Age-dependent labeling and imaging of insulin secretory granules

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Pages: 31
Title: 64 characters; Abstract: 155 words
Figures: 5; Tables: 3
Supplementary Tables: 1; Supplementary Figures: 9; Supplementary Movies: 4
Running title: Insulin granule aging in vitro and in vivo
Abstract

Insulin is stored within the secretory granules of pancreatic beta cells and impairment of its release is the hallmark of type 2 diabetes. Preferential exocytosis of newly synthesized insulin suggests that granule aging is a key factor influencing insulin secretion. Here we illustrate a technology that enables the study of granule aging in insulinoma cells and beta cells of knock-in mice through the conditional and unequivocal labeling of insulin fused to the SNAP tag. This approach, which overcomes the limits encountered with previous strategies based on radiolabeling or fluorescence timer proteins, allowed us to formally demonstrate the preferential release of newly-synthesized insulin and reveal that the motility of cortical granules significantly changes over time. Exploitation of this approach may enable the identification of molecular signatures associated with granule aging and unravel possible alterations of granule turnover in diabetic beta cells. Furthermore, the method is of general interest for the study of membrane traffic and aging.
Introduction

Insulin is the key hormone for glucose homeostasis. Pancreatic beta cells produce insulin and store it within secretory granules (SGs), until elevated glycaemia triggers SG exocytosis and insulin release. Seminal studies using pulse-chase labeling protocols indicated that newly synthesized insulin is preferentially released (1,2). Until today, however, a method for the reliable imaging and study of age-distinct insulin SGs has not been available. In this manuscript we present a new approach that enables the unequivocal imaging and study of insulin SGs of defined age.
Research design and methods

Constructs

The human preproinsulin cDNA was cloned in frame with the SNAP tag either into pEGFP-N1 (Clontech) void of the GFP cDNA or into the pShuttle-CMV vector (Qbiogene). The adenoviral construct pAdEasyn1-RIP-hIns-SNAP was transfected into QBI-293A cells (Qbiogene) to produce Adenovirus RIP-hIns-SNAP. To generate the SOFIA mouse, the SNAP cDNA was subcloned into a vector carrying the neo resistance gene, driven by PGK/\(gb3\) promoter and flanked by FRT sites (oligos: CCAATGCATTGGTCTGCACTTTGGAGATCCACCGGTC; GAAGATCCCTGGACTAGTCCCTAACCCAGCCGAG. This plasmid was used to generate a BAC tagging cassette (oligos: CAGTGCTGCACCAGCATCTGTGCTCCCTCTACCAGCTGGAGAACTGCACTGGGATCCACCGGTCGCCACCGAG, CTCATTCAAAGGTTTTATTCCATTTGCAAGGGGTAGGCTGGGTAGGTGGGTGCTC). This cassette was used for Red-ET recombineering as described (3). All constructs were verified by sequencing.

Islet isolation and cell culture

Islets from SOFIA and control C57Bl/6J mice were isolated and cultured as described (4). INS-1 and MIN6 cells were kind gifts from Claes Wolheim (Geneva, Switzerland) and Jun-ichi Miyazaki (Osaka, Japan) and were grown as described (5,6).

Cell transfection and transduction

INS-1 and MIN6 cells were transiently transfected with pEG-hIns-SNAP or the insulin2-SNAP-PGK-neo BAC, respectively, as described (7). Islet cells were transduced with Adenovirus RIP-hIns-SNAP.
Generation of the SOFIA mouse

Mouse JM8.F6 (C57Bl/6N) embryonic stem cells (mES) were cultured on Mitomycin-C inactivated mouse embryonic fibroblasts (MEFs) using ES-medium containing leukemia inhibitory factor. mES cells (1x10^7) were electroporated with 40 µg of the linearized insulin2-SNAP-PGK-neo construct using 250 V (BioRad) and selected with 200 µg/ml G418 (Invitrogen). Colonies were screened by Southern hybridization using a 5’-external probe (SpeI) or a 3’-external probe (BsrGI/MunI) and an internal probe (neo) using BsrGI/MunI. Insulin2-SNAP-PGK-neo mES cells were injected into C57Bl6 blastocysts (TCF, MPI-CBG, Dresden). Chimaeras were crossed to Bl6 mice and the progeny was genotyped. Positive mice were further crossed with a Flpo expressing mouse strain (8).

Measurement of TMR-Star^+-hIns-SNAP secretion

Secretion of Ins-SNAP-TMR-Star was measured in stable hIns-SNAP INS-1 cell clones 8 (expresses hIns-SNAP) and 5 (no detectable hIns-SNAP) seeded either in 12-well (2.33x 10^5 cells/well) or 384-well (3.5 x 10^4 cells/well) plates. The latter clone was used to subtract background fluorescence. The cells were sequentially incubated in: 1) 25 mM glucose and 55 mM KCl HEPES buffer (15 mM HEPES, pH 7.4, 70 mM NaCl, 24 mM NaHCO_3, 1 mM MgCl_2, 2 mM CaCl_2, 1 mg/ml albumin): 1 hour; 2) RPMI 1640 with 2.8 mM glucose and 20 µM BTP (blocking SNAP-substrate): 20 minutes; 3) RPMI 1640: 3 hours; 4) RPMI 1640 with 0.8 µM TMR-Star: 45 minutes; 5) RPMI 1640: 1-20 hours; 6) 0 mM glucose, 5 mM KCl in HEPES buffer with 120 mM NaCl: 1 hour; 7) 0 mM glucose, 5 mM KCl or 25 mM glucose, 55 mM KCl in HEPES with 120 mM NaCl: 1 hour. Media from resting and stimulated hIns-SNAP INS-1 cells and the corresponding cell extracts were scanned with an EnVision reader (Perkin Elmer).
Radioimmunoassays

Human Insulin, human C-peptide, human Proinsulin and Rat Insulin were measured by RIA according to the manufacturer’s recommendations (Linco Research). Cells were kept at rest or stimulated as described above.

Subcellular fractionation

Transiently transfected hIns-SNAP INS-1 cells were processed as described (7).

Western blotting

Total protein extracts or subcellular fractions of hIns-SNAP INS-1 cells and SOFIA mouse islets were immunoblotted with anti-Insulin (Sigma), anti-A-chain (Santa Cruz), anti-CPE (Chemicon), anti-GM-130 (BD Biosciences), and anti-γ-tubulin (Sigma) antibodies.

In vitro labeling of cultured cells with SNAP substrates for imaging studies

All labeling steps were carried in RPMI 1640 with 11 mM glucose, unless otherwise stated. Single labeling of transiently transfected hIns-SNAP INS-1 cells and insulin2-SNAP-PGK-neo MIN6 cells for confocal microscopy involved the following incubation steps: 1) 0.8 µM TMR-Star (NEB) or 10 µM BG-505 (NEB): 30 minutes; 2) RPMI 1640: 30 minutes; 3) 4% PFA in PBS. Double labeling of hIns-SNAP INS-1 cells for confocal and TIRF microscopy involved the following steps: 1) 0.8 µM TMR-Star: 15 minutes; 2) RPMI 1640: 30 minutes; 3) 11 µM BTP in DMSO or DMSO alone: 20 minutes; 4) RPMI 1640: 4 hours; 5) 10 µM BG-505: 30 minutes; 6) RPMI 1640: 30 minutes; 7) 4% PFA in PBS. For TIRF microscopy the last step was omitted. Alternatively, hIns-SNAP INS-1 cells were double labeled for confocal microscopy as follows: 1) 11 µM BTP: 20 minutes; 2) RPMI 1640: 2 hours; 3) 0.8 µM TMR-Star: 15 minutes; 4) 11 µM BTP: 20 minutes; 5) RPMI 1640: 2 hours; 6) 10 µM
BG-505: 30 minutes; 6) RPMI 1640: 30; 7) 4% PFA in PBS. Single labeling of hIns-SNAP INS-1 cells for TIRF microscopy involved the following steps: 1) HEPES with low glucose (2.8 mM) and high KCl (55 mM) (LGHP) and 70 mM NaCl: 30 minutes; 2) HEPES LGHP with 20 µM BTP: 30 minutes; 3) HEPES with 250 µM diazoxide: 2 hours; 4) HEPES with 250 µM diazoxide and 0.8 µM TMR-505: 45 minutes; 5) HEPES with 2.8 mM glucose and 5 mM KCl buffer: 1.5 to 4.5 hours. In some instances cells were fixed with 4% PFA in PBS while imaging. hIns-SNAP INS-1 cells cultured in 384-well plates (Aurora, 3.5 x 10^4 cells/well) were labeled as follows: 1) HEPES LGHP: 30 minutes; 2) HEPES LGHP with 20 µM BTP: 30 minutes; 3) RPMI 1640: 90 minutes; 4) 0.8 µM TMR-Star: 45 minutes; 5) HEPES with 5 mM glucose, 5 mM KCl: 90 minutes; 6) HEPES with 25 mM glucose, 55 KCl or 5 mM glucose, 5 mM KCl: 60 minutes; 7) 3.5% PFA in PBS.

**In vivo labeling of Ins-SNAP in mice with SNAP substrates for imaging studies**

SOFIA and C57Bl/6J mice were administered 15 nmol TMR-Star via tail-vein injection. After 30 minutes to 2 hours pancreata were explanted and processed in one of the following ways: 1) fixed with 4% PFA for cryosectioning and immunostaining; 2) digested with collagenase for islet isolation; 3) perfused with agarose for vibratome sectioning as reported (9).

**Immunocytochemistry**

INS-1 cells, hIns-SNAP INS-1 cells, insulin2-SNAP-PGK-neo MIN6 cells and pancreatic cryosections of SOFIA mice were immunolabeled using the following antibodies: anti-insulin (DAKO), anti-CgA (BD Biosciences), anti-TGN (BD Biosciences), anti-EEA1 (BD Biosciences), anti-GM130 (BD Biosciences), anti-PDI (Stressgen Biotechnologies), anti-glucagon (Sigma), anti-somatostatin (US Biomax).
Confocal microscopy

TMR-Star labeled hIns-SNAP INS-1 cells, insulin2-SNAP-PGK-neo MIN6 cells and pancreatic cryosections were imaged with a CLSM Zeiss LSM 510 equipped with: 488 nm, 561 nm lasers, Plan-APOCHROMAT 63x/1.4 oil, HFT 405/488/561, BP505-530 and LP575. For in situ imaging of islets in vibratome sections LSM780 CM (Zeiss) equipped with a 20x/1.0 WD objective was used. Tissue backscatter was recorded by detecting reflection of the 405nm laser at 395nm-415nm. Imaris 7.4 was used for image processing. To increase contrast linear stretch was applied to all images. High magnification images were deconvolved using blind deconvolution algorithm (AutoQuant). Confocal images of labeled hIns-SNAP INS-1 cells in 384-well plates were acquired with a Perkin Elmer OPERA at 60x.

TIRF microscopy

The data in Figure 4A, C and D was acquired with an Olympus IX71-based TIRF system with UApo150x/1.45 NA, BLHC617/73 (Semrock) and iXon+DU897 (0.106 µm/pixel) (AndorTechnology). For dual color (Suppl. Movie 1) a 100x/1.45 NA Oil (0.164 µm/pixel) objective was used. The remainder data was acquired using a NikonEclipse-Ti microscope, with 2 iXon+ DU897, 100x/1.49 Apo TIRF (0.161 µm/pixel), BP512/18 and BP647/57 (Semrock).

Light-sheet microscopy

SOFIA islets labeled in vivo for 2 hours by TMR-Star injection were isolated, fixed with 4% PFA) and imaged with a LSF Microscope (Zeiss) equipped with 561 nm laser, LBF405/488/561 and W PlanApochromat 63x/1.0.
**Immunoelectron microscopy**

*hIns-SNAP* INS-1 cells and SOFIA islets were processed for Tokuyasu-cryosectioning and immunolabeling as described (10-12) using mouse anti-SNAP (Chemicon), guinea pig anti-insulin (Abcam) and rabbit anti-mouse (bridging antibody, Sigma) antibodies followed by protein-A conjugated to 10 nm or 15 nm gold particles (Aurion).

**Imaging and statistical analyses**

All confocal images, unless otherwise stated, were analyzed with the Fiji software plug-ins “colocalization threshold” and “colocalization test” and subjected to the Costes colocalization significance test (13). Segmentation analysis of insulin⁺, CgA⁺ and TMR-Star⁺ objects in immunolabeled INS-1 and *hIns-SNAP* INS-1 cells was carried out with CellProfiler. Cells with fewer than 20 fluorescent objects and objects with fewer than 3 pixels, i.e. not fulfilling Nyquist's sampling requirement, were excluded from the analysis. Binary masks of all objects were measured for surface overlap. Threshold for object colocalization was an overlap ≥30%. TIRF data was analyzed using MotionTracking (14,15), which allows for the identification of single objects and their tracking through the dataset. All tracks with a minimum length of 10 frames (frame rate ~30 fps) were considered. The processivity criteria required that the tracks indicated unidirectional transport for at least 4 frames with maximum deviation of 30 degrees. We analyzed 6920, 4405, 4244 and 4800 tracks for the 2-4 hour (18 movies), 3-5 hour (10 movies), 4-6 hour (16 movies) and 5-7 hour (26 movies) time points, respectively. The decay of the TMR-SNAP⁺-SG mean speed was found by fitting a straight line by maximum-likelihood estimation. The fitting was done with the Pluk mathematical library (16). The statistical significance was assessed by a two-tailed Student’s t-test. The statistical significance of the immunoelectron microscopy data was assessed by a two-tailed Student’s t-test. Images of labeled *hIns-SNAP* INS-1 cells in 384-well plates were analyzed with CellProfiler.
Results

Generation and expression of human insulin-SNAP

To image age-distinct pools of insulin SGs we tagged human insulin with SNAP (hIns-SNAP) (Figure 1A) and transiently expressed it in rat insulinoma INS-1 cells (hIns-SNAP INS-1 cells). SNAP is a 22 kDa polypeptide derived from human O6-alkylguanine-DNA alkyltransferase (AGT), a suicidal enzyme involved in DNA repair. To generate SNAP, AGT was modified such that its catalytic cysteine covalently binds benzylguanine-containing substrates while having lost DNA binding capability (17-19). Cell permeable fluorescent SNAP-substrates include TMR-Star and BG-505. Immunoblotting of hIns-SNAP INS-1 cell lysates with an anti-insulin antibody detected a ~31 kDa protein, as expected for proinsulin-SNAP (hProIns-SNAP; Figure 1B, top panel). An anti-human A-chain antibody detected an additional ~24 kDa protein, as expected for A-chain-SNAP (Figure 1B, bottom panel). Both antibodies neither reacted with non-transfected INS-1 cell lysates nor with purified His-tagged SNAP, while endogenous rat proinsulin (~11 kDa) and insulin A-chain (~2 kDa) were outside the size resolution range of these gels.

Immunoblot quantifications indicated that in hIns-SNAP INS-1 cells incubated with 2.8 mM glucose the mean rat insulin:hIns-SNAP ratio was 1.12±0.33 (n=3), while hProIns-SNAP:hIns-SNAP was 2.02±0.18 (n=3) (Figure 1C). Stimulation of hIns-SNAP INS-1 cells with 11 mM glucose increased the hProIns-SNAP:hIns-SNAP ratio to 8.61±2.55 (n=3), mainly because the content of hIns-SNAP, similarly to endogenous insulin, was diminished, conceivably due to its release. Comparable changes were observed with the anti-A chain antibody (Suppl. Figure 1). Hence, INS-1 cells express hProIns-SNAP and convert it into hIns-SNAP, albeit less efficiently than endogenous proinsulin.
hIns-SNAP is targeted to and released from insulin granules

Conversion of hProIns-SNAP suggests its sorting into SGs (20,21). By confocal microscopy TMR-Star⁺-hProIns-SNAP/hIns-SNAP appeared concentrated in cytosolic puncta which were also positive for the SG marker Chromogranin A (CgA) (22) (Figure 2A). Spatial intensity correlation analysis (13,23) indicated a Pearson’s correlation coefficient for TMR-Star⁺-hIns-SNAP and CgA of 0.76±0.05 (n=13 cells); with thresholded Manders coefficients being tM1_{avg} = 0.91±0.05; tM2_{avg} = 0.95±0.05 (controls, Rcoloc_{avg} = 0.72±0.04, tM1_{avg} = 0.53±0.06; tM2_{avg} = 0.81±0.06). Co-localization of TMR-Star⁺-hIns-SNAP with SGs was further supported by segmentation analysis, which showed that 80.3%±3.6 of insulin⁺ objects (controls, 100%) and 70.4%±12.7 of TMR-Star⁺ hIns-SNAP objects (87.71% of controls) were also CgA⁺. Furthermore, TMR-Star⁺-hIns-SNAP did not co-localize with markers of the endoplasmic reticulum, the trans-Golgi network or early endosomes (Suppl. Figure 2-4).

Next, we assessed the localization of hIns-SNAP by cryoimmunoelectron microscopy. Immunostaining for SNAP alone or together with insulin showed their specific co-localization in SGs, with equivalent background levels for both proteins in the cytosol, mitochondria and nuclei (Figure 2B, Table 1).

Finally, we analyzed the intracellular distribution of hIns-SNAP in subcellular fractions of transiently transfected hIns-SNAP INS-1 cells (Figure 2C). One peak of hProIns-SNAP was detected in lighter fractions enriched in the Golgi marker GM130. Another peak was present in heavier fractions, which were also enriched for the 54 kDa form of carboxypeptidase E, a marker of mature insulin SGs (24). These fractions were also enriched in hA-chain-SNAP, although hProIns-SNAP remained the prevalent form.
Being sorted into SGs, hIns-SNAP should be released in a regulated fashion. We measured with RIA the static secretion of hIns-SNAP, hProIns-SNAP, hC-peptide and rat insulin from hIns-SNAP INS-1 cells stimulated with 25 mM glucose and 55 mM KCl for 1 hour (n=3). The RIA for human insulin had negligible cross-reactivity towards rat insulin and human proinsulin (25), while the RIA for human C-Peptide does not detect rat C-peptide. Stimulation triggered the release of hIns-SNAP, hProIns-SNAP and hC-peptide (Figure 2D, 2E and Table 2), indicating that INS-1 cells co-release hProIns-SNAP, hIns-SNAP and hC-peptide with endogenous insulin in a regulated fashion, although a large fraction of hProIns-SNAP had not been converted into hIns-SNAP.

Pulse-labeling experiments (1,2) could not distinguish between bona fide preferential release of newer insulin vs. heterogeneity among beta cells, with those cells displaying faster insulin turnover being labeled to a greater extent during the pulse and then preferentially secreting during the chase. To address this question, we plated hIns-SNAP INS-1 cells in 384-well plates and counted 3-4 hour±30 minutes old TMR-Star+ SGs by confocal microscopy. We counted 2,616,149 TMR-Star+ SGs in 33,795 resting cells (median: 70±40 MAD/cell) vs. 1,212,080 TMR-Star+ SGs in 24,262 stimulated cells (median: 44±31 MAD/cell) (Fig. 2G and H). The data was log normalized to obtain a nearly normal distribution (Fig. 2G), while its location was normalized by dividing the median of each population (Fig. 2F). A Kolmogorov-Smirnov two-tailed test was repeated 10 times on a sample of 100 data points drawn randomly from each population. The statistics were consistent with the populations being drawn from the same distribution (average D = 0.14, average p = 0.36 at alpha = 0.05). These results are consistent with the hypothesis of the secretory response being homogenous over the
entire cell population rather than being primarily sustained by a more active subpopulation.

**Unequivocal imaging of age-defined and -distinct insulin granule pools**

Next, two age-distinct SG pools were labeled in the same cells using a protocol including three main steps (Figure 3A and B). First, transiently transfected *hIns-SNAP* INS-1 cells were incubated with TMR-Star for 15 minutes to label hProIns-SNAP/hIns-SNAP expressed in the cells at that time. Second, 10 µM BTP was added for 20 minutes to block any unlabeled hIns-SNAP. Third, cells were cultured for 4 hours to allow for the synthesis of new SGs, which were then labeled with BG-505 for 30 minutes. The longer incubation with BG-505 was devised to compensate for its lower brightness compared to TMR-Star (18). After washes, the cells were either fixed or imaged live by TIRF microscopy. Hence, TMR-Star⁺-SGs were “older” than 5 hours and 20 minutes, while BG-505⁺-SGs were younger than 5 hours (Figure 3A and B and Suppl. Movie 1). BG505⁻-SGs, were spatially segregated from TMR-Star⁺-SGs (Rcoloc\_avg = 0.45±0.11, n=10 cells) (Figure 3A). If the intervening BTP blocking step was omitted BG-505 and TMR-Star signals considerably overlapped (Rcoloc\_avg = 0.70±0.05, n=7 cells) (Figure 3B). Addition of BTP prior to labeling with the first fluorophore allows the time-resolution of older SGs to be restricted and defined more accurately. More younger and fewer older SGs were observed when TMR-Star and BG-505 were applied in the reverse order (Suppl. Figure 5), reflecting differences between the two dyes with respect to brightness, photostability and membrane permeability (18). Hence, for quantitative analysis of age-distinct SG pools we opted to label separate samples with the same SNAP fluorophore and vary the time interval between the “pulse” and “chase” labeling.
We assessed the release of TMR-Star\(^+\)-hIns-SNAP from younger and older SGs by fluorimetry. Resting cells secreted comparable amounts of 4-6 hour (9.0%) and 24-26 hour (6.4%) old TMR-Star\(^+\)-hIns-SNAP (Figure 2F). Upon stimulation with 25 mM glucose and 55 mM KCl, however, only 11.0% 24-26 hour old TMR-Star\(^+\)-hIns-SNAP was released compared to 50.6% 4-6 hour old TMR-Star\(^+\)-hIns-SNAP (n=3). These data are consistent with newly-synthesized insulin being preferentially released (1,2). The release of 4-6 hour old TMR-Star\(^+\) hINS-SNAP from hIns-SNAP INS-1 cells in 384-well plates could be independently measured by fluorimetry (Suppl. Fig. 6). These data, which are consistent with the ~40% reduction in the number of 3-4 hour±30 minutes TMR-Star\(^+\) SGs measured in stimulated hIns-SNAP INS-1 cells by automated microscopy (Fig. 2G and H), indicate that the sensitivity of this approach is suitable for large-scale screenings.

**Age-distinct insulin granules differ in their mobility and processivity**

Next, we tracked the motility of age-distinct SGs by TIRF microscopy. In hIns-SNAP INS-1 cells exposed to 2.8 mM glucose the mean speed of processive SGs at the cell cortex steadily decreased with time (Figure 4A, Table 3 and Suppl. Movies 2 and 3 for 2-4 hour and 5-7 hour old TMR-Star\(^+\) SGs, respectively) (for processivity criteria see section: Statistical analysis). Decreased motility of processive SGs was accompanied by reduction of their track maximum displacement (defined as the distance between the two furthest points within a SG trajectory (Figure 4B), indicating that their processive transport is age-dependent (Figure 4C and Table 3). We further correlated the mean speed of age-distinct processive SGs with their fluorescence intensity (Figure 4D). Processive SGs brighter than 1,100 arbitrary units (AU), conceivably the closest to the plasma membrane, displayed the lowest mean speed regardless of their age, suggesting that SGs in proximity of the cell surface, within the analyzed age-
window, are similarly restrained. Conversely, for SGs with brightness <1,100 AU, the lower the intensity the higher the mean speed. Still, age-distinct SGs of comparable brightness differed in their motility, with the youngest being the fastest. Hence, SGs processivity depends not only on restraints imposed by the surrounding environment, but also on changes inherent to their aging. The molecular mechanisms underlying these differences remain to be uncovered.

Targeting of Ins-SNAP into mouse islets in vitro and in vivo

The suitability of this technology was further tested by targeting insulin-SNAP into mouse islets in vitro and in vivo. For the first aim we transduced mouse islets with a hIns-SNAP adenoviral vector (Figure 5A.1, Figure 5B and Suppl. Movie 4). For the second aim we generated the SOFIA (Study OF Insulin granule Aging) mouse, in which one Ins2 locus was targeted with a mouse Ins2-SNAP cassette (Figure 5A.2), previously validated in transfected mouse MIN6 cells (Suppl. Figure 7).

The correct integration of mIns2-SNAP in the chosen ES cell clone was verified by Southern blotting (Suppl. Figure 8), while the progeny of chimeric mice generated with this clone was genotyped by PCR. Immunoblottings for insulin on non-reduced islet extracts indicated that SOFIA mouse islets expressed both mProIns2-SNAP and mIns2-SNAP (Figure 5C). The mProIns2-SNAP:mIns2-SNAP and insulin:mIns2-SNAP ratios were 1.36±0.12 and 1.92±0.64, respectively, whereas the insulin ratio between SOFIA and C57Bl/6 islets was 0.12±0.06 (n=3). The sorting of mIns2-SNAP in SGs was verified by cryoimmunoelectron microscopy (Figure 5D, Suppl. Table 1). Next, we attempted the labeling of mIns2-SNAP in vivo. For this, pancreatic tissue slices and sections of SOFIA mice were prepared 30-120 minutes after intracardiac or intravenous injection of TMR-Star. The SNAP-labeling was
restricted to islets (Figure 5E), and specifically to β-cells, as α- and δ-cells (Figure 5F and Suppl. Figure 9) were TMR-Star®. Notably, 30 minutes after TMR-Star injection the fluorescence was mostly perinuclear (Figure 5F), while after 2 hours was mostly granular and scattered throughout the cells (Figure 5E) consistent with the temporal progression of mProIns2-SNAP/mIns2-SNAP from the Golgi complex to SGs, as reported for [3H] proinsulin/insulin in isolated islets in vitro (26). The TMR-Star signal was retained even after SOFIA islets labeled in vivo by tail-vein injection had been isolated by collagenase digestion (Figure 5G).
Discussion

The functional heterogeneity and turnover of insulin SGs in relationship to their spatial and age distribution deserves greater attention, as knowledge about the molecular basis of such heterogeneity could be relevant for the understanding and treatment of type 2 diabetes.

Hallmarks of the disease onset are a blunted first phase of insulin secretion and an increased proinsulin release (27). In the prevailing model, insulin SGs docked at the plasma membrane contribute preferentially to the first phase of insulin secretion (28). Mobilization of more distal insulin SGs is critical instead to sustain the second phase of insulin secretion. This model has been challenged by the detection of insulin SGs which crash and fuse with the plasma membrane immediately after their appearance in the TIRF microscopy field (29). As these “newcomer” insulin SGs contribute to both insulin secretory phases, and newly synthesized insulin is preferentially released, it is of great interest to gain insight into the age distribution of “docked” and “newcomer” insulin SGs. Progress in this area, however, has been hampered by the lack of a method for the unequivocal time-resolved labeling of SGs. This approach has now been developed by tagging insulin with the SNAP tag (17-19). This technology has several advantages. It allows for the conditional labeling of targets with probes suited not only for live-cell imaging, but also for affinity purification, thus greatly extending the range of feasible analyses compared to the classical radioisotope based “pulse-chase” approach. Unlike approaches based on fluorescent proteins, including the “timer” reporter Ds-Red (30), it also enables the setting of accurate experimental time-boundaries without resorting to photo-bleaching, -activation or -conversion. Being spatially restricted and potentially detrimental to cells, the latter approaches are inadequate to follow the dynamics of entire populations of organelles such as insulin SGs.
The choice of insulin as reporter was favorable, being the most abundant cargo in β-cell SGs. Its employment together with SNAP was not obvious, since the latter relies on the reactivity of a cysteine with the substrate. As cysteines of secretory proteins are oxidized, ProIns-SNAP could have all been degraded in the ER, conjugated to other secretory proteins via disulphide bridges with free cysteines or mistargeted as reported for insulin-GFP in some (31), albeit not all, cases (32). As ProIns-SNAP is converted less efficiently than endogenous proinsulin its folding may indeed be altered in part. Accordingly, preliminary evidence suggests that glucose tolerance in SOFIA mice is impaired. Our data, however, indicate also that a fraction of Ins-SNAP is correctly processed, sorted into and released from SGs.

The approach presented here allowed for the first time the simultaneous imaging of insulin SG pools with distinct and precisely defined ages. In principle, the number of age-distinct SG pools that may be visualized is only limited by the availability of SNAP fluorescent substrates with distinct spectra. From a biological perspective, we could exclude that greater release of newly-synthesized insulin reflects heterogeneity of cells in regard to their biosynthetic and secretory responsiveness, hence providing formal evidence in support of the original claim that “younger SGs” undergo preferential exocytosis. We could also show that insulin SGs located in proximity to the plasma membrane markedly decrease their speed and processivity as they age from 2 to 7 hours - an early time point considering a half-life of ~ 30 hours. This phenomenon may result from age-dependent changes in the coupling of insulin SGs to motors and/or competence for association with the cortical cytomatrix. Rab27a, its interacting proteins granuphilin and MyRIP/Slac2/Exophilin8 (33) as well as β2-syntrophin (34) may restrict the mobility of insulin SGs. Hence, it will be important to determine whether these or other factors change their association with insulin SGs overtime.
The release of an age-defined pool of TMR-Star\(^+\)-Ins-SNAP from INS-1 cells cultured in 384-well plates could be measured with fluorimetry. Implementation of this approach may therefore enable the development of high-throughput screenings for compounds differentially affecting the exocytosis of age-distinct insulin SGs. Ins-SNAP viral vectors and the SOFIA mouse, in turn, represent valuable tools to compare the turnover of insulin SGs in non-diabetic and diabetic human islets and mouse models.
Authors’ contribution

Anna Ivanova – performed most of the experiments, analyzed the data and wrote the manuscript; Yannis Kalaidzidis – Developed the MotionTracking software and performed statistical analysis of imaging data; Ronald Dirkx – measured insulin-SNAP by fluorimetry; Mihail Sarov – helped in the generation of the targeting cassette generation; Michael Gerlach – imaged islets in vibratome tissue slices; Britta Schroth-Diez – provided assistance with TIRF microscopy; Andreas Müller – generated immunoelectron microscopy data; Yanmei Liu – transduced human islets with adenoviral vector; Cordula Andree – performed the 384-well plate assays; Bernard Mulligan – provided assistance with statistical analysis of TIRF microscopy data; Carla Münster – isolated rodent islets; Thomas Kurth – collaborated for immunoelectron microscopy experiments; Marc Bickle – supervised the 384-well plate assays and performed the segmentation analyses; Stephan Speier – supervised imaging of islets in vibratome tissue slices; Konstantinos Anastasiadis – provided expertise for the generation of the knock-in mice; Michele Solimena – conceived the project, analyzed the data and wrote the manuscript.
Acknowledgements

The authors thank C. Wollheim (Univ. Geneva) for the gift of INS-1 cells, Dr. J. Miyazaki (Univ. Osaka) and Dr. S. Seino for the gift of MIN6 cells; A. Brecht (Covalys) for providing SNAP reagents; R. Naumann (MPI-CBG) for help with the generation of transgenic mice; D. Krastev (MPI-CBG), J. Schindelin (MPI-CBG) and D. White (MPI-CBG) for discussions; P. De Camilli (Yale Univ, New Haven, CT) and M. Zerial (MPI-CBG) for reading of the manuscript; A. Altkrueger (PLID), I. Baer (CRTD), S. Kretschmar (CRTD), K. Saydaminova (PLID), D. Richter (PLID) and A. Friedrich (PLID) for technical help; M. Chernykh (MPI-CBG) for MT software assistance; K. Pfriem (PLID) for administrative help. We thank the ZIH at TU Dresden for providing resources on their Deimos PC cluster. This work has been supported with funds from the German Ministry for Education and Research (BMBF) to the German Centre for Diabetes Research (DZD; http://www.dz-dyev.de), and the European Community's Seventh Framework Programme (FP7/2007-2013) for the Innovative Medicine Initiative under grant agreement n° 115005 (IMIDIA, http://www.imidia.org). A.I. has been supported with a MedDrive Grant from the Medical Faculty at TU Dresden. M.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors do not have any conflict of interest.
Figure legends

Figure 1. hIns-SNAP is expressed and converted in INS-1 cells.

A. pEG-hIns-SNAP construct. Vector for the expression of human pre-proinsulin fused to SNAP. Mature hIns-SNAP includes the B-chain and A-chain-SNAP connected by disulfide bridges. Dark blue: CMV (cytomegalovirus promoter); light blue: signal peptide; green: B-chain; yellow: C-peptide; red: A-chain; orange: SNAP. B. Expression of hIns-SNAP in INS-1 cells. Immunoblotting with anti-insulin (upper panel) and anti-A-chain (lower panel) antibodies on purified recombinant His-SNAP and reduced lysates of transiently transfected hIns-SNAP INS1-cells or non-transfected INS-1 cells, separated by SDS-PAGE. Mw: molecular weight markers. A dotted line indicates where the image of the original blot was cut to assemble the figure. C. Expression of hIns-SNAP and rat insulin in INS-1 cells. Immunoblotting with anti-insulin (upper panel) and anti-γ-tubulin (lower panel) antibodies on non-reduced lysates of transiently transfected hIns-SNAP INS1-cells (+) or non-transfected (-) and incubated with 2.8 or 11 mM glucose prior to extraction and separation on a Tris-tricine gel. Dotted lines indicate where images of the original blot were cut to assemble the figure. B and C are representative results of 3 independent experiments.

Figure 2. Sorting of hIns-SNAP into insulin SGs and preferential release of newly-synthesized hIns-SNAP.

A. Confocal immunomicroscopy. Transiently transfected hIns-SNAP INS-1 cells were labeled with TMR-Star and co-immunostained after fixation for CgA. The insets show cytoplasmic extensions, where SGs accumulate. Scale bar: 10 μm. The scatter plot in the right panel shows the pixel intensity distribution of the signals for TMR-Star (y-axis) and
CgA (x-axis). **B. Cryoimmunoelectronmicroscopy.** Cryosections of transiently transfected hIns-SNAP INS-1 cells immunogold-labeled for SNAP (left panel) or double immunogold-labeled for SNAP and insulin (right panel). Scale bar: 500 nm. **C. Subcellular fractionation.** Immunoblotting on subcellular fractions of transiently transfected hIns-SNAP INS-1 cells cultured in 11 mM glucose and separated on sucrose density gradients. CPE: carboxypeptidase E; GM130: Golgi matrix protein of 130 kDa. **D. Radioimmunoassay for human insulin and proinsulin and rat insulin.** Concentrations of human insulin and proinsulin and rat insulin in the media of resting (R: 0 mM glucose, 5 mM KCl in HEPES buffer with 120 mM NaCl) and stimulated (S: 25 mM glucose, 55 mM KCl in HEPES with 120 mM NaCl) transiently transfected hIns-SNAP INS-1 cells and control INS-1 cells for 1 hour (n=3). Y axis: ng/ml. **E. Radioimmunoassay for human C-peptide.** Concentrations of human C-peptide in the media of resting (R, as in D) and stimulated (S, as in D) transiently transfected hIns-SNAP INS-1 cells and control INS-1 cells (n=3). Y axis: ng/ml. **F. Release of age-distinct hIns-SNAP pools.** Percentages of the total amount of 4-6 hour and 24-26 hour old TMR-Star⁺-hIns-SNAP in the media and extracts of resting (R, as in D) or stimulated (S, as in D) INS-1 cells stably expressing hIns-SNAP, as measured by fluorimetry (n=3). Y axis: % of total. **G. Release of hIns-SNAP – 384-well plate format.** Log transformation of the granule distribution data in resting cells (R: 2.8 mM glucose, 5 mM KCl in HEPES buffer with 120 mM NaCl) or upon stimulation (S, as in D). Y axis: estimation of the probability. **H. Normalization of the data presented in Figure 2H.**

**Figure 3. Simultaneous detection of age-distinct insulin SGs.**

**A-B. Confocal microscopy.** Transiently transfected hIns-SNAP INS-1 cells were sequentially labelled with TMR-Star and BG-505 according to the indicated schemes. In **(A),** but not in **(B),** cells were incubated with the non-fluorescent SNAP substrate BTP (block) before being incubated with BG-505. Scale bar: 10 µm.
Figure 4. Mobility and processivity of insulin SGs are age-dependent.

TMR-Star labeled *hIns-SNAP* INS-1 cells were imaged by single-color TIRF-microscopy. Mobility and processivity of cortical insulin SGs were analysed using the Motion Tracking software. The data shown in A, C, D originated from the analysis of 20,369 tracks in total (results from 2-4 independent experiments are expressed as mean±S.E.M). **A.** Mean speed of processive, age-distinct insulin SGs. The red straight line shows the maximum likelihood fit. The mean speed decay coefficient was -0.0266 ± 0.0079 µm/s/h. Y axis: mean speed. **B.** Illustration of the maximum displacement of SGs. **C.** Track maximum displacement of processive, age-distinct insulin SGs. Y axis: track maximum displacement. **D.** Distribution of mean speed vs. intensity of processive, age-distinct insulin SGs. Y axis: mean speed.

Figure 5. Expression of Ins-SNAP in mouse islets in vitro and in vivo.

**A. 1.** Scheme of hIns-SNAP adenoviral vector, L: left inverted terminal repeats (white); E: encapsidation signal (grey); R: rat insulin promoter (dark blue); light blue: signal peptide; green: B-chain; yellow: C-peptide; red: A-chain; orange: SNAP; R: right inverted terminal repeats (white); **2.** Scheme of the targeted *INS-2* locus in ES cells prior to the removal of the region between the two FRT sites (magenta arrowheads). **B.** TIRF microscopy image of a single cell dissociated from mouse islets, infected with the hIns-SNAP adenoviral vector and labeled with TMR-Star. Scale bar: 5 µm. **C.** Immunoblot with the anti-insulin antibody on non-reduced extracts of islets isolated from *mIns-SNAP*^+/−_ SOFIA and C57BL/6 mice. **D.** Cryoimmunoelectron microscopy of pancreatic islets isolated from SOFIA mice and immunolabeled with the mouse anti-SNAP antibody, followed by rabbit anti-mouse IgG and 10 nm gold particle-conjugated protein A. Scale bar: 500 nm. **E.** Confocal image (maximum intensity projection) of a pancreatic tissue slice from a SOFIA mouse *i.v.* injected with TMR-Star. Bottom left panel: merge of images acquired by laser backscatter (top left panel) and
fluorescence (top right panel). Scale bar: 100 µm. Bottom right panel: High magnification single optical confocal plane of an islet within the pancreatic tissue slice. Scale bar: 20 µm.

F. Confocal image of a pancreatic cryosection from a SOFIA mouse \textit{i.v.} injected with TMR-Star. Immunolabeling with anti-glucagon antibody. Scale bar: 10 µm. Arrows point to the region of the trans-Golgi network. G. Light-sheet microscopy imaging (single focal planes) of isolated islets from SOFIA (top panel) and C57Bl/6 (bottom panel) mice \textit{i.v.} injected with TMR-Star. Scale bar: 50 µm.
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| Labeling         | anti-SNAP (single) | anti-SNAP (double) | anti-insulin (double) |
|------------------|--------------------|--------------------|-----------------------|
| # cells          | 11                 | 14                 | 14                    |
| SGs              | 812.7 gp/µm²       | 47.4 gp/µm²        | 261.2 gp/µm²          |
| background       | 1.2 gp/µm²         | 0.2 gp/µm²         | 1.8 gp/µm²            |
| P value          | 0.000948           | 0.002068           | 0.0000026             |

**Table 1. Quantification of immunoelectron microscopy.**

Single (anti-SNAP antibody) and double (anti-SNAP and anti-insulin antibodies) immunostaining of *hIns-SNAP* INS-1 cells was performed on cryosections. Dual labelings for insulin and SNAP were performed consecutively. The data is presented as number of gold particles per µm².
| RIA specificity                        | Resting [ng/ml] | Stimulation [ng/ml] |
|---------------------------------------|-----------------|---------------------|
| Human insulin (hIns-SNAP INS-1 cells) | 6.21 ± 1.21     | 81.75. ± 11.73      |
| Human proinsulin (hIns-SNAP INS-1 cells) | 25.53 ± 3.08   | 221.31 ± 31.20      |
| Rat insulin (hIns-SNAP INS-1 cells)   | 30.54 ± 8.59    | 517.11 ± 227.33     |
| Rat insulin (INS-1 cells)             | 26.19 ± 3.29    | 900.96 ± 282.61     |
| Human C-peptide (hIns-SNAP INS-1 cells) | 3.02 ± 0.76    | 49.77 ± 7.93        |

Table 2. Summary of Radioimmunoassay results.

Human insulin, human proinsulin, rat insulin and human C-peptide were measured by RIA. A set for measurements consisted of one well (in triplicate) of cells incubated in resting and one well (in triplicate) in stimulation conditions (n=3).
| Age   | Mean speed [µm/sec] | P value       | Track max. displ. [µm] | P value       |
|-------|---------------------|---------------|------------------------|---------------|
| 2-4 h | 0.53 ± 0.023        | vs. 5-7 h, 0.005 | 0.53 ± 0.020           | vs. 5-7 h, 0.014, vs. 4-6 h, 0.0007 |
| 3-5 h | 0.51 ± 0.015        | vs. 5-7 h, 0.024 | 0.51 ± 0.013           | vs. 2-4 h, 0.0007, vs. 4-6 h, 0.0006 |
| 4-6 h | 0.48 ± 0.021        | -             | 0.45 ± 0.009           | vs. 3-5 h, 0.0006 |
| 5-7 h | 0.45 ± 0.013        | vs. 2-4 h, 0.005, vs. 3-5 h, 0.02 | 0.47 ± 0.015           | vs. 2-4 h, 0.014 |

Table 3. Age-dependent changes of SG mean speed and track maximum displacement.

Images of 2-4 hour, 3-5 hour, 4-6 hour and 5-7 hour old TMR-Star⁺-SGs were acquired by TIRF microscopy, and the data analysed for calculation of the mean speed and track maximum displacement of processive granules.
Figure 1

A

Expression → Processing

B

anti-insulin

Mw 30 kDa 20 kDa

anti-A-chain

Mw 30 kDa 20 kDa

C

anti-insulin

Mw 40 kDa 30 kDa 20 kDa

2.8 mM 11 mM glucose

Mw 10 kDa 4.6 kDa 1.7 kDa 50 kDa

hProIns-SNAP

hins-SNAP

hC-peptide

γ-tubulin

hProIns

hins

DNA

Control

hProIns-SNAP

hins-SNAP
Figure 3

A

15 min
TMR-Star

30 min
BTP

20 min

4 hrs

30 min
BG-505

30 min
fixation

5 hrs

older, TMR-Star

younger, BG-505

merge

B

15 min
TMR-Star

30 min
no BTP

20 min

4 hrs

30 min
BG-505

30 min
fixation

5 hrs

older, TMR-Star

newer, BG-505

merge

143x159mm (300 x 300 DPI)
Figure 4

A

B

C

D

142x107mm (300 x 300 DPI)
Figure 5

A

1) L E R cDNA hins SNAP AS(5) SNAP L E R
2) Stop Acan SNAP

B

Ad hins-SNAP, TMR-Star

C

SOFIa C37Bl/6

D

SOFIA islet, anti-SNAP, 10 µm

E

Reflected light, TMR-Star

F

SOFIA islet, TMR-Star

G

C57Bl/6 islet, TMR-Star

150x188mm (300 x 300 DPI)
Supplementary Figure 1. hIns-SNAP is expressed and converted in INS-1 cells.

Immunoblotting with anti-A-chain antibody on reduced (red) and non-reduced (nred) lysates of transiently transfected Ins-SNAP INS-1 cells (+) or non-transfected (-) and separated on a Tris-tricine gel. The cells were grown in 2.8 or 11 mM glucose containing media prior to extraction and separation (n=3). The position of an intermediate processing product is indicated with an asterisk (*). It corresponds in size to hC-peptide-A-chain-SNAP (~ 27 kDa). Mw: molecular weight markers.
Supplementary Figure 2. hIns-SNAP is not retained in the ER. Immunocytochemistry.

A. INS-1 cells were co-stained with anti-insulin and anti-protein disulphide isomerase (PDI) antibodies. B. Transiently transfected *Ins-SNAP* INS-1 cells were co-stained with BG-505 and an anti-PDI antibody. Scale bar: 5 µm.
Supplementary Figure 3. Immunostaining of INS-1 cells expressing hIns-SNAP with trans-Golgi marker TGN38.

A. INS-1 cells were co-stained with anti-insulin and anti-TGN38 antibodies. B. Transiently transfected Ins-SNAP INS-1 cells were co-stained with the TMR-Star and an anti-TGN38 antibody. Scale bar: 5 µm.
Supplementary Figure 4. hIns-SNAP is not missorted to early endosomes.

A. INS-1 cells were co-stained with anti-insulin and anti-EEA1 antibodies. B. Transiently transfected Ins-SNAP INS-1 cells were co-stained with TMR-Star and an anti-EEA1 antibody. Scale bar: 5 µm.
Supplementary Figure 5. SNAP substrates exhibit different labelling efficiencies.

Double labelings of transiently transfected *Ins-SNAP* INS-1 cells with TMR-Star and BG-505. In A and B the two SNAP substrates were applied sequentially in the reverse order, as indicated in the respective schemes. A blocking step with BTP preceded and followed the first fluoroprobe. Scale bar: 5 µm.
Supplementary Figure 6. Fluorimetric measurement of 4-6 hour old TMR-Star$^+$-hIns-SNAP released from hIns-SNAP INS-1 cells cultured in 384-well plates. Secreted TMR-Star$^+$ hIns-SNAP is presented as % of the total amount of TMR-Star$^+$-hIns-SNAP (n=2).
Supplementary Figure 7. hIns-SNAP is targeted to the insulin SGs in mouse MIN6 cells.

A-B. Confocal microscopy of MIN6 cells transfected with the mIns2-SNAP modified BAC and labeled with TMR-Star (A) followed by immunostaining with a rabbit anti-CPE antibody (B). The secondary antibody was conjugated with Alexa488. C. Pixel distribution diagram (scatter plot) of the two fluorescent signals.
Supplementary Figure 8. Construct for the knockin of mIns2-SNAP in mice and southern blots of targeted ES clones.

A. Schematic representation of the mouse Ins2 gene and the fragments generated by its digestion with the indicated restriction enzymes. The domains of preproinsulin are indicated in colors. P1 and P2 indicate the location of the probes for southern blotting.

B. Expected structure of Ins2 following the targeting with the mIns2-SNAP cassette. The portion of the cassette including PGK/gb3 (eukaryotic/prokaryotic promoter) and neo (neomycin resistance gene) is flanked by FRT sites for its removal.

C. Southern blots of genomic DNA isolated from several candidate ES clones targeted with the mIns2-SNAP cassette and digested with SpeI. Clone 32 showed the expected bands of 5.1 Kb and 4.1 Kb for the wild type and targeted alleles, respectively.
Supplementary Figure 9. Immunohistochemical characterization of SOFIA mouse islets.

Confocal microscopy images of immunolabeled pancreatic tissue slices from a SOFIA mouse i.v. injected with TMR-Star. A-B. Images of islets immunolabeled with anti-insulin (A) or anti-somatostatin (B) antibodies and secondary antibodies conjugated to Alexa488. Scale bar: 25 µm.
Supplementary Table 1. Quantification of immunoelectron microscopy.

Single (anti-SNAP antibody) immunostaining of SOFIA and control C57Bl/6 islets was performed on cryosections.

| Cell type         | SGs        | background | P value  |
|-------------------|------------|------------|----------|
| SOFIA, n=18 cells | 26.5 gp/µm² | 0.7 gp/µm² | 1.26.10⁻¹⁵ |
| C57Bl/6, n=18 cells | 0.4 gp/µm² | 0.2 gp/µm² | 0.75     |
| P value            | 8.73.10⁻¹⁸ | 0.00006    |