

## Characterization of the Vacuolar Transporters for Amino Acid Recycling in Yeast Autophagy

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**Abstract**—Autophagy is induced in response to nitrogen starvation and delivers bulk of cytosolic proteins to the vacuole to degrade them. Amino acids generated in vacuoles by autophagy are recycled for survival in nitrogen-starved condition. Recently, various physiological roles of autophagy have been revealed. Studies on the marine mussel, *Mytilus edulis*, demonstrated that autophagy facilitates the recovery of digestive gland from environmentally-induced oxidative stress. It is inferred that autophagy removes oxidatively and pollutant damaged proteins. We here examined the genes for the amino acid recycling process in autophagy. In yeast *Saccharomyces cerevisiae*, three transporters have been demonstrated to involve in export of amino acids from vacuoles. Both Avt3 and Avt4 export neutral amino acids and Avt6 exports acidic amino acids. We found that the deletion of *AVT6* gene resulted in the loss of survivability and the increase of vacuolar glutamate concentration especially in response to nitrogen starvation. In addition, the cellular amount of Avt6-Myc<sup>9</sup> protein increased upon nitrogen starvation. These suggest that Avt6 functions in the recycling process of amino acids in autophagy. In addition, we examined the expression and subcellular localization of Avt5 and Avt7, which are closely related to Avt6, and found the distinct characteristics of these proteins.

**Keywords:** vacuole, amino acid, vacuolar amino acid transporter, autophagy, nitrogen starvation

### INTRODUCTION

Maintenance of the cytosolic amino acid concentration at a constant level is important for effective protein synthesis in all living organisms. The regulatory mechanism for the accumulation of amino acids from the external environment and biosynthesis has been extensively studied so far (Forsberg and Ljungdahl, 2001; Bernard and André, 2001; Magasanik and Kaiser, 2002; Hinnebusch, 2005). In addition to these sources, however, the significance of vacuolar compartmentalization of amino acids should be concerned. In budding yeast

*Saccharomyces cerevisiae*, vacuoles highly accumulate amino acids, especially basic amino acids. For example, more than 90% of arginine is compartmentalized in the vacuoles (Kitamoto *et al.*, 1988). In contrast, glutamate and aspartate are exclusively localized in the cytosol (Wiemken and Dürr, 1974). These observations imply that the vacuoles are a physiologically active compartment for certain amino acids and are potentially concerned with cellular amino acid metabolism.

The importance of vacuolar function in the amino acid metabolism was revealed by the studies of nitrogen-starved *S. cerevisiae* cells. Arginine accumulated in the vacuoles is utilized as a nitrogen source during nitrogen starvation (Kitamoto *et al.*, 1988). In addition, recent studies of autophagy revealed the role of vacuole as a supplier of bulk amino acids under nitrogen starvation (Onodera and Ohsumi, 2005). Autophagy is a conserved defensive response among eukaryotes to maintain cell survivability against nitrogen starvation. In this process, the formation of double membrane vesicles, called autophagosomes (AP), is induced in response to nitrogen starvation (Baba *et al.*, 1994). During AP formation, cytoplasmic proteins are nonselectively sequestered into the vesicles. After completion of AP formation, the outer membrane of AP fuses to the vacuolar membrane and the inner single membrane structure, called autophagic body (AB), is released into the vacuolar lumen (Takeshige *et al.*, 1992; Baba *et al.*, 1994). Subsequently, AB is degraded by vacuolar resident hydrolases. Amino acids generated by the degradation are recycled for the synthesis of proteins. The list of autophagy function is growing fast by extensive studies using mammalian system. In addition to providing the tolerance against starvation, autophagy is involved in various events, such as degradation of invading bacteria, programmed cell death, tumor suppression, and development (Mizushima, 2005). Low but substantial autophagic activity has also been demonstrated to be required for the intracellular clearance of abnormal proteins (Komatsu *et al.*, 2005; Hara *et al.*, 2006). Recently, it has been suggested that augmented autophagic removal of oxidatively damaged organelles and proteins provides a second tier of defense against oxidative stress (Moore, 2008). Lysosomal and autophagic reactions to pollutant stress are widely used as indicators of cell injury in bioindicator species, such as molluscs, earthworms and fish, for monitoring toxic impact of environmental contamination on the health of aquatic and terrestrial ecosystems (Köhler *et al.*, 1992; Cajaraville *et al.*, 1995, 2000; Marigómez and Baybay-Villacorta, 2003; Svendsen *et al.*, 2004; Moore *et al.*, 2007).

Amino acids generated by autophagy are exported to the cytosol across the vacuolar membrane to be recycled for protein synthesis. Since yeast mutants defective in AP formation extensively reduce the survivability under nitrogen starvation (Tsukada and Ohsumi, 1993), the amino acid recycle is thought to be an essential process for the maintenance of survivability under nitrogen starvation. For the amino acid recycle process, transporter proteins to export amino acids from vacuoles must work. Recent studies using *S. cerevisiae* successfully identified the vacuolar amino acid transporters (Rusnak *et al.*, 2001; Shimazu *et al.*, 2005) and thus open the way to assess the physiological significance of amino acid

Table 1. Yeast strains used in this study.

| Strain  | Genotype                                    | Reference                    |
|---------|---|------------------------------|
| BY4741  | <i>MATa leu2Δ0, his3Δ1, met15Δ0, ura3Δ0</i> | Shimazu <i>et al.</i> (2005) |
| STY3134 | BY4741 <i>Δavt6::kanMX</i>                  | This study                   |
| STY3135 | BY4741 <i>Δavt7::kanMX</i>                  | This study                   |
| STY3350 | BY4741 <i>AVT5-Myc<sup>9</sup>::kanMX</i>   | This study                   |
| STY3352 | BY4741 <i>AVT6-Myc<sup>9</sup>::kanMX</i>   | This study                   |
| STY3354 | BY4741 <i>AVT7-Myc<sup>9</sup>::kanMX</i>   | This study                   |
| STY3258 | BY4741 <i>AVT5-GFP::kanMX</i>               | This study                   |
| STY3259 | BY4741 <i>AVT6-GFP::kanMX</i>               | This study                   |
| STY3260 | BY4741 <i>AVT7-GFP::kanMX</i>               | This study                   |

recycling at the molecular level. So far, three vacuolar amino acid transporters, Avt3, Avt4, and Avt6 have been shown to export amino acids from vacuoles. Both Avt3 and Avt4 export neutral amino acids and Avt6 exports acidic amino acids. Interestingly, Avt3 and Avt4 have been shown to be required for the survivability under nitrogen starvation (Yang *et al.*, 2006).

We found that Avt6 is required for the survivability under nitrogen starvation. Our results herein strongly suggest that Avt6 is involved in the recycle process of amino acid generated by autophagy. We also present the results of brief characterization of two Avt6 homologues, Avt5 and Avt7, of which molecular property is unknown.

## MATERIALS AND METHODS

### *Strains and media*

The *S. cerevisiae* strains used in this study are listed in Table 1. Cells were grown in rich media (YPD; 1% yeast extract, 2% peptone, 2% glucose). Starvation experiments were conducted in synthetic medium lacking nitrogen (SD-N; 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose). For microscopic study, cells were grown in SD + casamino acid medium (0.17% yeast nitrogen base without amino acids, 2% glucose, 0.5% ammonium sulfate, 0.5% casamino acid, 20 mg/L tryptophan, 20 mg/L uracil, 20 mg/L adenine) to reduce the autofluorescence.

### *Manipulation of yeast strains*

For gene disruptions, the entire coding region was replaced with *kanMX* cassette amplified by using polymerase chain reaction (PCR) primers containing 55 bases of identity to the regions flanking the open reading frame (Longtine *et al.*, 1998). Avt5, Avt6, and Avt7 were endogenously tagged with Myc<sup>9</sup> or green fluorescent protein (GFP) at the C-terminus by the PCR-based epitope-tagging strategy (Janke *et al.*, 2004). The sequences encoding the tag were amplified with

Table 2.  $\Delta avt6$  cells displayed a loss of viability under nitrogen starvation. The wild type and  $\Delta atg6$  cells were incubated in SD-N and assayed the survivability at the indicated days as described in Materials and Methods.

| Days          | Survival (%) |        |        |       |       |
|---------------|--------------|--------|--------|-------|-------|
|               | 0            | 2      | 4      | 6     | 8     |
| WT            | 100          | 147.1  | 120.09 | 84.19 | 78.63 |
| $\Delta avt6$ | 100          | 128.42 | 49.73  | 27.87 | 8.74  |

the S2 and S3 primer pair from the plasmid pYM-18 and pYM-27 (for the Myc<sup>9</sup> and GFP, respectively; Janke *et al.*, 2004). The correct fusion of the C-terminal tag to *AVT* gene was verified by sequencing the region of the junction.

### Survivability assay

The wild type (BY4741),  $\Delta avt6$  (STY3134), and  $\Delta avt7$  (STY3135) cells were grown in YPD medium to OD<sub>660</sub> 1.0–1.5, and then they were shifted to SD-N. At the indicated day, viability was determined by removing aliquots, plating on YPD in duplicate, and counting the number of colonies per plate after 2–3 d of growth.

### Vacuolar amino acid analysis

The cupric ion treatment method (Ohsumi *et al.*, 1988) was used for extraction of vacuolar amino acid pools from yeast cells. In summary, cells cultured in either YPD or SD-N were harvested, washed twice with distilled water, resuspended in A buffer (2.5 mM potassium phosphate buffer, pH 6.0, 0.6 M sorbitol, 10 mM glucose, and 0.2 mM CuCl<sub>2</sub>), and incubated at 30°C for 30 min. Cell suspensions were collected by centrifuge at 500 g for 3 min and washed with the A buffer lacking 0.2 mM CuCl<sub>2</sub>. The cell pellets were resuspended in distilled water and boiled for 15 min. Then, they were subjected to centrifugation at 13,000 g for 5 min. The supernatant was collected as the vacuolar amino acid fraction and dried up by speed vacuum pump. The amino acids were resuspended in 150  $\mu$ l of 0.02 N HCl and centrifuged at 13000 g for 5 min to remove the debris. The supernatant was filtrated by Millex-HV filter (Millipore). The amino acid analysis was performed by using a HITACHI L-8800 amino acid analyzer.

Amino acid samples for whole cell were prepared by suspending cells in distilled water and boiling those for 15 min. After removing the debris by centrifugation at 13,000 g for 5 min, the supernatants were subjected to amino acid analysis as described above.

### Protein extraction and immunoblot analysis

Trichloroacetic acid precipitates of total yeast cell proteins were prepared by pelleting cells from YPD or SD-N culture as described before (Rothermel *et al.*,

Table 3. Amino acid concentration of whole cell and vacuolar fraction.

|     | Concentration of amino acid (nmol/5 × 10 <sup>7</sup> cells) with following medium |              |         |              |       |              |         |              |
|-----|--|--------------|---------|--------------|-------|--------------|---------|--------------|
|     | YPD  |              |         |              | SD-N  |              |         |              |
|     | Whole  |              | Vacuole |              | Whole |              | Vacuole |              |
|     | WT   | <i>Δavt6</i> | WT      | <i>Δavt6</i> | WT    | <i>Δavt6</i> | WT      | <i>Δavt6</i> |
| Glu | 323.1  | 348.5        | 7.7     | 15.0         | 135.9 | 132.1        | 15.1    | 52.4         |
| Arg | 180.4  | 163.5        | 102.8   | 114.6        | 32.9  | 23.6         | 27.5    | 21.4         |

1995). Equal volumes of extract dissolved in SDS-PAGE sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.002% bromphenol blue, and 0.1 M dithiothreitol) were loaded onto an SDS-PAGE gel with 8% of acrylamide and separated. Proteins were transferred to PVDF membranes (Millipore) by semidry transfer units (Bio-Rad). Immunodetection of proteins was carried out using 9E10 anti-Myc antibody.

#### *Fluorescent microscopy*

Intracellular localization of fluorescent proteins was examined using an inverted fluorescence microscope (IX81, Olympus) as described previously (Suzuki *et al.*, 2007)

## RESULTS

### *Avt6 is required for the survivability under nitrogen starvation*

In nitrogen-starved condition, bulk cytoplasmic proteins are degraded in vacuoles by autophagy (Takeshige *et al.*, 1992). The degradation products, amino acids, are recycled for protein synthesis (Onodera and Ohsumi, 2005). Since *atg* mutants that are defective in the formation of AP, are not able to survive under nitrogen starvation (Tsukada and Ohsumi, 1993), amino acid recycle process seems to be essential for the survivability in nitrogen-starved condition. If so, the vacuolar amino acid transporter which exports amino acids from the vacuoles to the cytoplasm, should be also required for the survivability under nitrogen starvation. Indeed, it has been reported that Avt3 and Avt4, vacuolar amino acid transporters to export neutral amino acids, are required for the survivability under nitrogen starvation (Yang *et al.*, 2006).

Then, we expected that Avt6, which exports acidic amino acids, such as glutamate and aspartate, from vacuoles (Russnak *et al.*, 2001), is required for the survivability under nitrogen-starved condition. To test this, *Δavt6* cells were incubated in nitrogen starvation medium (SD-N) and then spreaded onto nutrient-rich medium (YPD) plate to count the number of viable cells. As shown in Table 2, the number of *Δavt6* colonies grown on YPD plate was rapidly reduced after 4 days starvation compared with that of parent colonies. This indicates that Avt6

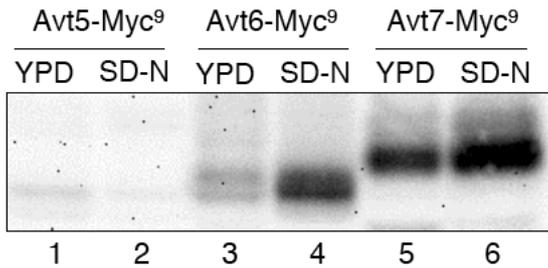


Fig. 1. Expression of Avt5, Avt6, and Avt7. Yeast cells expressing Avt5-Myc<sup>9</sup> (lanes 1 and 2), Avt6-Myc<sup>9</sup> (lanes 3 and 4) or Avt7-Myc<sup>9</sup> (lanes 5 and 6) were analyzed in exponential growth in rich YPD media (YPD) or after a nitrogen starvation (SD-N) by 4.5 h of incubation in SD-N starvation medium. Cell extracts were prepared and subjected to immunoblot analysis as described in Materials and Methods.

is required for the survivability under nitrogen starvation, and suggests that the amino acid export from vacuoles is a critical step to maintain the survivability under nitrogen starvation.

#### *Export of amino acids generated by autophagy from vacuoles is blocked in $\Delta$ avt6 cells*

To test whether the reduction of  $\Delta$ avt6 cell survivability under nitrogen starvation is due to defect in the amino acid recycle process, we compared the amino acid concentration of whole cell and vacuolar fraction between the wild type and  $\Delta$ avt6 cells. Based on the result from *in vitro* amino acid transport assay using isolated vacuoles, Russnak *et al.* (2001) suggested that Avt6 exports glutamate and aspartate *in vivo*. Thus,  $\Delta$ avt6 cells are expected to accumulate these amino acids in vacuoles more than the wild type cells. To examine *in vivo* vacuolar amino acid content, cells were subjected to cupric ion treatment, which disrupts the permeability barrier of the plasma membrane but remains the vacuolar membrane intact (Ohsumi *et al.*, 1988). As shown in Table 3, glutamate concentration in whole cell extract was almost identical between the wild type and  $\Delta$ avt6 cells in both nutrient-rich (YPD) and nitrogen-starved (SD-N) conditions. In contrast, the glutamate concentration in vacuoles remarkably increased in  $\Delta$ avt6 cells compared with the wild type cells, whereas the concentration of arginine, which was taken as a control, did not change between these cells (Table 2). The accumulation of glutamate in  $\Delta$ avt6 vacuoles was more apparent in nitrogen-starved condition, which is more than three-fold higher concentration of that in the wild type vacuoles. These indicate that Avt6 is required for the export of glutamate from vacuoles *in vivo* and supports our hypothesis that Avt6 is involved in recycling of amino acids generated by autophagy.

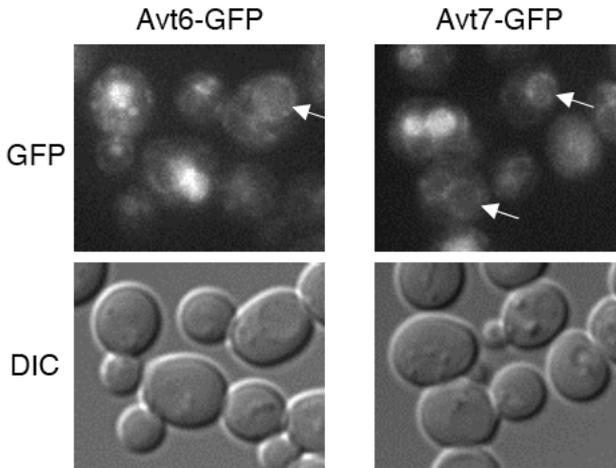


Fig. 2. Subcellular localization of Avt6 and Avt7. Cells expressing Avt6-GFP (STY3259) or Avt7-GFP (STY3260) were grown in synthetic casamino acid medium, and analyzed by fluorescence microscopy as described in Materials and Methods. Arrows indicate vacuolar membrane. DIC, differential interference contrast.

### Characterization of Avt5 and Avt7

In amino acid recycle process after autophagic protein degradation, multiple amino acid transporters must cooperatively function to recycle various kinds of amino acids from vacuoles. Thus, it is highly possible that there are yet unknown vacuolar amino acid transporters. For precise understanding of the amino acid recycling machinery, it is necessary to identify and characterize more amino acid transporters. Avt5 and Avt7 are closely related to Avt6 among AVT family members (Rusnak *et al.*, 2001). Thus, these are good candidates for the vacuolar amino acid transporter. To characterize these Avt proteins, we examined the endogenous expression of these proteins by immunoblot analysis. To this end, Avt5 and Avt7, as well as Avt6, are chromosomally fused to Myc<sup>9</sup> tag at the C-terminal end. Cells expressing the Myc<sup>9</sup>-tagged protein were collected from YPD culture at OD<sub>660</sub> 1.0–1.5 as a nutrient-rich sample. For nitrogen-starved sample, the YPD-grown cells were transferred to SD-N and further cultured for 4.5 hours. The cell extracts were subjected to immunoblot analysis with anti-Myc antibody. As shown in Fig. 1, the expression level of Avt6-Myc<sup>9</sup> was significantly increased in SD-N (compare lanes 3 and 4), supporting our notion that Avt6 carries out amino acid recycle, which is required for the survivability under nitrogen starvation. In contrast to Avt6-Myc<sup>9</sup>, the expression level of Avt5-Myc<sup>9</sup> was constantly low regardless the nutritional condition (lanes 1 and 2), and Avt7-Myc<sup>9</sup> level was abundant in YPD and further increased in SD-N (lanes 5 and 6).

Next, we examined whether Avt5 and Avt7 localize to the vacuolar membrane. To this end, we observed GFP fused to the C-terminal end of Avt proteins by

fluorescent microscope. The fluorescence of Avt5-GFP was not observable possibly due to the low expression as shown by immunoblot analysis described above (Fig. 1). The fluorescence of Avt7-GFP was observable and, interestingly, it stained the vacuolar membrane (Fig. 2). In addition, it stained nuclear envelope and peripheral patches, which is a typical localization pattern of ER protein. It is noted that Avt6-GFP protein, taken as a control, displayed a similar localization pattern to Avt7-GFP, a combination of vacuolar membrane and ER (Fig. 2). Avt6-GFP is functionally active, since it was able to maintain cell survivability under nitrogen starvation (data not shown). Although the physiological significance of the ER localization remains to be elucidated, at least a substantial population of Avt6 localizes to the vacuolar membrane. This is consistent with the function of Avt6 as a vacuolar amino acid transporter. We expected that the deletion of *AVT7* gene results in the loss of survivability in nitrogen-starved condition as seen for *AVT6* deletion. However,  $\Delta avt7$  cells maintained the survivability during nitrogen starvation (data not shown). Consistently, no particular accumulation of vacuolar amino acid was detected from the analysis of cupric-ion treated  $\Delta avt7$  cells (data not shown). Thus, we have not obtained the evidence showing that Avt7 functions as a vacuolar amino acid exporter. However, we cannot exclude out the possibility that the other amino acid transporter(s) function in a redundant manner with Avt7, which may mask the effect of *AVT7* deletion.

#### DISCUSSION

Autophagy-deficient (*atg*) cells reduce the total intracellular amino acid pool and the bulk protein synthesis under nitrogen starvation (Onodera and Ohsumi, 2005). Taking account that the *atg* mutants are unable to survive under nitrogen starvation (Tsukada and Ohsumi, 1993), amino acid recycling process by autophagy seems to be a critical event for the maintenance of survivability in the absence of nitrogen. In this process, amino acids must be exported from vacuoles to be utilized for protein synthesis. However, the physiological importance of this export step has not been elucidated in particular since the transporter protein responsible for this step had long been unknown. Russnak *et al.* (2001) identified a vacuolar amino acid transporter family, AVT family, in *S. cerevisiae* by reverse genetics approach. In their work, the amino acid transport activity of Avt protein was detected by *in vitro* assay using purified vacuoles. However, physiological effect of the amino acid transport activity remained unknown. In this study, we found that the vacuolar amino acid transporter, Avt6, is required for the survivability in nitrogen-starved condition (Table 2). Together with the fact that Avt3 and Avt4 are also required for the survivability under nitrogen starvation (Yang *et al.*, 2006), the reduction of survivability seems to be a common phenotype for cells disrupted the gene encoding vacuolar amino acid exporter. We also demonstrated that  $\Delta avt6$  cells accumulate glutamate in vacuoles especially upon nitrogen starvation (Table 3). This is the first indication of *in vivo* effect on the vacuolar amino acid pool by deleting *AVT6* gene and suggests that the reduction of  $\Delta avt6$  cell survivability is due to defect in the recycle of glutamate generated by autophagy.

The expression level of Avt6 highly increased upon nitrogen starvation (Fig. 1). Interestingly, the Avt4 level also increased in the same condition (data not shown). These results are consistent with the requirement of these proteins for the maintenance of survivability in this condition. In contrast to Avt4 and Avt6, the expression of Avt3 was reduced in nitrogen-starved condition (data not shown). This implies that Avt3 and Avt4 play distinct physiological roles, although both proteins export neutral amino acids from vacuoles. The change of Avt protein levels in response to nutritional condition totally corresponds to the result of microarray experiment by Gasch *et al.* (2000), suggesting the presence of the transcriptional regulation of *AVT* genes. Studying the molecular mechanism of such regulation and the upstream signal to modulate the expression will help to further understand the physiological role of vacuolar amino acid transporters.

Avt5 and Avt7 are especially closely related to Avt6 among the *AVT* family (Rusnak *et al.*, 2001). Then, we conducted the characterization of these Avt proteins to address the possibility that these also function as a vacuolar amino acid transporter. Immunoblot analysis indicated that the Avt5-Myc<sup>9</sup> level was quite low, whereas Avt7-Myc<sup>9</sup> was relatively abundant regardless nutritional condition (Fig. 1). The fluorescence of Avt5-GFP could not be observed by microscope possibly due to the low expression. Thus, the subcellular localization of Avt5 still remains unknown. It is noted that the level of Avt proteins tagged at the C-terminal end was not increased when expressed in  $\Delta pep4$  cells (data not shown), which largely lack the vacuolar protease activity, indicating that the C-terminal tag of Avt proteins is not subjected to vacuolar degradation and, thus, located at the cytosolic side. In contrast to Avt5-GFP, the fluorescence of Avt7-GFP was observable at the vacuolar membrane, although the significant population of Avt7-GFP localized at ER. Avt6-GFP, taken as a control, displayed the similar localization pattern. The ER localization of Avt6-GFP and Avt7-GFP could be due to partial inhibition of the vacuolar sorting, which is caused by GFP tagging. However, cells expressing Avt6-GFP were able to maintain the survivability during nitrogen starvation (data not shown), indicating that this GFP fusion protein is functionally active. Thus, it seems that the observed ER localization is physiologically relevant, implying that the ER population of Avt6 and Avt7 plays some, yet unknown, physiological role(s). Although our microscopic result indicated that the localization pattern of Avt7-GFP is similar to that of Avt6-GFP,  $\Delta avt7$  cells maintained the survivability during nitrogen starvation and displayed no difference in the vacuolar amino acid concentration from wild type cells. Thus, our results so far gave no indication showing that Avt7 functions as a vacuolar amino acid transporter. It is still possible that Avt7 may act in a redundant manner with other amino acid transporter(s). To address this possibility, characterization of cells deleted multiple *AVT* gene is in progress.

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