Endoplasmic reticulum acetyltransferases Atase1 and Atase2 differentially regulate reticulophagy, macroautophagy and cellular acetyl-CoA metabolism

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Nε-lysine acetylation in the ER lumen is a recently discovered quality control mechanism that ensures proteostasis within the secretory pathway. The acetyltransferase reaction is carried out by two type-II membrane proteins, ATase1/NAT8B and ATase2/NAT8. Prior studies have shown that reducing ER acetylation can induce reticulophagy, increase ER turnover, and alleviate proteotoxic states. Here, we report the generation of Atase1−/− and Atase2−/− mice and show that these two ER-based acetyltransferases play different roles in the regulation of reticulophagy and macroautophagy. Importantly, knockout of Atase1 alone results in activation of reticulophagy and rescue of the proteotoxic state associated with Alzheimer’s disease. Furthermore, loss of Atase1 or Atase2 results in widespread adaptive changes in the cell acetylome and acetyl-CoA metabolism. Overall, our study supports a divergent role of Atase1 and Atase2 in cellular biology, emphasizing ATase1 as a valid translational target for diseases characterized by toxic protein aggregation in the secretory pathway.
N-$\text{\textsuperscript{1}}$-lysine acetylation of nascent proteins within the lumen of the ER is a recently discovered quality control (QC) mechanism that ensures protein homeostasis (proteostasis) within the secretory pathway\textsuperscript{1-18}. ER-based N-$\text{\textsuperscript{1}}$-lysine acetylation is catalyzed by two ER-membrane bound acetyltransferases, ATase1/NAT8B and ATase2/NAT8L\textsuperscript{19,20}. Both ATases are type-II membrane proteins with the catalytic domain facing the cytosol. They work in tandem with AT-1/SLC33A1, an ER-membrane antipporter that moves acetyl-CoA from the cytosol into the lumen of the ER in exchange for free CoA\textsuperscript{8}. Dysfunctional ER acetylation, as caused by loss-of-function mutations or gene duplication events, is associated with severe inherited diseases\textsuperscript{13,19-21}.

The proteostatic functions of the ER acetylation machinery involve acetylation of correctly folded glycoproteins to ensure engagement of the secretory pathway as well as regulation of ER-autophagy (also referred to as reticulophagy or ER-phagy) to dispose of toxic protein aggregates\textsuperscript{13,22,23}. Mechanistically, the regulation of reticulophagy involves acetylation of ATG9A, the only integral membrane autophagy protein, on two lysine residues K359 and K363 that face the lumen of the ER\textsuperscript{11,14}. The acetylation status of ATG9A regulates its ability to interact with the reticulophagy receptors, FAM134B and SEC62, and engage cytosolic LC3\textsuperscript{15-17,25-28}. Importantly, mice with reduced ER acetylation display excessive induction of reticulophagy while mice with increased ER acetylation display the opposite\textsuperscript{15,17}. In both cases, lack of homeostatic balance causes severe disease phenotypes\textsuperscript{7,15,17}. Therefore, fluctuations in ER-based N-$\text{\textsuperscript{1}}$-lysine acetylation have dramatic impacts on glycoprotein flux, ER turnover, and cellular physiology.

Autophagy is an essential component of the cell degradation system that is responsible for the disposal of large protein aggregates within the cell. Malfunction of autophagy contributes to the progression of many diseases across lifespan, whereas increased levels of autophagy can be beneficial in mouse models of diseases characterized by increased accumulation of toxic protein aggregates\textsuperscript{6,14,17,29-37}.

Here, we report the generation of Atase1$^{-/-}$ and Atase2$^{-/-}$ mice and show that these two ER-based acetyltransferases play different roles in the regulation of reticulophagy and macroautophagy. We also show that knockout of Atase1 alone results in activation of reticulophagy and alleviated proteotoxicity in a mouse model of Alzheimer’s disease (AD). Furthermore, loss of either Atase1 or Atase2 resulted in widespread changes in the cellular acetylome but differential changes in acetyl-CoA metabolism. Overall, our study supports partially divergent functions for Atase1 and Atase2, emphasizing ATase1 as a valid translational target for diseases characterized by toxic protein aggregation in the secretory pathway.

**Results**

**Knockout of Atase2 resulted in a compensatory increase in Atase1 expression in multiple organs.** In order to elucidate the roles of Atase1 and Atase2 independently, we generated Atase1$^{-/-}$ and Atase2$^{-/-}$ mice on a C57BL/6J background. Both knockout mice were born with Mendelian ratio and did not exhibit any apparent physical abnormalities. The gene knockouts were detectable by traditional PCR (Fig. 1a,b). To confirm the gene was indeed knockout out, we designed reverse transcription quantitative PCR (RT-qPCR) primers specific for mouse Atase1 and Atase2, and in multiple tissue types, we were able to confirm that the respective gene was no longer expressed at the mRNA level (Fig. 1c).

Once the desired genetic changes were confirmed, we performed a phenotypic assessment of our Atase1$^{-/-}$ and Atase2$^{-/-}$ mice. First, we performed necropsy and histologic assessment of all organ and tissue types in both knockout male and female mice but did not observe any notable gross or histologic abnormalities. Next, we assessed the Atase1$^{-/-}$ and Atase2$^{-/-}$ mouse behavior compared to their WT littermates by several paradigms including open field, light/dark box exploration, novel object recognition, marble burying, and fear conditioning. We did not observe substantial changes in behavior in either Atase1$^{-/-}$ or Atase2$^{-/-}$ mice (Supplementary Fig. 1). There are reported GWAS associations between Atase2/NAT8 and chronic kidney disease in humans\textsuperscript{38-41}. Furthermore, one study put forward the hypothesis that ATase2/NAT8 might be responsible for mercapturic acid synthesis and excretion of xenobiotics in the urine\textsuperscript{42}. Thus, we assessed kidney health in our knockout mice by specifically evaluating kidney weight, plasma creatinine and urea nitrogen, which were all not different from WT littermates (Supplementary Fig. 2a-c). We attempted to measure the spot urine albumin to creatinine ratio, but in most urine samples, the albumin level was below the limit of detection and thus could not be reliably quantified. We also measured spot urine 1,4-dihydroxynonane mercapturic acid (DHN-MA), a mercapturic acid derivative naturally excreted by the kidney, and again found no difference from WT littermates (Supplementary Fig. 2d). Additional studies have shown that reduced ER-based acetylation in hypomorphic AT-1 mice results in chronic pancreatitis and fibrosis, presumably from disrupted excretion of pancreatic enzymes\textsuperscript{43}. In both our knockout mice, we did not observe any changes in the histologic appearance of the pancreas nor changes suggestive of pancreatic fibrosis (Supplementary Fig. 3). In conclusion, our Atase knockout mice appear healthy without evident behavioral abnormalities, organ dysfunction, or disease.

Using the primers specific for mouse Atase1 and Atase2 validated above, we performed RT-qPCR on several tissue types to examine the endogenous expression of the two genes. By using absolute quantification compared to a known amount of plasmid DNA containing either the mouse Atase1 or Atase2 gene, both genes were found to be ubiquitously expressed in the tissue types we examined; additionally, there was significantly more Atase2 expression observed in the kidney (Fig. 1d, e). By using relative quantification, we examined the expression of the Atase enzymes as well as the ER acetyl-CoA transporter AT-1 to assess for potential compensation when one of the ER acetyltransferases was knocked out. In multiple tissue types, we observed an increase in Atase1 expression in the Atase2$^{-/-}$ mouse; there were no substantial changes in Atase2 or AT-1 expression in either knockout mouse (Fig. 1f-h). The compensatory upregulation of Atase1 in the Atase2$^{-/-}$ mouse may play a role in the phenotype observed below.

**Knockout of Atase1 activated reticulophagy in the mouse.** Mice with reduced ER acetylation, as caused by hypomorphic AT-1, display excessive induction of reticulophagy while mice with increased ER acetylation, as caused by AT-1 overexpression, display reduced reticulophagy\textsuperscript{7,15,17}. Therefore, we predicted that knockout of the individual acetyltransferase enzymes in the ER would result in reduced ER-based acetylation of Atg9a and activation of reticulophagy. In the following reticulophagy-focused studies, we used both liver and mouse embryonic fibroblasts (MEFs). While the liver is more congenial for the isolation and studies, we used both liver and mouse embryonic fibroblasts (MEFs).
increased turnover of the ER (Fig. 2a). To verify this change, we performed immunocytochemistry on MEFs obtained from our knockout mice and observed a decrease in Fam134b puncta in the Atase1−/− MEFs (Fig. 2b). In addition, we observed an increase in Lc3β puncta in both knockout mice, which is consistent with activation of reticulophagy (Fig. 2b). Importantly, the change in Fam134b protein expression did not appear to be driven by a decrease in mRNA expression (Fig. 2c).

Next, we examined the acetylation status of Atg9a as well as Atg9a-interacting proteins by studying liver ER. Prior studies have shown that inhibition of the Atases results in decreased acetylation of Atg9a, increased interaction between Atg9a and reticulophagy receptors Fam134b and Sec62, and activation of reticulophagy.14,17 By performing an immunoprecipitation with an antibody specific for acetylated lysine residues, we found ER-based Atg9a to be hypoacetylated in Atase1−/− mice, when compared to WT (Fig. 2d). This finding was paralleled by increased Atg9a-Fam134b and Atg9a-Sec62 interaction on the ER in Atase1−/− mice (Fig. 2e, f). Interestingly, we did not observe changes in Atg9a acetylation status nor interaction with Fam134b/Sec62 in the Atase2−/− mice (Fig. 2d–f).

Finally, we employed ER-specific probes to track reticulophagy and ER turnover in our knockout MEFs. First, we transfected our MEFs with the ER tandem reporter mCherry-GFP-RAMP444,
which gives a yellow signal at a neutral pH and red signal in an acidic pH due to quenching of the GFP signal. By quantifying the number of red puncta per cell, which represents acidified ER from autophagolysosome formation, we observed an increase in the percentage of cells that contained more than one ER-containing autophagolysosome in the Atase1−/− MEFs compared to WT MEFs (Fig. 2g). Importantly, this percentage increased as expected when the MEFs were starved in Earl’s balanced salt solution (EBSS) and decreased to fed levels when EBSS was supplemented with folimycin, which prevents lysosomal acidification (Fig. 2g), thus providing functional validation to our assay. Next, we transfected our MEFs with RAMP4-mCherry to specifically label the ER followed by immunostaining for Lc3β. We observed an increase in co-localization between the RAMP4-mCherry and Lc3β signals in the Atase1−/− MEFs compared to WT MEFs, suggesting increased autophagosome formation from the ER (Fig. 2h). As expected, we did not observe changes between WT and Atase2−/− MEFs in either of these experiments.

When taken together, the above results show that knockout of Atase1 but not Atase2 in the mouse causes elevated reticulophagy and ER turnover.

**Atase1−/− and Atase2−/− mice exhibited increased macroautophagy and ER stress.** In addition to activation of reticulophagy, prior studies have demonstrated that reduced ER-based acetylation also causes an activation of general cellular autophagy.
Therefore, we investigated whether this also occurred in our Atase knockout mice. First, we performed Western blotting of several commonly used markers of autophagic flux in our knockout MEFs. We observed an increase in expression of Lc3β-1, Lc3β-II, and Beclin consistent with chronic activation of macroautophagy in the Atase1−/− and Atase2−/− MEFs compared to WT (Fig. 3a). In liver, we also observed increased expression of Lc3β-1 in both knockout mice as well as an increase in Beclin in Atase1−/− mice (Supplementary Fig. 4). We did not observe a change in p62 expression in our MEFs, but in liver, we did observe a decrease in expression levels in both knockout mice compared to WT supportive of increased autophagic flux (Fig. 3b). Finally, we performed live cell imaging in our MEFs with GFP-LC3β, which represents autophagosome trafficking throughout the cell (Fig. 3c). Overall, we observed an increase in organelle density in both knockout MEFs compared to WT consistent with prior work. In addition, in our Atase1−/− MEFs, we observed an increase in autophagosome speed and track displacement length compared to WT MEFs (Fig. 3c). Thus, these data show that both knockout mice have an increase in autophagic flux, with a more dramatic phenotype observed in the Atase1−/− mice. 

Next, we evaluated levels of ER stress in our Atase knockout mice. By Western blotting from liver lysates, we observed an increase in BiP/Grp-78 expression in both the Atase1−/− and Atase2−/− mice compared to WT (Fig. 3d). While not statistically significant, we did observe a trend in increased BiP mRNA expression in the liver of both knockouts compared to WT mice, with large variation in the Atase2−/− mice (Fig. 3f). We then evaluated for activation of the three canonical ER stress signaling pathways to assess for drivers of increased BiP expression. Both the Perk and Ire1 pathways did not appear activated as evident by the lack of change in the phosphorylation status of Perk, Elf2α, and Ire1; nuclear expression level of Atf4; and mRNA expression of spliced Xbp1 and Atf4 (Fig. 3d–f). However, we did observe an increase in the cleavage of Atf6 in the Atase2−/− mouse as evident by increased expression of the p50 fragment relative to the p90, full length form of the protein (Fig. 3d). In addition, by transfecting MEFs with an Atf6 transcriptional reporter that results in the expression of firefly luciferase, we observed an increase in luminescence in our Atase2−/− MEFs, consistent with an increase in p50-Atf6 activity (Fig. 3g). Overall, our results show activation of the Atf6 canonical ER stress signaling pathway in the Atase2−/− mouse together with stimulation of macroautophagy.

Knockout of Atase1 improves the proteotoxicity phenotype of the APP/PS1 mouse model of Alzheimer’s disease. Previous studies have shown that induction of reticulophagy, down-stream of the ER acetylation machinery in AT-1 hypomorphic (AT-1S113R/+) mice or in the presence of ATase1/ATase2 chemical inhibitors, can resolve the proteopathy associated with the AD phenotype. Since our Atase knockout mice exhibit activation of reticulophagy (Atase1−/− only) and macroautophagy (both Atase1−/− and Atase2−/−), we crossed our Atase knockout mice with the APPswe/PS1dE9 (henceforth referred to as APP/PS1) AD-like model to generate APP/PS1;Atase1−/− and APP/PS1;Atase2−/− mice. First, we tracked lifespan over 10 months, which was dramatically reduced in the APP/PS1 mouse. We observed increased survival in both APP/PS1;Atase1−/− and APP/PS1;Atase2−/− mice compared to APP/PS1 mice; however, the rescue of survival was more evident in the APP/PS1;Atase1−/− mouse (Fig. 4a). In addition, while male mice lifespan was partially rescued in both crosses, we did not observe a rescue in lifespan for the female APP/PS1;Atase2−/− mice compared to APP/PS1 female mice (Fig. 4a).

At 10 months of age, we assessed the male APP/PS1 phenotype compared to the APP/PS1;Atase1−/− and APP/PS1;Atase2−/− male mice, evaluating several pathologic hallmarks of AD including amyloid plaque deposition, gliosis, and synaptic loss. First, by using thiocillin-S to stain for dense plaques in the brain, we observed a reduction in plaque density and area coverage in our APP/PS1;Atase1−/−, most notable in the hippocampus; whereas we only observed a slight reduction in plaque area percentage in the APP/PS1;Atase2−/− mice (Fig. 4b). Next, we assessed astrocytic and microglial activation via immunofluorescence and Western blotting in the cortex. In our APP/PS1 mice, we observed a marked increase in number of astrocytes and microglia via Gfap and Iba1 immunofluorescence, respectively, compared to WT control mice (Fig. 4c). This change in immunostaining was also evident via Western blotting for Gfap and Iba1 in which the expression of these proteins was significantly increased in the APP/PS1 mice compared to WT (Fig. 4d). In our APP/PS1;Atase1−/− and APP/PS1;Atase2−/− mice, we observed a significant decrease in Gfap immunofluorescence signal as well as expression level while the Iba1 immunofluorescence signal and expression level were mostly unchanged (Fig. 4c, d). Finally, we evaluated for synaptic loss in the CA3 region of the hippocampus via immunofluorescent staining for the presynaptic protein synaptophysin and the postsynaptic protein Psd-95. In our APP/PS1 mice, we observed reduced synaptophysin immunofluorescent signal in the CA3 region as well as a marked reduction in the number of synaptophysin and Psd-95 co-localized puncta compared to WT mice (Fig. 4e). In both our APP/PS1;Atase1−/− and APP/PS1;Atase2−/− mice, there was an increase in the number of co-localized puncta compared to APP/PS1 mice, signifying more synapses retained at 10 months of age (Fig. 4e). Overall, our data show that knockout of either Atase1 or Atase2 in the mouse can result in improved proteotoxicity compared to wild-type controls.
Lysine acetylation stoichiometry revealed widespread changes reflecting overlapping and non-overlapping substrates of Atase1 and Atase2 in the secretory pathway. Both Atase enzymes are responsible for the acetylation of ER-resident and cargo proteins; however, it is currently unknown whether they have different substrates or whether they compete for the same substrates, either at the level of the protein or the specific lysine residue. To address both questions, we prepared enriched ER from the liver of WT, Atase1−/−, and Atase2−/− mice and used a mass spectrometry method that reveals the stoichiometry of lysine acetylation within the proteome. We detected a total of 6242 acetylpeptides, with 253 and 219 acetylpeptides in the Atase1−/− and Atase2−/−, respectively, that exhibited a stoichiometry value that was statistically different from WT.
We first evaluated acetylpeptides found within the secretory pathway as possible direct substrates of Atase1 and Atase2. Of the 6242 acetylpeptides detected, we filtered these based upon a cellular localization of "ER", "Golgi", or "secreted", yielding a total of 2396 acetylpeptides. Of these acetylpeptides, we found 88 and 82 sites in the Atase1−/− and Atase2−/−, respectively, that exhibited a stoichiometry value that was statistically different from WT (Fig. 5a). Interestingly, when evaluating these acetylpeptide stoichiometry changes from WT between the Atase1−/− and Atase2−/− mice, the overall distributions of the two were different. The Atase2−/− distribution centered below zero, suggesting a net decrease in lysine acetylation in the secretory pathway (Fig. 5b). When comparing the acetylpeptides that significantly changed from WT, only 19 sites overlapped between the two knockout mice, but all except 3 exhibited a stoichiometry change from WT in the same direction (Fig. 5c). In addition, when examining the non-overlapping sites, there was a significant positive correlation between the Atase1−/− and Atase2−/− changes in acetyl stoichiometry (Supplementary Fig. 5). Next, we examined the acetylpeptides that could be detected in the WT samples with confidence (n ≥ 3) but not in the Atase1−/− or Atase2−/− samples; we found 7 and 2 sites in the Atase1−/− and Atase2−/−, respectively, that we call "Atase1- or Atase2-dependent" (Fig. 5d). These acetylpeptides do not appear in the volcano plots shown in Fig. 5a since a stoichiometry difference cannot be computed. Importantly, we were able to detect the heavy-labeled acetylpeptides (in vitro acetylation) in the knockout mice, but not the light-labeled acetylpeptides (endogenous acetylation), verifying a stoichiometry value of effectively zero.

To evaluate the biologic significance of the above findings, we combined the sites with significantly different stoichiometry (Fig. 5a) with those that appeared Atase-dependent (Fig. 5d) and performed a gene ontology analysis of the proteins harboring those sites (Fig. 5e). In both knockouts, we found many overlapping cellular component categories such as ER, ER-Golgi-Intermediate Compartment, and Golgi-associated, which highlight known functions of the Atases within the secretory pathway. However, Atase1−/− mice displayed a preponderance of categories that are generally involved with the engagement as well as morphology of the secretory pathway, likely suggesting partially different regulatory functions of the two transferases (Fig. 5e). Interestingly, we also found ribosome-associated categories, which would suggest a previously uncharacterized role of the Atases in post-translational modification of ribosome-associated proteins. The representation of ribosomal-associated proteins within our enriched ER is not surprising as the great majority of ribosomal elements are tightly associated with the ER membrane and about 50–75% of the translational activity of the ER is directed toward cytosolic proteins6.}

**Atase1−/− and Atase2−/− affected non-secretory pathway protein acetylation and acetyl-CoA metabolism.** Since our acetyomic data revealed many changes in protein acetylation within the secretory pathway, we next decided to examine whether lack of one Atase in our knockout mice would also affect acetyl-CoA/CoA dynamics and cause adaptive responses beyond the ER. In fact, prior studies in our laboratory have shown that changes in At-1 activity, the ER membrane-based acetyl-CoA/CoA antiporter, also affect cellular acetylation and lipid metabolism beyond the ER2. We reasoned that reduced acetyl-CoA consumption within the ER lumen, as caused by the disruption of one of the acetyltransferases, would limit the generation and availability of free CoA, which is necessary for the antiporter-mediated influx of acetyl-CoA from the cytosol (Fig. 6a).

As mentioned above, when we decided to determine stoichiometric changes within the acetyome of Atase1−/− and Atase2−/− mice, we opted to use enriched- instead of purified-ER because, in addition to abundant ER membranes, the former retains sufficient residual cytosolic material that would allow us to explore possible perturbation of the acetyl-CoA/CoA antiporter system, as reflected by the "non-secretory pathway acetyome". Indeed, our strategy successfully yielded 3846 acetylpeptides not designated with a cellular localization of "ER", "Golgi", or "secreted" (henceforth called "non-secretory pathway"). Of these 3846 acetylpeptides, we found 165 and 134 sites in Atase1−/− and Atase2−/− mice, respectively, that exhibited a stoichiometry value that was statistically different from WT (Fig. 6b). When evaluating these acetylpeptide stoichiometry changes from WT between Atase1−/− and Atase2−/− mice, the Atase1−/− distribution centered well above zero, suggesting a net increase in lysine acetylation (Fig. 6c). When comparing the acetylpeptides that significantly changed from WT, 31 sites overlapped between the...
two knockout mice, and all except one site exhibited the same direction of stoichiometry change from WT (Fig. 6d). Again, the changes in acetyl stoichiometry from WT of non-overlapping sites in both mouse models displayed a positive correlation (Supplementary Fig. 5). When evaluating these significantly changed acetylpeptides with a gene ontology analysis on the proteins harboring the sites, we found several categories in both knockouts that highlight a broad cellular response (Fig. 6e). Interestingly, the Atase2<sup>−/−</sup> analysis revealed a heavy predominance of proteasome categories that did not appear in the Atase1<sup>−/−</sup> analysis.

We then examined the steady-state levels of acetyl-CoA and CoA in the liver cytosol and ER in fasted mice. We found a

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decrease in ER CoA levels in both Atase knockout mice (Fig. 6f). Since cytosolic acetyl-CoA is the substrate for fatty acid synthesis, we assessed whether our Atase knockout mice had an accumulation of lipids in the liver as seen when At-1 activity is reduced. While liver H&E staining did not reveal dramatic changes in liver microscopic anatomy, lipid-specific staining did reveal an increase in lipid droplet density in the Atase2−/− mouse (Fig. 6g, h).

When taken together, the above data support the argument that hyperacetylation of cytosolic proteins in Atase1−/− as well as the accumulation of lipid droplets in Atase2−/− reflect secondary adaptation that may be from disruption of At-1 acetyl-CoA/CoA antiporter activity. They also suggest that the Atases might be part of an intricate system that regulates metabolic crosstalk between the ER lumen and the cytosol, presumably through At-1.

Discussion

Nε-lysine acetylation within the lumen of the ER, which is carried out by the two acetyltransferases Atase1/NAT8B and Atase2/NAT8, plays an important role in the regulation of protein aggregation within the organelle. There is building evidence that ATase1 and ATase2 might serve different roles in cellular biology. Indeed, despite high sequence similarity and catalytic activity, recent work has shown that ATase1 is post-translationally regulated by acetylation while ATase2 is transcriptionally regulated by the immediate-early gene cascade. ATase2 might serve different roles in cellular biology. Indeed, despite high sequence similarity and catalytic activity, recent work has shown that ATase1 is post-translationally regulated by acetylation while ATase2 is transcriptionally regulated by the immediate-early gene cascade. The work presented here suggests that Atase1 plays a more substantial role in the regulation of reticulophagy and protein aggregation, as also revealed by the rescue of several phenotypic qualities in the APP/PS1 mouse that may in part be due to the acetylation status of Atg9a and downstream assembly of the reticulophagy machinery. We presume that Atase1−/− mice exhibit increased turnover of toxic protein aggregates within the secretory pathway that ameliorates the APP/ARβ-associated proteotoxicity in the APP/PS1 mouse. A similar effect was observed with APP695/715 mice, a different model of AD, following genetic or biochemical inhibition of ER acetylation. It is important to point out that the lack of activation of reticulophagy in the Atase2−/− mouse may be due to at least in part- the compensatory upregulation of Atase1 expression. These data also demonstrate the overlapping functions of Atase1 and Atase2 as knockout of a single ER-based acetyltransferase does not result in the same, dramatic phenotype as observed with the At-1S113R+/− mice. Although many aspects of the APP/PS1 AD-like phenotype improved in one or both knockout mice, the rescue was not as dramatic as seen with the At-1S113R+/− or compound 9 (Atase1/Atase2 inhibitor) treatment. However, it is imperative to point out that those prior studies targeted both Atases and used the less-severe APP695/715 mouse that does not harbor the PS1E9 mutation. Overall, this work emphasizes the validity of using inhibitors of Atase1 for translational medicine for diseases characterized by toxic protein aggregation in the secretory pathway. Importantly, there are natural ATase1 and ATase2 nonsense mutations in humans that do not appear to associate with pathologic consequences, further emphasizing the ability to use these enzymes as therapeutic targets.

While Atase1 appears to be heavily implicated in the induction of reticulophagy, our data suggest that Atase2 is more involved in protein QC as loss of the enzyme results in activation of ER stress via Atf6-mediated signaling. Interestingly, Atase1 is allosterically regulated by availability of acetyl-CoA, while Atase2 is not. Therefore, we could presume that Atase2 acts as a "constitutive" acetyltransferase, thus explaining the apparent activation of the ER stress response in the Atase2−/− mice, while Atase1 acts as "regulated" acetyltransferase. Atase2−/− mice also exhibited activated macroautophagy similar to the Atase1−/− mouse, with a mechanism appearing to be independent of the acetylation status of Atg9a and potentially reliant on the unfolded protein response. Activating cellular autophagy has been repeatedly shown to be beneficial in models of AD, which may explain the partial phenotypic rescue of the APP/PS1;Atase2−/− mice observed in this study. Again, it is likely that overlapping functions between Atase1 and Atase2 prevent widespread activation of ER stress and cellular pathology in the knockout mice, which is further supported by the short list of Atase1/2-dependent acetylation sites found in liver ER. Prior work in our laboratory has demonstrated that ER-based Nε-lysine acetylation as catalyzed by ATase1 and ATase2 plays a role in protein QC within the secretory pathway, and alterations in levels of ER acetylation can dramatically impact glycoprotein flux. Therefore, we have ongoing studies to assess the engagement of the secretory pathway in our knockout mice that may deepen our understanding about the differing and overlapping roles of ATase1 and ATase2.

Reduced activity of Atase1 or Atase2 are expected to have two important consequences: (1) reduced acetylation of ER-resident and -cargo proteins and (2) reduced ER acetyl-CoA consumption resulting in decreased CoA generation. AT-1, the ER-based acetyl-CoA transporter, is an antiporter that moves acetyl-CoA from the cytosol to ER in exchange for CoA from the ER to cytosol. Therefore, the concentration gradients of both acetyl-CoA and CoA in both cellular compartments would impact the function of AT-1. Prior work in our lab has shown that reducing At-1 activity can dramatically alter acetyl-CoA pools within the cell, drastically changing the proteome and acetyl-proteome as well as resulting in cytosolic lipid accumulation from excess acetyl-CoA availability. As a result, in our Atase knockout mice, we anticipated a similar effect due to reduced CoA generation in the ER, reduced At-1 activity, and a buildup of cytosolic acetyl-CoA. Our data demonstrated an expected decrease in steady-state ER CoA levels in both knockout mice, but acetyl-CoA levels did not change in either cellular compartment. Furthermore, Atase2−/− mice had evidence of cytosolic lipid accumulation whereas Atase1−/− did not. In addition, Atase1−/− mice exhibited robust hyperacetylation of non-secretory pathway proteins. Therefore, we presume that the excess cytosolic acetyl-CoA...
accumulating from slowdown of At-1 antiporter activity is being used to acetylate proteins in the Atase1−/− mouse and to build lipids in the Atase2−/− mouse. The underlying reasons for this different adaptive response remain to be explored.

Nε-lysine acetylation is a key regulatory mechanism of many cellular proteins in eukaryotic cells. In our acetylomic data, we observed changes in stoichiometry of acetylation both within and outside the secretory pathway with substantial overlap between Atase1−/− and Atase2−/− mice. Indeed, we only observed a handful Atase-dependent sites that are likely to only be substrates of Atase1 or Atase2, thus confirming that the vast majority of Nε-lysine acetylation in the ER can be carried out by both Atase1 and
Atase2. However, we did observe stoichiometry changes in one model that were not observed in the other suggesting that, under normal conditions, many sites are “preferentially” modified by only one Atase. These findings are likely explained by the different Km and affinity for the individual peptides and lysine sites. Interestingly, several of the proteins harboring unique sites exhibited changes in stoichiometry of acetylation at other sites; for example, the Nucbl K301 site was lost in the Atase1−/− mouse, but the K288 site exhibited increased stoichiometry in the Atase1−/− mouse compared to WT. Furthermore, we found 11 new acetylation sites in our knockout mice that were not detected in WT samples, which may be of importance for the phenotypes observed. For example, ATL2 plays a role in remodeling the ER membrane in preparation for reticulophagy44, and acetylation at K314 may impact the protein’s function in the Atase knockout mice. Overall these data point to complex changes occurring in protein acetylation, presumably from changes in protein folding, affinity for individual sites, or acetyl-CoA availability in the knockout mice. It is important to point out that our samples were enriched for ER-resident proteins, and thus the results of our analysis are likely to miss many cargo proteins transiting through the secretory pathway. In addition, the observed changes in acetylation in our knockout mice highlight the multifaceted adaptive response of the cell imparted upon by changes in Atase1 and Atase2 activity. Indeed, in addition to the immediate regulation of ER intraluminal events, namely the engagement of the secretory pathway and the induction of reticulophagy, the ER acetylation machinery is emerging as a vital metabolic switch that ensures crosstalk among different intracellular organelles and compartments.2,6,31

In conclusion, we have begun dissecting the differing roles of the ER-based Atase1 and Atase2 enzymes in cellular biology, demonstrating Atase1 to be a valid target for translational medicine in diseases characterized by toxic protein aggregation in the secretory pathway.

Methods
Atase knockout mouse generation
The mouse Nat8b gene was completely excised by the Genome Editing and Animal Models Core at the University of Wisconsin-Madison (Madison, WI, USA). Murine bacterial artificial chromosome (BAC) clones generated from C57BL/6 embryonic stem (ES) cell DNA and containing all of the Nat8b gene were purchased from Children’s Hospital Oakland Research Institute (Oakland, CA). Portions of these BAC clones were used to construct a Nat8b knockout (K0) targeting vector containing a floxed Neo selection cassette via traditional cloning techniques and recombining. The completed targeting vector was linearized and introduced by electroporation into murine JMRI3 (C57BL/6N-A1m1Brd) parental ES cells. Cells that integrated the targeting vector were screened using G418 and GANC selection, replicated, and expanded. The integrity of these clones was verified by Southern blot and DNA sequence analysis; chromosome counting performed at the Animal Genomics Service of Yale University confirmed that all clones were euploid. Clones were then microinjected into C57BL/6 blastocysts to produce chimeric founders. Highly chimeric male founders were mated to C57Bl/6 females, and F1 pups were genotyped to identify those carrying the gene-targeted allele with the following primers: forward, 5′-ATTAGACGATTGCTGGGGAT-3′ and reverse, 5′-GGCTCAGTAAAAACACAGGCC-3′ (amplicon of 359 bp). After expansion of correctly identified F1 pups, the loxp-flanked Neo cassette was excised by breeding with mice expressing Cre recombinase (Jackson Laboratory). Mice were screened for Neo cassette removal with the following genotyping primers: forward, 5′-GGACACAGACCTCCGCCATGAT-3′ and reverse, 5′-GGCTCAGTAAAAACACAGGCC-3′ (amplicons of 1000 and 437 bp). A single founder was used to establish our Atase2−/− colony with the aforementioned genotyping primers.

Animals. All animals used in this study are Mus musculus strain C57BL/6J (MMRC Stock No. 000664-JAX) with sex and age information listed in each figure legend. APP APPsw/Psen1ΔE9 (APP/PS1) double transgenic mice were obtained from Jackson Laboratory (MMRC Stock No. 34832-JAX) and crossed with our in-house Atase1−/− and Atase2−/− colonies. Mice were housed in standard cages provided by the University Laboratory Animal Resources and grouped by sex and age. Animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison (protocol #M005120).

Behavior testing. All behavioral testing was conducted within the Waisman Center Behavioral Testing Service (Madison, WI, USA). The experimenter was blind to the genotype of the mice during testing. All mice received a minimum of 30 min acclimation time to the testing room prior to each behavior test. The following behavioral assays have been previously described: novel object recognition, marble burying assay, and fear conditioning paradigm7.

Open field exploration. Open field exploration sessions lasted 30 min, and each mouse received 1 session. Each mouse was removed from its home cage and placed in the center of the arena. The Omnitech Fusion system used photo beams to continuously monitor and record the animal’s placement during the session. Testing variables included total distance traveled and time spent in the central portion of the open field vs. the periphery (a measure of anxiety). Data were recorded using the Omnitech Fusion system with a center ratio zone map.

Light/dark exploration. Each mouse was placed into a split arena for a 10 min assay. Time spent in the dark portion of the arena and entries into the dark portion of the arena were recorded.

Kidney function assessment. Plasma urea nitrogen, plasma creatinine, urine albumin, and urine creatinine were measured by the UW Health Clinical
Blood was collected by transcardial puncture of CO₂-euthanized animals into a 0.5 M ethylenediaminetetraacetic acid (EDTA)-flushed needle and syringe. Samples were immediately centrifuged at 10,000 × g for 5 min and the plasma supernatant was collected and flash frozen for storage at −80 °C before testing. Spot urine was collected by scruffing animals on a hydrophobic surface and was sent immediately for laboratory testing. Kidney to body mass ratios were calculated by weighing the total carcass mass and wet mass of left and right kidneys separately after CO₂ euthanasia.

**Cell culture**

Mouse embryonic fibroblasts (MEFs) preparation and culturing. MEFs were prepared from timed pregnant females on embryonic days E12.5–E14.5. After CO₂ euthanasia of the pregnant female, the embryo head and visceral organs were removed, body minced in cell culture grade trypsin-EDTA (0.25%; Gibco™, #25200-056), and incubated at 37 °C for 15 min. Trypsin was quenched by addition of complete media containing Dulbecco’s modified Eagle’s medium (DMEM; Corning, #10-017-CV) supplemented with 10% Fetal Bovine Serum (FBS; Corning, #10-017-CV).
whiskers at the 1st and 99th percentiles, and dots representing data points outside the 99th percentile. Statistical testing between the Atase1 included.

(abcam; #ab108338; 1:100) primary antibodies as previously described

anti-acetylated lysine (Cell Signal Technologies; #9441; 1:100) or anti-ATG9A

immunoprecipitation was performed with 500

ER-based acetylation machinery. The AT-1 antiporter brings in acetyl-CoA from the cytosol in exchange for CoA. Newly folded proteins within the ER

For experiments, MEFs were cultured in complete media in a 37 °C humidi

frozen in DMEM supplemented with 20% FBS and 10% cell culture grade DMSO.

incubator with 5% CO2 and used up to three passages.

Western blotting. Western blotting was conducted as previously described

Liver enrichment and immunoprecipitation. Enriched liver ER was prepared as previously described using the Endoplasmic Reticulum Enrichment Extraction Kit (Novus; # NB2P-29482).

For immunoprecipitation, the total ER pellet was resuspended in kit-provided 1X suspension buffer supplemented with protease inhibitor cocktail and 1% Triton X-100. For co-immunoprecipitation, no detergent was added after the final suspension buffer. Immunoprecipitation and co-

immunoprecipitation was performed with 500–1000 μg enriched liver ER using anti-acetylated lysine (Cell Signal Technologies; #9441; 1:100) or anti-ATG9A

(abcam; #ab193338; 1:100) primary antibodies as previously described.

Subcellular fractionation. Nuclear protein was extracted from liver using the Nuclear Extraction Kit (abcam; ab113474) according to kit instructions.

Liver ER transfected with LipofectamineTM Stem Transfection Reagent (Invitrogen; #STEM00015) according to kit instructions. Cells were incubated 24–48 h before analysis.

Transduction. MEFs were grown to 60–80% confluency and transduced with BacMam 2.0 PremoTM Autophagy Sensor LC3B-GFP (Invitrogen; #PM6235) according to kit instructions. Cells were cultured for 20–24 h before imaging.

Liver ER transfected with plerixaF-XI-hygro-m-Cherry-RAMPlasmid plasmid, a gift from Jacob Corn (Addgene plasmid #118393)45, and plated on #1.5 glass coverslips. After 24–48 h, cells were processed for endogenous LC3β staining as previously described using anti-LC3β (Cell Signal Technologies; #2775; 1:200)44. Cells were imaged using a Nikon A1 confocal microscope with a 100× oil immersion lens (NA 1.4), Z-stack images (1024 × 1024 pixels at 0.12 μm/pixel with 10 z-stacks at 0.3 μm step size) were acquired using NIS-Elements AR version 5.1.1.1 software with 405 nm (blue channel), 488 nm (green channel), and 561 nm (red channel) laser wavelengths at a pinhole size of 67.7 μm. Images were analyzed on Imaris version 9.5 (Bitplane) using voxel-by-voxel co-localization of the green and red channels, and the Pearson’s correlation coefficient in the dataset volume was reported.

Live cell imaging. Liver ER transfected with pEGFP-C1, a gift from Tamotsu Yoshimori (Addgene #21073)53, and plated on a glass bottom 35 mm dish (Millipore; #PI30040). After 4 h, cells were imaged using a live cell imaging chamber (37 °C with 5% CO2) on a Nikon A1 confocal microscope using a 10× oil immersion lens (NA 0.4). Cells were transfected with TetOn-mCherry-eGFP-RAMPlasmid plasmid, a gift from Jacob Corn (Addgene plasmid #109014)44, using LipofectamineTM Stem Transfection Reagent and maintained in 4 μg/mL doxycycline (Sigma-Aldrich; #D9891). After 24 h, media was replaced with the following conditions: fed, DMEM without phenol red (Gib- coTM; #21063-029) + 10% FBS + 1% penicillin/streptomycin, starved, Erl’s buffered saline solution (EBSS; GibcoTM; #14315-063); starved with folimycin, EBSS + 100 nM folimycin (Millipore; #344085). After 4 h, cells were imaged in a live cell imaging chamber (37 °C with 5% CO2) on a Nikon A1 confocal microscope using a 10× oil immersion lens (NA 0.4). Single z-slice images (1024 × 1024 pixels; 0.12 μm/pixel with 10 z-stacks at 0.3 μm step size) were acquired using NIS-Elements AR version 5.1.1.1 software with 488 nm (green channel) and 561 nm (red channel) laser wavelengths at a pinhole size of 67.7 μm. Images were analyzed on Imaris version 9.5 (Bitplane) by performing a red – green channel subtraction and quantifying the number of puncta using a surface reconstruction.

Autophagosomes trafficking. MEFs were nucleofected with pEGFP-LC3, a gift from Tatsumi Yoshimori (Addgene #21073)53, and plated on a glass bottom 35 mm dishes (MatTek; #P35G-1.5-C) or plated and transduced with BacMam LC3B-GFP as described above. After 20–24 h, cells were imaged live using the Andor Revolution XD spinning disc microscopy system as previously described.

Histology and immunostaining. Histology and immunostaining techniques were performed as described previously44,45. For thiolavin-S staining, deparaf

minated slides were incubated for 10 min in 1% thiolavin-S (Sigma-Aldrich; #T1892-25G) dissolved in 50% ethanol. Slides were rinsed in 80% ethanol and 50%
ethanol for 1 min each, briefly rinsed in distilled water, and mounted with aqueous mounting media with DAPI (Electron Microscopy Sciences; #17985-50). Picro- Sirius red staining was performed according to the protocol (5861). Liver LipidTOX staining was performed as previously described. The following primary antibodies were used: anti-GFAP (Thermo Fisher; # P35315203; 1:1,000), anti-IBA1 (Abcam; #ab178847; 1:1,000), anti-NeuN (EMD Millipore; #ABN91MI; 1:1,000), anti-synaptophysis (abcam; #ab31227; 1:200), anti-PSD95 (Thermo Fisher; #MA1-045; 1:200), and anti-collagen I (abcam; #ab34710; 1:50). For actin immunostaining, phallolidin-TRITC (Sigma-Aldrich; # P9155-1MG; 1:1,000) was used during the secondary antibody incubation step.

Bright-field images were acquired using a Leica DM4000 B microscope with a 10× or 40× objective using Image-Pro Plus version 6.3. All fluorescent slides were imaged on a Nikon A1 confocal microscope using NIS-Elements AR version 5.1.1.0 software with 405 nm (blue channel), 488 nm (green channel), 561 nm (red channel), and 640 nm (far red) laser wavelengths. For collagen/actin-stained pancreas slides, single z-slice images (1024 × 1024 pixels; 1.2 μm/pixel) were acquired using a 20× air objective (NA = 0.75) at a pinhole size of 25.4 μm. For acetyl-CoA and Coenzyme A assays

Acetyl-CoA and CoA were generated using plasmid DNA. Nat8-pCMV6 (Origene; #MR202704) was transfected into 293T cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions using the standard curve. Acetyl-CoA concentrations were determined with a CoA assay kit (BioVision; #K808) according to the manufacturer’s instructions. Acetyl-CoA concentrations were determined with an acetyl-CoA assay kit (Abcam; #ab87546) according to the manufacturer’s instructions using the standard curve. Fluorescence was measured on a GloMax plate reader (Promega) using a green fluorescence filter (Ex = 525 nm; Em = 580–640 nm). Interpolated concentrations (in pmol/well) were converted to pmol/μL (based on adding 50 μL of sample per well) then scaled by a dilution factor calculated from the deproteinization protocol (2.5 for cytosolic fraction and 1.25 for ER fraction). Coenzyme A (CoA) concentrations were determined with a CoA assay kit (Abcam; #ab38889) according to the manufacturer’s instructions using the standard curve. Fluorescence was measured on a GloMax plate reader (Promega) using a blue fluorescence filter (Ex = 490 nm; Em = 510–570 nm). Interpolated concentrations (in μM) were scaled by a dilution factor calculated from the deproteinization protocol (125 for cytosolic fraction and 1.25 for ER fraction).

Receptor expression was quantified using the following primers via traditional PCR with Phusion

Reverse transcription quantitative PCR (RT-qPCR). RNA extraction, cDNA synthesis, and RT-qPCR was performed as previously described. For absolute quantification, the number of copies for unknown cDNA samples is reported as number of copies per ng RNA added to cDNA reaction. Primer sequences not previously reported for RT-qPCR

For determination of the changes in acetyl stoichiometry values (Δ acetyl stoichiometry) were always reported as the Atase knockout value minus the WT value. The stoichiometry of an acetylation site was considered to be 0 if the ratios were met: (1) it had measurable stoichiometry in at least 3 of 4 control samples, but not in the experimental samples, and (2) we could detect only the heavy-labeled peptide (in vitro acetylation) but no light-labeled peptide (endogenous acetylation) in at least 3 of 4 experimental samples. Subcellular localization was annotated by Uniprot, Human Protein Atlas, and GO Terms; if there were discrepancies in localization between databases, secretory pathway-associated localizations (“ER”, “Golgi”, or “secreted”) were used. Pathway analysis and network plot construction was conducted using the R package enrichplot implementing an overrepresentation analysis (ORA) with the Mus musculus organism database. Relevant images included a minimum of two images per condition per genotype (derived from independent embryos). The only exception is the survival analysis for which we have determined requires at least 40–50 animals per group. Comparison of the means was performed using two-tailed Student’s t test for two groups and ordinary one-way or two-way ANOVA for ≥3 groups followed by either Tukey–Kramer (comparison between all groups) or Dunnett’s (comparison to one control group) multiple comparisons test. Statistical test details are described in the figure legends. Grubb’s test was used to remove outliers. Differences were declared statistically significant if p < 0.05 and the following statistical significance indicators are used: *p < 0.05; **p < 0.005; ***p < 0.0005.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data included in this study are stored and maintained by the corresponding author and are available from the corresponding author upon reasonable request. Source data underlying graphs shown in figures and supplementary figures are provided in Supplementary Data 1. The proteoXomics data that support the findings of this study have been deposited to the ProteomeXchange Consortium (ID number PXD026641) and the MassIVE partner repository (ID number MSV00086712).

Code availability

The R script that was used to process the acetyl-proteomics data have been deposited on Github with the identifier (https://doi.org/10.5281/zenodo.4447491). The README file found on Github describes how the input data for the scripts can be accessed through ProteomeXchange accession code PXD026641.

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Author contributions M.J.R., A.J.L., G.K., V.C.B., and W.E.K. performed the experiments and analyzed the data. A.L. I.M.D., and L.P. provided critical advice for the experiments. L.P. designed the overall study. M.J.R. and L.P. wrote the paper with input from all authors.
Competing interests
The authors declare the following competing interests: J.M.D. is a co-founder of Galilei BioScience Inc and a consultant for Evrys Bio. Remaining authors have no competing interests to disclose.

Additional information
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