

Dimerization of proICA512/IA-2 ectodomain in the ER

1 **Stability and targeting of proICA512/IA-2 to insulin secretory granules requires β 4-**
2 **sheet mediated dimerization of its ectodomain in the endoplasmic reticulum**

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15 *Running title: Dimerization of proICA512/IA-2 ectodomain in the ER*

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27 **ABSTRACT**

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29 The type-1 diabetes autoantigen ICA512/IA-2/RPTPN is a receptor protein tyrosine
30 phosphatase of the insulin secretory granules, which regulates the size of granule stores,
31 possibly via cleavage/signaling of its cytosolic tail. The role of its extracellular region,
32 instead, remains unknown. Structural studies indicated that β 2- or β 4-strands in the
33 mature ectodomain (ME ICA512) form dimers *in vitro*. Here we show that ME ICA512
34 prompts proICA512 dimerization in the endoplasmic reticulum. Perturbation of ME
35 ICA512 β 2-strand N-glycosylation upon S508A replacement allows for proICA512
36 dimerization, *O*-glycosylation, targeting to granules and conversion, which are instead
37 precluded upon G553D replacement in the ME ICA512 β 4-strand. S508A/G553D or
38 N506A/G553D double mutants dimerize, but remain in the endoplasmic reticulum.
39 Removal of the N-terminal fragment (ICA512-NTF) preceding ME ICA512 allows
40 instead an ICA512- Δ NTF G553D mutant to exit the endoplasmic reticulum and ICA512-
41 Δ NTF is constitutively delivered to the cell surface. The signal for SG sorting is located
42 within the NTF RESP18-homology domain (RESP18-HD), whereas soluble NTF is
43 retained in the endoplasmic reticulum. Hence, we propose that the ME ICA512 β 2-strand
44 fosters proICA512 dimerization until NTF prevents N506 glycosylation. Removal of this
45 constraint allows for proICA512 β 4-strand induced dimerization, exit from the
46 endoplasmic reticulum, *O*-glycosylation and RESP18-HD-mediated targeting to granules.

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49 **INTRODUCTION**

50

51 Receptor protein tyrosine phosphatases (RPTPs) form a large family of transmembrane
52 proteins, which counteract protein tyrosine kinases, and are therefore involved in many
53 signaling pathways. Dimerization of RPTPs is regarded as a main mechanism to regulate
54 their constitutive intracellular phosphatase activity. The contribution of extracellular,
55 transmembrane and cytoplasmic PTP regions to dimerization varies among different
56 RPTPs (1-4).

57 The extracellular domains of RPTPs can promote the receptor dimerization in several
58 ways. As for receptor protein tyrosine kinases, RPTP oligomerization and clustering can
59 be induced upon heterophilic binding to extracellular ligands. RPTPs for which such
60 mechanism has been demonstrated include the type I RPTP CD45/RPTPC, the type IIa
61 RPTP σ , the type III RPTP β and the type V RPTP ζ (5). In the case of CD45 and RPTP β
62 changes in their glycosylation pattern, including *O*-glycosylation, were shown to
63 modulate their binding to galectin-1, conceivably increasing clustering and affecting
64 phosphatase activity (6, 7). Homophilic interactions among ectodomains of RPTPs in the
65 same or apposing cells, as it is the case for the type IIb RPTP μ and RPTP κ or the type IV
66 RPTP α , likely represent another mechanism for inducing receptor oligomerization and
67 regulating phosphatase activity (8-10).

68 The type VII RPTPs ICA512/IA-2/PTPRN and its homologue phogrin/IA-
69 2 β /PTPRN2 are 'pseudophosphatases' with a large ectodomain followed by a
70 transmembrane region and a single catalytically inactive PTP domain (11, 12). They are
71 mainly expressed in neuropeptidergic neurons and peptide-secreting endocrine cells,

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72 including insulin producing pancreatic β cells, where they are enriched in secretory
73 granules (SGs) (13, 14). Upon expression in *Escherichia coli* the transmembrane regions
74 of phogrin and ICA512 showed only weak or no ability to interact (2). On the other hand,
75 in transfected fibroblasts the cytosolic juxtamembrane and PTP domains of ICA512 and
76 phogrin were shown to form homodimers as well as heterodimers with each other and
77 with the PTP domains of other conventional RPTPs, possibly inhibiting their phosphatase
78 activity (15). In insulinoma cells, homodimerization of the ICA512 PTP domain was
79 found to disrupt the interaction of the latter with the cortical actin cytoskeleton, thereby
80 affecting insulin SG dynamics and exocytosis (16).

81 Recent structural studies on the membrane-proximal, recombinant mature,
82 ectodomain of ICA512 (ME ICA512/IA-2), which results from cleavage of proICA512
83 by prohormone convertases during SG maturation (13, 17), show that this region readily
84 adopts various dimeric assemblies both in the protein crystals and in solution (18, 19). In
85 contrast, the corresponding recombinant mature ectodomain of phogrin, despite its
86 structural similarity to ICA512 (20, 21), was found to fold as a monomer in solution (20,
87 21). In this study, therefore, we investigated the ability of ME ICA512 to induce receptor
88 homodimerization in insulin-producing cells.

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90 **MATERIALS AND METHODS**

91

92 **Generation of ICA512 constructs and site-directed mutagenesis.** pEGFP-N1
93 constructs encoding full length human ICA512 (amino acids 1-979), ICA512 N-terminal
94 fragment (NTF, amino acids 35-446), ICA512 Δ NTF (amino acids 449-979), ICA512
95 Regulated endocrine-specific protein 18 homology domain (RESP18-HD, amino acids
96 35-131) or ME (amino acids 449-575) were fused at their C-termini to green fluorescent
97 protein (GFP) or a hemagglutinin (HA) epitope tag. These constructs included either the
98 native signal peptide or that of CD33, while an 11-mer linker was placed between ME
99 ICA512 and GFP. Conventional cloning strategies were also used to generate ICA512-
100 NTF or a secretory GFP by inclusion of the CD33 signal peptide at the N-terminus. Site-
101 directed mutagenesis, using these constructs as template, was performed with the
102 QuikChangeTM kit (Stratagene). All constructs and mutations were verified by
103 sequencing.

104 **Antibodies against ICA512-NTF, ME ICA512, and ICA512 CT.** Mouse
105 monoclonal antibodies directed against a recombinant fusion protein between GST and
106 amino acids 389-575 (anti-ectodomain) or amino acids 935-974 (anti-ICA512
107 cytoplasmic tail; CT) of human ICA512 were generated in the Antibody Facility at the
108 Max Planck Institute of Molecular Cell Biology and Genetics (MPI-CBG, Dresden,
109 Germany). The ectodomain antibodies were further selected for recognition of an epitope
110 within amino acids 389-446 of the NTF (anti-ICA512 NTF ab) or amino acids 449-575 of
111 ME ICA512 (anti-ME ICA512 ab) using a MesoScale Discovery platform and by
112 Western blotting against ICA512-NTF-GFP, ICA512-GFP Δ NTF and GST.

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113 **Culture and transfection of insulinoma INS-1 cells.** Rat INS-1 cells were grown in
114 RPMI 1640 medium as described (22). Cells were split every 4-days, plated onto
115 coverslips on 35 mm dishes (Costar[®] 3516) and transfected 3 days after with plasmid-
116 DNA diluted into Cytoporation^R medium using Cyto Pulse electroporator (Cyto Pulse
117 Sciences, Inc.). Four days after transfection, cells were pre-incubated for 1 hour in resting
118 buffer (5 mM KCl, 120 mM NaCl, 24 mM NaHCO₃, 1 mM MgCl₂, 2 mM CaCl₂, 1
119 mg/ml ovalbumin, 5 mM HEPES pH 7.5) and then either kept at rest or in stimulation
120 buffer [25 mM glucose (high glucose, HG) and 55 mM KCl (high K⁺, HK) in 70 mM
121 NaCl, 24 mM NaHCO₃, 1 mM MgCl₂, 2 mM CaCl₂, 1 mg/ml ovalbumin, 5 mM HEPES
122 pH 7.5] for additional 2 hours. In some instances, 30 μM Calpeptin or MG-115
123 (Calbiochem) was added to the stimulation buffer. After 2 hours of incubation, cells were
124 washed twice with ice-cold PBS and harvested in ice-cold lysis buffer [(20 mM Tris-Cl
125 pH 8.0, 140 mM NaCl, 1 mM EDTA, 1 mM Triton X-100 and 1% protease inhibitor
126 cocktail (Sigma P8340)]. Cell lysates were centrifuged at 4°C 12,000 rpm for 5 min in an
127 Eppendorf 5415C centrifuge. The pre-cleared soluble protein fraction was separated from
128 the pellet and prepared in SDS-PAGE sample buffer for Western blotting analysis.

129 **Immunoprecipitation (IP).** Equal amount of cell lysates were mixed with 20 μl
130 (1/10 vol/vol) Protein G Sepharose beads (GE Healthcare), and incubated on a rotating
131 wheel at 4°C for 30 min to pre-clear the lysates of the IgG bound protein fraction. The
132 lysates were centrifuged at 2,000 rpm for 3 min in the Eppendorf centrifuge and the pellet
133 of beads removed. Goat anti-GFP antibodies (MPI-CBG, Dresden, Germany) or IgG
134 from goat serum (Sigma I5256), 1:100-dilution, respectively, were then incubated with
135 the pre-cleared lysates overnight prior to the addition of 20 μl (1/10 vol/vol) Protein G

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136 Sepharose beads. The lysates were centrifuged at 2,000 rpm for 3 min and the soluble
137 fraction was removed. The beads were washed twice with PBS, centrifuged as above and
138 re-suspended in SDS-PAGE sample buffer for Western blotting analysis.

139 **Enzymatic de-glycosylation assays.** INS-1 cells transfected for expression of
140 different ICA512 variants, or mouse or human islet cells, were incubated in resting or
141 stimulation buffer for 2 hours. Cells were lysed as above and incubated in denaturation
142 buffer (New England Biolabs) at 95°C for 5 min. The denatured samples of the cell
143 lysates were then incubated with PNGaseF, Endoglycosidase H or *O*-glycosidases, endo-
144 α -N-acetylgalactosaminidase and neuramidase in 1x reaction buffer (New England
145 Biolabs) at 37°C for 3 hours. Glycosylated fetuin from fetal calf serum (Sigma F2379)
146 was used as a control to confirm the performance of glycosidases. Non- and enzyme-
147 treated cell lysates were prepared in SDS-PAGE sample buffer for Western blotting
148 analysis.

149 **Western blotting.** Cell lysates and IPs in SDS-PAGE sample buffer were heated to
150 95°C for 5 min, and then subjected to SDS-PAGE and Western blotting. The following
151 primary antibodies were employed: mouse monoclonal anti-GFP antibody (Clontech
152 #632381); mouse anti-ICA512 antibody, HM-1 (23); mouse anti- γ -tubulin antibody
153 (Sigma T6557) and rabbit anti-HA antibody (Abcam ab9110). Primary antibodies were
154 detected with goat anti-mouse or anti-rabbit HRP-conjugated IgGs (Bio-Rad) followed by
155 addition of substrates for chemiluminescence (Thermo Scientific, Pierce Biotechnology)
156 and a LAS-3000 Imaging System (Fuji). Black dividing lines indicates cutting of the
157 same or different Western blots or their groupings.

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158 **Immunostaining and confocal microscopy.** INS-1 cells transfected for the
159 expression of different ICA512 variants and grown on coverslips on 35 mm dishes for 4
160 days post-transfection were incubated in resting or stimulation buffer as described above,
161 then rinsed 3x in PBS and fixed with 4% paraformaldehyde. After aldehyde quenching
162 and permeabilization (200 mM glycine, 0.1% Triton X-100 in PBS) for 20 min, cells
163 were incubated in blocking buffer (0.2% Gelatin, 0.5% albumin in PBS) for 1 hour, and
164 thereafter in blocking buffer with guinea pig anti-insulin antibody (Abcam ab7842) or
165 rabbit anti-calnexin antibody (Sigma C4731) at 4°C overnight. Next, cells were washed
166 5x with PBS, and then incubated in blocking buffer with goat anti-guinea pig or anti-
167 rabbit AlexaFluor568-conjugated IgGs (Molecular Probes) for 2 hours at room
168 temperature. After additional washing 5x with PBS, immunolabeled coverslips were
169 mounted onto slides with Mowiol™.

170 INS-1 live cell staining with the ER tracker red dye (glibenclamide BODIPY®-TR;
171 Molecular Probes) was done according to the manufacturer's protocol. ICA512 mature
172 ectodomain or cytosolic domain specific antibodies (see above), the anti-ME ICA512
173 antibody or the anti-ICA512 CT antibody was incubated with living INS-1 cells to assess
174 ICA512 extracellular domain exposure at the cell surface. Images of labeled cells were
175 acquired with an Olympus FluoView-1000 laser scanning confocal microscope equipped
176 with a 60x PlanoApo OLSM lens (NA=1.10).

177 **Cell sorting.** Four days post-transfection with ICA512-GFP, ICA512-GFP G553D or
178 ICA512-GFP N506A/N524A cells were detached by trypsin digestion and re-suspended
179 in 1x PBS, 25 mM HEPES (pH 7.0), 1 mM EDTA and 1% albumin. The cell suspension
180 was filtered through cell-strained cap in 5 ml round-bottom Falcon® tubes (Corning

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181 352235) prior to sorting for GFP expression with a Becton Dickinson BD FACS Aria II.
182 Sorted cells expressing the ICA512-GFP variants were cultured in 24 mm culture dishes
183 (Costar[®] 3513) in RPMI 1640 medium.

184 **Insulin gene expression, contents and secretion.** After two days in culture, the total
185 RNA of 1×10^5 cells sorted for the expression of each ICA512-GFP variant was purified
186 with Qiagen RNeasy Kit and used as RNA template for the First-Strand cDNA synthesis
187 with SuperScript[™] II reverse transcriptase (Invitrogen) and oligo (dT) primer. Levels of
188 insulin mRNA were analysed by Quantitative real-time PCR with the qPCR GoTaq kit
189 (Promega) and an MX4000 Thermocycler (Stratagene). β -actin mRNA was amplified in
190 parallel for normalization of the insulin mRNA levels in the individual samples.
191 Alternatively, 3×10^5 ICA512-GFP⁺ sorted cells were switched to resting buffer for pre-
192 incubation for 1 hour and then to resting or stimulation buffer for additional 2 hours
193 before being harvested for analyses for proinsulin/ insulin content and insulin secretion in
194 static conditions with Rat/Mouse Proinsulin/ Rat Insulin ELISA (Merckodia).

195 **Statistical analysis.** All data are representative of three or more independent
196 experiments, with the exception of the analysis in human islets, which was performed
197 two times. Statistical significance was assessed with student's T-test or ANOVA.

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200 **RESULTS**

201

202 **ProICA512 dimerizes in INS-1 cells.** ME ICA512 encompasses amino acids 449-575 of
203 human ICA512 (Fig. 1A). The X-ray structure of recombinant ME ICA512 (Fig. 1B) (18)
204 encompassing amino acids 468-558 revealed that this region displays a ferredoxin-like
205 fold related to the SEA (sea urchin sperm protein, enterokinase, agrin) domain, which is
206 known to promote oligodimerization (24-28). Accordingly, both crystallized (18) and
207 soluble (19) ME ICA512 form dimers resulting from the antiparallel pairing of β 2- β 2 or
208 β 4- β 4 strands (Fig. 1B) (19).

209 To verify the occurrence of ICA512 dimers in insulin-producing cells, full-length
210 ICA512 constructs differentially tagged at their C-termini with GFP (ICA512-GFP) or a
211 HA epitope (ICA512-HA) (Fig. 2A) were transiently expressed alone or together in rat
212 insulinoma INS-1 cells. Consistent with previous findings (29, 30), the mature
213 transmembrane fragments (TMF) of ICA512-GFP (ICA512-TMF-GFP; ~100 kDa) and
214 ICA512-HA (ICA512-TMF-HA; ~75 kDa) were the major ICA512 species detected in
215 cell lysates of resting (R) INS-1 cells (Fig. 2A). In cells stimulated (S) with 25 mM
216 glucose with or without 55 mM KCl, the respective proICA512 species became the most
217 prominent species, while the levels of the corresponding ICA512-TMF, which undergo
218 calpain mediated cleavage upon SG exocytosis (29, 30), were drastically reduced.
219 Stimulation with 55 mM KCl alone, which prompts SG exocytosis, reduced also the
220 levels of ICA512-TMF, but barely up-regulated those of proICA512. The detection of
221 multiple proICA512 species reflects the different degree of glycosylation of the protein
222 during its maturation along the secretory pathway prior to cleavage and conversion into

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223 ICA512-TMF. Notably, in cells stimulated with 55 mM KCl alone, the major proICA512
224 form migrated faster than the proICA512 species detected in cells concomitantly exposed
225 to high glucose. This discrepancy conceivably reflects differences in the efficiency of
226 proICA512 N-glycosylation in the endoplasmic reticulum (ER) depending on the glucose
227 levels.

228 ProICA512-HA and ICA512-TMF-HA were readily detectable, albeit at lower levels
229 when co-expressed with ICA512-GFP (Fig. 2B). Immunoprecipitation with anti-GFP
230 antibodies led to the recovery of ICA512-TMF-GFP or proICA512-GFP from resting or
231 stimulated cells, respectively (Fig. 2C, top panel). Co-immunoprecipitation of
232 proICA512-HA, but not of ICA512-TMF-HA, suggests that proICA512, but not ICA512-
233 TMF form dimers (Fig. 2C, bottom panel). Neither ICA512-GFP nor ICA512-HA co-
234 immunoprecipitated with control IgGs.

235 **ME ICA512 mediates the dimerization of proICA512 in INS-1 cells.** Previous
236 studies *in vitro* and in transfected INS-1 cells indicated that the intracellular PTP domain
237 of ICA512 can dimerize (15, 16). To verify whether ME ICA512 could account for
238 proICA512 dimerization independently of the cytoplasmic domain, INS-1 cells were
239 transfected with ICA512-HA either alone or in combination with soluble ME ICA512-
240 GFP (Fig. 2D, left panel). The slowest electrophoretic species of ME ICA512 was
241 upregulated in stimulated cells and migrated at the expected size of ca. 50 kDa, taking
242 into account also its N-glycosylation. The fastest migrating species corresponds instead to
243 the non-glycosylated form of ME ICA512-GFP (see also below, Fig. 5D).
244 Immunoprecipitation of ME ICA512-GFP led to the specific recovery of proICA512-HA
245 from both resting and stimulated cells (Fig. 2D, right panel). Hence, consistent with *in*

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246 *vitro* structural and biochemical analyses (18, 19), ME ICA512 is sufficient to induce the
247 dimerization of proICA512 in INS-1 cells.

248 **Lack of N-glycosylation or perturbation of the β 2- β 2 association interface of ME**
249 **ICA512 do not preclude SG sorting and conversion of ICA512.** Electrophoretic
250 mobility shift of ICA512 upon digestion with PNGaseF indicated that the protein is N-
251 glycosylated (17, see also below Fig. 4C and 8D). ICA512 contains only two consensus
252 sites for N-glycosylation at N506 and N524, both within ME ICA512 (Fig. 1A and 1B).
253 N506 is located in the β 2-strand (Fig. 1B), a critical structural element for β 2- β 2
254 dimerization (19). The consensus sequence for N506 glycosylation includes S508, which
255 was shown to mediate β 2- β 2 dimerization of ME ICA512 *in vitro* (19). N524 is instead
256 remote from the identified dimerization interfaces (Fig. 1B).

257 To verify the glycosylation of both N506 and N524, INS-1 cells were independently
258 transfected with the single mutants ICA512-GFP N506A, ICA512-GFP S508A and
259 ICA512-GFP N524A, or with the double mutant ICA512-GFP N506A/N524A. ICA512-
260 TMF of all ICA512-GFP mutants, as detected with the anti-GFP or the anti-ICA512 HM-
261 1 antibody, displayed a faster electrophoretic mobility than wild type ICA512 in resting
262 cells (Fig. 3A). A similar behavior was observed for the corresponding proICA512-GFP
263 species in resting and stimulated cells (Fig. 3A and 3B). Thus, both N506 and N524 are
264 glycosylated. The expression of proICA512-GFP N506A/N524A was significantly higher
265 than that of ICA512-GFP or single N-glycosylation ICA512-GFP mutants (Fig. 3C),
266 conceivably due to its delayed folding, traffic and conversion. Lack of N-glycosylation,
267 however, did not prevent the sorting of ICA512-GFP N506A/N524A into SGs, as
268 indicated by its co-localization with insulin at the cell periphery of INS-1 cells similar to

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269 ICA512-GFP (Fig. 3D). Notably, ICA512-GFP S508A, in which the $\beta 2$ - $\beta 2$ dimerization
270 should be perturbed (19), was also sorted into SGs (Fig. 3D).

271 The total levels of insulin mRNA in ICA512-GFP N506A/N524A⁺ sorted cells was
272 not significantly changed compared to ICA512-GFP⁺ sorted cells (Fig. 3E). Likewise, in
273 ICA512-GFP N506A/N524A⁺ sorted cells the proinsulin (Fig. 3F) and insulin (Fig. 3G)
274 content as well as the fractional stimulated insulin secretion (Fig. 3H) were not
275 significantly reduced compared to ICA512-GFP⁺ sorted cells.

276 **Perturbation of the ME ICA512 $\beta 4$ - $\beta 4$ association interface precludes SG sorting**
277 **and conversion of ICA512.** The replacement of G553, which sterically and
278 electrostatically hinders $\beta 4$ - $\beta 4$ dimerization (19), differently from the replacement of
279 N506 or S508 in the $\beta 2$ - $\beta 2$ interface, profoundly altered the maturation and stability of
280 ICA512. Specifically, the expression of ICA512-TMF-GFP G553D was dramatically
281 reduced (Fig. 4A and 4B, lane 4). This was also the case upon cell treatment with the
282 proteasome inhibitor MG-115 (Fig. 4A, lane 6) or calpeptin (Fig. 4B lane 6), which
283 prevents the calpain-mediated cleavage of ICA512-TMF and thus its disappearance (Fig.
284 4B, lane 3). These observations suggested that the G553D replacement prevents the
285 conversion of proICA512-GFP into ICA512-TMF-GFP. Conversely, expression of
286 ICA512-GFP G553D correlated with the enrichment of proteolytic fragments, mostly in
287 resting cells (Fig. 4A and 4B, lanes 1 and 4), but also in stimulated cells treated with MG-
288 115 (Fig. 4A, lanes 3 and 6) or calpeptin (Fig. 4B, lanes 3 and 6). Notably, proICA512-
289 GFP G553D appeared as a single band with electrophoretic mobility comparable to the
290 fastest migrating species of proICA512-GFP (Fig. 4A and 4B, lane 2 vs. 5). Sensitivity to
291 PNGaseF treatment indicated, that this phenotype is not attributed to lack of N-

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292 glycosylation (Fig. 4C). Analysis by confocal microscopy further indicated that ICA512-
293 GFP G553D (Fig. 4D left panel), unlike ICA512-GFP and ICA512-GFP S508A (Fig.
294 3D), did not co-localize with insulin (Fig. 4D, left panel), but was dispersed throughout
295 the cytoplasm with a reticular pattern resembling that of ER tracker red dye (Fig. 4D,
296 right panel). These data suggested that ICA512-GFP G553D is retained within the ER.

297 In ICA512-GFP G553D⁺ sorted cells, similar to ICA512-GFP N506A/N524A⁺ sorted
298 cells (see above, Fig. 3), the total levels of insulin mRNA was not changed (Fig. 4E). The
299 proinsulin (Fig. 4F) and insulin content (Fig. 4G) as well as the fractional stimulated
300 insulin release (Fig. 4H) were also not significantly changed relative to ICA512-GFP⁺
301 sorted cells.

302 **ICA512 is O-glycosylated.** ME ICA512 possesses a SEA domain fold, which is
303 present in some mucins and other membrane proteins. SEA domain containing proteins
304 are commonly extensively O-glycosylated nearby this domain, possibly to hinder its
305 proteolysis (25, 27, 28). We wondered therefore, whether ICA512 is also O-glycosylated
306 and whether this modification, which mainly occurs in the Golgi, would be affected by
307 the G553D replacement.

308 O-glycosidase treatment increased the electrophoretic mobility of the O-glycosylated
309 control protein fetuin (Fig. 5A), as well as that of ICA512-TMF-GFP in extracts of
310 resting cells (Fig. 5B, lane 1 vs. 2). De-O-glycosylation of lysates from stimulated cells
311 led to the almost complete disappearance of the slowest migrating species of proICA512-
312 GFP (Fig. 5B, lane 3 vs. 4). A comparable mobility shift of ICA512-TMF was observed
313 upon O-glycosidase treatment of lysates of mouse and human islets (Fig. 5C), thus
314 indicating that this modification, similar to N-glycosylation (17), occurs also in non-

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315 transformed ICA512 expressing tissues. ProICA512-GFP N506A (Fig. 5B, lane 5 vs. 6
316 and lane 7 vs. 8) and proICA512-GFP S508A (Fig. 5B, lane 9 vs. 10 and lane 11 vs. 12),
317 as well as the corresponding ICA512-TMF forms (Fig. 5B, lane 5 vs. 6 and lane 9 vs. 10),
318 were also *O*-glycosidase sensitive. Conversely, the single proICA512-GFP G553D
319 species was *O*-glycosidase insensitive (Fig. 5B, lane 15 vs. 16), consistent with its
320 retention in the ER (Fig. 4D).

321 **Relevance of the NTF domain for *O*-glycosylation and export of proICA512**
322 **from ER.** Soluble ME ICA512-GFP, ME ICA512-GFP S508A and ME ICA512-GFP
323 G553D (Fig. 5D, lanes 1, 5 and 9) were reduced to a single band upon treatment with N-
324 glycosidases, Endoglycosidase H (Fig. 5D, lanes 2, 6 and 10) or PNGaseF (Fig. 5D, lanes
325 3, 7 and 11), but were insensitive to *O*-glycosidases (Fig. 5D, lanes 4, 8 and 12). Thus,
326 multiple ME ICA512-GFP species result from N-glycosylation alone. However, the
327 Endoglycosidase H sensitivity of all these ME ICA512 variants further indicated these
328 retain the high-mannose structure N-oligosaccharide chains, *i.e.* they are not exported
329 from the ER. Indeed, confocal microscopy indicated that ME ICA512-GFP or its G553D
330 variant was found to accumulate in the ER (Fig. 5E), concurrent with the lack of *O*-
331 glycosylation.

332 Sequence analysis of ICA512 with NetOGlyc 3.1 (31) pointed to serines and
333 threonines clustered within amino acid residues 400-440, *i.e.* in the NTF domain (13, 17),
334 preceding ME ICA512, as the most likely sites for *O*-glycosylation (Fig. 1A).
335 Nonetheless, the deletion mutant ICA512-GFP Δ NTF, which lacks amino acid residues
336 35-448 (Fig. 1A), *i.e.* the entire NTF domain, as well as the corresponding ICA512-GFP
337 Δ NTF S508A and ICA512-GFP Δ NTF G553D mutants, were both PNGaseF (Fig. 5F,

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338 lanes 3, 7 and 11) and *O*-glycosidase (Fig. 5F, lanes 4, 8 and 12) sensitive. Notably,
339 ICA512-GFP Δ NTF and ICA512-GFP Δ NTF G553D displayed comparably slower
340 electrophoretic mobility than ICA512-GFP Δ NTF S508A, suggesting their similar
341 glycosylation at N506 and N524. Moreover, ICA512-GFP Δ NTF G553D, similar to all
342 other constructs lacking NTF, was Endoglycosidase H resistant (Fig. 5F, lanes 2, 6 and
343 10) and unlike ICA512-GFP G553D did not undergo extensive proteolysis. Evidence that
344 proICA512 mutants lacking NTF domain can fold and exit from the ER, even if the ME
345 ICA512 β 4- β 4 dimerization interface is perturbed suggests that this region contains
346 information for retention of unfolded proICA512 in the ER.

347 **ICA512-NTF is required for targeting of proICA512 to the SGs.** To further verify
348 the progression of ICA512-GFP Δ NTF along the secretory pathway, we imaged its
349 localization in non-permeabilized resting (Fig. 6A and 6B) or stimulated (Fig. 6C) INS-1
350 cells kept at 4°C to block endocytosis. Both ICA512-GFP and ICA512-GFP Δ NTF, as
351 detected with their GFP fluorescence, appeared enriched at the cell periphery (Fig. 6A,
352 6B and 6C). However, labeling with a novel mouse anti-ME ICA512 antibody revealed
353 that ICA512-GFP Δ NTF, unlike ICA512-GFP, was exposed at the cell surface even in
354 resting conditions (Fig. 6A). As expected, the concomitant control labeling of resting
355 cells for insulin, which like ICA512-GFP should be mainly confined to SGs and not be
356 exposed at the surface, was negative (Fig. 6A). Surface labeling with a mouse anti-
357 ICA512 CT antibody was also negative (Fig. 6B). Conversely, the anti-ME ICA512
358 antibody labeled the cell surface of both ICA512-GFP and ICA512-GFP Δ NTF
359 stimulated cells, albeit more prominently in the case of the latter cells (Fig. 6C). Thus,
360 ICA512-GFP Δ NTF, unlike ICA512-GFP, is constitutively targeted to the cell surface.

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361 Immunoblotting with anti-GFP (Fig. 6D) or anti-ME ICA512 (Fig. 6E) antibodies
362 indicated that unlike ICA512-GFP (Fig. 6D and 6E, lanes 1 vs. 2), ICA512-GFP Δ NTF
363 (Fig. 6D and 6E, lanes 3 vs. 4) is not susceptible to Ca^{2+} /calpain-dependent cleavage
364 upon stimulation of INS-1 cells. Hence, the NTF domain is not only required to retain
365 unfolded proICA512 in the ER, but also for targeting the latter to SGs, thus enabling the
366 cleavage of ICA512-TMF upon granule exocytosis (29, 30).

367 **The RESP18 homology domain targets proICA512 to SGs.** The most N-terminal
368 portion of ICA512-NTF corresponds to the RESP18 homology domain (RESP18-HD)
369 (32), which begins at amino acid residue 35, following the signal peptide and ends at a
370 putative cleavage site for protein convertases, between residues 133 and 134 (17) (Fig.
371 1A). Similar to RESP18 (32, 33), ICA512-RESP18-HD is characterized by a cysteine-
372 rich motif (Fig. 1A), which may be relevant for sorting into SGs. In particular, other
373 characteristic neuroendocrine proteins, such as Chromogranin B and Secretogranin II,
374 have been shown to depend on disulfide-bonded loops for their targeting to SGs (34-36).

375 Consistent with the enrichment of RESP18 in SGs (32, 33), also soluble ICA512-
376 RESP18-HD-GFP co-localized extensively with insulin in SG like structures (Fig. 7A)
377 and its recovery in the media was enhanced upon stimulation of the cells (Fig. 7B).
378 Conversely, soluble ICA512-NTF-GFP, despite the inclusion of the RESP18-HD, rather
379 displayed a diffuse ER like pattern (Figs. 7C and D), resembling that of the ER marker
380 calnexin (Fig. 7D). A similar expression pattern was observed for ICA512-NTF (Fig. 7F),
381 as detected with a novel antibody that recognizes human NTF, but not the equivalent
382 domain of the endogenous rat protein (Fig. 7E, lane 1 vs. 2). These data exclude the
383 possibility that retention of soluble ICA512-NTF in the ER is secondary to its misfolding

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384 upon fusion with GFP. Intriguingly, in cells expressing soluble ICA512-NTF-GFP insulin
385 immunoreactivity was also more diffuse, rather than being enriched in SGs, as in cells
386 expressing ICA512-RESP18-HD-GFP (Fig. 7A). Thus, RESP18-HD contains
387 information that is sufficient for SG targeting, while the remaining portion of ICA512-
388 NTF exerts a dominant role in ER retention, unless associated with the remaining C-
389 terminal transmembrane portion of the protein.

390 **The ME ICA512 β 4- β 4 strand interface is involved in dimerization.** Finally, we
391 investigated in cells the occurrence of the main dimerization modes identified in the X-
392 ray structure of non-glycosylated ME ICA512 (18, 19). The association through a β 2- β 2
393 interface was tested by the S508A mutation, which impedes dimerization *in vitro* (albeit
394 not in the crystal) of recombinant non-glycosylated ME ICA512. The second
395 dimerization mode, through the β 4- β 4 interface, was tested by the G553D replacement,
396 which is sterically incompatible with it (19). ICA512-HA and ME ICA512-GFP variants
397 with the symmetric single or combined mutations were co-expressed in INS-1 cells, and
398 the lysates of the stimulated cells and the corresponding immunoprecipitates were
399 analyzed by immunoblotting (Fig. 8A and 8B). While symmetric replacement of S508
400 did not preclude the recovery of proICA512-HA S508A with ME ICA512-GFP S508A
401 (Fig. 8B, lane 9), the symmetric replacement of G553 prevented the co-
402 immunoprecipitation of proICA512-HA G553D with ME ICA512-GFP G553D (Fig. 8C,
403 lane 10). The recovery of proICA512-HA mutants with the corresponding ME ICA512-
404 GFP variants was specific, as co-immunoprecipitations with control IgG were
405 significantly less effective compared to those with the anti-GFP antibody (Fig. 8B, lanes
406 13-18 and Fig. 8C). These findings suggest that dimerization of proICA512 occurs

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407 through $\beta 4$ - $\beta 4$ interactions. Remarkably, however, the combined replacement of
408 S508A/G553D was still permissive for ME ICA512 dimerization, albeit the recovered
409 proICA512-HA S508A/G553D displayed a faster electrophoretic mobility (Fig. 8B, lane
410 11), consistent with lack of N506 glycosylation. Hence, even upon perturbation of the
411 $\beta 4$ - $\beta 4$ interface, the dimerization may still occur via the $\beta 2$ - $\beta 2$ interaction, but only in
412 conditions which prevent/precede N506 glycosylation. Glycosylation, because of its
413 bulkiness, would impede the physical contact between $\beta 2$ -strands. Consistent with this
414 interpretation was the greater yield of co-immunoprecipitated proICA512-HA upon
415 replacement of N506 rather than S508 in combination with G553 (Fig. 8B, lane 12
416 and Fig. 8C).

417 According to the structural data N506 is less critical than S508 for the
418 establishment/retention of $\beta 2$ - $\beta 2$ dimer interface (19), and thus for protein folding and
419 stability, as corroborated by the greater amount of proICA512-HA N506A/G553D
420 compared to proICA512-HA S508A/G553D in the cell lysate (Fig. 8A, lane 6 vs. 5).
421 Notably, $\beta 2$ - $\beta 2$ proICA512 dimers are mainly retained in the ER, as shown in the specific
422 case of proICA512-HA S508A/G553D, which for the most part remains Endoglycosidase
423 H sensitive (Fig. 8D, lower panel) and is not converted into the corresponding mature
424 ICA512-TMF species (Fig. 8D, upper panel). Hence, preclusion of N506 glycosylation
425 does not rescue the replacement of G553, but it allows the detection of $\beta 2$ - $\beta 2$ mediated
426 dimerization of ME ICA512, which in normal conditions may only represent a transient
427 state of the receptor. Once N506 glycosylation occurs, folding of the $\beta 4$ - $\beta 4$ interface
428 would become critical for stabilization/dimerization of proICA512 and for overcoming
429 the dominant ER retention signal within ICA512-NTF, thus enabling the further

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430 progression of the protein along the secretory pathway and its RESP18-HD mediated
431 targeting to the SGs (Fig. 8E).
432

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433 **DISCUSSION**

434

435 Dimerization of RPTPs has been generally found to occur at the cell surface. This process
436 is driven by the interaction of ligands to the extracellular domain and can affect the
437 phosphatase activity of the cytoplasmic PTP domain. In the case of ICA512, however, no
438 extracellular ligands have yet been identified. Its signaling pathway remains also unclear,
439 albeit the calpain cleaved cytosolic fragment generated upon granule exocytosis has been
440 suggested to act as a retrograde signal to modulate insulin SG mobility and biogenesis in
441 relationship to the size and consumption of the SG stores (30, 23, 16). In this work we
442 have begun to unravel some of the functional properties of the ICA512 SG luminal/
443 extracellular region.

444 Specifically, we show that ME ICA512 is sufficient to promote ICA512 dimerization
445 in cells (Fig. 2D), as suggested by previous structural studies of the corresponding
446 recombinant portion of the receptor (18, 19). Notably, we only detected dimers of
447 proICA512, but not of ICA512-TMF (Fig. 2C and D), implying that maturation of the
448 protein resolves this interaction such that in the SGs ICA512-TMF is present as a
449 monomer. On the other hand we cannot exclude that in the granule lumen ICA512-TMF
450 forms instead a heterodimer with soluble ICA512-NTF generated from convertase-
451 mediated cleavage of proICA512 between residues 448 and 449 (Fig. 1A). Repeated
452 attempts to co-immunoprecipitate ICA512-NTF with ICA512-TMF, however, have been
453 unsuccessful. Since ICA512-NTF contains two additional likely sites for convertase
454 cleavage between residues 133-134 and 387-388 (Fig. 1A), it is also possible that two
455 putative fragments of ~28 kDa and ~7 kDa resulting from these cleavages might have not

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456 been detected on SDS-PAGE and Coomassie blue/silver staining, either being masked by
457 the IgG light chain or due to their small size. Other factors that may disrupt ME ICA512
458 dimers include post-translational modifications occurring while the protein transits from
459 the ER to the SGs, the progressive intraluminal acidification of the Golgi and SG
460 compartment and/or binding to other secretory proteins. Intriguingly, it has been reported
461 that phogrin/IA-2 β interacts with carboxypeptidase E/H (37). Finally, we cannot rule out
462 that ME ICA512 prompts again homodimerization of ICA512-TMF once the latter is
463 transiently inserted into the plasma membrane upon granule exocytosis.

464 Our data suggest that dimerization of proICA512 occurs in the ER. Supporting this
465 conclusion is the fact that proICA512-GFP G553D accumulates in the ER (Fig. 4D) and
466 does not undergo *O*-glycosylation (Fig. 5B), a post-translation modification that most
467 commonly occurs in the Golgi. However, ICA512-GFP Δ NTF G553D is
468 Endoglycosidase H resistant and *O*-glycosylated (Fig. 5F). These findings together with
469 the evidence that soluble NTF accumulates instead in the ER (Fig. 7C, 7D and 7F),
470 suggest that NTF contains an ER retention signal. In contrast, soluble RESP18-HD-GFP,
471 similar to its paralogue RESP18 (32, 33, 38) is targeted to the SGs (Fig. 7A) and released
472 in a regulated fashion from the cells (Fig. 7B). Accordingly, ICA512-GFP Δ NTF is
473 neither retained in the ER nor targeted to SGs, but constitutively delivered to the cell
474 surface (Fig. 6A-C). Intriguingly, in cells overexpressing soluble NTF, but not soluble
475 RESP18-HD, insulin immunoreactivity is also more diffused and less granular (Fig. 7C).
476 Additional studies on the structure of ICA512-NTF and RESP18-HD and their roles in
477 SG biogenesis will be necessary to elucidate the reason for these phenomena.

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478 Extending previous findings (17, 39, 40), we show that N506 and N524 of ICA512
479 are both glycosylated (Fig. 3A and 3B). Lack of glycosylation at these sites does not
480 prevent targeting of proICA512 to SGs (Fig. 3D) and its conversion to ICA512-TMF
481 (Fig. 3A). Simultaneous inhibition of N506 glycosylation and perturbation of the ME
482 ICA512 β 2- β 2 association interface by replacement of S508A is also insufficient to
483 prevent proICA512 conversion to ICA512-TMF (Fig. 3A) and ME ICA512 mediated
484 homodimerization (Fig. 8B). Conversely, perturbation of the ME ICA512 β 4-strand by
485 G553D replacement hinders the stability, SG targeting and conversion of proICA512
486 (Fig. 4A-D), as well as its recovery as a homodimer (Fig. 8B). On the other hand,
487 combined S508A/G553D or N506A/G553D replacements are still compatible with
488 proICA512 dimerization (Fig. 8B). A possible explanation for the latter finding is that the
489 extended β 2- β 2 association interface (19, Fig. 1A and 1B) is sufficient for stable
490 proICA512 dimerization, provided that glycosylation of N506, as upon S508A
491 replacement, is precluded. This conclusion is corroborated by the greater yield of pro-
492 ICA512 homodimer recovered when the G553D mutation is combined with N506A
493 replacement, which structural studies predict to be less detrimental for ME ICA512 β 2-
494 β 2 mediated dimerization than S508A replacement (Fig. 8B and 8C). Notably, such β 2-
495 β 2 mediated proICA512 dimers are restricted to the ER, as indicated by the
496 Endoglycosidase H sensitivity and lack of conversion of proICA512-GFP S508A/G553D
497 (Fig. 8D).

498 In conclusion, we propose a model whereby proICA512 β 2- β 2 dimers prevail over
499 β 4- β 4 dimers in the ER as long as the β 2-strand N506 is not glycosylated possibly due to
500 occlusion of this region by NTF. Dislocation of NTF, as upon its binding to another

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501 molecule, would allow for N506 glycosylation, thus irreversibly shifting the equilibrium
502 towards proICA512 β 4- β 4 dimers, consequently allowing ICA512 to leave the ER (Fig.
503 8E). Importantly, this model does not assume that β 4- β 4 dimerization is *per se* the signal
504 for ER export. Indeed, ICA512-GFP Δ NTF G553D, in which concomitant glycosylation
505 of N506 and G553D replacement should hinder with the formation of both ME ICA512
506 β 2- β 2 and β 4- β 4 dimers, is nevertheless exported from the ER, conceivably as a
507 monomer. Hence, it is plausible that folding and/or homodimerization of ME ICA512 β 4-
508 strand are instrumental to stabilize proICA512 and overcome the NTF ER retention
509 signal.
510

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512

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531

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532 **ABBREVIATIONS**

533

534 **ER** endoplasmic reticulum535 **IP** immunoprecipitation536 **ME** mature ectodomain537 **NTF** N-terminal fragment538 **RESP18** Regulated endocrine-specific protein 18539 **SG** secretory granule540 **TMF** transmembrane fragment

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702 **FIGURE LEGENDS**

703

704 FIG 1 Sequence of the ICA512 extracellular domain and model of ME ICA512
705 dimerization through β 2- β 2 and β 4- β 4 interfaces. (A) Primary amino acid sequence of
706 human ICA512 extracellular domain (amino acid residues 1-575), including the signal
707 peptide (SP, residues 1-34), the RESP18 homology domain (RESP18-HD, residues 35-
708 133), as a part of the N-terminal fragment (NTF, residues 35-448) and the mature
709 ectodomain (ME, residues 449-575, in bold). Residues in α -helices and β -strands of ME
710 ICA512, as resolved in (18) are highlighted in orange and yellow, respectively. The N-
711 glycosylation sites N506 and N524 are indicated in red and cleavage sites for protein
712 convertases (KR 132-133, KK 386-387 and KK 447-448) are underlined. Clustered
713 cysteines within the RESP18-HD (C40, C47, C53 and C62) are indicated in italics, while
714 threonines and serines predicted as putative *O*-glycosylation sites are indicated with
715 asterisks. Critical residues for β 2- β 2 (S508) or β 4- β 4 (G553) mediated dimerization of
716 recombinant ME ICA512 *in vitro* are shown in purple. (B) Modeled β 2- β 2 (left) or β 4- β 4
717 (right) ME ICA512 dimers (the symmetry axes shown by dotted lines), as resolved by X-
718 ray crystallography (18). Color code for the relevant residues and secondary structures
719 are as described in (A). Schematic drawing of ICA512 below shows the extracellular
720 domain regions as in (A), followed by the transmembrane (TM) region and the cytosolic
721 PTP domain. The arrow and arrowhead indicate the cleavage site for conversion of
722 proICA512 into ICA512-TMF and the more distal cleavage site for calpain in the
723 cytoplasmic domain of ICA512-TMF, respectively.

724

Dimerization of proICA512/IA-2 ectodomain in the ER

725 FIG 2 ME ICA512 is sufficient for dimerization of proICA512 in INS-1 cells. (A)
726 Schematic drawing of ICA512-GFP and ICA512-HA, and their detection by
727 immunoblotting with the mouse anti-GFP or rabbit anti-HA antibody in lysates of single
728 transfected INS-1 cells. The arrows indicate the cleavage site for conversion of
729 proICA512 into ICA512-TMF, while the arrowheads indicate the more distal calpain
730 cleavage site in the cytoplasmic domain of ICA512-TMF. Prior to lysis cells were kept
731 either at rest (R) in 0 mM glucose and 5 mM KCl or stimulated (S) for 2 hours either with
732 55 mM KCl (High K⁺) or 25 mM glucose (High Glucose) or both (High Glucose and
733 High K⁺, HGHK) (n≥3). (B) Immunoblottings for HA or GFP on lysates of INS-1 cells
734 transfected with ICA512-HA alone or together with ICA512-GFP. Prior to lysis the cells
735 were either kept at rest (R) or stimulated (S) for 2 hours with HGHK (n≥3). For
736 normalization, the same cell lysates were also immunoblotted for γ -tubulin. (C)
737 Immunoblottings for HA or GFP on immunoprecipitates with goat anti-GFP antibody or
738 goat IgG from the lysates shown in (B) (n≥3). (D) Schematic drawing of ME ICA512-
739 GFP and immunoblottings for HA or GFP on lysates and corresponding
740 immunoprecipitates with goat anti-GFP antibody from INS-1 cells transfected with
741 ICA512-HA alone or together with ME ICA512-GFP. Prior to lysis the cells were kept at
742 rest (R) or stimulated (S) for 2 hours with HGHK (n≥3). For normalization, the same cell
743 lysates were also immunoblotted for γ -tubulin.
744

745 FIG 3 Perturbing N-glycosylation or ME ICA512 β 2-strand does not prevent ICA512
746 targeting to SGs. (A, B) Immunoblottings for GFP or ICA512 on lysates of resting (A) or
747 stimulated (B) INS-1 cells transfected either with ICA512-GFP wt or the mutants N506A,

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748 N524A, N506A/N524A or S508A ($n \geq 3$). For normalization, the same cell lysates were
749 also immunoblotted for γ -tubulin. (C) Quantification by ANOVA of proICA512-GFP
750 species (top panel) and of the ratios of the corresponding proICA512-GFP/ICA512-TMF-
751 GFP species (bottom panel). Values \pm SEM for each ICA512 species were normalized to
752 those of γ -tubulin in the same lysates; *, $p < 0.005$ ($n = 3$). (D) Confocal microscopy images
753 of HGHK stimulated INS-1 cells transfected with ICA512-GFP or the corresponding
754 N506A/N524A or S508A mutants (in green) and immunostained for insulin (in red)
755 ($n \geq 3$). Nuclei were labeled with DAPI (in blue). Scale bars = 5 μ m. (E) Levels of insulin
756 mRNA in ICA512-GFP wt and ICA512-GFP N506A/N524A sorted cells as measured by
757 RT-PCR and normalized for β -actin mRNA levels ($n = 3$). Levels of (F) proinsulin and (G)
758 insulin content in ICA512-GFP wt and ICA512-GFP N506A/N524A sorted cells kept at
759 rest (R) or stimulated (S) for 2 hours with HGHK as measured by ELISA ($n = 3$). (H)
760 Insulin secretion measured from ICA512-GFP wt and ICA512-GFP N506A/N524A
761 sorted cells kept at rest (R) or stimulated (S) for 2 hours with HGHK ($n = 3$).

762

763 FIG 4 Perturbing the ME ICA512 β 4-strand impairs proICA512 stability and targeting to
764 SGs. (A, B) Immunoblottings for GFP on lysates of resting (R) or HGHK stimulated (S)
765 INS-1 cells transfected with ICA512-GFP or ICA512-GFP G553D and untreated (-) or
766 treated (+) with the protease inhibitors MG-115 (A) or calpeptin (B) ($n \geq 3$). For
767 normalization, the same cell lysates were also immunoblotted for γ -tubulin. (C)
768 Immunoblotting for GFP on lysates of HGHK stimulated INS-1 cells transfected with
769 ICA512-GFP or ICA512-GFP G553D and untreated (-) or treated (+) with N-glycosidase
770 PNGaseF ($n \geq 3$). (D) Confocal microscopy images of HGHK stimulated INS-1 cells

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771 transfected with ICA512-GFP (top panels) or the corresponding G553D mutant (bottom
772 panels), and immunostained for insulin (in red, left panels) or co-labeled with the ER
773 tracker red (in red, right panels) ($n \geq 3$). Nuclei were labeled with DAPI (in blue). Scale
774 bars = 5 μm .

775

776 FIG 5 ME ICA512 is *O*-glycosylated. (A) Ponceau S staining of recombinant fetuin
777 untreated (-) or treated (+) with *O*-glycosidases. (B) Immunoblottings for GFP on lysates
778 of resting (R) or HGHK stimulated (S) INS-1 cells transfected with ICA512-GFP,
779 ICA512-GFP N506A, ICA512-GFP S508A or ICA512-GFP G553D, untreated (-) or
780 treated (+) with *O*-glycosidases ($n \geq 3$). (C) Immunoblotting for ICA512 on lysates of
781 resting (R) or HGHK stimulated (S) mouse and human pancreatic islets, untreated (-) or
782 treated (+) with *O*-glycosidases (upper and lower panels, $n=3$ and $n=2$, respectively). For
783 normalization, the same lysates were also immunoblotted for γ -tubulin. (D)
784 Immunoblottings for GFP on lysates of HGHK stimulated INS-1 cells transfected with
785 ME ICA512-GFP, ME ICA512-GFP S508A or ME ICA512-GFP G553D, untreated (-)
786 or treated (+) with *O*-glycosidases, PNGaseF or Endoglycosidase H ($n=3$). (E) Merge
787 confocal microscopy images of INS-1 cells transfected with ME ICA512-GFP or ME
788 ICA512-GFP G553D (in green) and co-labeled with the ER tracker red (in red) and DAPI
789 for nuclei (in blue) ($n=3$). Scale bars = 10 μm . (F) Schematic drawing of ICA512-GFP
790 Δ NTF, and immunoblotting for GFP on lysates of HGHK stimulated INS-1 cells
791 transfected with ICA512-GFP Δ NTF, ICA512-GFP Δ NTF S508A or ICA512-GFP Δ NTF
792 G553D, untreated (-) or treated (+) with *O*-glycosidases, PNGaseF or Endoglycosidase H

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793 (n=3). Fetuin (A) and the ICA512 related protein fragments (B, C, F) sensitive to *O*-
794 glycosidase treatment (+) are indicated by arrowheads (<).

795

796 FIG 6 NTF targets ICA512 to SGs. (A-C) Confocal microscopy images of resting (A, B)
797 or HGHK stimulated (C) INS-1 cells transfected with ICA512-GFP (top panels, green) or
798 ICA512-GFP Δ NTF (bottom panels, green). Live cells were incubated at 4 °C with the
799 mouse anti-ME ICA512 antibody (A, C) together with the guinea pig anti-insulin
800 antibody (A), and also alternatively with the mouse anti-ICA512 CT antibody (B). After
801 fixation, the binding of the primary antibodies to the cell surface was detected by
802 incubation with Alexa-conjugated anti-mouse (A, B, C; in red) or anti-guinea pig (A; in
803 white) IgGs. Merge images are additionally shown for (A) and (C). Scale bars = 10 μ m
804 (n \geq 3). (D, E) Immunoblottings for GFP (D) and ME ICA512 (E) on lysates of resting (R)
805 or HGHK stimulated (S) INS-1 cells transfected with ICA512-GFP or ICA512-GFP
806 Δ NTF (n \geq 3). For normalization, the same lysates were also immunoblotted for γ -tubulin.

807

808 FIG 7 NTF contains information for both ER retention and SG targeting. (A) Schematic
809 drawing of ICA512-RESP18-HD-GFP, and confocal microscopy images of resting INS-1
810 cells transfected with ICA512-RESP18-HD-GFP (in green) and immunostained for
811 insulin (in red) (n \geq 3). Nuclei were labeled with DAPI (in blue). Scale bars = 5 μ m. (B)
812 Immunoblottings for GFP on lysates (left panel) or immunoprecipitates obtained with a
813 different, goat anti-GFP antibody from incubation media (right panel) of resting (R) and
814 HGHK stimulated (S) INS-1 cells transfected with ICA512-RESP18-HD-GFP (n \geq 3). The
815 cell lysates were immunoblotted also for γ -tubulin. (C, D) Schematic drawing of ICA512-

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816 NTF-GFP, and confocal microscopy images of resting INS-1 cells transfected with
817 ICA512-NTF-GFP (in green), and immunostained for insulin (C, in red) or calnexin (D,
818 in red) ($n \geq 3$). Nuclei were labeled with DAPI (in blue). Scale bars = 5 μm . (E)
819 Immunoblottings for GFP (left panel) or NTF (right panel), on lysates of INS-1 cells
820 transfected with either CD33-GFP or ICA512-NTF-GFP (lanes 1 vs. 2) ($n \geq 3$). (F)
821 Schematic drawing of ICA512-NTF, and confocal microscopy images of INS-1 cells
822 transfected with ICA512-NTF, and immunostained for NTF (in green) and calnexin (in
823 red) ($n \geq 3$). Scale bars = 5 μm .

824

825 FIG 8 Establishment of ME ICA512 $\beta 4$ - $\beta 4$ dimers overcome the NTF ER retention
826 signal. (A) Immunoblottings for GFP (top panel) or HA (bottom panel) in the lysates of
827 HGHK stimulated INS-1 cells transfected with ICA512-HA alone (lane 1) or double
828 transfected with ICA512-HA and ME ICA512-GFP (lane 2) or with the respective
829 ICA512-HA and ME ICA512-GFP mutants S508A, G553D, S508A/G553D or
830 N506A/G553D (lanes 3-6) ($n \geq 3$). (B) Immunoblottings for GFP (top panel) or HA
831 (bottom panel) on immunoprecipitates obtained with goat anti-GFP antibody (lanes 7-12)
832 or control IgG (lanes 13-18) from the corresponding cell lysates shown in (B) ($n \geq 3$). (C)
833 Quantification of the immunoprecipitates in (B), for all comparisons ($n=5$), $p < 0.05$, *.
834 (D) Immunoblottings for HA on Endoglycosidase H or PNGaseF untreated (-) or treated
835 (+) lysates from resting (top panel) or HGHK stimulated (bottom panel) INS-1 cells
836 transfected with ICA512-HA, ICA512-HA S508A, G553D or S508A/G553D ($n \geq 3$). (E)
837 Model on how N506 glycosylation affects the folding and dimerization of ME ICA512
838 $\beta 2$ - $\beta 2$ and $\beta 4$ - $\beta 4$ association interfaces and the export of proICA512 from the ER.

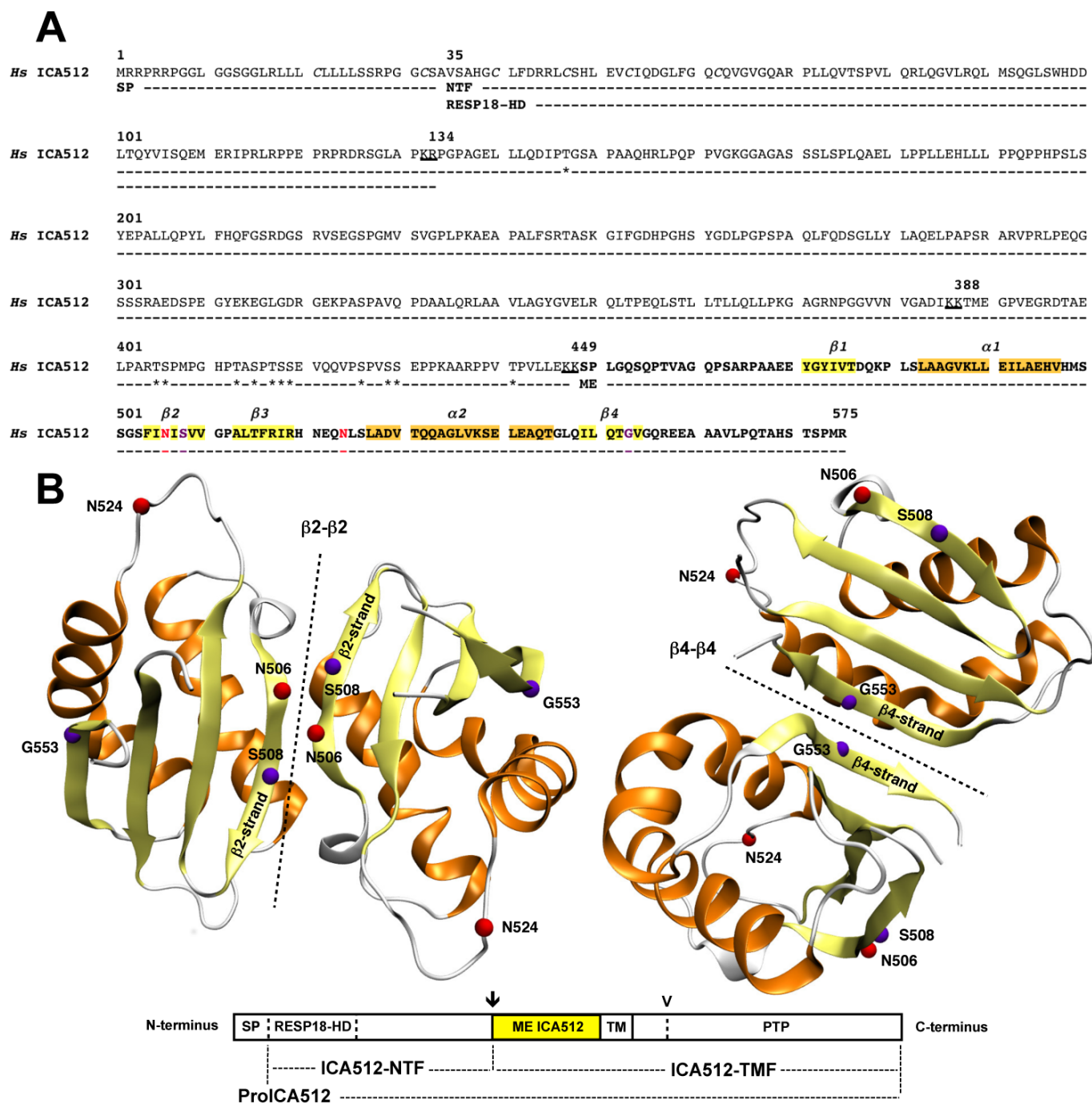


FIG 1 Torkko et al. 2014

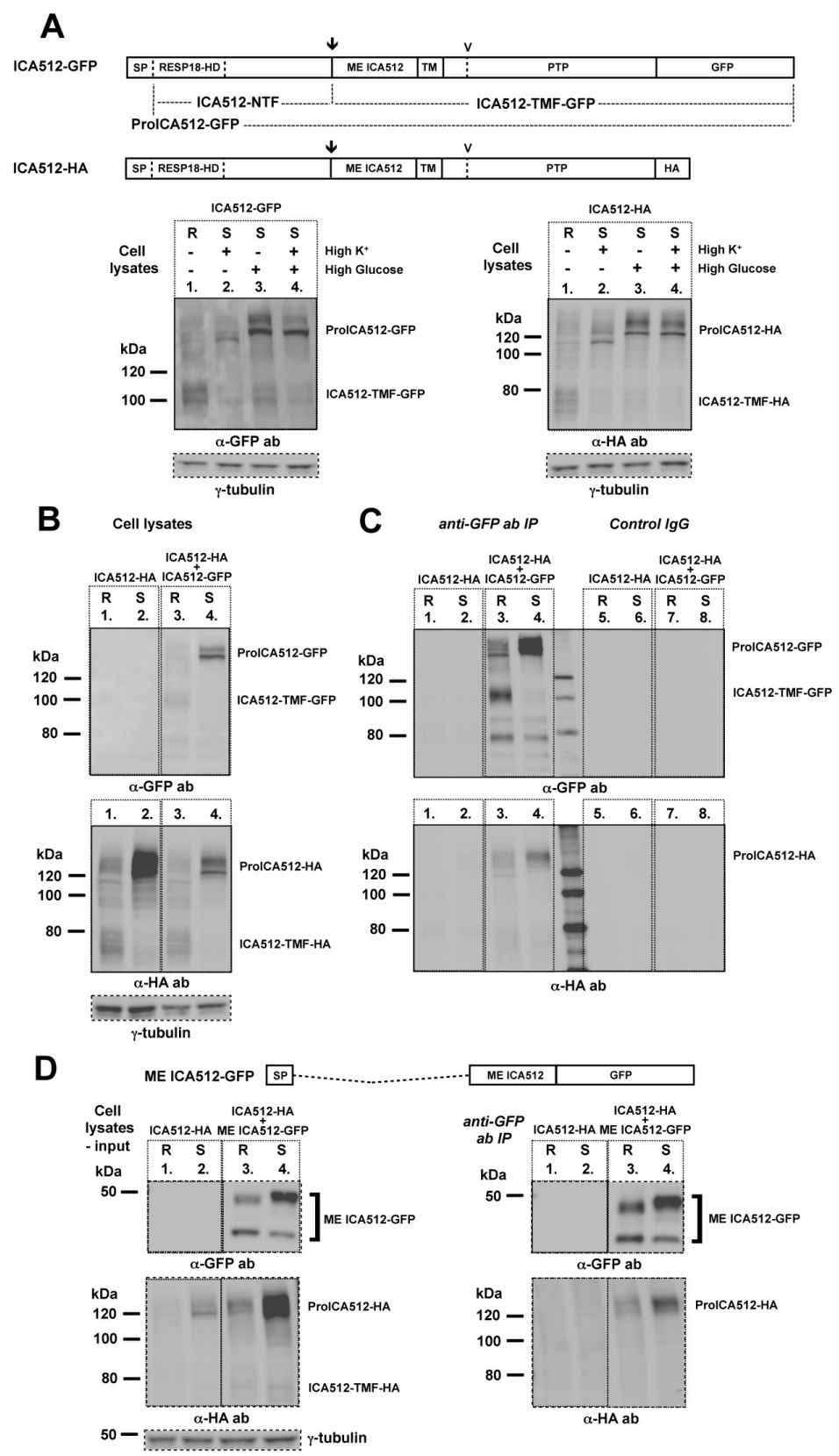


FIG 2 Torkko et al. 2014

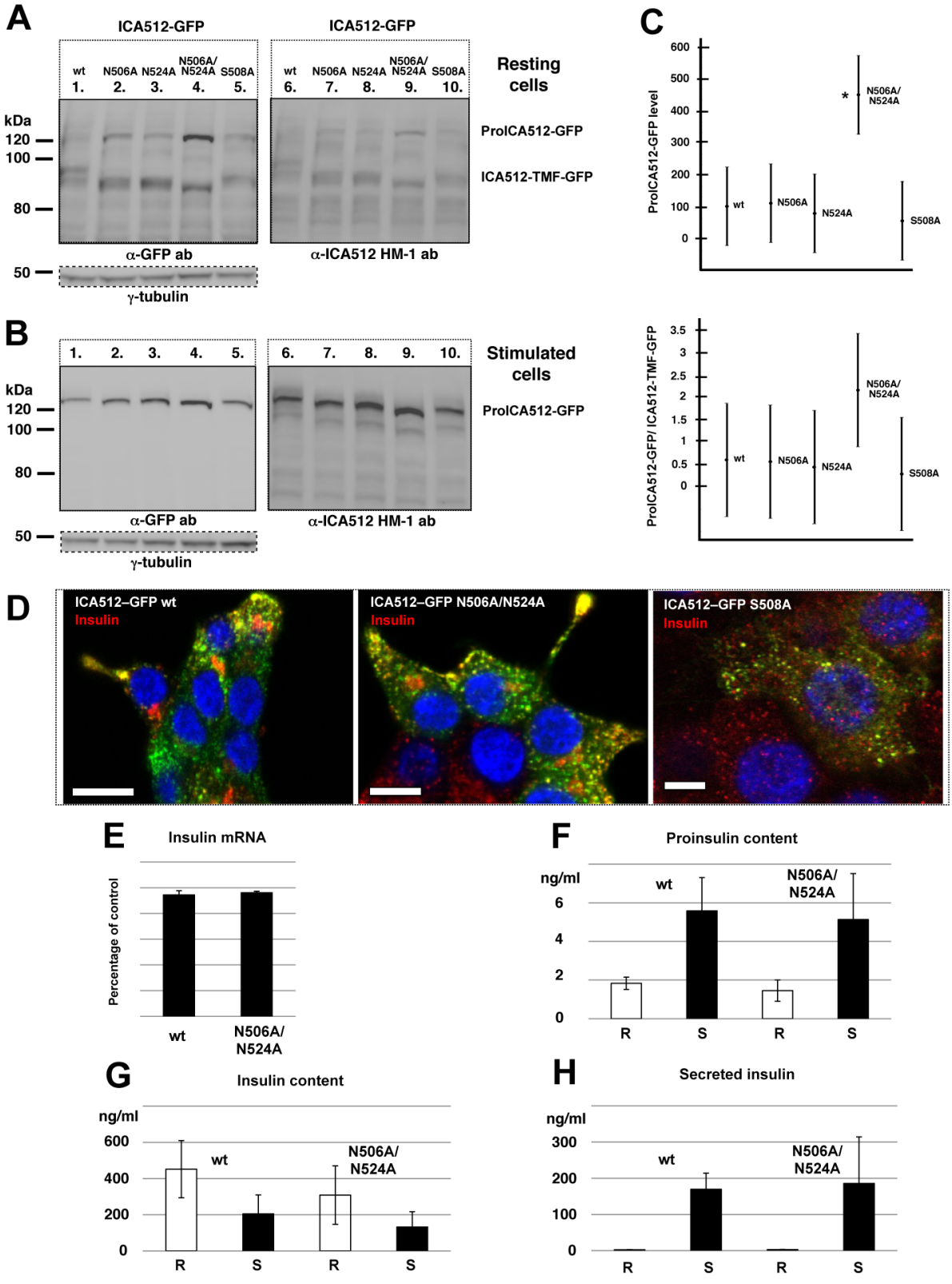


FIG 3 Torkko et al. 2014

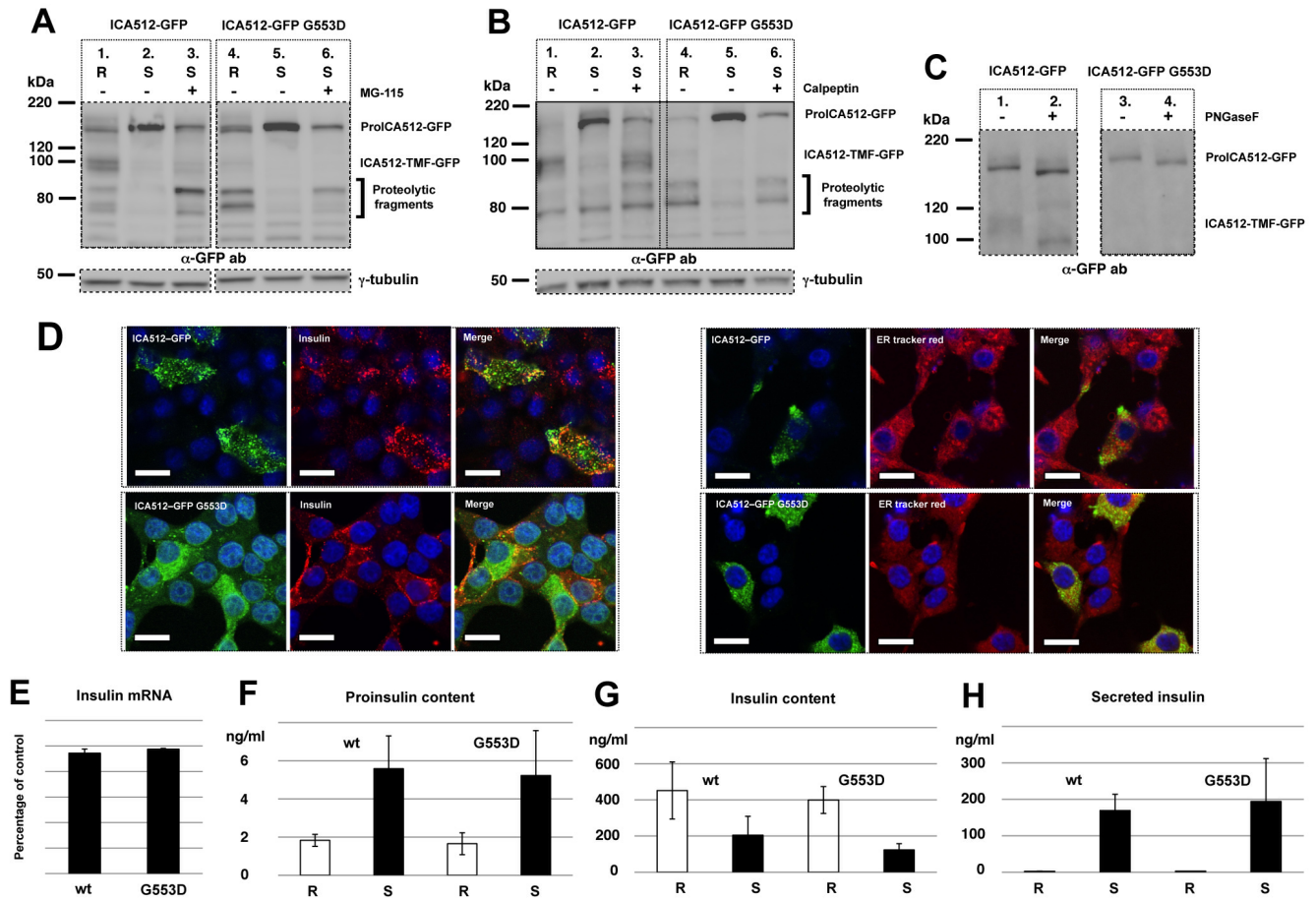


FIG 4 Torkko et al. 2014

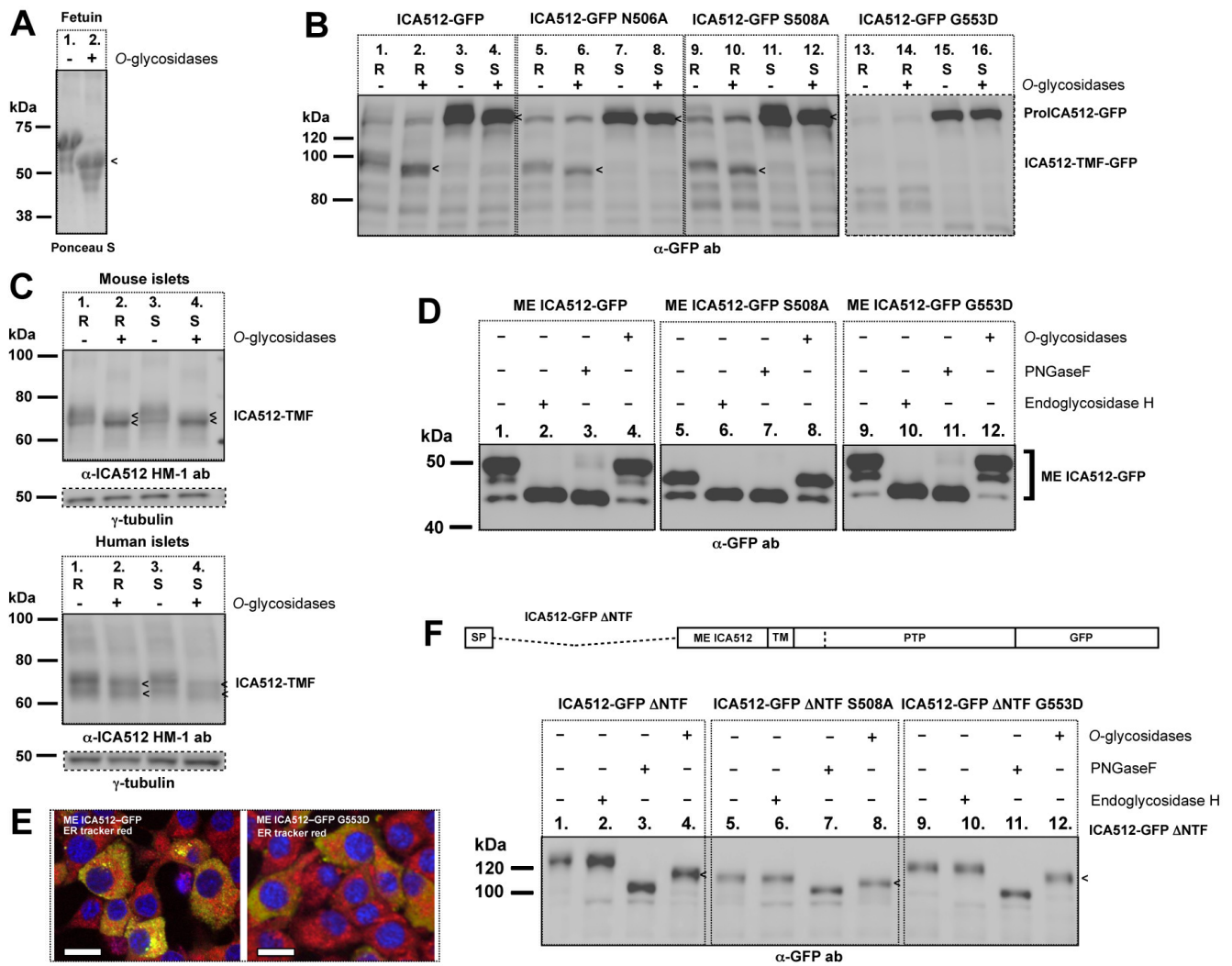


FIG 5 Torkko et al. 2014

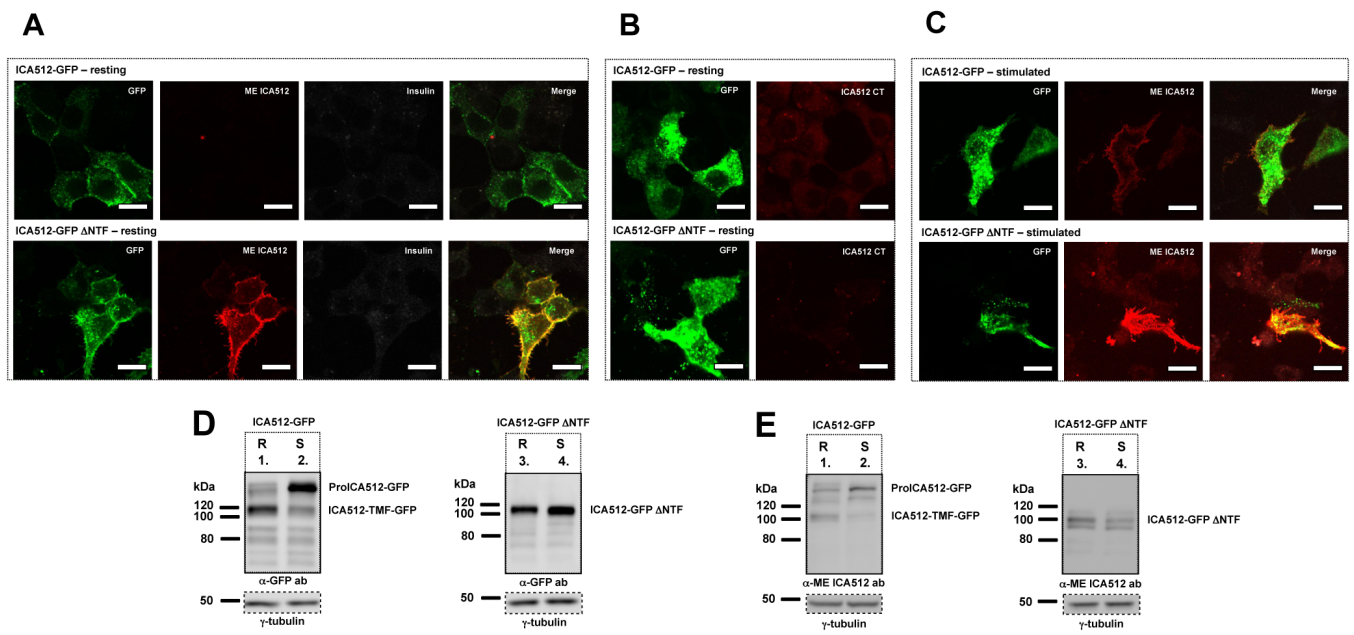


FIG 6 Torkko et al. 2014

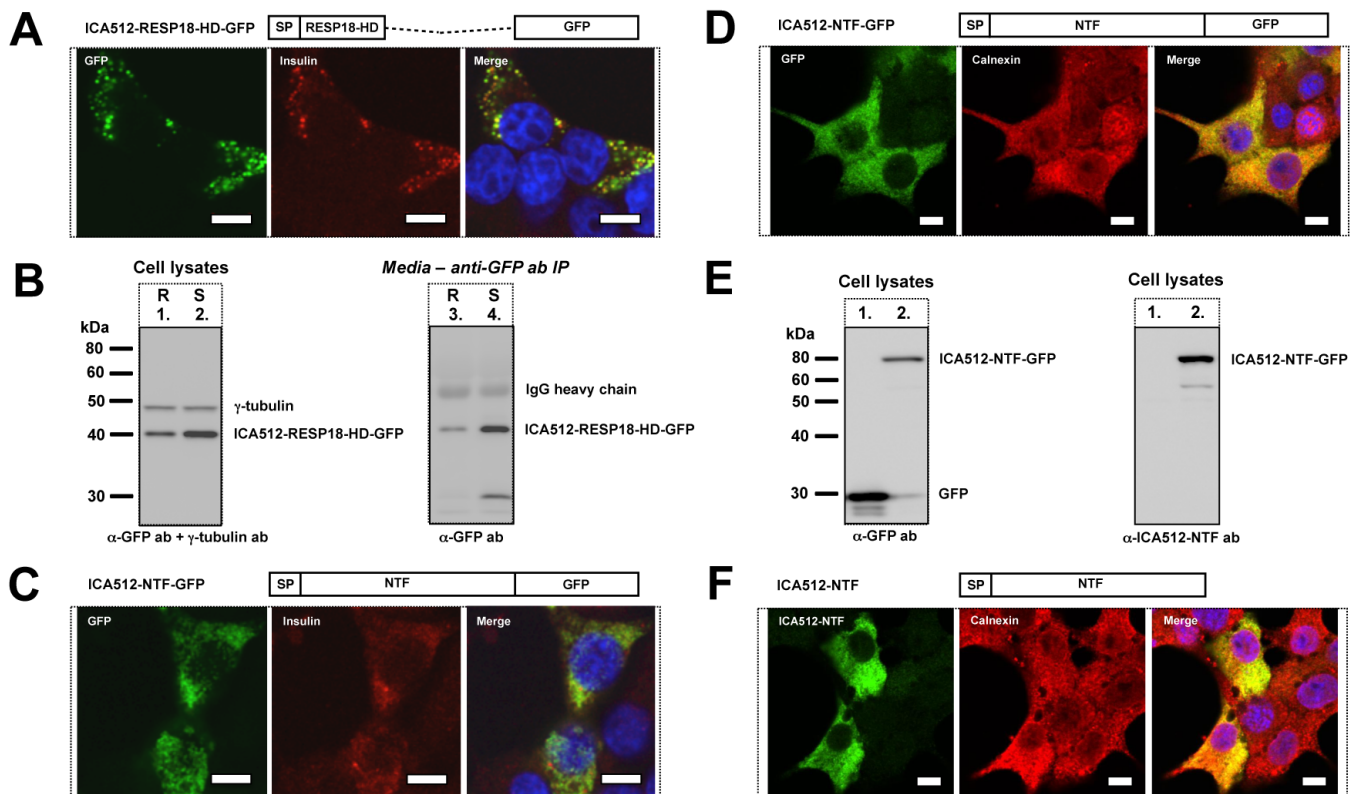


FIG 7 Torkko et al. 2014

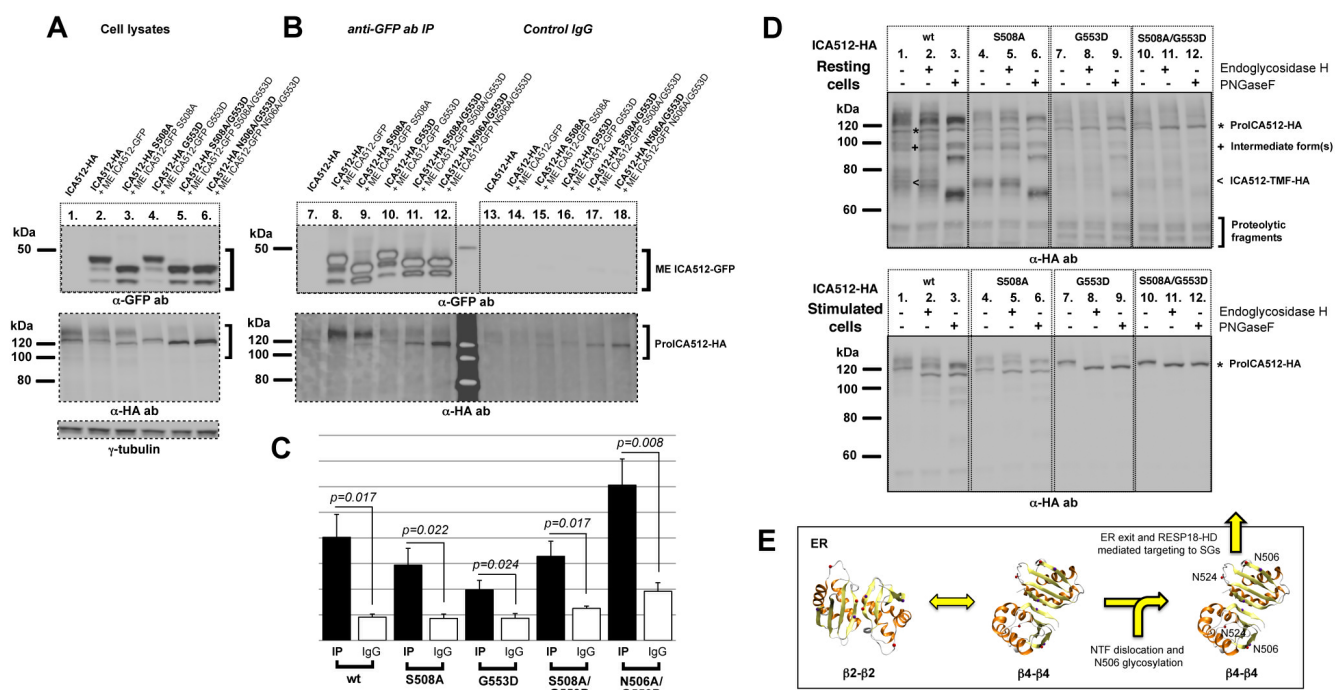


FIG 8 Torkko et al. 2014