1 **Lipid flippase dysfunction as a novel therapeutic target for endosomal anomalies in**
2 **Alzheimer's disease**
3
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26 **Abstract**
27   β-amyloid precursor protein (APP) and their metabolites are deeply involved in the
28 development of Alzheimer's disease (AD). Upon the upregulation of β-site APP cleaving
29 enzyme 1 (BACE1), its product, the β-carboxyl-terminal fragment of APP (βCTF), is
30 accumulated in the early stage of sporadic AD brains. βCTF accumulation is currently
31 considered the trigger for endosomal anomalies to form enlarged endosomes, one of the
32 earliest pathologies in AD. However, the details of the underlying mechanism remain largely
33 unclear. In this study, using BACE1 stably-overexpressing cells, we describe that lipid
34 flippase subcomponent TMEM30A interacts with accumulated βCTF. Among the lipid
35 flippases in endosomes, those composed of TMEM30A and active subcomponent ATP8A1
36 transports phospholipid, phosphatidylserine (PS), to the cytosolic side of the endosomes.
37 The lipid flippase activity and cytosolic PS distribution are critical for membrane fission and
38 vesicle transport. Intriguingly, accumulated βCTF in model cells impaired lipid flippase
39 physiological formation and activity, along with endosome enlargement. Moreover, in the
40 brains of AD model mice before the amyloid-β (Aβ) deposition, the TMEM30A/βCTF complex
41 formation occurred, followed by lipid flippase dysfunction. Importantly, our novel Aβ/βCTF
42 interacting TMEM30A-derived peptide "T-RAP" improved endosome enlargement and
43 reduced βCTF levels. These T-RAP effects could result from the recovery of lipid flippase
44 activity. Therefore, we propose lipid flippase dysfunction as a key pathogenic event and a
45 novel therapeutic target for AD.

Introduction

Amyloid-β (Aβ) peptides are accumulated in the brains of patients with Alzheimer's disease (AD)[1] and are produced from the sequential cleavage of the β-amyloid precursor protein (APP) by the β-site APP cleaving enzyme 1 (BACE1)[2,3,4] and γ-secretase[5]. Although pieces of genetic and biological evidence suggest the link between Aβ and AD pathogenesis, several clinical trials based on the "amyloid hypothesis" have failed[6]. Therefore, new ideas and therapeutic targets that complement the hypothesis are required for this field.

A recent report identified that traffic impairment, showing endosomal anomalies, is an early pathogenic event in AD before Aβ deposition[7]. In line with this, several studies in AD model mice[8], human iPSC-derived neurons[9], and AD brains[10] have shown that the accumulated β-carboxyl-terminal fragment of APP (βCTF), the product of BACE1 and direct precursor of Aβ, is the cause of endosomal anomalies. Indeed, BACE1 expression and activity are upregulated at the early stage of sporadic AD[11,12]. However, the details of the mechanism underlying the βCTF mediated endosomal anomalies remain unclear.

Previously, we identified TMEM30A (CDC50A), a subcomponent of lipid flippases, as a candidate partner for βCTF. This complex mediates the formation of enlarged endosomes[13]. Most lipid flippases consist of TMEM30A and active subcomponents, P4-ATPases. These enzymes translocate phospholipids from the exoplasmic/luminal side to the cytoplasmic leaflet of the lipid bilayer to regulate phospholipids asymmetry[14].

Phosphatidylserine (PS), one of the phospholipids, is a component of lipid bilayer and mainly localizes at the cytoplasmic leaflet[14]. PS level on the cytosolic side in endosomes determines the recruitment of PS-binding proteins to trigger membrane budding, which promotes subsequent vesicle fission and transport[15,16]. This PS asymmetry is regulated by one of the endosomal lipid flippases, those composed of TMEM30A and active subcomponent ATP8A1, which has a high affinity for PS[14]. These lines of evidence indicate that lipid flippase activity is essential for vesicular trafficking.

In this study, we investigated whether lipid flippase activity contributes to the βCTF-mediated endosomal anomalies and could be a novel therapeutic target for AD treatment.

Results

BACE1 upregulation promotes complex formation between endogenous TMEM30A and accumulated βCTF, and endosomal anomalies

To gain insight into the early pathology of AD, we established BACE1 stably-overexpressing SH-SY5Y cell lines (SH-BACE1 cells). BACE1 produces β1 or β11CTF depending on APP cleavage sites[17]. BACE1 upregulation increased the β-secretase products sAPPβ, βCTF, and Aβ, although it severely reduced α-secretase-cleaved carboxyl-terminal fragment (αCTF), in good agreement with previous reports[18] (Fig. 1A and Supplementary Fig. 1A). We previously demonstrated that TMEM30A is a candidate partner for βCTF (β1/β11CTF)-mediated endosomal anomalies[13]. Supportively, CFP-TMEM30A, SC100[19], and SC89 (artificial β1 and β11CTF) co-transfection revealed that TMEM30A could interact with these βCTF (Supplementary Fig. 1B). Although βCTF in non-treated SH-BACE1 cells might deserve further analysis, we found that endogenous βCTF exhibited resistance to mild detergents such as CHAPS (Supplementary Fig. 1C and D). Indeed, βCTF insolubility in mild detergents was reported elsewhere[20]. Our result suggests that the environment surrounding βCTF is particularly unique. Therefore, to verify whether endogenous TMEM30A interacts with accumulated βCTF in SH-BACE1 cells, we treated γ-secretase inhibitor DAPT to accumulate APP-CTF, then performed co-immunoprecipitation analysis. In line with our previous findings, we observed an interaction between the endogenous TMEM30A and accumulated βCTF, but not αCTF, and the BACE1 inhibitor co-treatment abolished this interaction (Fig. 1B).

Next, a stepwise iodixanol gradient organelle fractionation was performed as described previously[21] to analyze the protein distributions. The distribution of LAMP2 (lysosome marker) and calreticulin (ER marker) showed no obvious change between SH-control and SH-BACE1 cells (Supplementary Fig. 2A). In contrast, Rab5A, an early endosome marker,

100 was distributed in fractions (Fr) 7~12 in SH-control cells (Fig. 1C) and its distribution was
101 broadened to the heavier fractions (Fr 14~16) in SH-BACE1 cells. Similarly, βCTF, enriched
102 in Fr 6~9 in SH-control cells, changed its distribution in the heavier fractions (Fr 14~16) in
103 SH-BACE1 cells (Fig. 1C). Concomitantly, BACE1 was distributed in Fr 14~16 in SH-BACE1
104 cells (Supplementary Fig. 2A). Intriguingly, TMEM30A was mainly distributed in Fr 7~12 in
105 SH-control cells. However, in SH-BACE1 cells, its distribution drastically changed to
106 Fr 14~16 where Rab5, βCTF, and BACE1 abnormally co-localized (Fig. 1C). We observed
107 no alteration in the TMEM30A and Rab5A protein levels between SH-control and SH-BACE1
108 cells (Fig. 1A and Supplementary Fig. 2B).
109   Next, we performed immunofluorescence analysis using BACE1 stably-overexpressing
110 COS-7 cells (COS-BACE1), well-characterized in organelle morphology observations[13,22,23].
111 In contrast to the lack of alteration in the Rab5A protein level (Supplementary Fig. 2C), the
112 mean of the Rab5A-positive puncta area and the frequency of size distribution ($\geq 0.1\ \mu m^2$)
113 were significantly increased in COS-BACE1 cells (Fig. 1D, E, and Supplementary Fig. 2D).
114   Our findings suggest that BACE1 increases βCTF level, which interacts with TMEM30A
115 and mediates endosomal anomalies.
116
117 **βCTF accumulation triggers the lipid flippase activity impairment**
118   TMEM30A interacts with active subunits, P4-ATPases, to form lipid flippase, and
119 contributes to stability, distribution, and activity of P4-ATPases[14,24]. Therefore, we
120 hypothesized that lipid flippase activity is associated with TMEM30A/βCTF-mediated
121 endosomal anomalies.
122   Immunoprecipitation analysis in the membrane fractions revealed that the physiological
123 complex formation between TMEM30A and ATP8A1, an endosomal P4-ATPase which has
124 a high affinity for PS, significantly decreased in SH-BACE1 cells. The BACE1 inhibitor
125 treatment recovered this interaction in SH-BACE1 cells
126 (Fig. 2A, B, and Supplementary Fig. 3A), indicating that this deficit is BACE1 activity-
127 dependent.
128   Since it is difficult to evaluate lipid flippase activity in organelles such as endosomes, we
129 attempted to exploit the endosomal PS binding property of Evectin2, a promoting factor of
130 membrane fission in vesicle transport to analyze the lipid flippase activity. Evectin-2 has a
131 PS-specific pleckstrin-homology (PH) lipid-binding domain, and its association with the
132 endosomal membrane depends on lipid flippase activity[23,25]. As previously reported[23], the
133 predominant distribution of Evectin-2 in the membrane fractions was abolished by TMEM30A
134 knockdown (Fig. 2C, D, and Supplementary Fig. 3B). Intriguingly, Evectin-2 dissociated from
135 the membrane fractions in SH-BACE1 cells and got redistributed upon the BACE1 inhibitor
136 treatment without altering total Evectin-2 level (Fig. 2E and F).
137   To further quantify Evectin-2 localization in the endosomes, we used a NanoBiT
138 luciferase reconstitution system. Large BiT (LgBiT) and Small BiT (SmBiT) are the parts of
139 the *Oplophorus gracilirostris*-derived Nanoluc luciferase. As SmBiT has a low affinity
140 (Kd = 190 μM) to the LgBiT, their interaction is fragile and reversible, and the luciferase
141 activity is not reconstituted without their forced proximity by fused proteins[26]. We fused these
142 subunits to Rab5A (LgBiT-Rab5) and Evectin-2 (SmBiT-Evectin-2) to monitor the Evectin-2
143 localization in the endosomes (Fig. 3A). Immunofluorescence analysis showed that LgBiT-
144 Rab5 localized in the endosomes (Supplementary Fig. 4A). Moreover, similar to our
145 biochemical analysis (Fig. 2C and D), TMEM30A knockdown significantly decreased the
146 luciferase activity (Fig. 3B, C and Supplementary Fig. 4B). For further validation, we
147 investigated the effect of Evectin-2 mutant on the PH domain (K20E), lacking the binding
148 affinity to PS[23]. We observed significantly reduced luciferase activity in the K20E mutant
149 (Supplementary Fig. 4C and D). These results clearly show that our reporter system could
150 be used for semi-quantitative estimation of lipid flippase activity in endosomes.
151   Next, we applied this system to SH-BACE1 cells. Intriguingly, we observed a significant
152 reduction in the lipid flippase activity, rescued by the BACE1 inhibitor treatment (Fig. 3D and
153 E). To confirm that the BACE1-mediated APP cleavage is a prerequisite for this event, we

154 performed APP knockdown in SH-BACE1 cells and detected lipid flippase activity recovery
155 (Fig. 3F, G and Supplementary Fig. 5A-C). Interestingly, the APP knockdown failed to affect
156 the lipid flippase activity in SH-control cells (Supplementary Fig. 5D). Furthermore, lipid
157 flippase activity significantly decreased in SC100 stably overexpressing SH-SY5Y cells
158 (Fig. 3H and I).
159 Our data strongly support the hypothesis that βCTF accumulation leads to lipid flippase
160 dysfunction via abnormal complex formation with TMEM30A.
161
162 **TMEM30A/βCTF complex formation and subsequent lipid flippase dysfunction**
163 **precede Aβ deposition in AD model mice**
164 Next, we explored the TMEM30A/βCTF complex formation and lipid flippase dysfunction
165 at the early stage in AD model mice. As A7 model mice show a relatively slow Aβ deposition,
166 starting at approximately 9 months of age, we used these AD model mice as an appropriate
167 model to observe the precursory phenomenon[27]. At 3 and 6 months, the ATP8A1, TMEM30A,
168 and Evectin-2 protein levels were not significantly different between the WT and transgenic
169 (Tg) mice (Supplementary Fig. 6A and B). Moreover, we observed no significant difference
170 in the αCTF and βCTF levels between the 3- and 6-month-old Tg mice
171 (Supplementary Fig. 6A and B). Intriguingly, TMEM30A interacted with βCTF in both 3- and
172 6-month-old model mice (Fig. 6A and B). However, TMEM30A failed to interact with Aβ
173 oligomers, previously described as another factor in vesicular traffic impairment[28], and with
174 Aβ monomers (Supplementary Fig. 7). Importantly, both lipid flippase formation
175 (Fig. 4A and C) and Evectin-2 localization in the membrane fractions (Fig. 4A and D)
176 significantly decreased in 6-month-old A7 mice. We confirmed that the indicated band is
177 Evectin-2 (Supplementary Fig. 6C). Our results suggest that the TMEM30A/βCTF complex
178 induces lipid flippase dysfunction, which precedes Aβ deposition.
179
180 **βCTF/Aβ interactive peptide "T-RAP" improves endosome enlargement**
181 We expected that the inhibition of the interaction between TMEM30A and βCTF could
182 improve lipid flippase dysfunction and endosomal anomalies. Previously, we identified that
183 the extracellular domain of TMEM30A (TmEx) interacts with the Aβ sequence of βCTF[13]. We
184 explored the interacting domain by sequential deletion of TmEx using GST-pulldown assay
185 (Fig. 5A-C) and found that the 117–166 AA region contains the critical residues for the
186 interaction with βCTF (Fig. 5B-D). Using RaptorX prediction (http://raptorx.uchicago.edu/),
187 we noticed that the conformation of the α-helices and β-sheet exists in the well-conserved
188 125–150 AA region. We named this sequence "T-RAP" (Tmem30A related amyloid-
189 beta interacting peptide) (Fig. 5A). We verified that GST-fused T-RAP efficiently pulled down
190 βCTF and Aβ, but not αCTF (Fig. 5B, D and Supplementary Fig. 8), indicating T-RAP has a
191 high affinity for Aβ N-terminal sequence.
192 To assess whether the synthetic T-RAP peptide influences lipid flippase activity, we
193 treated SH-SY5Y cells with T-RAP and performed a flippase activity assay. In advance, we
194 confirmed that T-RAP displayed no toxicity at the density used in this study
195 (Supplementary Fig. 9A). Although not significant, T-RAP showed a trend to improve lipid
196 flippase activity in SH-BACE1 cells (Fig. 6A and B). On the other hand, T-RAP (50 μM) fused
197 with the Trans-Activator of Transcription Protein (TAT) which is easily introduced into cells
198 significantly rescued the lipid flippase activity in SH-BACE1 cells (Supplementary Fig. 9B).
199 We consider that TAT-T-RAP could penetrate membranes more efficiently than T-RAP.
200 As lipid flippase dysfunction is βCTF level-dependent, we investigated the involvement
201 of T-RAP in APP metabolism. After a 48 h T-RAP peptide treatment, the sAPPβ and βCTF
202 levels significantly decreased without altering the full-length APP and TMEM30A protein
203 levels (Fig. 6C and D). Next, we analyzed the T-RAP effect on endosomal morphology in
204 COS-7 cells. Importantly, T-RAP significantly decreased the mean of the Rab5A-positive
205 puncta area in COS-BACE1 cells without altering the total Rab5A protein level
206 (Fig. 6E, F, and Supplementary Fig. 9C). These lines of evidence explain that T-RAP peptide
207 improves endosomal anomalies by rescuing the lipid flippase activity.

Discussion

βCTF-mediated endosomal anomalies in forming enlarged endosomes are considered early AD pathogenic events[10]. In this study, we showed that lipid flippase activity in endosomes decreased by elevated βCTF level and it contributes to endosomal anomalies. Moreover, in the AD model mice brain, age-dependent lipid flippase dysfunction occurs before Aβ deposition, supporting their strong link with the early pathogenic event. Importantly, we found that a novel βCTF/Aβ-interacting peptide "T-RAP" could recover the lipid flippase activity and endosomal anomalies. Therefore, our findings suggest that lipid flippase impairment is a driving mechanism of βCTF-mediated endosomal anomalies and present a novel therapeutic strategy for AD treatment.

Endosomal anomalies are the signature for vesicular traffic impairment and could be found in the early phase of AD and Down syndrome (DS) prior to Aβ deposition[7]. As DS displays trisomy on chromosome 21, upregulating APP expression, many studies have focused on APP metabolites toxicity. Among these metabolites, the BACE1 product βCTF is considered the driver for endosomal anomalies. Supportively, βCTF accumulation is observed in AD brains[10], concomitant with upregulated BACE1 expression and activity in AD brains[12]. Several studies using human APP/Presenilin-1 familial AD mutant knock-in iPSC-derived neurons[9] or 3xTg-AD model mice[8] have shown that endosome enlargement depends on elevated βCTF but not Aβ. Moreover, analysis of DS fibroblasts or Ts65Dn model mice has demonstrated that accumulated βCTF induces endosome enlargement[29]. However, the underlying mechanisms are not fully explored.

To model the early pathogenic event of AD, we established a BACE1 stably overexpressing neuroblast cell line (SH-BACE1) and observed an increase in BACE1 products, β1CTF (C99) and β11CTF (C89) (Fig. 1A). Interestingly, we found that a subcomponent of lipid flippase, TMEM30A, is the interacting partner for these βCTF (Fig. 1B and Supplementary Fig. 1B). Concomitantly, our organelle fractionation analysis showed a broadened distribution of the endosomal marker protein Rab5A to heavier fractions (Fr 14~16) in SH-BACE1 cells compared with normal endosome fractions (Fr 7~12) in control cells. Moreover, TMEM30A, βCTF, and BACE1 were co-distributed in these abnormal heavier fractions (Fig. 1C and Supplementary Fig. 2A). We hypothesize the vicious cycle, in which increased βCTF triggers the interaction with TMEM30A to promote endosomal anomalies, and then the abnormal distribution of BACE1 further promotes βCTF production. In connection with these results, immunofluorescence analysis using COS-7 cells showed the formation of enlarged endosomes in COS-BACE1 cells (Fig. 1D and E). Supportively, our previous study showed that βCTF co-localized with TMEM30A in enlarged endosomes in COS-7 cells[13]. Another study proposed that intracellular Aβ, such as Aβ oligomers, cause vesicular traffic imparment[28]. However, we failed to detect the interaction between TMEM30A and Aβ monomers or oligomers in A7 mice (Supplementary Fig. 7). Although we cannot fully rule out the possibility of the Aβ oligomer involvement in a more advanced stage of AD, our data indicate that the major partner of TMEM30A, which mediated endosome enlargement, is βCTF.

There are two possible mechanisms underlying the TMEM30A/βCTF complex-mediated endosome enlargement. The first is lipid flippase dysfunction and the second is Rab5 overactivation.

Most lipid flippases consist of TMEM30A and active subcomponents, P4-ATPases[30,31], and regulate phospholipid asymmetry in the lipid bilayer[14]. TMEM30A regulates proper cellular localization and activity of partners, P4-ATPases[24,32]. Intriguingly, we showed the complex formation of TMEM30A and ATP8A1, a brain-enriched endosomal P4-ATPase[31], decreased in SH-BACE1 cells, and the inhibition of the BACE1 activity recovered the physiological complex formation (Fig. 2A and B). Our data suggest the hypothesis that upregulated BACE1 activity accumulates βCTF, which interacts with TMEM30A to interrupt the complex formation between TMEM30A and ATP8A1. Indeed, the age-dependent disruption of this lipid flippase formation was followed by TMEM30A/βCTF complex formation

262   in AD model mice (Fig. 4A-C). We consider that the TMEM30A/βCTF complex further
263   accumulates βCTF in a vicious cycle, disrupting lipid flippase formation. Supporting this idea,
264   TMEM30A overexpression in COS-7 cells accumulates βCTF, concomitantly with the
265   complex formation of TMEM30A and βCTF[13].

266   Endosomal lipid flippases transport phospholipids, like PS, to the cytosolic leaflet[14]. PS
267   on the cytosolic side in endosomes recruits PS-binding proteins, such as Evectin-2, to
268   promote membrane fission and trigger vesicle transport[15,16]. In this study, we focused on
269   Evectin-2 distribution in the endosomes to indirectly monitor lipid flippase activity. We clarified
270   that Evectin-2 distribution decreased depending on the increased βCTF level in SH-BACE1
271   cells using biochemical analysis (Fig. 2) and the split-luciferase system (Fig. 3). These
272   findings suggest that accumulated βCTF decreases the lipid flippase activity in endosomes
273   to reduce PS level on the cytosolic side. Moreover, the AD model mice analysis indicated
274   decreased Evectin-2 distribution in the membrane fractions (Fig. 4A and D). Indeed, the
275   ATP8A1 knockdown induces endosome-mediated membrane traffic defects[22]. Therefore, we
276   propose the hypothesis that the TMEM30A/βCTF complex impairs lipid flippase formation
277   and its activity to develop endosomal anomalies. It is important to note that our split-luciferase
278   system can be applied to various organelle markers to estimate the localization of proteins
279   and may be developed into a semi-quantitative method for measuring vesicular trafficking.
280   Further validation is needed as a future study.

281   The second possible mechanism is Rab5 activation. Previous reports, using Rab5A
282   constitutively active mutant (Q79L)[33] and overexpressing mice[34], showed Rab5 positive
283   enlarged endosomes. Additionally, elevated βCTF can form a complex with APPL1, a Rab5
284   effector protein, which mediates Rab5 activation and endosome enlargement in DS
285   fibroblasts and AD brains[10]. Consistent with these reports, we observed the upregulation of
286   BACE1 mediated the abnormal co-distribution of Rab5A and βCTF to alter Rab5A-positive
287   endosomal morphology (Fig. 1C-E). Therefore, accelerated βCTF accumulation by its
288   complex formation with TMEM30A might activate Rab5 to induce endosome enlargement. It
289   would deserve further investigation that lipid flippase dysfunction contributes to Rab5
290   activation-dependent or independent endosomal anomalies.

291   Questions still remain concerning how lipid flippase-mediated endosomal anomalies
292   could contribute to AD pathogenesis. Previously, APP-dependent endosomal anomalies and
293   the traffic impairment of nerve growth factor (NGF), which resulted in neuronal atrophy, were
294   observed in rodent neuron models[29]. Another study demonstrated that TMEM30A deficiency
295   influenced sAPPβ and βCTF levels, as well as Aβ/p3 production via the increased β/α-
296   secretase processing of APP[35]. Therefore, lipid flippase dysfunction possibly mediates APP
297   metabolic changes or vesicle transport deficit such as NGF to contribute to AD pathogenesis.

298   Since vesicular traffic impairment has been implicated as the contributor of AD pathology,
299   βCTF-mediated endosomal anomalies might be promising therapeutic targets for treating AD.
300   The BACE1 inhibitor might be a candidate. According to this notion, β-secretase inhibitor IV
301   recovered lipid flippase function (Fig. 2E, F, and Fig. 3E). However, the study of BACE1
302   knockout mice showed unexpected neuronal phenotypes, such as schizophrenia
303   endophenotypes or spine density reduction, originating from abrogated β-secretase
304   processing of different substrates such as neuregulin 1 (NRG1)[36,37]. Further therapeutic
305   candidates are pharmacological chaperons to stabilize retromers and limit APP processing
306   in the endosomes by enhancing vesicle transport[38]. There is also a concern that the
307   compound displays a too wide effect range and side effects[39]. These lines of evidence remind
308   us to develop more specific therapeutic targets reflecting AD pathology.

309   We observed a progressive decline of lipid flippase function in AD model mice
310   (Fig. 4A and D). Moreover, a genome-wide association study identified AD risk variants in
311   one of the P4-ATPases, *ATP8B4*[40]. Therefore, lipid flippase dysfunction could be associated
312   with AD pathogenesis and the optimal target based on AD pathology. Intriguingly, we
313   identified a TMEM30A-derived peptide, "T-RAP," specifically interacting βCTF and Aβ. T-
314   RAP trended to rescue the lipid flippase activity and improved endosome enlargement in
315   BACE1 stably overexpressing cells (Fig. 6B, E, F, and Supplementary Fig. 9B). We

316 hypothesize that T-RAP enfolds βCTF to inhibit the interaction with TMEM30A, then recovers
317 the lipid flippase physiological formation and activity. This functional lipid flippase recovery
318 could prevent βCTF-mediated endosomal anomalies, enhancing vesicle transport (Fig. 7). T-
319 RAP also decreased sAPPβ and βCTF levels (Fig. 6C and D), which means the peptide
320 corrects vesicle transport to normalize APP processing. Another possibility is that T-RAP
321 directly binds to the proximal site of BACE1 cleavage of APP. Moreover, T-RAP is hydrophilic
322 and easy to handle biochemically. Although further T-RAP specificity analyses would be
323 required, we propose that T-RAP and related molecules might be the optimal candidates for
324 AD therapeutics.
325 Three main problems could be distinguished in AD treatment from the perspective of
326 vesicular traffic impairment. First, the details of the mechanisms underlying the endosomal
327 anomalies were unclear. Second, the effective measurement of vesicular trafficking has not
328 been established. Third, endosomal anomaly-related drug treatment has not yet been
329 developed. In this study, we propose lipid flippase dysfunction as a mechanistic contributor
330 for βCTF-mediated endosomal anomalies. Next, our split-luciferase system for measuring
331 endosomal lipid flippase activity could be used for the development of quantitative vesicle
332 transport measurements. Finally, we identified a novel therapeutic candidate for βCTF-
333 mediated endosomal anomalies. Therefore, we present new insights into AD treatment for
334 targeting the early pathogenesis, endosomal anomalies.
335

336 **Methods**
337 **Usage of mouse brains**
338 APP transgenic mice (A7 line) have been previously generated[27] and genotyped using
339 specific primers[41]. All mice were kept under specific pathogen-free conditions and fed a
340 regular diet (Oriental Yeast). The animal care and use procedures were approved by the
341 Institutional Animal Care and Use Committees of the University of Tokyo (18-P-108).
342

343 **Statistical analysis**
344 All experimental data are expressed as the mean ± SEM. The experiments were analyzed
345 with two-tailed Student's t-test or one-way ANOVA with Bonferroni's multiple comparisons
346 test using the GraphPad Prism8 software. The statistical significance is indicated as follows:
347 $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.
348

349 **Supplementary information**
350 Detailed information of the materials and methods, original western blot data of Fig. 1-5, as
351 well as the supplementary figures are available in Supplementary information file.
352

360

361 **Author contributions**
362 N.K., and N.T., designed the research; N.K., R.I., M.K., A.I., and N.T. performed the
363 experiments and analyzed data; T.H. and T.S. prepared animal samples and provided
364 antibodies, respectively; N.K., and N.T., wrote the paper; Y.K., T.H., T.I., T.S., and T.U.
365 provided advice and helped write the article. All authors read and approved the final
366 manuscript.
367

368 **Conflict of interest**
369 The authors declare that they have no conflict of interest.

370

371 **References**

372 1. Hardy, J. & Selkoe, D. J. the Amyloid Hypothesis of Alzheimer 's Disease: Progress and
373   Problems on the. *Science* **297**, 353–6 (2002).
374 2. Vassar, R. *et al.* β-Secretase cleavage of Alzheimer's amyloid precursor protein by the
375   transmembrane aspartic protease BACE. *Science* **286**, 735–741 (1999).
376 3. Fairbanks, M. *et al.* Purification and cloning of amyloid precursor protein β-secretase
377   from human brain. *Nature* **705**, 537–540 (1999).
378 4. Yan, R. *et al.* Membrane-anchored aspartyl protease with Alzheimer's disease β-
379   secretase activity. *Cereb. Cortex* **2558**, 533–537 (1999).
380 5. Takasugi, N. *et al.* The role of presenilin cofactors in the γ-secratase complex. *Nature*
381   **422**, 438–441 (2003).
382 6. Huang, L. K., Chao, S. P. & Hu, C. J. Clinical trials of new drugs for Alzheimer disease.
383   *J. Biomed. Sci.* **27**, 1–13 (2020).
384 7. Cataldo, A. M. *et al.* Endocytic pathway abnormalities precede amyloid β deposition in
385   sporadic alzheimer's disease and down syndrome: Differential effects of APOE genotype
386   and presenilin mutations. *Am. J. Pathol.* **157**, 277–286 (2000).
387 8. Lauritzen, I. *et al.* The β-secretase-derived C-terminal fragment of βAPP, C99, but not
388   Aβ, is a key contributor to early intraneuronal lesions in triple-transgenic mouse
389   hippocampus. *J. Neurosci.* **32**, 16243–16255 (2012).
390 9. Kwart, D. *et al.* A Large Panel of Isogenic APP and PSEN1 Mutant Human iPSC
391   Neurons Reveals Shared Endosomal Abnormalities Mediated by APP β-CTFs, Not Aβ.
392   *Neuron* **104**, 256-270.e5 (2019).
393 10. Kim, S. *et al.* Evidence that the rab5 effector APPL1 mediates APP-βCTF-induced
394   dysfunction of endosomes in Down syndrome and Alzheimer's disease. *Mol. Psychiatry*
395   **21**, 707–716 (2016).
396 11. Zetterberg, H. *et al.* Elevated cerebrospinal fluid BACE1 activity in incipient alzheimer
397   disease. *Arch. Neurol.* **65**, 1102–1107 (2008).
398 12. Ahmed, R. R. *et al.* BACE1 and BACE2 enzymatic activities in Alzheimer's disease. *J.*
399   *Neurochem.* **112**, 1045–1053 (2010).
400 13. Takasugi, N. *et al.* TMEM30A is a candidate interacting partner for the β-carboxyl-
401   terminal fragment of amyloid-β precursor protein in endosomes. *PLoS One* (2018).
402 14. Andersen, J. P. *et al.* P4-ATPases as Phospholipid Flippases—Structure, Function, and
403   Enigmas. *Front. Physiol.* **7**, 275 (2016).
404 15. Leventis, P. A. & Grinstein, S. The distribution and function of phosphatidylserine in
405   cellular membranes. *Annual Review of Biophysics* vol. 39 407–427 (2010).
406 16. Varga, K., Jiang, Z. J. & Gong, L. W. Phosphatidylserine is critical for vesicle fission
407   during clathrin-mediated endocytosis. *J. Neurochem.* **152**, 48–60 (2020).
408 17. Deng, Y. *et al.* Amyloid-β protein (Aβ) Glu11 is the major β-secretase site of β-site
409   amyloid-β precursor protein-cleaving enzyme 1(BACE1), and shifting the cleavage site to
410   Aβ Asp1 contributes to Alzheimer pathogenesis. *Eur. J. Neurosci.* **37**, 1962–1969
411   (2013).
412 18. Vetrivel, K. S. *et al.* Loss of cleavage at β′-site contributes to apparent increase in β-
413   amyloid peptide (Aβ) secretion by β-secretase (BACE1)-glycosylphosphatidylinositol
414   (GPI) processing of amyloid precursor protein. *J. Biol. Chem.* **286**, 26166–26177 (2011).
415 19. Iwata, H., Tomita, T., Maruyama, K. & Iwatsubo, T. Subcellular Compartment and
416   Molecular Subdomain of β-Amyloid Precursor Protein Relevant to the Aβ42-promoting
417   Effects of Alzheimer Mutant Presenilin 2. *J. Biol. Chem.* **276**, 21678–21685 (2001).
418 20. Sakurai, T. *et al.* Membrane microdomain switching: A regulatory mechanism of amyloid
419   precursor protein processing. *J. Cell Biol.* **183**, 339–352 (2008).
420 21. Lee, M. S. *et al.* APP processing is regulated by cytoplasmic phosphorylation. *J. Cell*
421   *Biol.* **163**, 83–95 (2003).
422 22. Lee, S. *et al.* Transport through recycling endosomes requires EHD 1 recruitment by a
423   phosphatidylserine translocase. *EMBO J.* **34**, 669–688 (2015).
424 23. Matsudaira, T. *et al.* Endosomal phosphatidylserine is critical for the YAP signalling
425   pathway in proliferating cells. *Nat. Commun.* **8**, (2017).
426 24. Coleman, J. a & Molday, R. S. Critical Role of the β-Subunit CDC50A in the Stable

Expression, Assembly, Subcellular Localization, and Lipid Transport Activity of the P4-ATPase ATP8A2. *J. Biol. Chem.* **286**, 17205–17216 (2011).

25. Lee, S. *et al.* Impaired retrograde membrane traffic through endosomes in a mutant CHO cell defective in phosphatidylserine synthesis. *Genes to Cells* **17**, 728–736 (2012).

26. Dixon, A. S. *et al.* NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chem. Biol.* **11**, 400–408 (2016).

27. Yamada, K. *et al.* Aβ immunotherapy: Intracerebral sequestration of Aβ by an anti-Aβ monoclonal antibody 266 with high affinity to soluble Aβ. *J. Neurosci.* **29**, 11393–11398 (2009).

28. Umeda, T. *et al.* Intracellular amyloid β oligomers impair organelle transport and induce dendritic spine loss in primary neurons. *Acta Neuropathol. Commun.* **3**, 51 (2015).

29. Xu, W. *et al.* Amyloid precursor protein-mediated endocytic pathway disruption induces axonal dysfunction and neurodegeneration. *J. Clin. Invest.* **126**, 1815–1833 (2016).

30. Bryde, S. *et al.* CDC50 proteins are critical components of the human class-1 P 4-ATPase transport machinery. *J. Biol. Chem.* **285**, 40562–40572 (2010).

31. Wang, J. *et al.* Proteomic Analysis and Functional Characterization of P4-ATPase Phospholipid Flippases from Murine Tissues. *Sci. Rep.* **8**, 10795 (2018).

32. Van Der Velden, L. M. *et al.* Heteromeric interactions required for abundance and subcellular localization of human CDC50 proteins and class 1 P4-ATPases. *J. Biol. Chem.* **285**, 40088–40096 (2010).

33. Wegener, C. S. *et al.* Ultrastructural characterization of giant endosomes induced by GTPase-deficient Rab5. *Histochem. Cell Biol.* **133**, 41–55 (2010).

34. Pensalfini, A. *et al.* Endosomal Dysfunction Induced by Directly Overactivating Rab5 Recapitulates Prodromal and Neurodegenerative Features of Alzheimer's Disease. *Cell Rep.* **33**, 108420 (2020).

35. Tambini, M. D. & D'Adamio, L. Loss of CDC50A function drives Aβ/p3 production via increased β/α-secretase processing of APP. *bioRxiv* (2020). doi:10.1101/2020.11.12.379636.

36. Vassar, R. BACE1 inhibitor drugs in clinical trials for Alzheimer's disease. *Alzheimer's Res. Ther.* **6**, 1–14 (2014).

37. Savonenko, A. V *et al.* Alteration of BACE1-dependent NRG1/ErbB4 signaling and schizophrenia-like phenotypes in BACE1-null mice. **105**, 10–15 (2008).

38. Mecozzi, V. J. *et al.* Pharmacological chaperones stabilize retromer to limit APP processing. *Nat. Chem. Biol.* **10**, 443–449 (2014).

39. Berman, D. E., Ringe, D., Petsko, G. A. & Small, S. A. The Use of Pharmacological Retromer Chaperones in Alzheimer's Disease and other Endosomal-related Disorders. *Neurotherapeutics* **12**, 12–18 (2015).

40. Holstege, H., Hulsman, M., Charbonnier, C. & Grenier-boley, B. Exome sequencing identifies novel AD-associated Abstract Background: With the development of next-generation sequencing technologies. *medRxiv* (2020). doi:10.1101/2020.07.22.20159251.

41. Hashimoto, T. *et al.* Collagenous Alzheimer amyloid plaque component impacts on the compaction of amyloid-β plaques. *Acta Neuropathol. Commun.* **8**, (2020).

Figure 1. BACE1 upregulation induces the complex formation between TMEM30A and βCTF and endosomal anomalies. (**A**) Immunoblotting analysis for APP metabolites and TMEM30A in SH-control and SH-BACE1 cells. β-secretase inhibitor IV (10 μM) was treated to SH-BACE1 cells. (**B**) Co-immunoprecipitation analysis using TMEM30A antibody in the accumulation of βCTF by the DAPT (10 μM) treatment or co-treatment with β-secretase inhibitor IV (10 μM) for 24 h. (**C**) Iodixanol gradient fractionation of the homogenates from SH-control and SH-BACE1 cells. For the endosome marker, Rab5A was used. (**D**) COS-control and COS-BACE1 cells were immunostained for Rab5A (Red). DAPI stained the nucleus (Blue). Scale bars: 10 μm. Representative z-stack images were captured using a 60x objective lens (zoom x2.6). (**E**) Quantitation of size distribution (≥ 0.1 μm^2) and mean area of Rab5A-positive puncta (*n*=3, mean ± SEM, two-tailed Student's t-test, *P<0.05, **P<0.01).

Figure 2. BACE1 upregulation induces lipid flippase dysfunction depending on the BACE1 activity. (**A**) The membrane fractions from SH-control and SH-BACE1 cells applied to co-immunoprecipitation analysis using TMEM30A antibody. Cells were treated with the β-secretase inhibitor IV (10 μM) for 48 h. Calreticulin was used as a loading control of membrane fractions. (**B**) Quantification of ATP8A1 co-immunoprecipitated by TMEM30A antibody in Fig. 2A (*n*=3, mean ± SEM, one-way ANOVA Bonferroni's multiple comparisons test, *P<0.05, **P<0.01). (**C**) Immunoblotting analysis for Evectin-2 in total cell lysates and membrane fractions (MF) in the knockdown of TMEM30A in SH-SY5Y cells. (**D**) Quantification of Evectin-2 localization in whole cell lysates or MF (*n*=3, mean ± SEM, two-tailed Student's t-test, *P<0.05). (**E**) Immunoblotting analysis for Evectin-2 in total cell lysates and MF in the treatment of the β-secretase inhibitor IV (10 μM) for 48 h. (**F**) Quantification of the Evectin-2 localization in total lysates or MF (*n*=3, mean ± SEM, one-way ANOVA Bonferroni's multiple comparisons test, *P<0.05, ** *P*<0.01).

Figure 3. The semi-quantitative analysis shows the lipid flippase activity in endosomes is decreased depending on the levels of βCTFs. (**A**) Schematic view of the measurement methods for endosomal lipid flippase activity using a split-luciferase assay system. Luciferase subunits, LgBiT or SmBiT, were fused with Rab5A or Evectin-2, respectively. The reconstitution of luciferase activity depends on the endosomal distribution of Evectin-2, which reflects the endosomal lipid flippase activity (Fig.2). (**B**) Immunoblotting analysis for LgBiT-Rab5, SmBiT-Evectin-2, and TMEM30A using HA, FLAG, and TMEM30A antibodies in the knockdown of TMEM30A for 72 h in SH-SY5Y cells. (**C**) Quantification of the luciferase activity in the knockdown of TMEM30A for 72 h in SH-SY5Y cells (*n*=3, mean ± SEM, two-tailed Student's t-test, *P<0.05). (**D**) Immunoblotting analysis for LgBiT-Rab5, SmBiT-Evectin-2, and APP-CTF in the treatment of β-secretase inhibitor IV (10 μM) for 48 h. (**E**) Quantification of the luciferase activity for treating β-secretase inhibitor IV (10 μM) for 48 h (*n*=3, mean ± SEM, one-way ANOVA Bonferroni's multiple comparisons test, *P<0.05, ****P<0.0001). (**F**) Immunoblotting analysis for LgBiT-Rab5, SmBiT-Evectin-2, APP, and APP-CTF in the knockdown of APP for 72 h. (**G**) Quantification of the luciferase activity in the knockdown of APP for 72 h (*n*=4, mean ± SEM, one-way ANOVA Bonferroni's multiple comparisons test, *P<0.05). (**H**) Immunoblotting analysis for SC100, LgBiT-Rab5, and SmBiT-Evetin-2. P.C is the control for SC100 and αCTF. (**I**) Quantification of the luciferase activity in SH-control and SH-SC100 cells 48 h after the transfection of luciferase subunits (*n*=3, mean ± SEM, two-tailed Student's t-test, **P<0.01).

Figure 4. TMEM30A interacts with βCTF in A7 mice, which follows by lipid flippase dysfunction. (**A**) The membrane fractions from WT and A7 mice brain at 3- or 6-month-old

524   applied to co-immunoprecipitation analysis using TMEM30A antibody. Na+/K+-ATPase was
525   used as a loading control of membrane fractions. (**B, C**) Quantification of the complex
526   formation of (**B**) TMEM30A and βCTF or (**C**) TMEM30A and ATP8A1 (*n*=3, mean ± SEM,
527   two-tailed Student's t-test, *P<0.05, **P<0.01). (**D**) Quantification of Evectin-2 localization in
528   membrane fractions (*n*=3, mean ± SEM, two-tailed Student's t-test, **P<0.01).
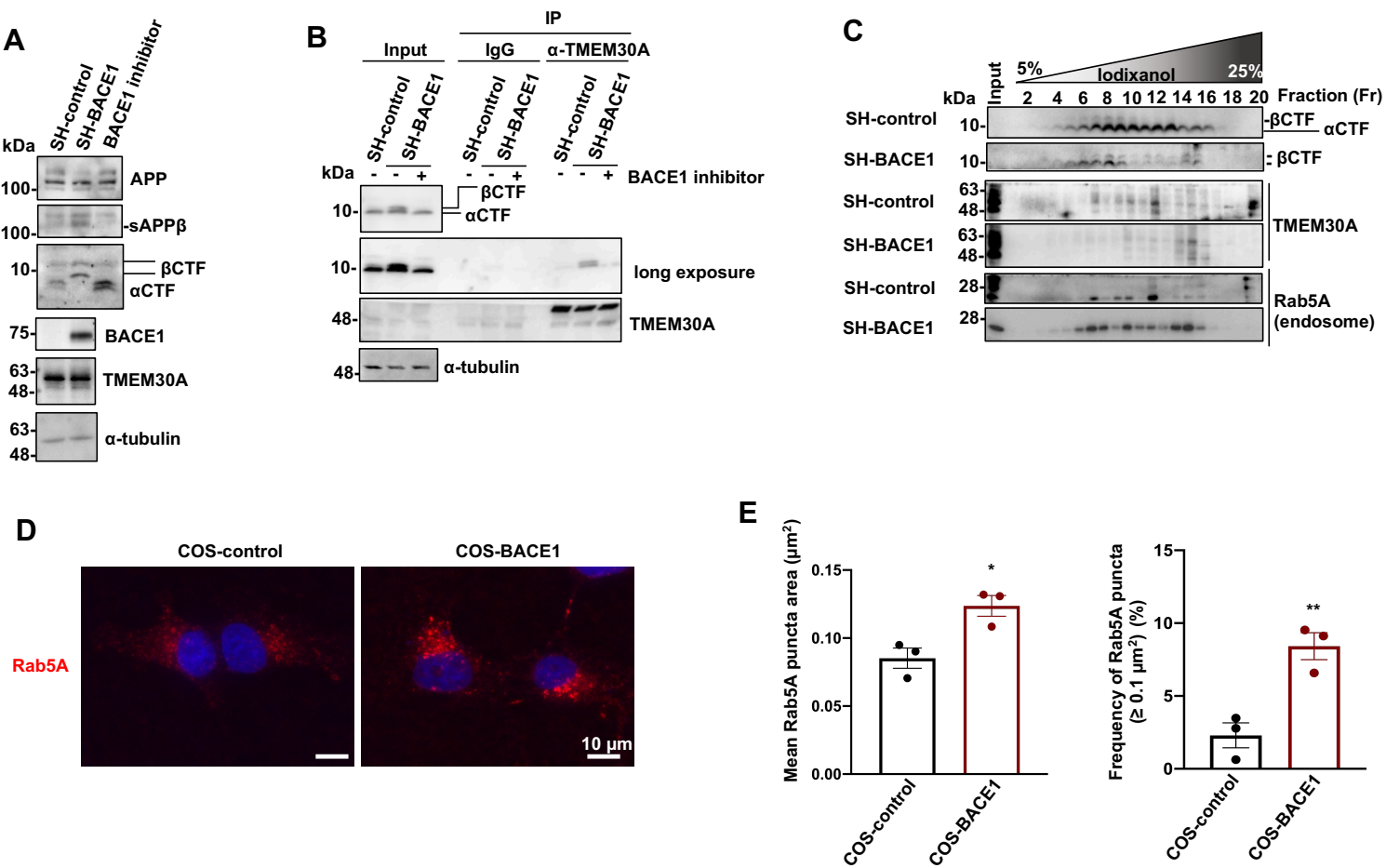
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531   **Figure 5. Identification of T-RAP peptide.** (**A**) Schematic view of the sequential deletion
532   constructs of GST-TmEx (Extracellular-domain of TMEM30A) and GST fused TMEM30A
533   (117-166 AA) and "T-RAP" sequence. Lowest panel: The conservation of the T-RAP
534   sequence among indicated species and predicted structure by Raptor-X. (**B**) Coomassie's
535   brilliant blue staining of purified GST-fusion proteins in Fig 5A. (**C, D**) GST-pull down from
536   HEK293 lysate transfected with SC100.

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539   **Figure 6. βCTF interacting peptide 'T-RAP' shows a trend to rescue lipid flippase**
540   **activity and improves the endosomal anomalies in BACE1 upregulation.** (**A**)
541   Immunoblotting analysis for SmBiT-Evectin-2 and LgBiT-Rab5 after the treatment of T-RAP
542   (10 μM) for 48h. (**B**) Quantification of the luciferase activity in T-RAP (10 μM) treatment for
543   48 h (*n*=5, mean ± SEM, one-way ANOVA Bonferroni's multiple comparisons test).
544   (**C**) Immunoblotting analysis for APP metabolites and TMEM30A in T-RAP (10 μM) treatment
545   for 48 h. (**D**) Quantification of sAPPβ and βCTF in Fig. 6C (*n*=3, mean ± SEM, one-way
546   ANOVA Bonferroni's multiple comparisons test, *P<0.05, **P<0.01, ***P<0.001). (**E**) COS-
547   control and COS-BACE1 cells were immunostained for Rab5A after T-RAP (10 μM)
548   treatment for 48 h. Scale bars: 20 μm. Representative z-stack images were captured using
549   a 60x objective lens. (**F**) Quantification of the mean Rab5A positive puncta area (*n*=4, mean
550   ± SEM, one-way ANOVA Bonferroni's multiple comparisons test, *P<0.05, **P<0.01).

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553   **Figure 7. Schematic view of the predicted mechanism of vesicular traffic impairment**
554   **in AD, and therapeutic effect of T-RAP.**
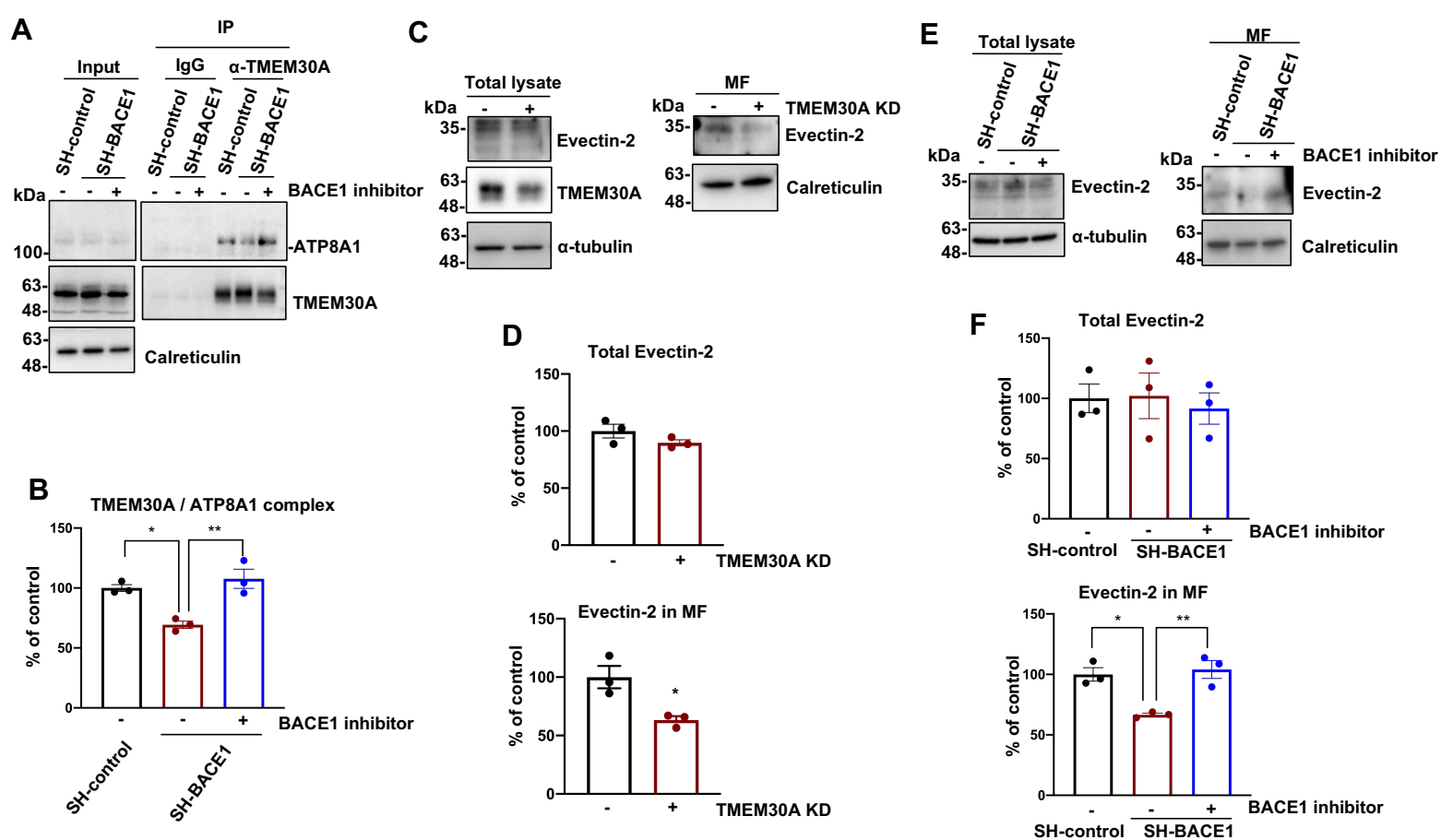555   In AD, the complex formation between TMEM30A and accumulated βCTF can interrupt lipid
556   flippase physiological formation and its activity in endosomes, decreasing cytosolic PS levels
557   required for membrane fission. Thus, the defect of membrane fission induces endosome
558   enlargement and inhibits vesicle transport. On the other hand, the treatment of βCTF
559   interacting peptide, T-RAP, can prevent the TMEM30A/βCTF interaction, resulting in
560   improved lipid flippase function and endosome enlargement. Therefore, T-RAP treatment
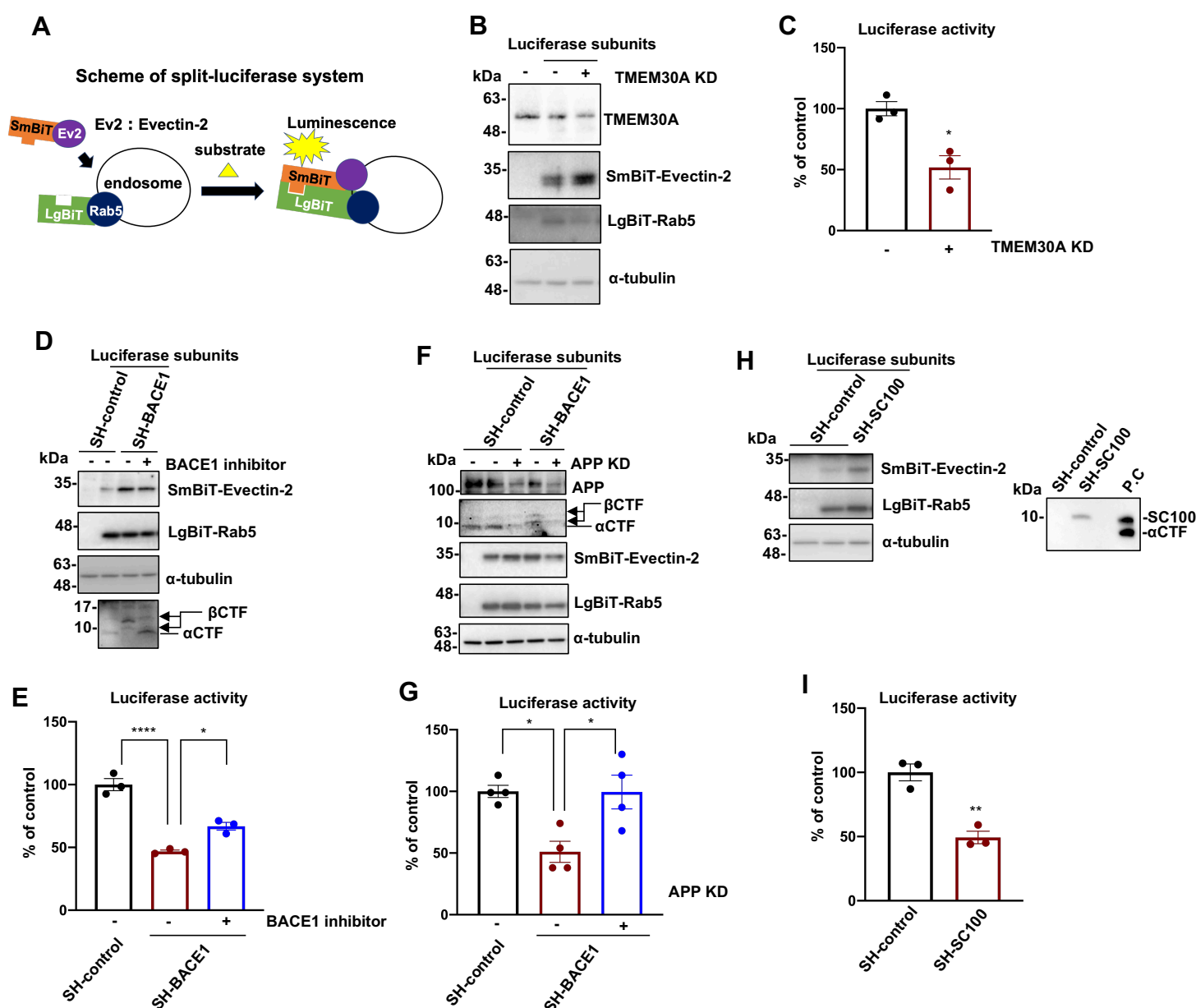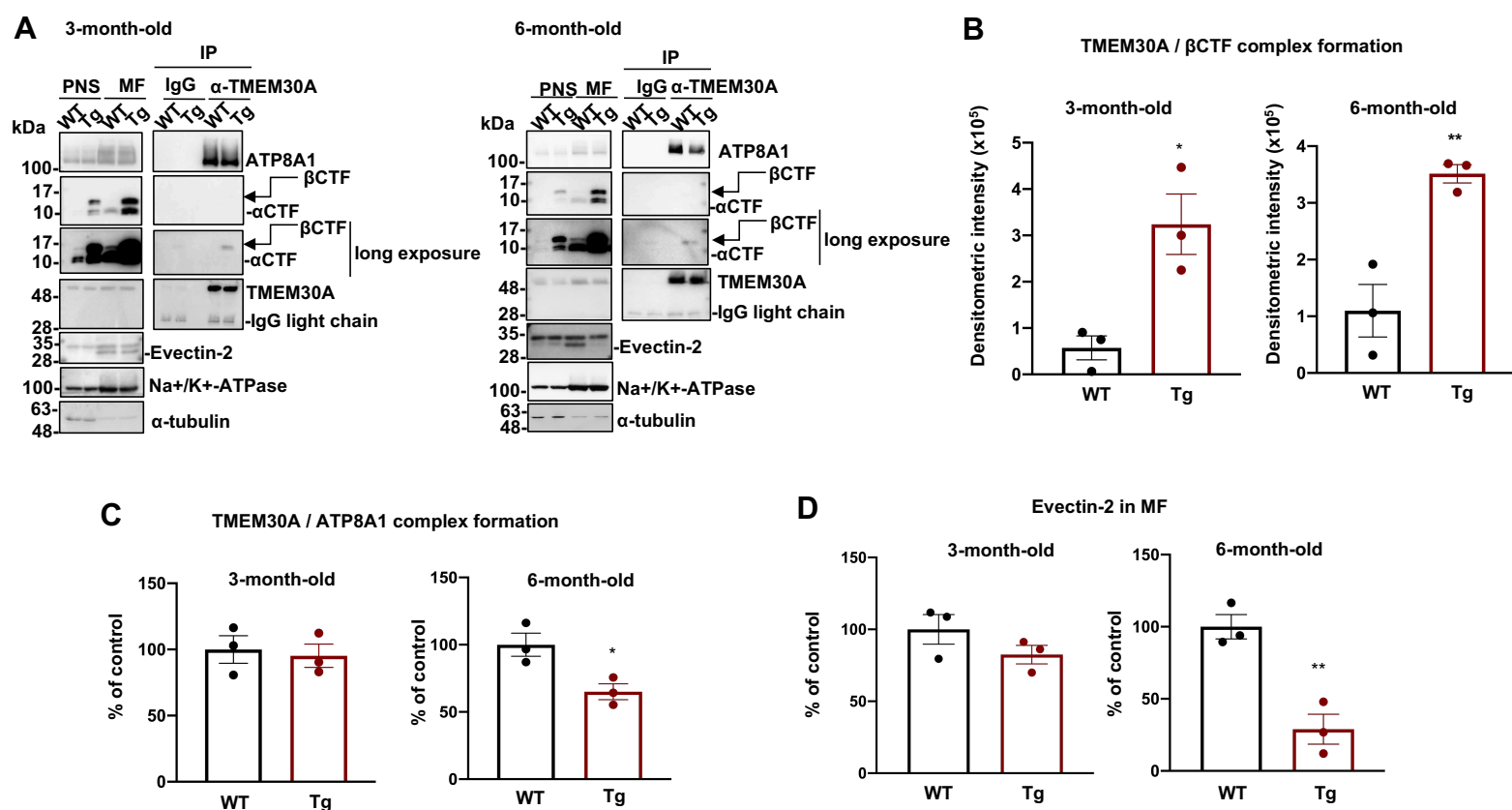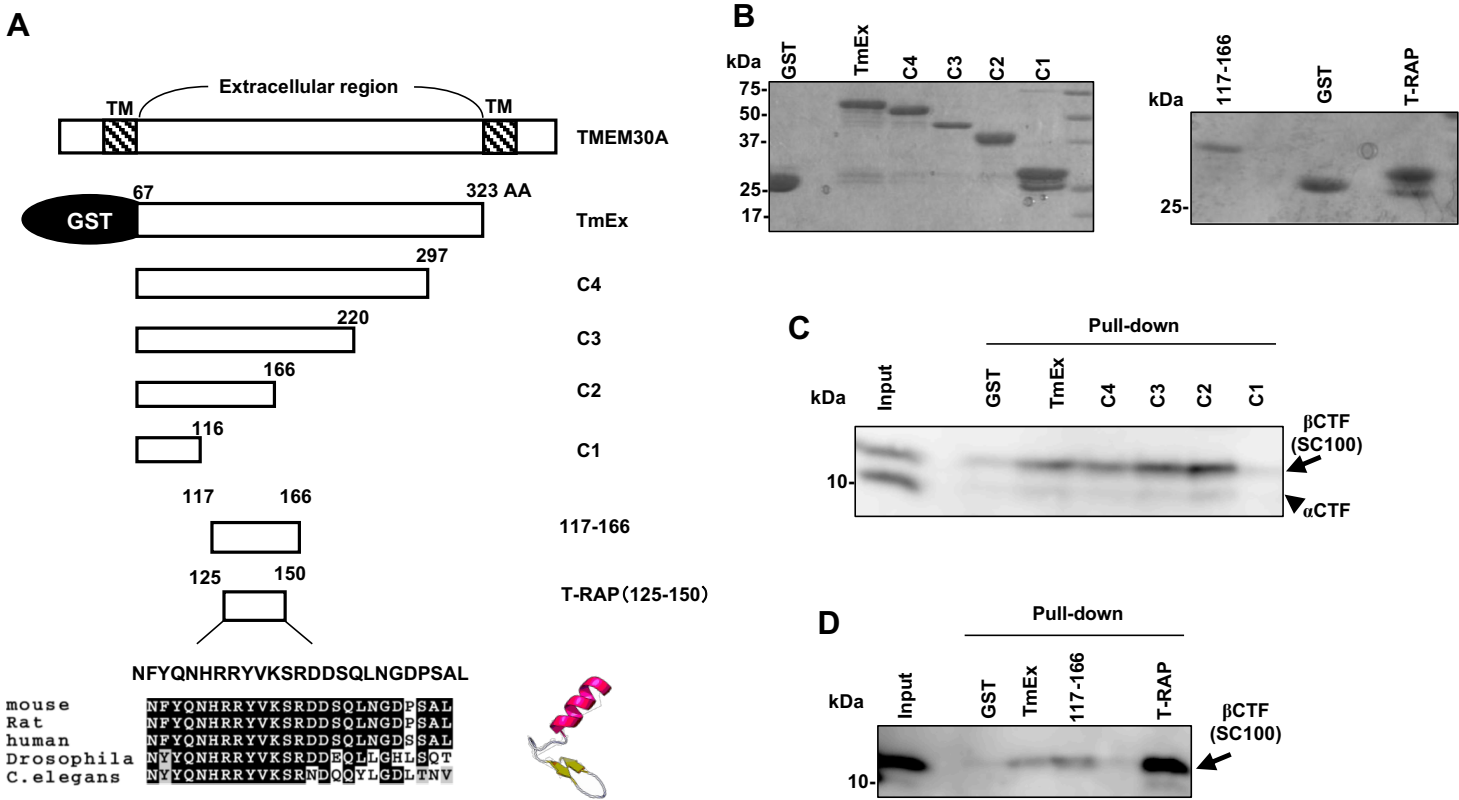561   can promote vesicle transport.
562

Fig.1

Fig.2

Fig.3

Fig.4

A 3-month-old / 6-month-old — western blot panels (PNS, MF, IP: IgG, α-TMEM30A) showing ATP8A1, βCTF, αCTF (long exposure), TMEM30A, IgG light chain, Evectin-2, Na+/K+-ATPase, α-tubulin

B TMEM30A / βCTF complex formation (3-month-old and 6-month-old; Densitometric intensity (×10^5); WT vs Tg; * , **)

C TMEM30A / ATP8A1 complex formation (3-month-old and 6-month-old; % of control; WT vs Tg; *)
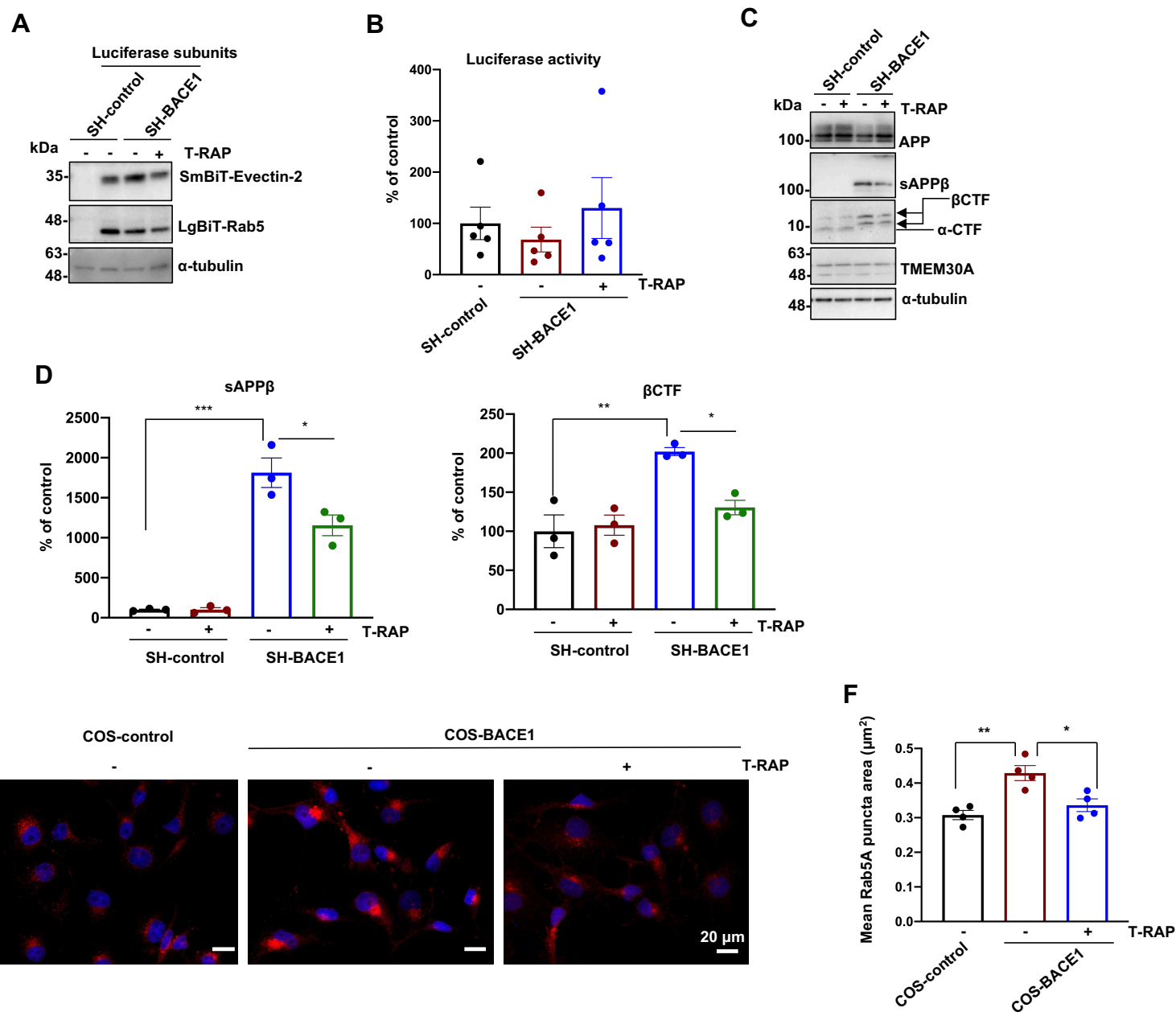
D Evectin-2 in MF (3-month-old and 6-month-old; % of control; WT vs Tg; **)

Fig.5

Fig.6

Fig.7