted in BioEdit (Hall, 1999).

7

8 **3. RESULTS**

9

10 **3.1 Homologues of yeast Atg2 protein**

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

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

1 72.4. using BLAST) and the corresponding region of the Atg2B protein (aa 1644-2078)
2 encoded by the *C14orf103* gene (Fig. 1 and not shown).

3

4 **3.2. *KIAA0404* gene can be expressed in yeast**

5 The data about the cellular function of the hAtg2A protein encoded by the *KIAA0404* gene
6 was lacking, thus we used yeast to express the *KIAA0404* gene and to study its potential
7 function in autophagy. Plasmid p415-GFP-KIAA0404 encoding hAtg2A protein N-terminally
8 fused to GFP was constructed. The fusion gene was expressed from a strong constitutive
9 *GPD1* promoter. A plasmid encoding yeast Atg2 fused to GFP was also constructed to serve
10 as a control. *atg2Δ* yeast cells were transformed with p415-GFP-KIAA0404 and control
11 plasmids (p415-GFP, p415-GFP-ATG2), grown in medium containing glucose, shifted or not
12 to nitrogen starvation medium for 4 h, and protein extracts were prepared and analyzed by
13 Western blotting using anti-GFP antibody. As expected, when *GFP-ATG2* was expressed an
14 abundant protein product of about 220 kDa was observed. When the *GFP-KIAA0404* gene
15 was expressed the GFP-hAtg2A fusion protein of about 315 kDa was present but its level was
16 low and two smaller proteins of 130 kDa and about 30 kDa were observed, probably
17 representing partial degradation products, the smaller one resembling GFP (Fig. 2(a) and data
18 not shown). The *atg2Δ* transformants expressing *GFP-ATG2* or *GFP-KIAA0404* shifted to
19 nitrogen starvation medium showed protein products of similar size as in growth conditions,
20 but less abundant (not shown).

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

1

2 **3.3. hAtg2A is localized in a few cytoplasmic dots and one of these dots colocalizes with**
3 **Ape1-RFP, so it is recruited to the PAS**

4 Atg2 localizes in yeast cells to one or two punctate cytoplasmic structures in growth
5 conditions and to one such structure adjacent to a vacuole called PAS in starvation condition
6 (Shintani *et al.*, 2001; Suzuki *et al.*, 2007). PAS is the site of recruitment of membranes and
7 cargo proteins, such as Ape1, to the growing autophagosome. So, Ape1 fused to a fluorescent
8 tag such as red fluorescent protein (Ape1-RFP) can be used as a marker for PAS in growing
9 cells (Kawamata *et al.*, 2005; Suzuki *et al.*, 2007). Ape1-RFP is localized to the vacuole in
10 starved cells. Similarly as published, in our experiments in *atg2Δ* cells in growth conditions
11 we observed GFP-Atg2 in one to three cytoplasmic dots, one of which colocalized with Ape1-
12 RFP, the PAS marker. Also slight diffused cytoplasmic staining was observed probably due to
13 overproduction of the protein (Fig. 2(b)). In starvation conditions GFP-Atg2 was cytoplasmic
14 in most cells and some cells showed also a single dot while Ape1-RFP was vacuolar and in
15 some cells also localized to the PAS (Fig. 2(b)).

16 Cellular localization of the human GFP-hAtg2A protein was observed in *atg2Δ* cells,
17 and was compared with the localization of Ape1-RFP, GFP-Atg2 and of GFP alone. In
18 growth conditions GFP was diffused in the cytoplasm while the GFP-hAtg2A protein was
19 localized to 1-3 punctate structures (Fig. 2(b)). One of them colocalized with the Ape1-RFP
20 punctum in about 60% of *atg2Δ* cells in which both GFP and RFP signals were visible,
21 suggesting that GFP-hAtg2A could be recruited to the PAS. In control experiments
22 employing *atg1Δ* cells in which PAS does not form we did not find colocalization of GFP-
23 Atg2A and Ape1-RFP, which supports this conclusion (not shown). The identity of the
24 punctate structures other than PAS containing GFP-Atg2A is unknown. In starvation
25 conditions GFP-hAtg2A was cytoplasmic in 95% of cells and 5% of cells showed it in one

1 punctate structure (Fig. 2(b)). The cytoplasmic staining may also derive from the partial
2 degradation product, GFP. In these cells Ape1-RFP was found in the PAS, not in the vacuole,
3 indicating a lack of complementation of the autophagy defects of *atg2Δ* by *GFP-KIAA0404*.

4

5 **3.4. N-terminal fragments of Atg2 and hAtg2A are sufficient for PAS recruitment but do**
6 ***not support Cvt transport of Ape1-RFP to the vacuole***

7

8 Since hAtg2A protein, despite being partially recruited to the PAS, did not complement the
9 Cvt defect of *atg2Δ* cells, in which it differed from the yeast Atg2, several questions arose.
10 Which part of Atg2 is required for PAS recruitment and for functioning in the Cvt pathway?
11 Is the function of the C-terminus of Atg2 conserved? Can the C-terminal part of Atg2 be
12 replaced with a homologous fragment of hAtg2A? To answer these questions several
13 plasmids were constructed and the cellular level and localization of protein products encoded
14 was analyzed. These were plasmids expressing: *GFP-ATG2-N* (aa 1-1184) devoid of a
15 fragment encoding the C-terminus of Atg2, *GFP-KIAA0404-N* (aa 1-1561) devoid of a
16 fragment encoding the C-terminus of hAtg2A, *GFP-ATG2-N-KIAA0404-C* fusion in which
17 the fragment encoding the C-terminus of Atg2 (aa 1185-1592) was replaced by a fragment
18 encoding the C-terminus of hAtg2A (aa 1540-1938), and *GFP-KIAA0404-N-ATG2-C*
19 encoding a fusion protein of the N-terminal part of hAtg2A (aa 1-1539) fused to the C-
20 terminal part of Atg2 (aa 1185-1592) (Fig. 3). The site of fusion of the Atg2 and hAtg2A
21 proteins was chosen between amino acids KR which are identical in both proteins and are
22 located in the first most conserved block of amino acids (see Fig. 1). Expression of these
23 fusion genes in *atg2Δ* strain was analyzed by Western blotting and compared with the
24 expression level of *GFP-ATG2* and *GFP-KIAA0404* in growth and starvation conditions (Fig.
25 4 and not shown). The expression of *GFP-ATG2-N* was very efficient and comparable to that

1 of *GFP-ATG2*, and Atg2-N protein of expected size of about 170 kDa was observed. The
2 expression of *GFP-KIAA0404-N* was less efficient and hAtg2A-N migrated faster than
3 expected, of about 130 kDa instead of 220 kDa. This size was similar to the major band of
4 GFP-hAtg2A representing partial degradation product, indicating that the C-terminal part of
5 hAtg2A could be unstable in yeast. The apparent molecular weight of the protein product of
6 *GFP-ATG2-N-KIAA0404-C* was about 180 kDa and was lower than expected (220 kDa),
7 again indicating an instability of the C-terminus of hAtg2A. The full length product of *GFP-*
8 *KIAA0404-N-ATG2-C* showed the expected size of about 260 kDa but a partial degradation
9 product of about 130 kDa was also observed. We cannot exclude the possibility that some of
10 these proteins migrate aberrantly and in fact are not even partially degraded. After a shift to
11 starvation conditions all these proteins were present but much less abundant (not shown).

12 The localization of the N-terminal parts of the proteins, Atg2-N and hAtg2A-N,
13 expressed from plasmids described above was observed in *atg2Δ* strain in growth and
14 nitrogen starvation conditions and compared with the localization of GFP-Atg2, GFP-hAtg2A
15 and Ape1-RFP. GFP-Atg2-N was present in 1-3 dots and slightly diffused in the cytoplasm,
16 similarly as GFP-Atg2. One of these dots colocalized with Ape1-RFP in about 50% of cells
17 indicating that the N-terminal fragment of Atg2 is sufficient for PAS recruitment.
18 Interestingly, about 20% of cells showed more than one Ape1-RFP dot indicating that more
19 than one PAS could be present in some cells or Ape2-RFP could be located also in other
20 punctate structure in this condition (Fig. 5). Observation of GFP-Atg2-N in starvation
21 conditions showed cytoplasmic localization or in 1-5 dots, and Ape1-RFP was localized in the
22 PAS. On the other hand, GFP-hAtg2A-N was localized in one or two dots and slightly
23 diffused in the cytoplasm and colocalized with Ape1-RFP in 60% of cells indicating that the
24 N-terminal part of hAtg2A is also sufficient for PAS recruitment. Ape1-RFP was localized in
25 two dots in some of these cells.

1 Fusion proteins GFP-Atg2-N-hAtg2A-C and GFP-hAtg2A-N-Atg2-C were localized in
2 few dots or one, respectively, and slightly diffused in the cytoplasm in *atg2Δ* cells in growth
3 conditions (Fig. 5). One of these dots colocalized with Ape1-RFP, and the recruitment to PAS
4 was more efficient for the hAtg2A-N-Atg2-C fusion. In starvation conditions GFP-Atg2-N-
5 hAtg2A-C was found in few cytoplasmic dots while GFP-hAtg2A-N-Atg2-C was in the dots
6 and the cytoplasm. Ape1-RFP localized exclusively to the PAS, not to the vacuole, in cells
7 expressing both fusion proteins showing a lack of complementation of Cvt defects of *atg2Δ*
8 (Fig. 5).

9

10 **3.5. Intact KIAA0404 and fusions containing fragments of KIAA0404 do not complement**
11 **defects of atg2Δ cells in survival upon starvation, preApe1 maturation and nonselective**
12 **autophagy**

13 The involvement of hAtg2A protein in yeast authophagy could be assessed by
14 investigating complementation of individual phenotypes of *atg2Δ* cells by *GFP-KIAA0404* or
15 its derivatives. The *atg2Δ* cells do not survive for longer than five days under nitrogen
16 starvation conditions when glucose is present in the medium, they show a block in preApe1
17 maturation and a defect in nonselective autophagy measured by Pho8Δ60 test (Shintani et al.,
18 2001; Wang et al., 2001). First we analyzed the complementation of the *atg2Δ* viability
19 defects by *GFP-KIAA0404*, its N-terminal fragment *GFP-KIAA-0404-N*, and the fusions
20 *GFP-KIAA0404-N-ATG2-C* and *GFP-ATG2-N-KIAA0404-C* relative to the complementation
21 by *GFP-ATG2* and *GFP-ATG2-N*. The respective transformants were grown in SC-leu,
22 transferred to nitrogen starvation medium for seven days and tested for viability by scoring
23 colony forming units after each day. The wild type control strain remained viable throughout
24 the experiment while the *atg2Δ* cells bearing the empty plasmid died after six days, in good
25 agreement with published data. Plasmids expressing *GFP-ATG2* fully complemented the

1 $atg2\Delta$ defect, cells remained viable, similarly as wild type strain, after 7 days of starvation.
2 Survival of transformants bearing plasmids expressing *GFP-KIAA0404*, *GFP-KIAA0404-N* or
3 the fusion genes declined during starvation and was similar to the $atg2\Delta$ control cells after 7
4 days of starvation (Fig. 6(a)). This result indicates that expressed *KIAA0404* gene, its 5'
5 fragment or fusions do not complement the $atg2\Delta$ defects in survival. Surprisingly, *GFP-*
6 *ATG2-N* was very toxic to $atg2\Delta$ cells, they did not survive even the first day of starvation.
7 However, the *KIAA0404* fragment encoding homologous C-terminal part of hAtg2A fused
8 with *GFP-ATG2-N* (resulting in *GFP-ATG2-N-KIAA0404-C*) prevented this toxicity,
9 similarly as the respective native fragment of *ATG2*. Although we do not observe full length
10 fusion protein expressed from *GFP-ATG2-N-KIAA0404-C* in our Western blot analysis (Fig.
11 4), this protein may be present and function in the cells and be partially degraded during
12 protein extraction.

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

1 preApe1 maturation was not restored in *atg2Δ* cells expressing *GFP-ATG2-N*, showing that
2 the Atg2-N fragment is not sufficient for function.

3 We also tested Pho8Δ60 activity, a measure of non-selective autophagy, in the set of
4 transformants described above. Wild type cells transformed with empty vector responded to
5 nitrogen starvation by increasing the activity of Pho8Δ60 more than 2-fold while in *atg2Δ*
6 cells this activity was similar as in growth conditions (Fig. 6(c)), in agreement with previously
7 published data (Shintani *et al.*, 2001). Expression of *GFP-ATG2* complemented this
8 phenotype, the Pho8Δ60 activity was significantly increased upon starvation. This
9 complementation was lost when *GFP-ATG2-N* was expressed, indicating that the N-terminal
10 fragment of Atg2 is not sufficient to function in nonselective autophagy. Complementation
11 was also not observed in *atg2Δ* cells expressing *GFP-KIAA0404*, its fragment *GFP-*
12 *KIAA0404-N* or fusions with fragments of *ATG2*, showing that hAtg2A does not function in
13 nonselective autophagy in yeast.

14

15 **3.6. hAtg2A interacts with Atg18 but does not interact with Atg9 in two-hybrid system**

16 Atg2 was shown to interact with the membrane protein Atg9 (Wang *et al.*, 2001) and lipid
17 binding protein Atg18 (Obara *et al.*, 2008b; Nair *et al.*, 2010) and to be required for Atg9
18 cycling between PAS and non-PAS peripheral compartment (Reggiori *et al.*, 2004).
19 Therefore, we tested if these interactions of Atg2 could be reproduced in yeast two-hybrid
20 system and if hAtg2A also interacted with the same proteins. Transformation of a tester strain
21 with plasmids bearing gene fusions of *GAL4_{AD}* with *ATG9* or *ATG18* and plasmids bearing
22 *GAL4_{BD}* fused with *ATG2* or *KIAA0404* followed by the analysis of transformants for growth
23 on SC-his plates showed that Atg2 interacted with both Atg9 and Atg18 in this system (Fig.
24 7(a)). hAtg2A did not interact with Atg9 but an interaction with Atg18 was observed (Fig.
25 7(a)). The interaction of hAtg2A with Atg18 suggests that Atg18 is probably one of the

1 proteins mediating recruitment of hAtg2A to PAS. Consistent with the fluorescence
2 microscopy studies showing hAtg2A-N recruitment to the PAS, the N-terminal fragment of
3 hAtg2A was also able to interact with Atg18 in two-hybrid system (Fig. 7(a)).

4 To analyze the involvement of Atg18 in hAtg2A recruitment to PAS we observed the
5 cellular localization of GFP-hAtg2A in the *atg18Δ* strain in growth and nitrogen starvation
6 conditions and compared it with localization of Atg2. We found that in growth conditions
7 Atg2 was present in one or two cytoplasmic dots and colocalized with Ape1-RFP1 in most
8 cells. In some cells Ape1-RFP was found in two dots, as before. In contrast, hAtg2A was
9 found in one to five dots, but none of those dots colocalized with Ape1-RFP. Upon nitrogen
10 starvation Atg2 and hAtg2A diffused in the cytoplasm while Ape1-RFP was in PAS and not
11 in the vacuole (Fig. 7(b)). These results indicate that *ATG18* is important for PAS localization
12 of hAtg2A in both growth and starvation conditions. It was previously reported that *ATG18* is
13 required for PAS recruitment of Atg2 in autophagy-inducing conditions (rapamycin
14 treatment)(Suzuki *et al.*, 2007) and our results are consistent with those findings. We also
15 indicated that some factors may help to recruit Atg2 to Ape1-RFP-containing dots in growing
16 *atg18Δ* cells.

17

18 **4. DISCUSSION**

19

20 The function of human hAtg2A protein encoded by *KIAA0404* has not been reported
21 to date. When this work was in progress several proteins interacting with hAtg2A were
22 identified, including some proteins involved in autophagy in humans (Behrends *et al.*, 2010).
23 Here we studied the homology of Atg2 and hAtg2A and used yeast as a model organism to
24 test the possible involvement of hAtg2A in autophagy. We showed that *GFP-KIAA0404*
25 could be expressed in yeast and its protein product, GFP-hAtg2A, was recruited to the PAS.

1 This recruitment is probably mediated by Atg18 which interacted with hAtg2A in the two
2 hybrid system. Human hAtg2A did not support growth of *atg2Δ* cells in starvation conditions
3 and could not substitute for the yeast Atg2 protein in the Cvt pathway or non-selective
4 autophagy. Moreover, we showed that the N-terminal fragments of Atg2 or of hAtg2A were
5 sufficient for the recruitment to the PAS but they did not function in autophagy. Also, the C-
6 terminal fragment of Atg2 could not be functionally substituted for homologous C-terminal
7 fragment of hAtg2A and vice versa. Surprisingly, we also found that Atg2-N was toxic to
8 yeast cells in starvation conditions and this toxicity was prevented by fusion with the hAtg2A
9 C-terminus.

10 Human hAtg2A and yeast Atg2 show only limited homology, restricted to the C-
11 terminal domain. However, the hAtg2A protein did localize to cytoplasmic punctate
12 structures, some of which colocalized with Ape1-RFP-marked PAS, similarly as did yeast
13 Atg2. One of the proteins mediating PAS recruitment of hAtg2A could be Atg18 since these
14 proteins interacted in the two-hybrid assay in our experiments and deletion of *ATG18*
15 abolished PAS localization of hAtg2A. Atg18 binds Atg2 independently of its
16 phosphatidylinositol-3-phosphate (PI3P)-binding ability (Obara *et al.*, 2008b), and this
17 complex is recruited to PAS by PI3P, in which PAS is enriched (Obara *et al.*, 2008a) and by
18 other factors (Suzuki *et al.*, 2007). One such factor is Atg9 (Wang *et al.*, 2001). However, in
19 contrast to Atg2, hAtg2A did not bind Atg9 in two-hybrid system suggesting that other
20 proteins must also contribute to the hAtg2A recruitment to PAS. Very recently, studies of
21 proteomic network organization of the human autophagy system have identified nine proteins
22 interacting with hAtg2A (Behrends *et al.*, 2010). Among them are Atg2B, and WIPI1 and
23 WDR45, two WD40 domain-containing proteins homologous to Atg18. This supports our
24 finding and indicates that the Atg2-Atg18 interaction is conserved in evolution from yeast to
25 humans. Moreover, hAtg2A was found among interacting proteins when any of the Atg8

1 human homologues (GABARAP, GABARAPL1, GABARAPL2, MAP1LC3B, MAP1LC3B)
2 was used as a bait in these proteomic studies (Behrends *et al.*, 2010). This suggests that Atg8
3 may contribute to the PAS recruitment of hAtg2A in yeast. However, PAS assembly studies
4 showed that Atg8 is not required for Atg2 PAS localization (Suzuki *et al.*, 2007) and rather
5 the Atg18-Atg2 complex facilitates PAS recruitment of lipidated Atg8 (Nair *et al.*, 2010), not
6 the opposite. We therefore hypothesize that Atg18 could be the only protein required for the
7 PAS localization of hAtg2A.

8 The N-terminal part of hAtg2A, also similarly as the N-terminal part of Atg2, was
9 sufficient for the punctate structure-localization and PAS recruitment, indicating that this
10 sequence, although not showing homology, has retained its PAS-addressing function.
11 However, this PAS localization was not sufficient to support hAtg2A, hAtg2A-N or Atg2-N
12 function in selective and nonselective autophagy. Therefore, the C-terminal domain of Atg2
13 must be important for the function in autophagy but not necessary for Atg2 localization. The
14 C-terminal domain of hAtg2A seems rather unstable in yeast cells since it was removed in
15 some pool of hAtg2A and a shorter form, similar in size to hAtg2A-N, predominated on
16 Western blots. This could be one of the reasons of the lack of complementation of *atg2Δ*
17 defects by *GFP-KIAA0404*. On the other hand, the amount of full length hAtg2A could be
18 nominally sufficient to function but the C-terminal domain of hAtg2A could lack some
19 important protein interactions. However, the presence of hAtg2A-C in fusion with Atg2-N
20 prevented its toxicity and kept Atg2-N-hAtg2A-C in the punctate structures in starvation
21 conditions. We tested if replacing the C-terminal domain of hAtg2A affected the function.
22 Although the fusion protein hAtg2A-N-Atg2-C was more stable than hAtg2A and was
23 recruited to the PAS, it still was not functional. So, the C-terminal Atg2 domain can properly
24 function only when attached to the N-terminus of Atg2. Similarly, the C-terminal domain of
25 hAtg2A can not functionally substitute for the C-terminal domain of Atg2 even though these

1 domains can regulate each other's function. Some human proteins, GAS41 for example, are
2 unable to complement phenotypes of the loss of a yeast homologue but can provide a
3 conserved domain which is able to functionally substitute for the yeast domain (Wang *et al.*,
4 2009). In our case only the combination of the Atg2-N and Atg2-C domains provides the
5 essential functions necessary for autophagy and cell survival upon starvation.

6 Our results on the survival of *atg2Δ* expressing *GFP-ATG2-N* pointed to previously
7 unreported effects of this overproduced Atg2 fragment on cells upon starvation. These cells
8 did not survive even one day of starvation, they also grew slower in growth conditions in
9 liquid medium compared to empty plasmid-bearing cells (not shown). The mechanism of this
10 toxicity is unknown at present but is an interesting subject for further investigation.

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

21 Recycling between PAS and an Atg9-containing compartment adjacent to
22 mitochondria has been observed for transmembrane domain-containing Atg9 protein which
23 interacts with Atg2, and Atg2 is required for this recycling (Reggiori *et al.*, 2005; Mari &
24 Reggiori, 2007; Mari *et al.*, 2010). We observed the Atg2-Atg9 interaction by using the two-
25 hybrid system. However, we were unable to map the interaction site in Atg2 since both C- and

1 N-terminal truncations abolished the binding (not shown). Considering our finding that
2 hAtg2A did not interact with Atg9 in yeast two-hybrid system it is possible that hAtg2A, even
3 recruited to PAS, cannot perform the Atg9-recycling function. This could be a possible reason
4 for the lack of complementation of *atg2Δ* by *KIAA0404*.

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

10

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15

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30

31 LEGENDS TO FIGURES

32

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

38

39 **Fig. 2** Expression of *KIAA0404* in yeast.

1 (a) Western blot analysis. *atg2Δ* strain (YPJ1) was transformed with plasmids expressing
2 *GFP*, *GFP-ATG2* or *GFP-KIAA0404* genes and grown in SD-leu medium to OD₆₀₀ of 0.4-0.6.
3 Extracts were prepared and Western blot analysis was performed using anti-GFP antibody.
4 (b) Localization of human hAtg2A in yeast cells. *atg2Δ* strain (YPJ1) was transformed with
5 plasmids expressing *GFP*, *GFP-ATG2* or *GFP-KIAA0404* and with plasmid expressing
6 *APE1-RFP*. Transformants were grown in SC-leu-his medium to OD₆₀₀ of 0.4-0.6 and were
7 shifted to nitrogen starvation medium for 4 hours. Cells were viewed by epifluorescence or by
8 DIC optics. Arrows indicate punctate structures where GFP-hAtg2A and Ape1-RFP
9 colocalize.

10

11 **Fig. 3** Schematic representation of Atg2 and hAtg2A domain structure and their constructs
12 expressed with N-terminally fused GFP.

13 Fragments and domains are as in Fig.1.

14

15 **Fig. 4** Cellular levels of GFP-Atg2-N, GFP-hAtg2A-N, and fusions of Atg2 with hAtg2A.
16 *atg2Δ* strain was transformed with plasmids expressing *GFP-ATG2-N*, *GFP-KIAA0404-N*,
17 *GFP-ATG2-N-KIAA0404-C* or *GFP-KIAA0404-N-ATG2-C* and grown in SD-leu medium to
18 OD₆₀₀ ~1. Extracts were prepared and Western blot analysis was performed using anti-GFP
19 antibody. Extracts from cells expressing *GFP-ATG2* or *GFP-KIAA0404* were used as a
20 control.

21

22 **Fig. 5** Localization of GFP-Atg2-N and GFP-hAtg2A-N fragments, and GFP-Atg2-N-
23 hAtg2A-C and GFP-hAtg2A-N-Atg2-C fusions.

24 *atg2Δ* strain was transformed with plasmids expressing *GFP*, *GFP-ATG2-N*, *GFP-*
25 *KIAA0404-N*, *GFP-ATG2-N-KIAA0404-C* or *GFP-KIAA0404-N-ATG2-C* and with plasmid

1 expressing *APE1-RFP*. Transformants were grown in SC-leu-his medium to OD₆₀₀ of 0.4-0.6
2 and were shifted to nitrogen starvation medium for 4 hours. Cells were viewed by
3 epifluorescence or by DIC optics. Arrows indicate punctate structures where GFP and RFP
4 signal colocalize. Scale bar, 10 μm.

5

6 **Fig. 6** Lack of complementation of *atg2Δ* autophagy defects by *GFP-ATG2-N*, *GFP-*
7 *KIAA0404*, *GFP-KIAA0404-N* and fusions of *KIAA0404* with *ATG2*.

8 **(a)** Survival of *atg2Δ* (YPJ1) trasformants during starvation. Wild type strain transformed
9 with empty vector for control and *atg2Δ* cells transformed with plasmids expressing *GFP*,
10 *GFP-ATG2*, *GFP-ATG2-N*, *GFP-KIAA0404* , *GFP-KIAA0404-N*, *GFP-ATG2-N-KIAA0404-*
11 *C* or *GFP-KIAA0404-N-ATG2-C* were grown in SC-leu, shifted to YB-N, and tested for
12 viability after 1 to 7 days of incubation. Colony forming units (CFU) were scored.

13 **(b)** Complementation of *atg2Δ* (YPJ1) defect of Cvt pathway. Same set of transformants as in
14 A was grown in SD-leu, shifted to YB-N for 4 hours, then extracts were prepared and
15 analyzed by Western blotting using anti-Ape1 antibody.

16 **(c)** Complementation of *atg2Δ* (YPJ1) defect of nonselective autophagy. Wild type cells
17 transformed with empty vector and *atg2Δ* cells expressing *GFP*, *GFP-ATG2*, *GFP-ATG2-N*,
18 *GFP-KIAA0404* , *GFP-KIAA0404-N*, *GFP-ATG2-N-KIAA0404-C* or *GFP-KIAA0404-N-*
19 *ATG2-C* were grown in SD-leu, shifted or not to YB-N for 4 hours, then extracts were
20 prepared and Pho8Δ60 test was performed.

21

22 **Fig. 7** hAtg2A interacts with Atg18 and requires Atg18 for recruitment to the PAS.

23 **(a)** Interaction of Atg2 and hAtg2A with Atg9 and Atg18. Two-hybrid tester strain (PJ69-
24 4A) was co-transformed with plasmids expressing activating domain (AD)-fused Atg9 or
25 Atg18 and Atg2, hAtg2A or hAtg2A-N fused with the DNA binding domain (BD) or with

1 empty vectors (-). Interactions were monitored by the ability of cells to grow on plates lacking
2 histidine and containing 5-10 mM 3-aminotriazol (SC-his).

3 **(b)** Localization of Atg2 and hAtg2A in *atg18Δ* strain.

4 *atg18Δ* strain was transformed with plasmid expressing *GFP-ATG2* or *GFP-KIAA0404* and
5 with plasmid bearing *APE1-RFP*. Transformants were grown in SC-leu-his medium to OD₆₀₀
6 of 0.4-0.6 and were shifted to nitrogen starvation medium for 4 hours. Cells were viewed by
7 epifluorescence or by DIC optics. Arrows indicate punctate structures where GFP and RFP
8 signals colocalize.

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