nature cell biology

ARTICLES

Synergy of glucose and growth hormone signalling in islet cells through ICA512 and STAT5

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Nutrients and growth hormones promote insulin production and the proliferation of pancreatic β-cells. An imbalance between ever-increasing metabolic demands and insulin output causes diabetes. Recent evidence indicates that β-cells enhance insulin gene expression depending on their secretory activity. This signalling pathway involves a catalytically inactive receptor tyrosine phosphatase, ICA512, whose cytoplasmic tail is cleaved on glucose-stimulated exocytosis of insulin secretory granules and then moves into the nucleus, where it upregulates insulin transcription. Here, we show that the cleaved cytosolic fragment of ICA512 enhances the transcription of secretory granule genes (including its own gene) by binding to tyrosine phosphorylated signal transducers and activators of transcription (STAT) 5 and preventing its dephosphorylation. Sumoylation of ICA512 by the E3 SUMO ligase PIASy, in turn, may reverse this process by decreasing the binding of ICA512 to STAT5. These findings illustrate how the exocytosis of secretory granules, through a retrograde pathway that sustains STAT activity, converges with growth hormone signalling to induce adaptive changes in β-cells in response to metabolic demands.

Glucose stimulates the release of insulin from the secretory granules of pancreatic β-cells. Deficits in β-cell mass and in insulin secretion relative to metabolic demands cause diabetes. Currently, it is unclear how β-cells, and neuroendocrine cells in general, adjust the production of secretory granules according to their secretory activity. Previous studies have shown that glucose sequentially enhances the post-transcriptional and transcriptional expression of insulin and other secretory granule components[1,2]. Although it has been proposed that insulin promotes its own transcription through an autocrine feedback loop[3], recent findings argue against this possibility[4–6]. More recently, we have shown that transcription of the rat *insulin 1* gene is enhanced by a retrograde signalling pathway involving islet cell autoantigen 512 (ICA512, also known as IA-2)[7].

ICA512 is an evolutionarily conserved member of the receptor protein tyrosine phosphatase (PTP) family[8,9] but it lacks PTP activity[10]. Knockout of *ICA512* in mice elevates blood glucose levels and impairs insulin secretion[11], whereas its over-expression in insulinoma cells enhances insulin secretion and the number of secretory granules[12]. However, a mechanistic explanation for these phenotypes does not exist. ICA512 originates as a pro-protein with a molecular weight (M_r) of 110 K. Intracellular processing of its ectodomain by a furin-like convertase generates the mature transmembrane form (65 K; ICA512-TMF), which is enriched in the membrane of secretory granules[13,14]. On exocytosis of secretory granules, ICA512-TMF is transiently inserted into the plasma membrane, and its intracellular domain is cleaved by the Ca^{2+}-activated protease, calpain-1 (refs 7, 15). The resulting cleaved cytosolic fragment (ICA512-CCF), which includes the entire catalytically inactive PTP domain (see Supplementary Information, Fig. S1a), is then targeted to the nucleus where it up-regulates *insulin 1* gene expression[7]. Hence, Ca^{2+} acts as a dual signal that triggers the exocytosis of secretory granules and the activation of a retrograde pathway promoting *insulin* gene transcription. To understand how secretory granule exocytosis regulates gene expression in β-cells, we have examined the events downstream of the nuclear translocation of ICA512-CCF.

RESULTS

ICA512 enhances the transcription of secretory granule genes

To establish whether the retrograde pathway involving ICA512 influences the transcription of secretory granule genes other than insulin, the effect of ICA512 cleavage on its own expression was analysed. As previously shown[16], stimulation of rat insulinoma INS-1 cells with 25 mM glucose and 55 mM KCl for 2 h increases the levels of pro-ICA512, whereas it reduces those of ICA512-TMF because of its cleavage by calpain-1 (see Supplementary Information, Fig. S1b). This rapid increase in pro-ICA512 is due to the activation of post-transcriptional mechanisms[16,17], and is insensitive to treatments with the transcription inhibitor actinomycin D[16] or the calpain inhibitor, calpeptin (see Supplementary Information, Fig. S1b). Calpeptin, however, partially diminished the increased induction of pro-ICA512 and the decrease in levels of ICA512-TMF in cells

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Received 10 November 2005; accepted 27 March 2006; published online 16 April 2006; DOI: 10.1038/ncb1395

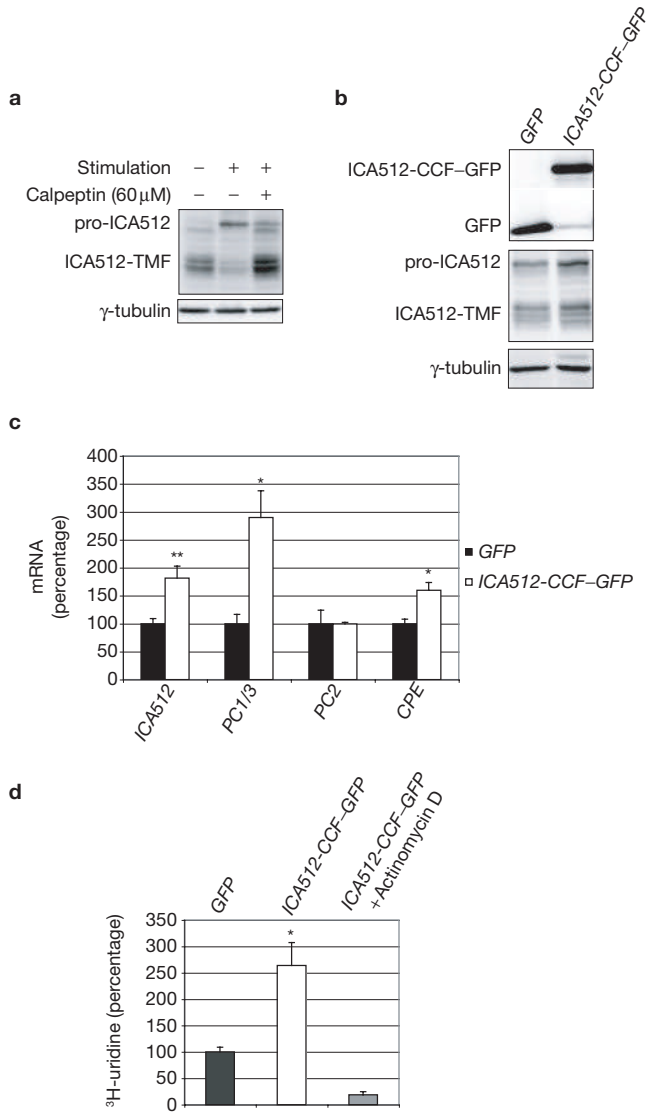

Figure 1 ICA512 enhances the transcription of secretory granule genes. (**a**) Immunoblot for ICA512 and γ-tubulin of extracts from INS-1 cells kept at rest or stimulated for 6 h with or without 60 μM calpeptin. (**b**) Immunoblots for GFP, ICA512 and γ-tubulin of extracts from *GFP*- or *ICA512-CCF–GFP*-transfected INS-1 cells. (**c**) The levels of rat *ICA512*, *PC1/3*, *PC2* and *CPE* mRNAs in *GFP*- or *ICA512-CCF–GFP*-transfected INS-1 cells were quantified by real-time PCR and normalized for *β-actin* mRNA. Values were compared with *GFP* transfected INS-1 cells (100%). (**d**) ^{3}H-uridine RNA recovered from *GFP*- or *ICA512-CCF–GFP*-transfected INS-1 cells, treated or untreated with 5 μg ml^{-1} actinomycin D. Values were normalized for *β-actin* mRNA. Results shown in **c** and **d** are from at least two independent experiments performed in triplicate. Single asterisk indicates $P<0.05$; double asterisks indicates $P<0.01$.

stimulated for 2 h and then incubated in resting buffer for an additional 4 h (Fig. 1a). The reduced ICA512 turnover in these conditions explains the accumulation of the 110 K and 130 K pro-ICA512 species[14]. Direct evidence that ICA512 enhances its own expression was supplied by the observation that in INS-1 cells expressing *ICA512-CCF–GFP* the levels of *ICA512* mRNA (Fig. 1c) and pro-ICA512 (Fig. 1b) were increased by 81% and 40%, respectively, compared with GFP-expressing INS-1 cells. The greater increase in levels of *ICA512* mRNA relative to pro-ICA512 can be explained by the rapid conversion of the latter into ICA512-TMF, whose levels were also increased by *ICA512-CCF–GFP* (Fig. 1b). Expression of *ICA512-CCF–GFP* correlated with significant increases in the mRNA levels of the secretory granule genes pro-hormone convertase 1/3 (*PC1/3*) and carboxypeptidase E/H (*CPE*), but not of pro-hormone convertase 2 (*PC2*; Fig. 1c). Moreover, *ICA512-CCF–GFP* increased ^{3}H-uridine incorporation by 270% (Fig. 1d) in an actinomycin D-sensitive manner. These data indicate that ICA512-CCF, in addition to promoting *insulin 1* gene transcription[7], enhances its own expression as well as that of other secretory granule genes.

ICA512 enhances the nuclear levels of STATs

Previous evidence suggested that in the nucleus ICA512-CCF binds to protein inhibitor of activated STAT-y (PIASy)[7]. STATs are tyrosine phosphorylated by Janus kinases when cells are stimulated with cytokines and growth hormones[18]. Tyrosine phosphorylated STATs (PY-STATs) form dimers in the cytosol and then move into the nucleus, where they bind to chromatin and activate gene transcription. Among the seven STAT genes expressed in mammals, STAT5a/b and STAT3 are the most relevant in β-cells[19,20]. Activation of STAT5a/b by growth hormone, prolactin or placental lactogen during pregnancy, enhances the expression of key β-cell genes, including insulin, glucose transporter-2, glucokinase and cyclin D2 (ref. 21, 22), as well as β-cell proliferation[22]. Therefore, whether or not ICA512 modulates STAT activity was examined. Expression of *ICA512-CCF–GFP* caused a dose-dependent increase in nuclear STAT5b and STAT3 (Fig. 2a, b). Nuclear enrichment of STAT3 (see Supplementary Information, Fig. S1c) and STAT5b (data not shown) was also evident in stable *ICA512–GFP* INS-1 cells, which overexpress ICA512 compared with INS-1 cells[7]. These findings were corroborated by imaging and biochemical analyses of pancreatic islets isolated from *ICA512*$^{-/-}$ mice and control littermates. Following costimulation of islets with 20 nM growth hormone and 25 mM glucose, which induces secretory granule exocytosis and the generation of ICA512-CCF [7], the nuclear levels of STAT5b in *ICA512*$^{-/-}$ islets relative to *ICA512*$^{+/+}$ islets was reduced to 61 ± 7%, as measured by immunoblotting (Fig. 2c, d), and to 62 ± 24% as measured by immunofluorescence microscopy (see Supplementary Information, Fig. S2a, b), whereas the ratio of nuclear versus total STAT5b was decreased from 45 ± 5% to 30 ± 3.5% (Fig. 2e). Notably, costimulation of *ICA512*$^{+/+}$ islets with growth hormone and glucose (see Supplementary Information, Fig. S2d) led to a greater nuclear accumulation of STAT5 than stimulation with growth hormone alone (see Supplementary Information, Fig. S2c). However, this glucose-mediated enhancement of STAT5 localization in the nucleus was not observed in *ICA512*$^{-/-}$ islets.

ICA512 enhances the nuclear levels of PY-STATs

We next investigated whether ICA512-CCF, whose PTP domain lacks phosphatase activity[10], affects the phosphorylation of STAT5b, and hence its nuclear targeting and activity. To enhance the detection of PY-STAT5, *GFP*- and *ICA512-CCF–GFP*-transfected INS-1 cells were stimulated with growth hormone for 20 min. Nuclear STAT5b and PY-STAT5, but not total STAT5b, were clearly increased in *ICA512-CCF–GFP* INS-1 cells relative to *GFP* INS-1 cells (Fig. 3a). In *ICA512-CCF–GFP* INS-1 cells there was a corresponding accumulation of PY-STAT3 (see Supplementary Information, Fig. S1d). Strikingly, the phosphatase active ICA512-CCF$^{A877D–D911A}$–GFP[10], in which Ala 877 and Asp 911 (see Supplementary Information, Fig. S1a) were replaced by Asp and Ala, respectively, was

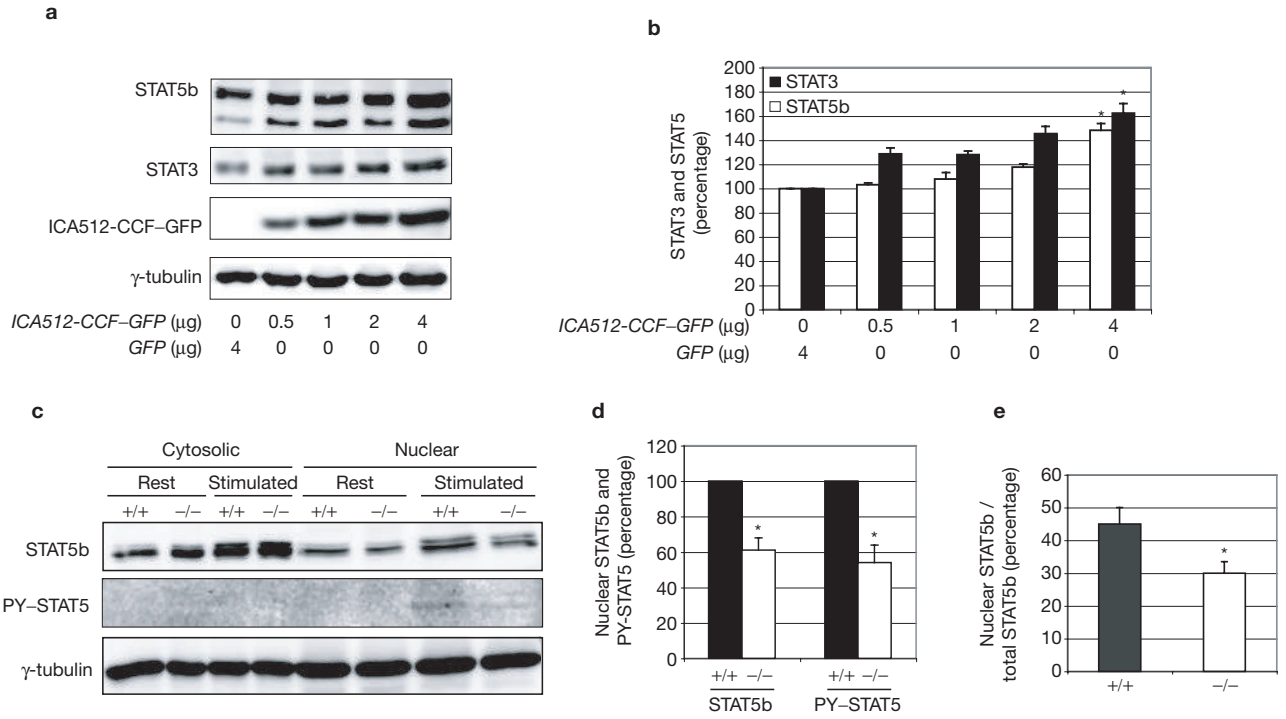

Figure 2 ICA512 enhances the nuclear levels of STATs. (**a**) Immunoblots for STAT5b, STAT3, GFP and γ-tubulin on nuclear extracts from INS-1 cells transfected with the indicated amount of *GFP* or *ICA512-CCF–GFP* plasmids. (**b**) Quantification of STAT5 and STAT3 as detected in **a**. Values were compared with *GFP*-transfected INS-1 cells (100%). (**c**) Western blots for STAT5b, PY-STAT5 and γ-tubulin on cytosolic and nuclear extracts from 3×10^2 islets from *ICA512*$^{+/+}$ and *ICA512*$^{-/-}$ mice. Islets were either kept at rest or stimulated with 25 mM glucose and 20 nM growth hormone for 2 h, followed by incubation in serum free media for 4 h before being harvested. (**d**) Nuclear levels of STAT5b and PY-STAT5 in *ICA512*$^{-/-}$ islets as a percentage of the corresponding levels in *ICA512*$^{+/+}$ islets. (**e**) Nuclear levels of STAT5b as a percentage of the corresponding total levels in *ICA512*$^{+/+}$ and *ICA512*$^{-/-}$ islets. Values in **d** and **e** were determined by immunoblotting as in **c**. Results shown in **b**, **d** and **e** are from three independent experiments. The asterisk indicates $P < 0.05$. Full scans of the blots shown in **a** and **c** can be found in the Supplementary Information, Fig. S5.

much less abundant in the nucleus than ICA512-CCF–GFP (Fig. 3b, c). Correspondingly, the nuclear levels of STAT5b (Fig. 3c) and PY-STAT5 (Fig. 3d, e) were reduced by approximately twofold in *ICA512-CCF*$^{A877D-D911A}$*–GFP* INS-1 cells compared with *ICA512-CCF–GFP* INS-1 cells and so were the levels of *insulin 1* mRNA (Fig. 3f). Furthermore, the nuclear levels of PY-STAT5 in *ICA512*$^{-/-}$ islets costimulated with growth hormone and glucose were reduced to 54% relative to *ICA512*$^{+/+}$ islets (Fig. 2c, d). Taken together, these data indicate a close quantitative correlation between levels of ICA512-CCF, nuclear accumulation of phosphorylated STATs and increased expression of the *insulin 1* gene.

ICA512 binds to STAT5b and prevents its dephosphorylation

Enhancement of PY-STAT5 and PY-STAT3 levels by ICA512-CCF raised the possibility that ICA512, presumably through its inactive PTP domain, binds to STATs and prevents their dephosphorylation. Consistent with this hypothesis, STAT5b and PY-STAT5 were coimmunoprecipitated with ICA512-CCF–GFP, but not with GFP (Fig. 4a). Recovery of STAT5b with ICA512-CCF–GFP from total, but not from cytosolic extracts of growth hormone-stimulated INS-1 cells, suggested that the complex including the two proteins is restricted to the nucleus, even though its assembly in the cytosol cannot be excluded. The direct interaction of ICA512-CCF and STAT5 in the nucleus was corroborated by fluorescence resonance energy transfer (FRET) analyses. The photobleaching of STAT5b-YFP increased the signal of ICA512-CFP in the nucleus of INS-1 cells costimulated with 20 nM growth hormone, 25 mM glucose and 55 mM KCl (conditions promoting the activation of STAT5b-YFP and the cleavage of ICA512-CFP; Fig. 4b). Conversely, photobleaching of STAT5b-YFP did not increase the fluorescence signal of the control nuclear protein Lsm4-CFP in resting or in costimulated INS-1 cells. Immunoprecipitations from extracts of costimulated INS-1 cells with the monoclonal antibody mICA512–HM1, which recognizes ICA512-CCF (see Supplementary Information, Fig. S3a–c), confirmed that endogenous ICA512 binds to PY-STAT5 (Fig. 4c). Finally, whether ICA512 inhibits the dephosphorylation of PY-STAT5 by a conventional PTP was examined. Supporting this hypothesis, GST–ICA512$^{601-979}$ prevented the *in vitro* dephosphorylation of immunoprecipitated PY-STAT5 by recombinant TC45 (Fig. 4d), a nuclear PTP that dephosphorylates STAT1 in various cell types[23]. Although it is unlikely that TC45 dephosphorylates PY-STAT5 in β-cells, these data illustrate how ICA512-CCF may modulate gene expression.

ICA512 increases STAT5b activity

ICA512-CCF association with chromatin was also examined. For this purpose, anti-GFP and anti-ICA512 antibodies, or control IgG, were used for chromatin immunoprecipitation assays using extracts either from *GFP*- or *ICA512-CCF–GFP* INS-1 cells stimulated with growth hormone (Fig. 5a) or from INS-1 cells costimulated with growth hormone, glucose and KCl (Fig. 5a). PCR analysis revealed that the γ-interferon activated sequence-like element (GLE) of the rat *insulin 1* gene promoter, which binds STAT5b[24], was coimmunoprecipitated with the anti-GFP antibody, but not with control IgG, and only from *ICA512-CCF–GFP* INS-1 cells (Fig. 5a). The same GLE was also weakly, but specifically

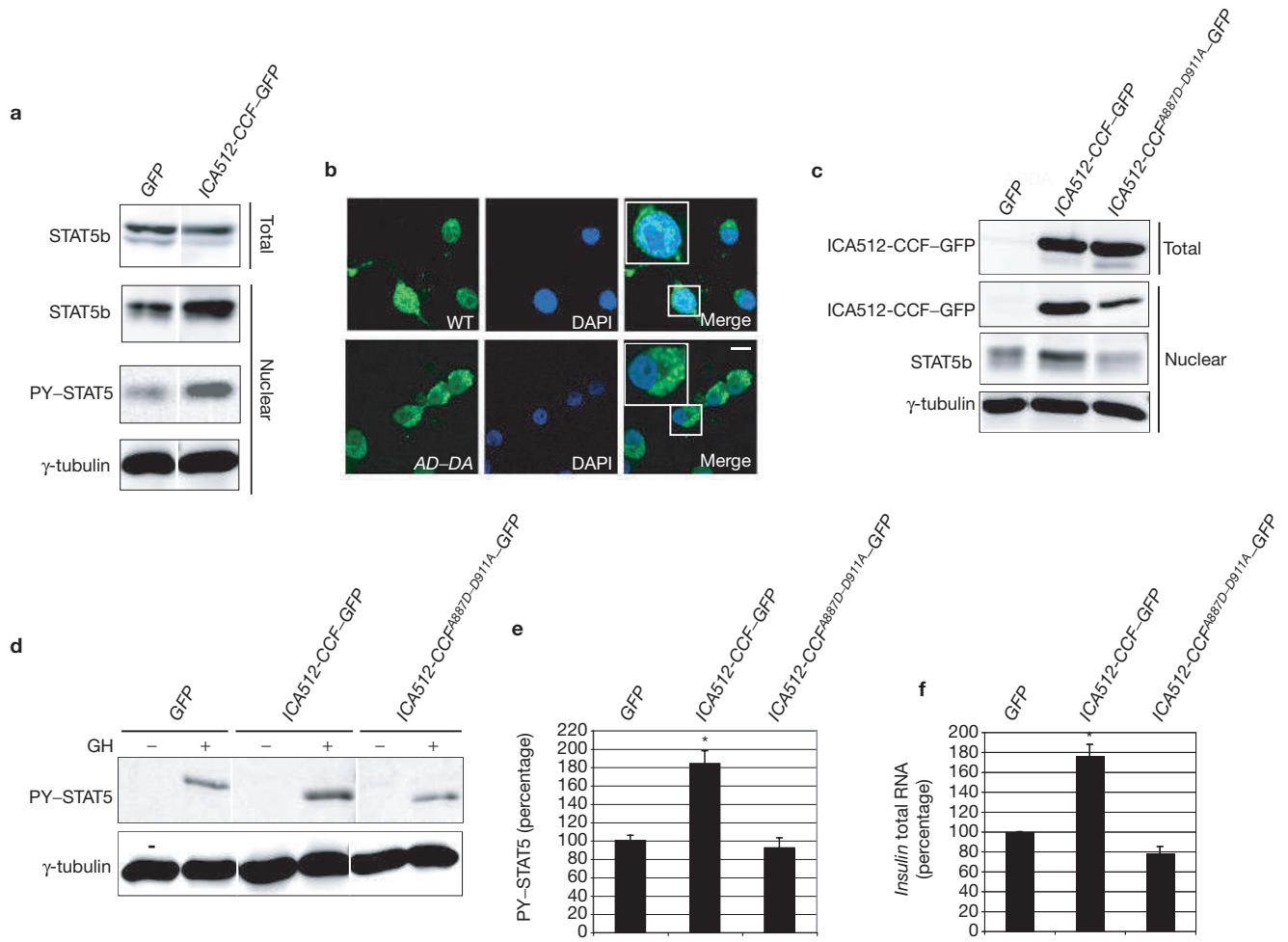

Figure 3 ICA512 enhances the nuclear levels of PY-STAT5. (**a**) Immunoblots for STAT5b on total extracts and for STAT5b, PY-STAT5 and γ-tubulin on nuclear extracts from growth hormone-stimulated INS-1 cells expressing *GFP*- or *ICA512-CCF–GFP*. (**b**) Confocal microscopy images for GFP in INS-1 cells expressing *ICA512-CCF–GFP* (WT) or *ICA512-CCF*$^{A877D–D911A}$*–GFP* (*AD–DA*). Nuclei were costained with DAPI (blue). Large insets in the panels on the right show 2× magnifications of the areas marked with a small square. The scale bar represents 5 μm. (**c**) Immunoblot for GFP of total extracts and for GFP, STAT5b and γ-tubulin on nuclear extracts from INS-1 cells expressing *GFP-*, *ICA512-CCF–GFP*- or *ICA512-CCF*$^{A877D–D911A}$*–GFP*. (**d**) Immunoblot for PY-STAT5 and γ-tubulin on nuclear extracts from growth hormone-stimulated INS-1 cells transfected as in **c**. (**e**) Levels of PY-STAT5 as detected in **d**. Values were compared with *GFP*-transfected INS-1 cells (100%). (**f**) *Insulin* mRNA levels in growth hormone-stimulated INS-1 cells expressing *GFP-*, *ICA512-CCF–GFP* or *ICA512-CCF*$^{A877D–D911A}$*–GFP* as quantified by real-time PCR. After normalization for *β-actin* mRNA, values were compared with *GFP*-transfected INS-1 cells (100%). Values in **e** and **f** are from three independent experiments. The asterisk indicates $P < 0.05$. All bands shown in **a** and **d** are vertical cropping from a single gel.

recovered in immunoprecipitates obtained with mICA512–HM1 from INS-1 cells (Fig. 5a). Nuclear extracts from growth hormone-stimulated INS-1 cells expressing increasing amount of *ICA512-CCF–GFP* were then incubated with a biotinylated oligonucleotide including the GLE of rat *insulin 1*. Electrophoretic mobility shift assays showed a positive correlation between increments in the levels of *ICA512-CCF–GFP* and binding of a factor, possibly STAT5b, to this oligonucleotide (Fig. 5b, c). The specificity of this assay was confirmed by competitive inhibition with the corresponding non-biotinylated oligonucleotide. To directly test whether ICA512-CCF enhances the transcription activity of STAT5, INS-1 cells were cotransfected with either *GFP* or *ICA512-CCF–GFP* and firefly *luciferase* driven by a bovine *β-casein* promoter with eight GLEs (*8GLE–CAS–FL*; Fig. 5d). Following growth hormone stimulation, luciferase activity increased >500% in *ICA512-CCF–GFP–8GLE–CAS–FL* INS-1 cells relative to *GFP–8GLE–CAS–FL* INS-1 cells. As expected, PIASy reduced this activity. However, luciferase expression was partially restored by ICA512-CCF–GFP. ICA512-CCF $^{A877D–D911A}$–GFP, which is not enriched in the nucleus, stimulated luciferase activity less than ICA512-CCF–GFP and did not prevent inhibition of transcription by PIASy. These data conclusively demonstrate that ICA512-CCF enhances STAT5 transcription activity, which, in turn, is inhibited by PIASy.

ICA512 mediates the binding of PIASy to STAT5

To gain insight into the interaction of PIASy with ICA512-CCF[7] the domain of ICA512 that binds to PIASy was mapped. Portions of recombinant ICA512 cytoplasmic domain fused to GST (see Supplementary Information, Fig. S3d) were tested by pulldown assays for their binding to *in vitro* transcribed and translated ^{35}S-methionine-PIASy (Fig. 6a). GST–ICA512$^{700–979}$ (which only contains the PTP domain) bound to ^{35}S-methionine-PIASy similarly to GST–ICA512$^{601–979}$ (which encompasses the entire ICA512 cytoplasmic domain). ^{35}S-methionine-PIASy did not bind to GST–ICA512$^{800–979}$, which lacks the first 100 residues of the PTP

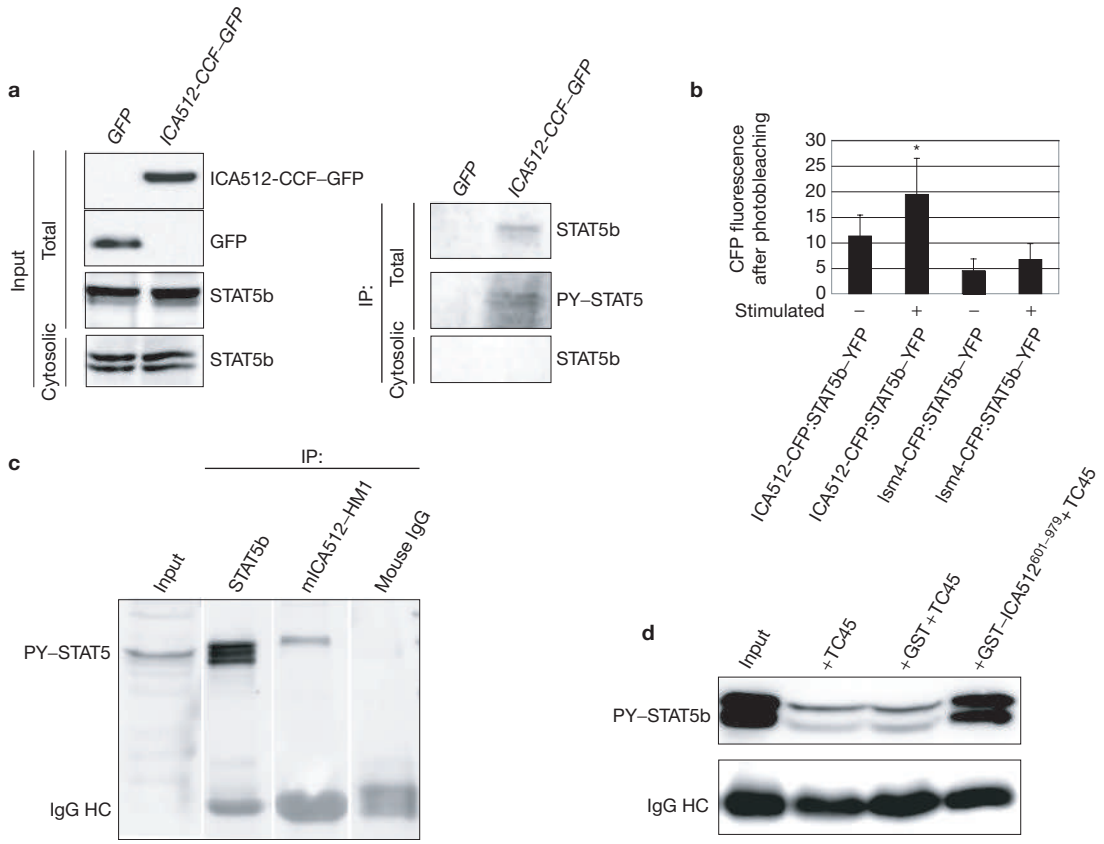

Figure 4 ICA512 binds to STAT5b and prevents its dephosphorylation. (**a**) Immunoblots for GFP and STAT5b on total and cytosolic extracts from growth hormone-stimulated INS-1 cells expressing *GFP*- or *ICA512-CCF–GFP* (left). Equal amounts of these extracts were used as the input for immunoprecipitations with anti-GFP antibodies. Immunoblots for STAT5b and PY-STAT5 on immunoprecipitates obtained with anti-GFP antibodies (right). (**b**) FRET in the nucleus of INS-1 cells co-expressing STAT5b–YFP with either ICA512-CCF–CFP or Ism4–CFP. Cells were either kept at rest or co-stimulated with growth hormone, glucose and KCl before measuring the intensity of the CFP signal as previously described[7]. Values are from two independent experiments, each including measurements from 15 photobleached cells. (**c**) Immunoblot for PY-STAT5 on immunoprecipitates obtained with antibodies against STAT5b, ICA512 or control mouse IgG from total extracts of INS-1 cells co-stimulated with growth hormone, glucose and KCl. (**d**) Immunoblot for PY-STAT5b on immunoprecipitates obtained with anti-STAT5b antibodies from growth hormone-stimulated INS-1 cells. Before immunoblotting, equal aliquots of the immunoprecipitates were incubated with or without (input) recombinant GST–TC45, plus or minus GST or GST–ICA512$^{601–979}$. The asterisk indicates P <0.05. All bands shown in **c** are vertical cropping from a single gel. Full scans of the blots shown in **c** and **d** can be found in the Supplementary Information, Fig. S5.

domain, suggesting that the association of the two proteins requires the integrity of the PTP domain. The reciprocal interaction of ICA512 with PIASy and STAT5 was also analysed. GST–PIASy was not required for the association of ^{35}S-methionine-ICA512$^{601–979}$ with STAT5, but could disrupt this interaction when added in high amounts (Fig. 6b). In contrast, ^{35}S-methionine-PIASy did not bind to STAT5 unless a recombinant histidine-tagged ICA512 cytoplasmic domain was added (Fig. 6c). The interaction of endogenous PIASy with ICA512 was confirmed by coimmunoprecipitation from extracts of INS-1 cells costimulated with growth hormone, glucose and KCl (Fig. 6d) using the anti-IA-2$^{771–979}$ antibodies that recognize an epitope between residues 771–979 of ICA512. PIASy was only weakly present in immunoprecipitates obtained with anti-STAT5b antibodies, and was not detected in immunoprecipitates generated with mICA512–HM1 or control mouse IgG.

Sumoylation of ICA512 regulates its binding to STAT5

Lys 754 in the ICA512 PTP domain lies within a consensus site for sumoylation (ΨKXE[25]; see Supplementary Information, Fig. S1a). Therefore, whether ICA512 is sumoylated by PIASy, an E3 SUMO-ligase[26], was tested. ^{35}S-methionine-ICA512$^{601–979}$, similar to ^{35}S-methionine-p53, binds to the E2 SUMO-conjugating enzyme, Ubc9, that was fused to a maltose binding protein (Fig. 7a). Sumoylated proteins (like p53) typically interact with both E2 SUMO-conjugating enzyme and E3 SUMO-ligases[27]. Addition of PIASy to an *in vitro* sumoylation assay correlated with the appearance of two GST–ICA512 species of 86 K and 105 K (see Supplementary Information, Fig. S4a). The slower mobility of these GST–ICA512 species compared with GST–ICA512$^{601–979}$, which migrates as a 70 K protein, was compatible with the addition of one and two SUMO-1 peptides, respectively. Likewise, two sumoylated species were generated in bacteria cotransformed with GST–ICA512$^{601–979}$ and a tri-cistronic plasmid encoding the minimal machinery for sumoylation (Aos–Uba2, Ubc9 and activated SUMO-1 or SUMO-2[28]; Fig. 7b, c). Sumoylation of ICA512 within INS-1 cells was assessed by co-expression of ICA512–GFP and HA-tagged SUMO-1 followed by immunoprecipitation with the anti-GFP antibody and immunoblotting for GFP and the HA-epitope (see Supplementary Fig. S4b, c). On replacement of Lys 754 with Ala, only one sumoylated ICA512-CCF–GFP species was detected instead of two, indicating that Lys 754 is sumoylated (see Supplementary Information, Fig. S4c), although the location of the second sumoylation site remains to be established. These data prove that ICA512-CCF undergoes sumoylation.

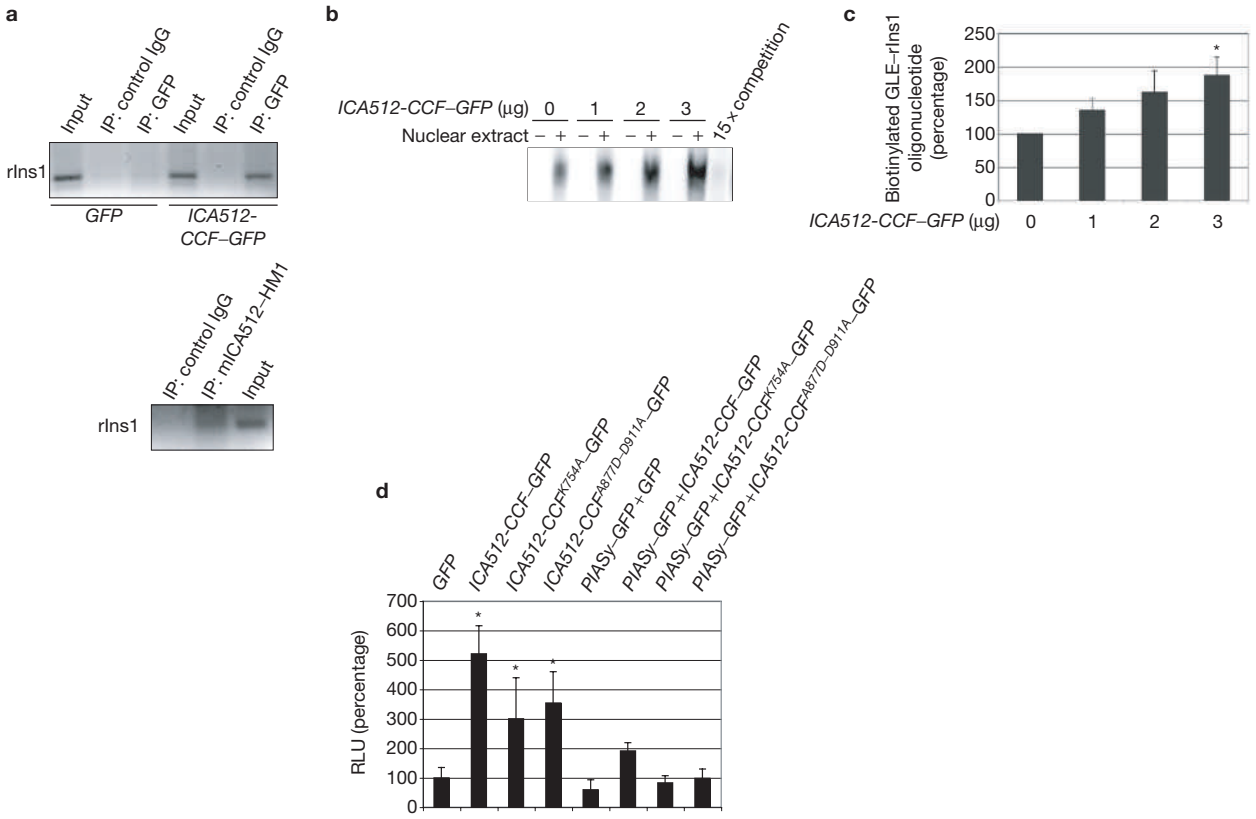

Figure 5 ICA512 increases STAT5b activity. (**a**) Ethidium bromide staining following agarose gel electrophoresis of the PCR product including the GLE in the rat insulin 1 (*rINS1*) gene promoter. PCRs were performed using the chromatin immunoprecipitates obtained with anti-GFP antibody or control IgG from extracts of stable *GFP* or *ICA512-CCF–GFP* INS-1 cells or with anti-ICA512 antibody and control IgG from extracts of INS-1 cells, as template. (**b**) Electrophoretic mobility shift assays with a biotinylated oligonucleotide including the GLE of *rINS1* promoter (bGLE–rINS-1). Before electrophoresis, bGLE–rINS-1 was incubated with (+) or without (–) 5 μg nuclear extracts from INS-1 cells transfected with increasing amount of *ICA512-CCF–GFP* plasmid. Competition assay (lane 9) was performed by adding 15-fold molar excess of the non-biotinylated GLE–rINS-1 oligonucleotide. (**c**) Quantification of the chemiluminescence from five independent experiments as shown in **b**. (**d**) Reporter assays for STAT activity in INS-1 cells cotransfected with *β-casein* promoter including eight GLEs upstream of firefly luciferase (*8GLE–CAS–FL*) and vectors encoding the indicated proteins. RLU: relative light units. Values were normalized to GFP expression and were collected from three independent experiments. Values were compared with the RLU detected in INS-1 cells co-expressing *8GLE–CAS–FL* and *GFP* (100%). The asterisk indicates $P < 0.05$. Full scans of the gel and EMSA shown in **a** and **b** can be found in the Supplementary Information, Fig. S5 and S6, respectively.

Lys 754 is adjacent to the 'phosphotyrosine recognition motif' that participates in the interaction of PTPs with their substrates[29, 30] (see Supplementary Information, Fig. S1a). Therefore, whether sumoylation affects the binding of ICA512-CCF to STAT5b was examined. Pulldown assays (Fig. 7e) showed that STAT5 binds more effectively to purified recombinant GST–ICA512$^{601–979}$ than to purified single, and especially double, SUMO-1-conjugated GST–ICA512$^{601–979}$ (Fig. 7d). Intriguingly, ICA512-CCF^{K754A}–GFP interacted with STAT5 in a similar manner to ICA512-CCF–GFP (see Supplementary Information, Fig. S4d), but it enhanced less STAT transcriptional activity (Fig. 5d). Mutation of Lys 754, however, correlated with a compensatory increase in sumoylation of the remaining site (see Supplementary Information, Fig. S4c). Taken together, these data suggest that sumoylation of ICA512 by PIASy affects its association with STAT5.

STAT5 promotes the transcription of ICA512

Over-expression of PIASy–GFP reduced the levels of *ICA512* mRNA (Fig. 8a) and protein (Fig. 8b), raising the possibility that STAT5b regulates the transcription of the *ICA512* gene in β-cells. Knockdown of STAT5b expression in INS-1 cells with small interference RNA (siRNA) oligonucleotides (Fig. 8c, d) decreased the levels of pro-ICA512 and ICA512-TMF by approximatley 50% relative to cells transfected with a control scrambled siRNA oligo (Fig. 8d, e), whereas the expression of STAT3 or γ-tubulin was unchanged. Knockdown of transfected firefly luciferase did not alter instead the expression of STAT5b and pro-ICA512 (see Supplementary Information, Fig. S4e, f). These data indicate that ICA512, in addition to insulin, upregulates its own expression, and that of other secretory granule components, through a retrograde feedback loop that enhances STAT5b activity.

DISCUSSION

Previous studies have shown that STAT5 is a potent inducer of insulin gene expression and β-cell proliferation in physiological conditions such as pregnancy[19]. Its transcriptional activity is activated by tyrosine phosphorylation in response to stimulation by various hormones, including growth hormone, prolactin and placental lactogen. Here, we demonstrate that the cleaved cytosolic fragment of ICA512, which is generated following glucose-stimulated exocytosis of insulin secretory granules, promotes the transcription of secretory granule genes by enhancing the activity of STAT5.

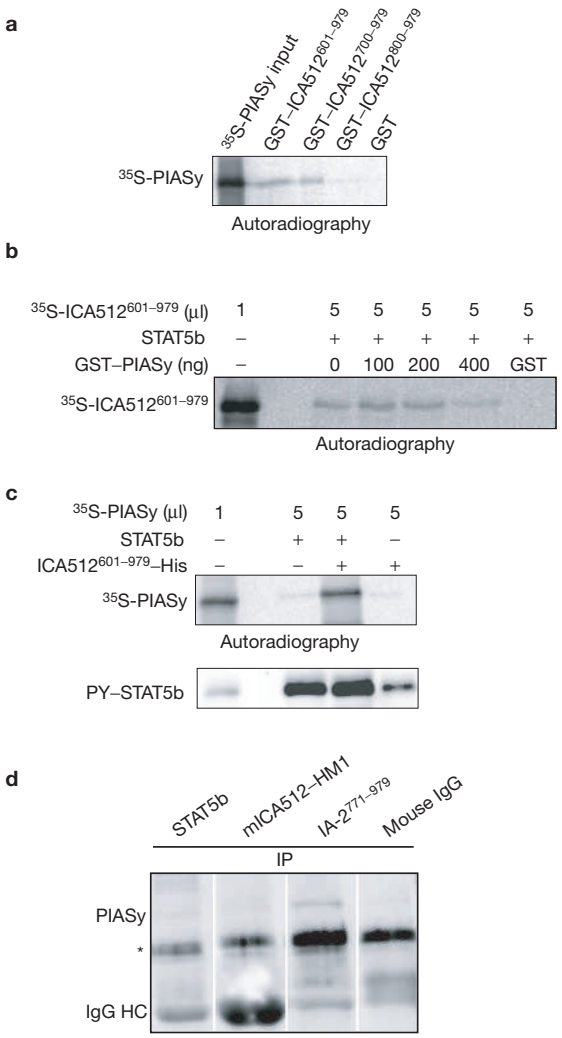

Figure 6 ICA512-mediated binding of PIASy to STAT5.
(**a**) Autoradiography of *in vitro* transcribed and translated ^{35}S-PIASy pulled down with GST–ICA512 constructs bound to glutathione–Sepharose beads. (**b**) Autoradiography of ^{35}S-ICA512$^{601–979}$ pulled down with immunoprecipitated STAT5b bound to protein G-Sepharose beads in the presence of increasing amount of purified recombinant GST–PIASy. STAT5b was immunoprecipitated from extracts of growth hormone-stimulated INS-1 cells. (**c**) Autoradiography of ^{35}S-PIASy pulled down with immunoprecipitated STAT5b bound to protein G–Sepharose beads in presence or absence of recombinant purified ICA512$^{601–979}$–His. STAT5b was immunoprecipitated as in **b**. The bottom panel shows the immunoblot for PY-STAT5 on the same membrane exposed for autoradiography. (**d**) Immunoblot for PIASy on immunoprecipitates obtained with antibodies anti-STAT5b, anti-ICA512 or control mouse IgG from total extracts of INS-1 cells costimulated with growth hormone, glucose and KCl. The asterisk indicates an unspecific protein. This membrane was first used in Fig. 4c and then stripped and reprobed for PIASy. Full scans of the autoradiography shown in **b** and **c** can be found in the Supplementary Information, Fig. S6.

Specifically, we show that ICA512-CCF and PY-STAT5 are associated in the nucleus and that STAT5 phosphorylation and transcriptional activity positively correlate with the levels of ICA512-CCF. Our immunoprecipitation experiments do not exclude the possibility that ICA512-CCF binds to STAT5 independently of its tyrosine phosphorylation. The association of several PTPs to their substrates, independently of tyrosine phosphorylation, has previously been shown[31,32]. The results of *in vitro* dephosphorylation assays further indicate that ICA512-CCF, which includes a catalytically inactive PTP domain, prevents the dephosphorylation of PY-STAT5 by a conventional PTP, thereby accounting for the ability of ICA512-CCF to enhance nuclear retention and transcriptional activity of STAT5 in β-cells. Although several PTPs have been shown to inhibit STATs[23,33–35], the identity of the nuclear PTP that dephosphorylates STATs in β-cells remains unknown. STATs are also inhibited by protein inhibitor of activated STAT (PIAS) proteins[36]. In the case of STAT1 this inhibition is due, at least in part, to its sumoylation by PIASs[37]. However, evidence for STAT5 directly interacting with, and being a substrate of, PIASy, is still lacking. Here, we show that PIASy induces the sumoylation of ICA512-CCF on at least two sites, one of which is adjacent to the phosphotyrosine recognition motif. Moreover, *in vitro* binding assays indicate that ICA512-CCF expressed and sumoylated in bacteria binds less to STAT5 than its nonsumoylated counterpart. Hence, our findings raise the possibility that PIASy inhibits STAT5, at least in part, by negatively affecting the binding of an enhancing factor. We further demonstrate that ICA512-CCF binds directly to PIASy and promotes its association with STAT5. Thus, an intriguing possibility is that PIASy, when recruited by ICA512-CCF, induces the sumoylation of STAT5. According to this model, ICA512-CCF would first increase gene transcription by preventing the dephosphorylation of PY-STAT5. Sumoylation of ICA512-CCF, and possibly STAT5, by PIASy would then cause the dissociation of ICA512 from STAT5 and thus down-regulate transcription (Fig. 8f). Through similar mechanisms ICA512-CCF may modulate the activity of other transcription factors. For instance, we have shown that ICA512-CCF increases the nuclear levels of STAT3 and PY-STAT3. Additional potential regulatory targets of ICA512-CCF include the transcription factors RIPE3b1–Act–C1 and PDX-1, whose ability to activate insulin gene transcription is affected by tyrosine phosphorylation[38] and sumoylation[39], respectively.

In addition to glucose, other stimuli that trigger secretory granule exocytosis could activate the ICA512 pathway. GLP-1 is an enteric hormone that stimulates both insulin gene expression and secretory granule exocytosis[40]. Enhancement of rat *insulin 1* gene transcription by GLP-1 requires the region of the insulin promoter that includes the STAT5 binding element[41], suggesting a potential link between GLP-1 and STAT5. In particular, GLP-1 may converge with STAT5 signalling through the generation of ICA512-CCF.

Our findings provide a mechanistic explanation for the ability of different stimuli to act together to promote insulin expression. First, convergent regulation of β-cell gene expression by glucose and growth hormones may be particularly important in conditions of increased metabolic demands (such as pregnancy or obesity). Second, although several families of STAT inhibitors are known (including PTPs, PIASs and suppressor of cytokine signalling (SOCSs) [18]), ICA512 is the first enhancer of STAT activity to be identified. In particular, our data show that the transcription activity of STAT could be increased through its interaction with a decoy tyrosine phosphatase. As catalytically inactive phosphatase domains are found in most receptor tyrosine phosphatases, such mechanism for preventing STAT dephosphorylation could be of general significance. Third, we demonstrate that the cytoplasmic domain of a transmembrane protein undergoes sumoylation when it is released into the cytosol and translocated to the nucleus. Moreover, our findings suggest that sumoylation affects the ability of this domain to modulate gene expression. Finally, we provide direct

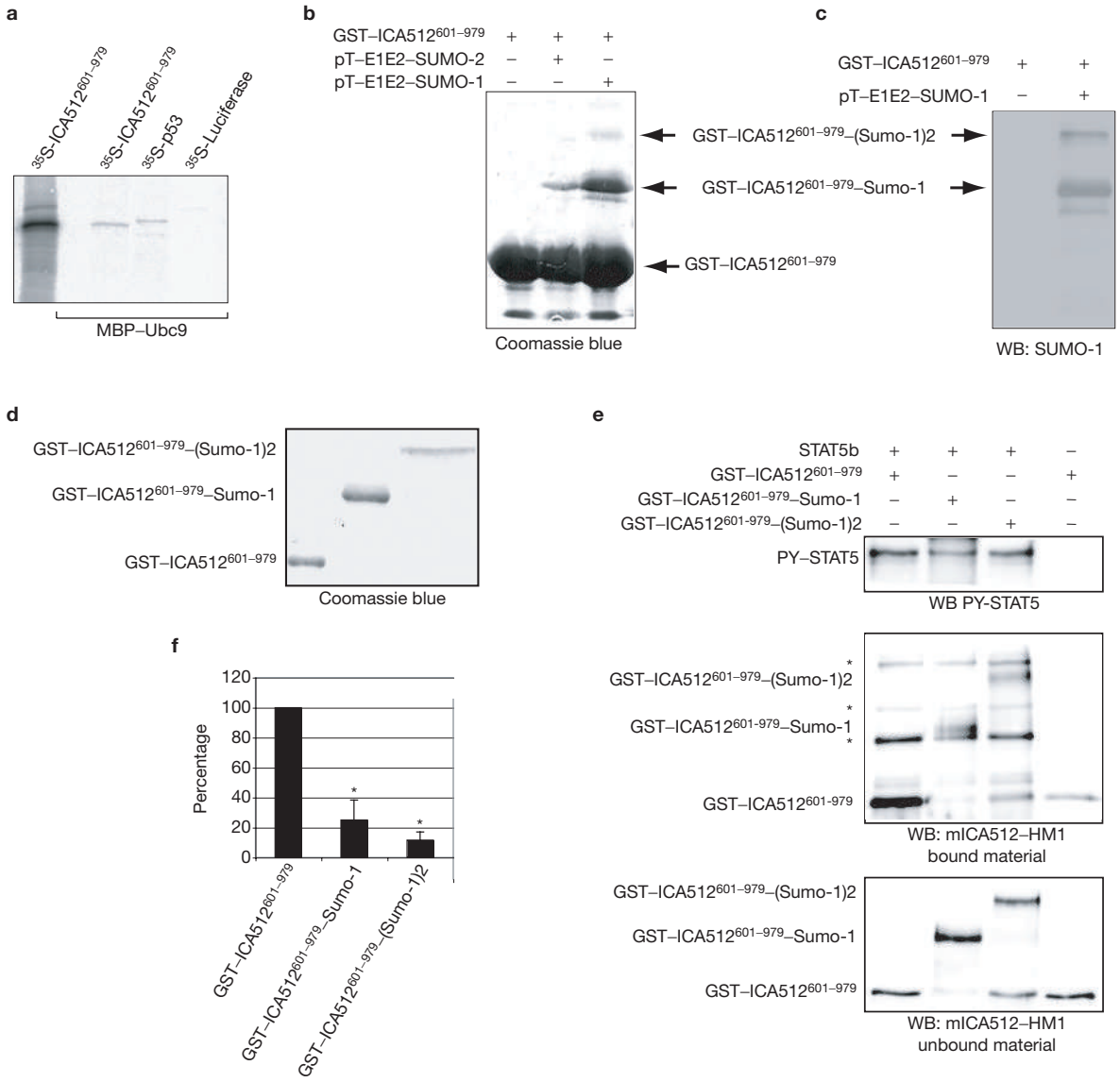

Figure 7 Sumoylation of ICA512 regulates its binding to STAT5. (**a**) Autoradiography of *in vitro* transcribed and translated ^{35}S-methionine-ICA512$^{601–979}$, p53, and firefly luciferase pulled down with recombinant purified MBP–Ubc9. (**b**) SDS–PAGE and Coomassie blue staining of recombinant GST–ICA512$^{601–979}$ purified from *Escherichia coli* cotransformed with or without *pT–E1E2–SUMO-1* or *pT–E1E2–SUMO-2* for SUMO-1 or SUMO-2 conjugation[28], respectively. (**c**) Immunoblot for SUMO-1 on recombinant GST–ICA512$^{601–979}$ purified from *E. coli* cotransformed with or without pT–E1E2–SUMO-1. (**d**) Coomassie blue staining of purified GST–ICA512$^{601–979}$, GST–ICA512$^{601–979}$–Sumo-1 and GST–ICA512$^{601–979}$–(Sumo-1)2 after separation by SDS–PAGE and recovery by electroelution. (**e**) Immunoblots for ICA512 on bound and unbound material following the pull down of purified GST–ICA512$^{601–979}$, GST–ICA512$^{601–979}$–Sumo-1 and GST–ICA512$^{601–979}$–(Sumo-1)2 with immunoprecipitated STAT5 bound to protein G–Sepharose beads. The last lane of the right show the amount of GST–ICA512$^{601–979}$ that binds non-specifically to the beads in the absence of STAT5. Asterisks indicate unidentified immunoprecipitated proteins. (**f**) Quantification of GST–ICA512$^{601–979}$ species pulled down with STAT5, measured from three immunoblots as in **e**. Values were compared with the signal intensity of GST–ICA512$^{601–979}$ (100%). The asterisk indicates P <0.05. Full scans of the autoradiography and of the blot shown in **a** and **e**, respectively, can be found in the Supplementary Information, Fig. S6.

evidence that a member of the PIAS protein family, namely PIASy, inhibits STAT5.

We have previously shown that calpain-1 is responsible for the Ca^{2+}-dependent cleavage of ICA512 cytoplasmic domain following the exocytosis of secretory granules[7,15]. Calpain-10, another member of the calpain family, has been identified as a type 2 diabetes susceptibility gene in several ethnic groups[42,43]. The association of polymorphisms in the calpain-10 gene with type 2 diabetes has yet to be explained, but it has been proposed that this enzyme regulates insulin secretion and fuel metabolism in β-cells[44,45]. As our results point to the involvement of ICA512 in the regulation of insulin gene expression and secretion, it would be interesting to establish whether its cleavage is also modulated by calpain-10 and to explore the potential significance of its retrograde signalling activity in the pathogenesis of type 2 diabetes. In conclusion, our study illustrates how the retrograde signalling pathway associated with ICA512 converges with the STAT signalling pathway to promote insulin secretory granule biogenesis, and possibly other adaptive responses of β-cells, in conditions of increased metabolic demands.

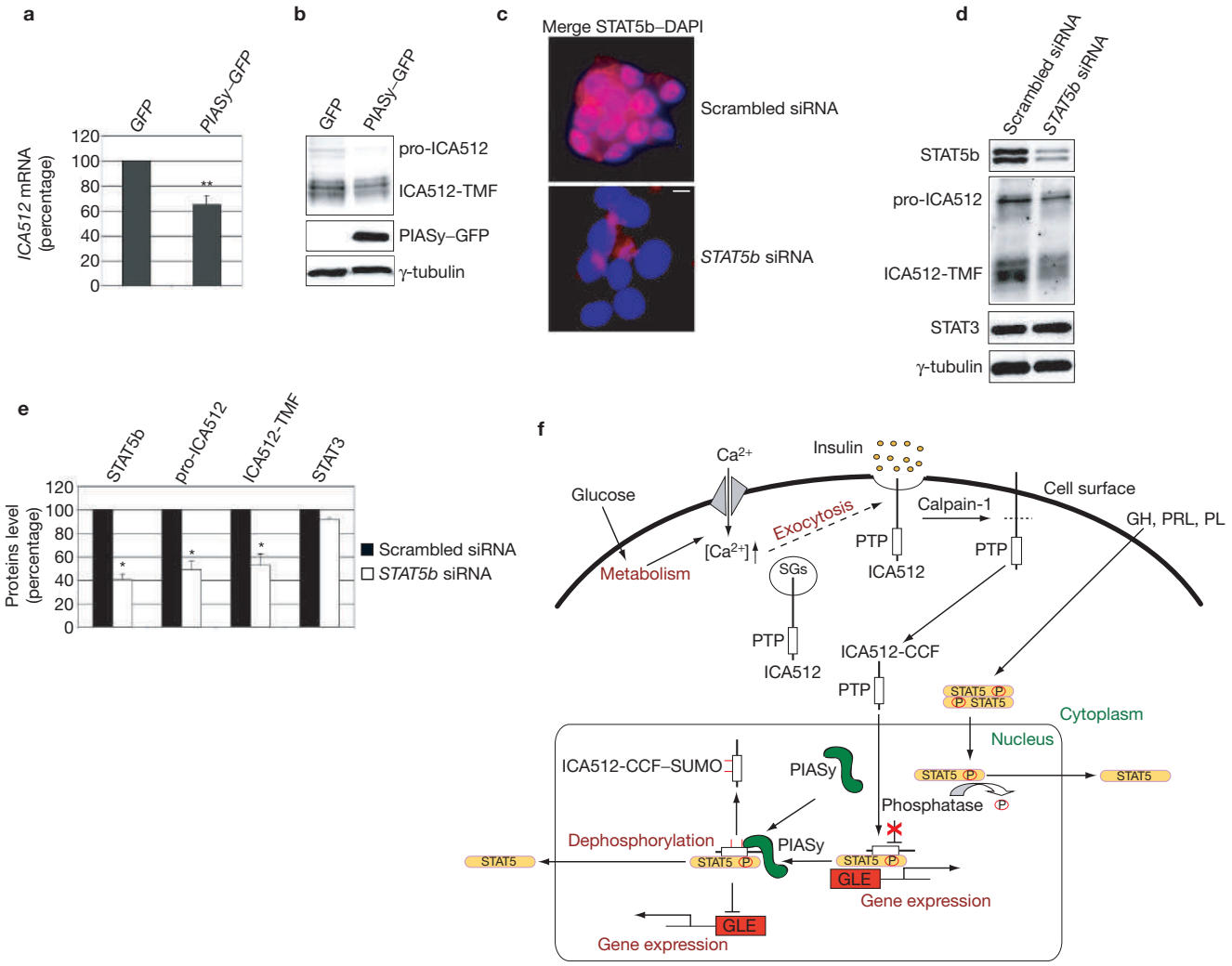

Figure 8 STAT5b promotes the transcription of *ICA512*. (**a**) Quantification of *ICA512* mRNA in *PIASy–GFP* INS-1 cells as percentage of *ICA512* mRNA in *GFP* INS-1 cells. Values were normalized for *β-actin* mRNA and were from three independent experiments. (**b**) Immunoblots for ICA512, PIASy–GFP and γ-tubulin on total extracts from INS-1 cells expressing GFP or PIASy–GFP. (**c**) Detection of STAT5b (red) by confocal microscopy in INS-1 cells treated with control or *STAT5b* siRNA oligonucleotides. Nuclei were counterstained with DAPI (blue). Scale bars represent 5 μm. (**d**) Immunoblots for STAT5b, ICA512, STAT3 and γ-tubulin on total extracts from INS-1 cells treated with control or *STAT5b* siRNA oligonucleotides. (**e**) Quantification of STAT5b, pro-ICA512, ICA512-TMF and STAT3 levels from three independent experiments as shown in **d**. The asterisk indicates $P < 0.05$. (**f**) A schematic representation of a model illustrating how stimuli inducing the exocytosis of insulin secretory granules (such as glucose) promote β-cell gene expression through the ICA512-mediated enhancement of STAT5 activity. In the nucleus ICA512-CCF binds to PY-STAT5, which in turn recognizes GLE-containing promoters. PY-STAT5 bound to ICA512-CCF is protected from dephosphorylation by PTPs and accumulates in the nucleus. ICA512-CCF-mediated recruitment of PIASy to the ICA512-CCF–PY-STAT5 complex leads to the sumoylation of ICA512-CCF, thus decreasing binding of the latter to PY-STAT5. This mechanism may explain how PIASy inhibits STAT5 activity. GH, growth hormone; PRL, prolactin; PL, placental lactogen.

METHODS

Generation of *ICA512*$^{-/-}$ mice. A construct for targeting the *ICA512* gene by homologous recombination in mouse 129/Sv ES cells was generated according to standard procedures. ES cells were electroporated with the targeting vector and injected into blastocysts of C57BL/6 donor mice to generate chimeric mice. Germline transmission was assessed by Southern blotting and PCR using primers specific for the *neo* transgene (reverse' CTATCGCCTTCTTGACGAGTTC) and the adjacent *ICA512* coding region (forward, CTGCTGGTCTGCCTGCTGTTG). *ICA512*$^{+/-}$ and *ICA512*$^{-/-}$ mice were identified by Southern blotting and PCR using primers specific for the deleted *ICA512* genomic region (forward, CTGTTTGACCGCAGACTTTGTTC; reverse, CTTGTAGGCGCTGGAGAACTG). Lack of *ICA512* expression was verified by RT–PCR on RNA from pancreatic islets with *ICA512*-specific primers (forward, CTGTTTGACCGCAGACTTTGTTC; reverse, CATTAGCAGATGCTCCAAGAGAG) and immunohistochemistry with anti-ICA512 antibodies.

Culture of INS-1 cells. INS-1 cells were grown as previously described[46]. In some instances cells were pre-incubated for 1 h in resting buffer (0 mM glucose, 5 mM KCl) and then for 2 or 6 h in resting or stimulating (25 mM glucose, 55 mM KCl) buffers as previously described[15]. Calpeptin (Calbiochem, San Diego, CA) was added for the last 15 min of the pre-incubation, and then until cells were harvested. Cells were stimulated with 20 nM human growth hormone for 20 min following 18 h incubation in low serum medium (LSM) containing RPMI-1640, 11 mM glucose, 0.5% FBS, 25 mM HEPES, 1% penicillin–streptomycin and 0.05 mM β-mercaptoethanol. After washing, cells were either harvested or incubated in LSM for another 6 h. For immunoprecipitation, cells were collected 1 h after stimulation with growth hormone.

Islet culture. Pancreatic islets were isolated from 15–17 week-old *ICA512*$^{+/+}$ or *ICA512*$^{-/-}$ mice as previously described[47]. After 48 h in culture, islets were incubated for 18 h in LSM before to stimulation with 25 mM glucose and 20 nM growth hormone or growth hormone alone for 2 h, followed by incubation in LSM for another 4 h.

cDNA constructs. The following constructs were previously described: *ICA512–CCF–GFP*[7]; *GST–ICA512*$^{601–979}$, *GST–ICA5127*$^{00–979}$ and *GST–ICA512*$^{800–979}$ (ref. 48); *p53*, activated SUMO-1(*SUMO–1GG*), E2–SUMO conjugating enzyme *MBP–Ubc9*, and *His–Uba2* and *GST–Aos1* to generate recombinant heterodimeric E1–SUMO activating enzyme[49]. *GST–PIASy* and HA-tagged *SUMO-1* cDNAs were gifts from R. Grosschedl (Univeristy of Munich, Germany) and R.Janknecht (Mayo Clinic, Rochester, MN), respectively. The *β-casein* promoter, including eight GLEs upstream of firefly luciferase (*8GLE–CAS–FL*) in pGL-3 (Promega, Madison, WI) was a gift from A. Barthel. The pT-E1E2S1–S2 construct, as previously described[28], was a gift from H. Saitoh. ICA512-CCF Lys 754, Ala 877 and Asp 911 were mutated with the QuickChange Mutagenesis kit (Stratagene, La Jolla, CA) into Ala, Asp and Ala, respectively, The *ICA512-CCF*^{K754A} and *ICA512-CCF*$^{A877D–D911A}$ cDNAs were fused at their carboxy (C)-termini to GFP by subcloning them as *Eco*RI–*Age*I fragments into pEGFP-N1 (Clontech, Mountain View, CA). The mouse *PIASy* cDNA was subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) and fused at its C-terminus to GFP as a *BglII–EcoRI* fragment into pEGFP-N2. Human *TC45* cDNA was amplified by PCR from IMAGE consortium clone 1894955 and cloned in pGEX4T-1.

Transfection of INS-1 cells. INS-1 cells were electroporated as previously described[7]. The generation of stable GFP and ICA512-CCF–GFP INS-1 cell clones was previously described[7].

Cell extraction and immunoblotting. INS-1 cells and pancreatic islets were harvested at 4 °C in RIPA buffer (50 mM Tris–HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate and 1× protease inhibitor cocktail; Sigma) to obtain total extracts. Insoluble material was removed by centrifugation. Cytosolic and nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). Proteins were separated by 8–10% SDS–PAGE and immunoblotted with the following antibodies: mouse monoclonal anti-ICA512[7], mICA512-HM1 and IA-2$^{771–979}$ (LAD, Berlin, Germany), anti-STAT5 and anti-PY-STAT3 (Santa Cruz, Santa Cruz, CA), anti-γ-tubulin and anti-HA (Sigma); rabbit anti-PY-STAT5 and anti-STAT3 (Cell Signaling, Danvers, MA), anti-PIASy (Abcam, Cambridge, MA); affinity purified goat anti-GFP IgGs. Immunoblotting was performed on half the amount of nuclear proteins relative to total and cytosolic proteins. Chemiluminescence was developed using the Supersignal West Pico or Femto kits (Pierce) and detected with a LAS 3000 Bioimaging System (Fuji, Tokyo, Japan). Protein signals were quantified with the Image Gauge v3.45 software (Fuji).

Immunoprecipitation. INS-1 cells were transiently transfected with one or more of the following constructs: *GFP*, *ICA512-CCF–GFP*, *ICA512-CCF*^{K754A}*–GFP*, *SUMO-1* and *HA–SUMO-1*. Anti-GFP IgGs were used for immunoprecipitations from total or cytosolic extracts. Immunoprecipitates were immunoblotted with anti-STAT5b, anti-PY-STAT5, anti-GFP or anti-HA antibodies. For immunoprecipitation of sumoylated ICA512 and STAT5, 600 μg and 200 μg protein were used as starting material, respectively.

***In vitro* phosphatase assay.** PY-STAT5 was immunoprecipitated with anti-STAT5 antibody from INS-1 cells stimulated with 20 nM of growth hormone for 20 min. After extensive washes, sepharose beads were resuspended in phosphatase assay buffer (PAB; 25 mM Tris at pH 7.5, 5 mM DTT, 0.5 mg ml^{-1} bovine serum albumin, 2.5 mM EDTA, 1 mM EGTA and 1× protease inhibitor cocktail), and divided into equal aliquots. An aliquot was assessed for the presence of any contaminating protease or phosphatase by incubation at 30 °C for 2 h in PAB alone, while the remaining aliquots in PAB were incubated with 0.5 μg GST–TC45 alone or in combination with 0.5 μg GST or GST–ICA512-CCF in the same conditions as the control reactions. GST and GST–ICA512-CCF were added 15 min before the addition of GST–TC45. Beads were subjected to SDS–PAGE and immunobloting.

Pulldown assays. GST–ICA512$^{601–979}$, GST–ICA512$^{700–979}$ and GST–ICA512$^{800–979}$ were bacterially expressed as previously described[48]. GST–PIASy was also expressed as previously described[47]. ^{35}S-methionine-PIASy, -ICA512$^{601–979}$, -p53 and -firefly luciferase were *in vitro* transcribed and translated with the T7-coupled transcription-translation system (Promega) according to the manufacturer's instructions. STAT5 was immunoprecipitated with anti-STAT5b antibodies from INS-1 cells stimulated with 20 nM growth hormone for 20 min. ^{35}S-methionine-PIASy was incubated with GST–ICA512 proteins, immunoprecipitated STAT5, GST or protein G-sepharose. ^{35}S-methionine-ICA512$^{601–979}$ was incubated with immunoprecipitated STAT5 and GST–PIASy, or, similarly to ^{35}S-methionine-p53 and ^{35}S-methionine-firefly luciferase, with maltose binding protein (MBP)–Ubc9. Proteins were collected with glutathione- or protein G-Sepharose beads (Amersham, Uppsala, Sweden), or amylose Sepharose beads (New England Biolabs, Ipswich, MA), as previously described[7]. Pulled down proteins were detected by immunoblotting or autoradiography following SDS–PAGE.

Cell imaging. INS-1 cells expressing ICA512-CCF–GFP or ICA512-CCF$^{A877D–D911A}$–GFP, or treated with siRNA oligonucleotides, were fixed with 2% paraformaldehyde. To detect STAT5b, cells were permeabilized with 0.3% Triton X-100 in 20 mM phosphate buffer at pH 7.4, 450 mM NaCl and 16% filtered goat serum, and incubated sequentially with mouse monoclonal anti-STAT5b and goat-anti-mouse Alexa^{568}-conjugated IgG (Molecular Probes, Carlsbad, CA). Nuclei were counterstained with DAPI (Sigma). Confocal images were acquired with an inverted LSM Meta 405nm confocal microscope (Zeiss, Jena, Germany).

RNA interference. RNA interference was performed as previously described[7] using the following forward and reverse oligonucleotides: *STAT5b* (F1, 5′- AAT TGGTTGATCTGAAGGTGCCCTGTCTC–3′; R1, 5′- AAGCACCTTCAGATC AACCAACCTGTCTC–3′; F2, 5′- AAGTTCATGTGCACGTTCAGCCCTGTC TC–3′; R2, 5′-AAGCTGAACGTGCACATGAACCCTGTCTC–3′). After seeding, cells were consecutively transfected on day 2 and 3 with 1 μg and 0.5 μg siRNA oligos per well in Lipofectamine (Invitrogen), respectively. On day 4 cells were harvested and processed for immunoblotting or immunocytochemistry.

Firefly luciferase assays. INS-1 cells were cotransfected with *8GLE–CAS–FL* and one or two of the following plasmids: pEGFP, *ICA512-CCF–GFP*, ICA512-CCF^{K754A}–GFP, ICA512-CCF$^{A877D–D911A}$–GFP and PIASy–GFP. Two days after transfection, cells were incubated in low serum medium (LSM) for 18 h, then stimulated with 20 nM human growth hormone for 20 min, and finally left in resting buffer for another 6 h. Cells were harvested in 500 μl lysis reagent for luciferase assays (Promega) and 10 μl were used for measurement of luciferase activity in a Berthold luminometer. Each sample was analysed in triplicate and the data represent the mean of at least 3 independent experiments.

EMSA. After three days in culture, INS-1 cells transfected with *GFP* or different amount of *ICA512-CCF–GFP* were incubated for 18 h in LSM, then stimulated with human growth hormone and harvested. Nuclear extracts were prepared as described above. Binding reactions with 20 fmol of double strand biotinylated oligonucleotide (5′-AGCTATGTTCTGAGAAAATC-3′) including the GLE motif in rat *insulin 1* gene promoter (*GLE–rIns1*) and 5 μg nuclear protein were performed following the manufacturers' instructions. For competition experiments, 300 fmol of the non-biotinylated *GLE–rIns1* oligonucleotide were added to the binding reaction. The reactions were separated on 6% polyacrylamide gels, then transferred to nylon membranes and blotted with streptavidin-conjugated horseradish peroxidase for detection by chemiluminescence.

Chromatin immunoprecipitations. Stable GFP or ICA512-CCF–GFP expressing INS-1 cells were crosslinked by addition of 1% formaldehyde to the medium for 15 min. Precleared nuclear extracts for chromatin immunoprecipitations, with 2 μg control IgG or anti-GFP antibodies, were prepared as previously described[50]. Immunocomplexes were recovered with protein G–Sepharose beads. Co-immunoprecipitated DNA fragments were eluted and analysed by PCR and agarose gel electrophoresis, and by quantitative real time PCR.

PCR and real-time PCR. For quantification of *ICA512* mRNA by real time PCR, RNA was isolated with the Oligotex direct mRNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Poly A$^+$-enriched RNA was reverse transcribed with specific antisense primers for *ICA512* and *β-actin* as previously described[16]. Amplification of a 200 base pair fragment flanking the GLE in the rat *insulin1* (*rINS1*) gene promoter by PCR and real-time PCR was performed with the following primers: sense, 5′-CTCAGCTGAGCTAAGAATCCA-3′; antisense, 5′- ATTAACAAGGGGCCAGATGCC-3′. Amplification of *ICA512* and *β-actin* was performed as previously described[16]. Real time PCR to quantify *insulin* mRNA was performed as previously described[16].

^{3}H-uridine incorporation. Three days after transfection with *GFP* or *ICA512-CCF–GFP*, INS-1 cells were incubated in LSM for 18 h and then radiolabelled with

25 μCi 5-^{3}H-uridine per well for 2 h in presence or absence of 5 μg ml^{-1} actinomycin D (Sigma). mRNA was purified with the Oligotex direct mRNA kit (Qiagen). Uridine incorporation was measured with a liquid scintillation counter (Wallac, Shelton, CA). For normalization, poly A^{+}-enriched RNA from each sample were reverse transcribed using antisense primers for *β-actin*[16] and the corresponding mRNA was quantified by real-time PCR.

Statistics and graphics. Statistical analyses were performed as previously described. Error bars show standard deviations from at least two independent experiments. Histograms were prepared with Microsoft Excel (Microsoft, Redmond, WA).

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGMENTS

We are grateful to P. De Camilli, M. Matteoli and M. Zerial for critical reading of the manuscript; A. Barthel, R. Grosschedl, R. Janknech, F. White and H. Saitoh for reagents; F. Ehehalt, K. Erdmann and M. Jäger for help in islet isolation; K. Pfriem for excellent assistance; and G. Nikolova and all members of the Solimena lab for advice. This work was supported by funds from the Alexander von Humboldt Foundation, the German Ministry for Education and Research (BMBF), the European Foundation for the Study of Diabetes and the Juvenile Diabetes Research Foundation to M.S., and a MedDrive Grant from the Medical School of Dresden Univsity of Technology to H.M. and M.T.

AUTHOR CONTRIBUTIONS

H.M. and M.S. wrote the manuscript. M-S.L. generated the *ICA512* knockout mice. H.M., M.T. and M.S. performed the data analysis. H.M, M.T., H-D.S, D.N.D, S.M. and M.S were responsible for project planning. H.M., M.T., S.K., A.E., A.A., R.P.L. and D.S. carried out all the experimental work.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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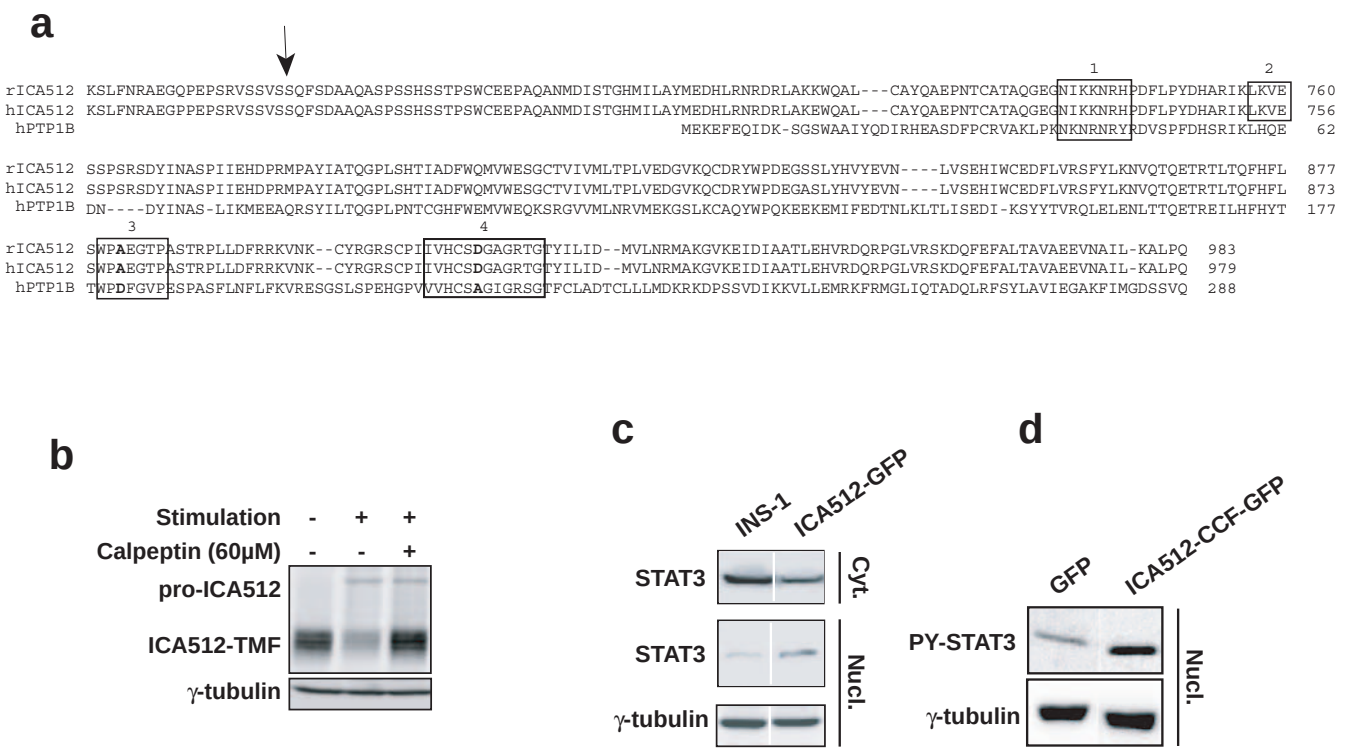

Figure S1a. **Alignment of the PTP domains of rat and human ICA512 and human PTB1B.** Regions of interests are identified with boxes. Box 1: phosphotyrosine recognition motif; Box 2: consensus motif for sumoylation (ΨKXE**)**. Lys^{754} is shown in bold. Box 3: WPD loop, which includes the essential aspartate (in bold) for phosphatase activity. In ICA512 this aspartate is replaced by an alanine (A^{877}). Box 4: PTP "signature motif", which includes the critical alanine (in bold) for phosphatase activity at position +2 from the catalytic cysteine. In ICA512 this alanine is replaced by an aspartate (D^{911}). The vertical arrowhead points to the location of the calpain-1 cleavage site in vitro[15]. **Figure S1b**. **Rapid induction of pro-ICA512 expression is calpeptin-insensitive.** Immunoblots for ICA512 (top panels) and γ-tubulin (bottom panels) on extracts from INS-1 cells kept at rest or stimulated for 2 hours with or without 60μM Calpeptin. **Figure S1c-d**. **ICA512 enhances the nuclear levels of STAT3 and PY-STAT3.** a) Immunoblots for STAT3 and γ-tubulin on cytosolic and nuclear extracts from INS-1 cells and stable ICA512-GFP INS-1 cells. b) Immunoblots for PY-STAT3 and γ-tubulin on nuclear fractions from GH-stimulated INS-1 cells expressing GFP or ICA512-CCF-GFP.

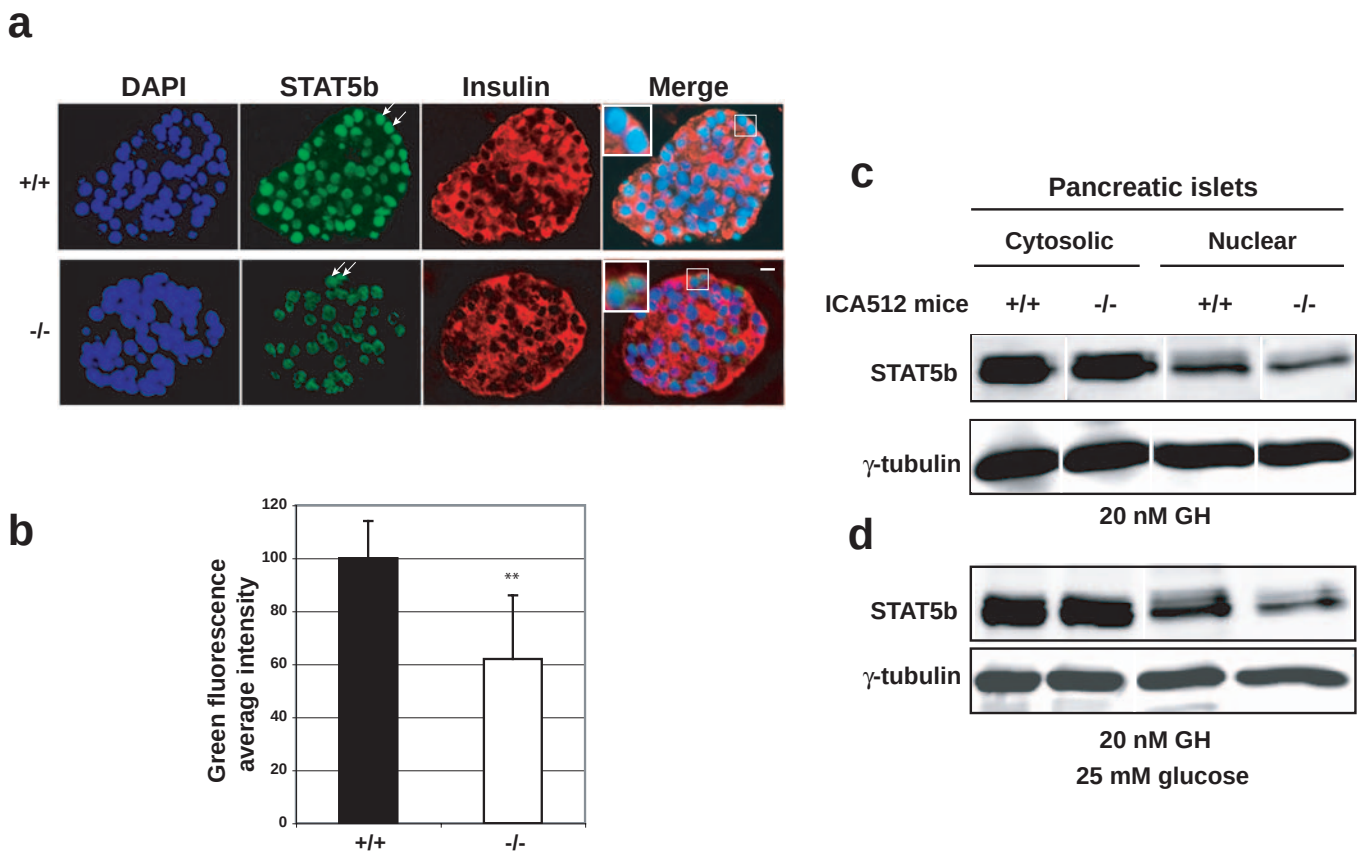

Figure S2a-b. The nuclear levels of STAT5 are decreased in stimulated ICA512 -/- islets. a) Immunomicroscopy of pancreatic islets isolated from ICA512 +/+ and -/- mice and stimulated as described in Figure 2c. Islets were immunostained for STAT5b (green) and insulin (red). Nuclei were co-stained with DAPI. Arrows point to the nuclei of some ICA512 +/+ and -/- β-cells positive for STAT5b. The insets in the merge panels show a 2x magnification of the same cells. Bars, 50 μm. b) Intensity of nuclear STAT5b signal in stimulated ICA512 +/+ and -/- islets as measured with the region measurement tool of MetaMorph 4.6 software (Universal Imaging). β-cells were identified by insulin staining, while nuclei were labeled with DAPI. Values are from 15 islets in each group. **: $p<0.01$. **Figure S2c-d. The nuclear levels of STAT5 in ICA512 -/- islets co-stimulated with GH and glucose are reduced relative to ICA512 +/+ islets.** (c, d) Immunoblots for STAT5b, PY-STAT5 and γ-tubulin on cytosolic and nuclear extracts from $3x10^2$ islets from ICA512 +/+ and -/- islets. Islets were stimulated with GH (c) or with GH and glucose (d) as described in Figure 2c.

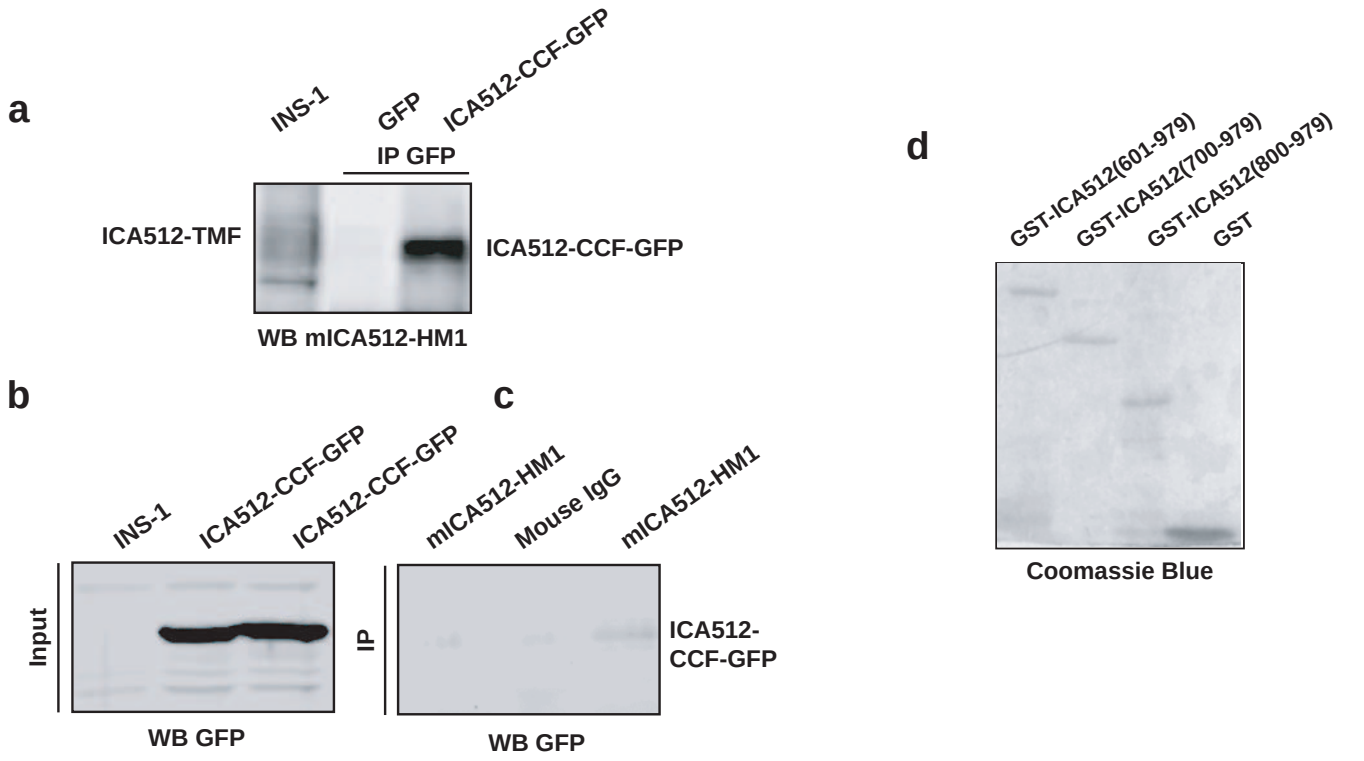

Figure S3a-c. The antibody mICA512-HM1 recognizes an epitope within ICA512-CCF. a) Immunoblot for ICA512 with the mouse monoclonal antibody mICA512-HM1 on total extracts from INS-1 cells and on immunoprecipitates obtained with the anti-GFP antibody from extracts of INS-1 cells expressing GFP or ICA512-CCF-GFP. b, c) Immunoblots for GFP on total extracts (b) or on immunoprecipitates (c) from INS-1 cells or ICA512-CCF-GFP INS-1 cells. Immunoprecipitations were performed with mICA512-HM1 or control mouse IgG. **Figure S3d**. **Purified truncated GST-ICA512 constructs.** Coomassie blue staining after separation by SDS-PAGE of GST and GST-ICA512 constructs expressed in bacteria and used as input for pull-down assays with in vitro transcribed-translated [^{35}S]-PIASy.

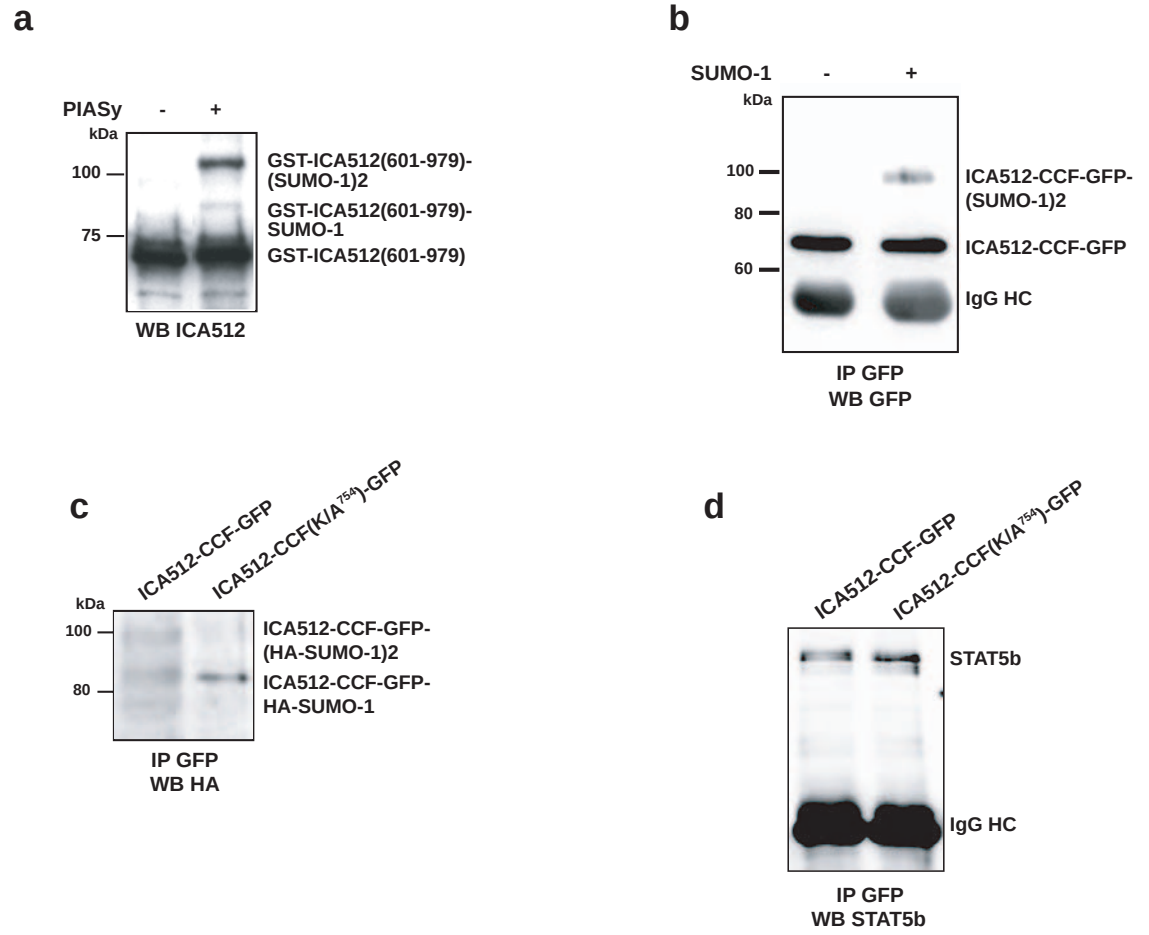

Figure S4a-c. **PIASy induces the sumoylation of ICA512.** a) Immunoblot for ICA512 following in vitro incubation of GST-ICA512 (601-979) with ATP, SUMO-1, the Aos1/Uba2 complex (E1-SUMO activating enzyme), Ubc9 (E2- SUMO-conjugating enzyme), and with (+) or without (-) GST-PIASy (E3-SUMO ligase). b) Immunoblot for GFP on immunoprecipitates with the anti-GFP antibody from total extracts of ICA512-CCF-GFP INS-1 cells co-transfected with (+) or without (-) SUMO-1. c) Immunoblot for the HA-epitope tag on immunoprecipitates obtained with the anti-GFP antibody from extracts of ICA512-CCF-GFP or ICA512-CCF(K/A^{754})-GFP INS-1 cells co-transfected with HA-SUMO-1. **Figure S4d. ICA512-CCF-GFP and ICA512-CCF(K/A^{754})-GFP interact with STAT5b.** Western blot for STAT5b on immunoprecipitates with the anti-GFP antibody from total extracts of INS-1 cells expressing ICA512-CCF-GFP or ICA512-CCF(K/A^{754})-GFP. **Figure S4e-f. Knockdown of firefly luciferase by RNAi does not affect the expression of STAT5b and ICA512.** e) Immunoblots for firefly luciferase, STAT5b, pro-ICA512, and γ-tubulin on total extracts from INS-1 cells transfected with scrambled or firefly luciferase siRNA oligonucleotides as described previously [16]. f) Firefly luciferase activity and protein levels of firefly luciferase, STAT5b and pro-ICA512 from three independent experiments as show in (a).

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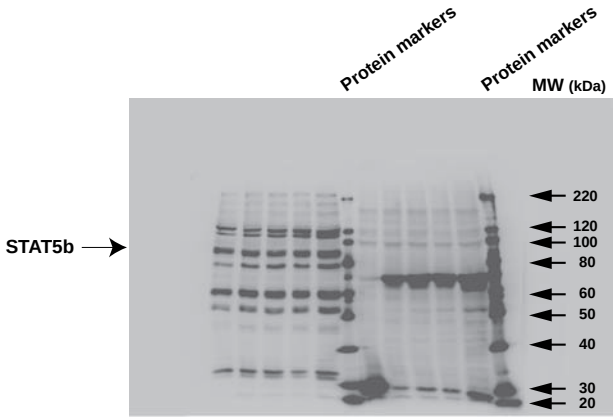

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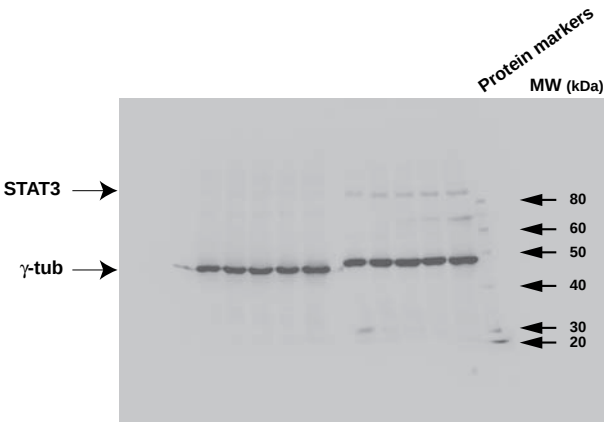

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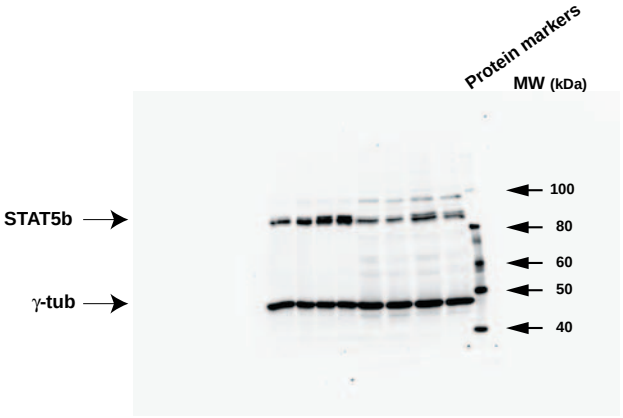

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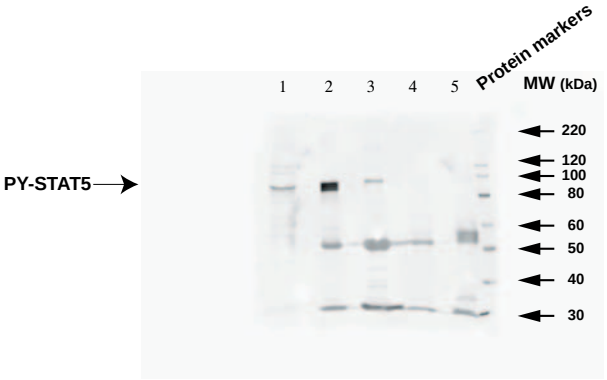

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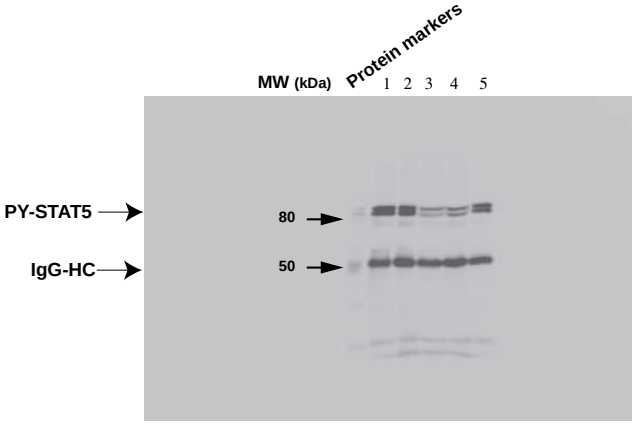

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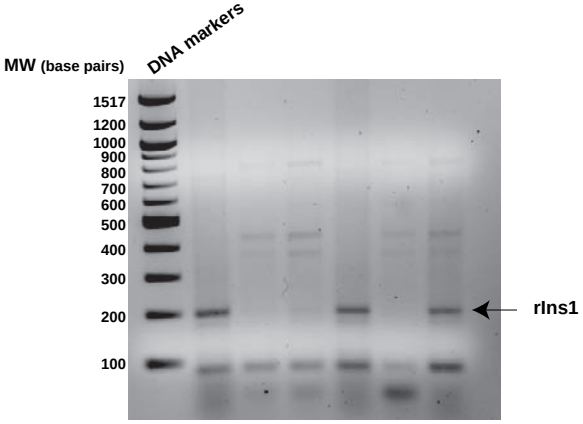

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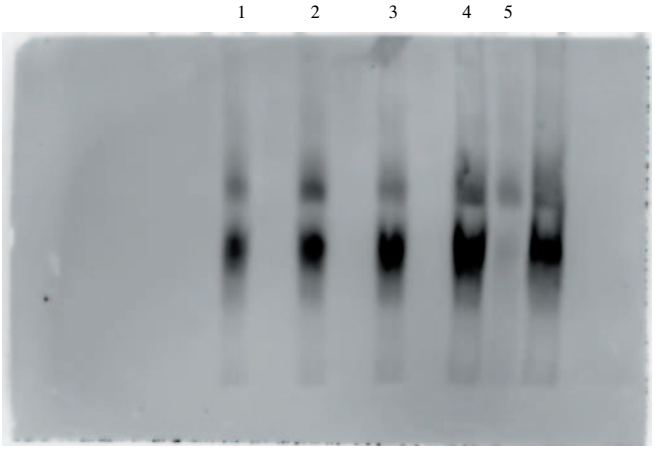

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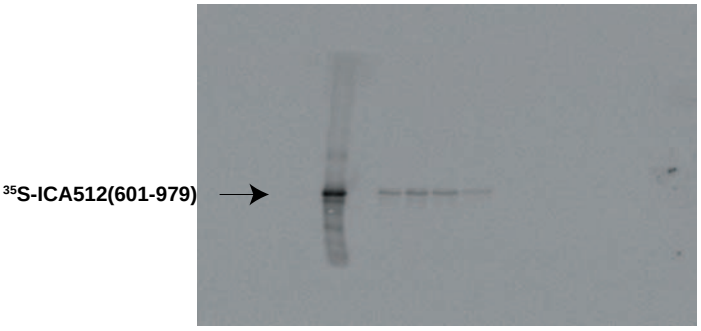

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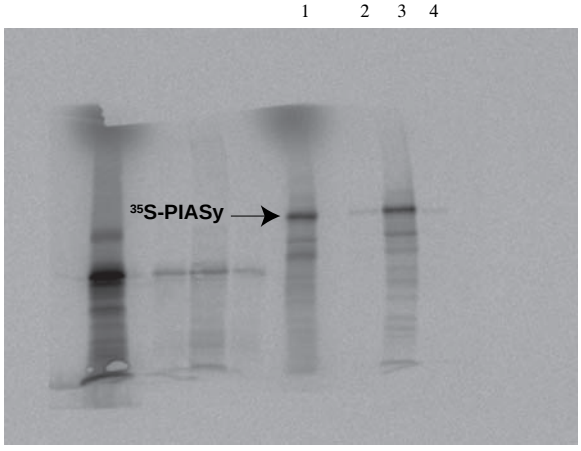

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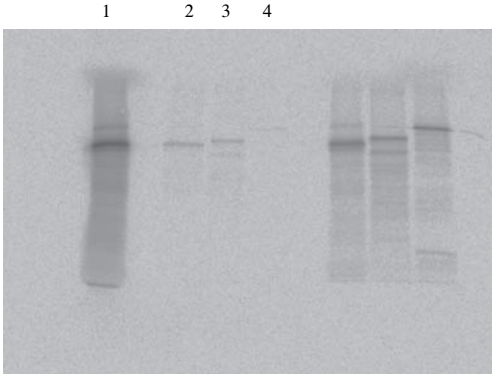

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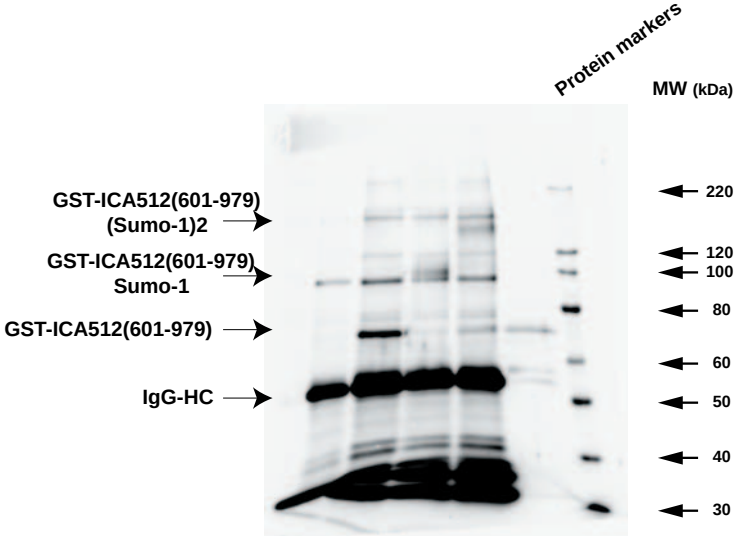

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