A fluorescent timer reporter enables sorting of insulin secretory granules by age

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Keywords: insulin, beta-cell, vesicles, insulin secretion, Type 2 diabetes, insulin secretory granule (SG), exocytosis, granule aging, fluorescent timer, dsRed-E5
ABSTRACT

Within the pancreatic β-cells, insulin secretory granules (SG) exist in functionally distinct pools, displaying variations in motility, as well as docking and fusion capability. Current therapies that increase insulin secretion do not consider the existence of these distinct SG pools. Accordingly, these approaches are effective only for a short period, with a worsening of glycaemia associated with continued decline in β-cell function. Insulin granule age is underappreciated as a determinant for why an insulin granule is selected for secretion and may explain why newly synthesised insulin is preferentially secreted from β-cells. Here, using a novel fluorescent timer protein, we aimed to investigate the preferential secretion model of insulin secretion and identify how granule aging is affected by variation in the β-cell environment, such as hyperglycemia. We demonstrate the use of a fluorescent timer construct, syncollin-dsRedE5TIMER, which changes its fluorescence from green to red over 18 h, in both microscopy and fluorescence-assisted organelle sorting techniques. We confirm that the SG-targeting construct localises to insulin granules in β-cells and does not interfere with normal insulin SG behavior. We visualise insulin SG aging behavior in MIN6 and INS1 beta-cell lines, and in primary C57BL/6J mouse and non-diabetic human islet cells. Finally, we separated young and old insulin SGs, revealing that preferential secretion of younger granules occurs in glucose-stimulated insulin secretion. We also show that SG population age is modulated by the β-cell environment in vivo in the db/db mouse islets and ex vivo in C57BL/6J islets exposed to different glucose environments.

The pancreatic beta-cell plays a central role in glucose homeostasis, and beta-cell dysfunction is key to the pathogenesis of Type 2 Diabetes (T2D). This manifests mainly as a reduction in glucose-stimulated insulin secretion (GSIS). In beta-cells, insulin is stored in secretory granules (SGs) and in response to stimulation, SGs mobilise and fuse with the plasma membrane, delivering insulin to the bloodstream. Models of sequential secretion, in which SGs are targeted for secretion based on their proximity to the plasma membrane, have been considered for many years [1-3]. However, the discovery of ‘newcomer’ insulin SG [4], “kiss-and-run” dynamics [5, 6], compound insulin SG secretion [7], and complexities surrounding the relationship between SG docking and exocytosis [8], suggests that either multiple, or more intricate, mechanisms dictate SG selection for GSIS.

Granule aging represents another facet of SG selection for insulin secretion. Evidence accumulating over the last 30 years, including pulse-chase radiolabeling as early as the 1960s [9-11] suggests that the age of SGs may play a significant role in dividing SGs into functionally distinct pools. More recently, fluorescent marker techniques such as the TMR-Star have allowed high resolution imaging of these uniquely aged pools [9, 12-16]. In parallel, it was observed that upon glucose stimulation, a highly mobile pool of SG rapidly moves from the interior of the beta-cell to be secreted with minimal to no residence time at the plasma membrane [17, 18]. This pool of SGs has been referred to as the “newcomer” or “restless” SG pool and is thought to account for all of the 2nd phase of GSIS and a substantial portion of 1st phase GSIS [19, 20]. Since then, characterisation of young SGs using fluorescent marker techniques has revealed that they are composed of newly synthesised insulin, exhibit high mobility [14, 15], and may account for this newcomer pool. Interestingly, in direct contrast to the classical model, recent studies have shown that aged SGs, including those that are pre-docked, lose mobility and fusion competency as a function of age [14].

In beta-cells, insulin is produced as a precursor, proinsulin, which is processed to
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Mature insulin combines with Zn$^{2+}$ and forms a hexameric crystal within the SGs. These SGs can be stored for days in the cytosol and the estimated half-life of insulin SGs is 3-5 days [21]. On average, a functional beta-cell contains ~10,000 SGs [22, 23]. In response to a single stimulus, a very small percentage of these insulin SGs (1%–5% of total insulin content, often less, over the course of an hour) are released through exocytosis. Older insulin SGs that do not undergo exocytosis are targeted to the lysosome for degradation [21, 24], but how a cell can distinguish young SGs from old is unclear. Interestingly, under conditions of metabolic stress, beta-cells could potentially lose their ability to distinguish young SGs from old; in diabetes, beta-cells hypersecrete insulin to compensate for insulin resistance and eventually become de-granulated [25-27]. Whether these changes are intrinsic to the SG or regulated by the beta-cell environment is not defined.

The use of fluorescent timer proteins such as DsRed-E5 [28], to segregate vesicles by age has been previously demonstrated in bovine chromaffin cells [13] and in PC12 neuroendocrine cells [29]. In both studies, preferential secretion of younger pools of vesicles was identified. Moreover, age-distinct vesicle pools identified in these studies were found not only to display dissimilar motility and localisation, but also differentially respond to secretagogue challenges [13].

In the present study we aimed to characterise young and old pools of insulin granules, and investigate their behaviours in a model of type 2 diabetes. We employed an established fluorescent timer [28] construct that is targeted within the lumen of insulin SG, syncollin-dsRedE5TIMER [30], to visualise insulin SGs as a function of age. The dsRedE5TIMER is a coral fluorescent protein that changes its emission characteristics from a green 505 – 550 nm to a red 590 – 700 nm with time. Therefore, the green to red fluorescence ratio indicates the age of the protein, and a yellow/orange fluorescence results from dsRedE5TIMER protein in an intermediate state of folding, reflective of a “middle-aged” granule [28]. Syncollin is a soluble intra-granular protein, which is not normally expressed in beta-cells. However, when syncollin is tagged with a fluorescent protein it is exclusively targeted to the lumen of beta-cell granules [31]. Using this syncollin-dsRedE5TIMER adenoviral construct, we have successfully expressed the protein in beta-cell granules [30]. Here, we show fluorescent timer maturation from green to red in the mouse MIN6 pancreatic beta-cell line, the INS1 insulinoma rat beta-cell line, as well as in primary mouse and human pancreatic islet cells. We demonstrate that insulin SGs differentially localise within the beta-cell cytoplasm by age, with younger ISGs positioned closer to the plasma membrane. Moreover, using flow cytometer-assisted organelle sorting (FAOS) analysis [32, 33], we identify age-distinct populations of insulin SGs while excluding large multi-granular bodies and establish that younger insulin SG selection is a glucose-mediated phenomenon. Finally, with islets from wildtype and obese type 2 diabetic db/db mice, we show that the granule population age is regulated by the beta-cell environment under conditions of chronic metabolic stress.
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Results

Syncollin-dsRedE5TIMER targets insulin SGs and differentiates younger and older SG populations

Acquisition of a 10 nm-step excitation lambda scan in adenovirus syncollin-dsRedE5TIMER-infected dispersed mouse primary islets, 48 h post-infection, identified two distinct excitation peaks at 490 and 570 nm corresponding to dsRed-E5 protein’s expected immature green, then mature red, fluorescence wavelengths respectively (Figure 1A). As with previous studies employing fluorescent timer constructs [34], a single excitation of 490 nm was used to minimize photobleaching, with sequential detection of 505 – 550 nm and 590 – 700 nm bands used to capture the immature and mature forms of dsRed-E5 respectively.

By confocal microscopy, transduction of syncollin-dsRedE5TIMER into beta-cells and dispersed islets clearly resulted in fluorescent punctate granule-like structures within the cytoplasm of cells (Figure 1B). As previously shown in both a rat insulinoma beta-cell line and in primary rat islets, adenoviral-induced expression of fluorescently-labelled syncollin leads to its targeting to the insulin SG lumen within beta-cells [30, 35]. To confirm these findings in mice, we stained the MIN6 mouse insulinoma cell line with a guinea pig anti-insulin antibody 24, 48 and 72 h post transduction with syncollin-dsRedE5TIMER (Figure 1B). It was apparent that though not all insulin SG expressed syncollin-dsRedE5TIMER, colocalisation of insulin and dsRed-E5 fluorescence was clear, with a Mander’s overlap coefficient of 0.917 ± 0.033 SEM and 0.920 ± 0.034 for immature and mature forms of syncollin-dsRedE5TIMER respectively by 48 h (Figure 1C).

To further confirm the presence of syncollin-dsRedE5TIMER in mouse insulin SGs, we performed sucrose gradient subcellular fractionation of syncollin-dsRedE5TIMER-infected MIN6 cells at 72 h post-transduction and demonstrated that syncollin and insulin co-fractionated in compartments positive for granule markers Vamp8 and PCSK2, but not beta-actin (Figure 1D and 1E). Additionally, to ensure that syncollin-dsRedE5TIMER expression was compatible with beta-cell function, we performed glucose-stimulated insulin secretion (GSIS) assays in syncollin-dsRedE5TIMER-infected MIN6 cells 72 h post infection, and confirmed high glucose-stimulated secretion of syncollin alongside PCSK2 (Figure 1F). We additionally performed GSIS in primary dispersed mouse islets with and without syncollin-dsRedE5TIMER infection, to confirm that insulin secretion and content was not affected by adenovirus transduction of the syncollin construct (Figure 1G).

To characterise the dynamics of syncollin-dsRedE5TIMER within insulin SGs, dispersed primary islet cells from normal C57BL/6J mice and non-diabetic human organ donors were infected to observe over a 72 h time course. Substantial immature green dsRed-E5 fluorescence was observed by 18 h post-infection, with maturation into red primarily observed as an overlapping yellow (Figure 1H). These yellow granules were termed “middle-aged” SGs, in that they contained the maturing dsRedE5 protein displaying emission at both green and red fluorophores [28]. Most interestingly, as new green-fluorescing granules appeared in the beta-cell, red older SGs localised in populations within the cell distinct from the younger, newly synthesised granules by 72 h post-transduction (Figure 1H), which appeared to maintain a closer proximity to the plasma membrane. Using fixed TIRF microscopy, we were also able to visualise more green young granules localised to the plasma membrane at 72 h, and not 24 h (Supplementary Figure 1).

To address functional dynamics of young and old populations, Brefeldin A was added to syncollin-dsRedE5TIMER-transduced INS1 rat beta-cells to halt vesicular transport from Golgi
and this resulted in an observable increase in red fluorescence, corresponding to the loss of SG biogenesis (Figure 2A), and this change in fluorescence ratio was statistically significant (Figure 2B). In contrast, addition of Taxol to stabilise microtubules resulted in small but non-significant increase in green fluorescence, presumably a result of immature SG accumulation due to decreased SG motility and exocytosis (Figure 2C and D). Altogether these data confirm that syncollin-dsRedE5TIMER transduction of both beta-cell models, as well as primary islet cells, results in functionally relevant time-dependent dynamic fluorescence in insulin granules, allowing differentiation of younger and older SG populations for further analyses.

**Flow cytometry-assisted organelle sorting (FAOS) separates insulin SGs by age**

Concurrent to confocal imaging, we used flow cytometry analysis of subcellular particles to characterize our syncollin-dsRedE5TIMER expressing SGs. FAOS analysis allowed the advantage of measuring both the immature (green) and mature (red) dsRed-E5 fluorescence intensities of individual insulin SG relative to the entire subcellular particle population. Gating for a typical granule size 100 – 500 nm [22], and then single particles (Figure 3A), we aimed to exclude the large multi-granular bodies previously measured by Hoboth and colleagues at > 500 nm [14].

Analysis of a time course of fluorescent granules from syncollin-dsRedE5TIMER-transduced mouse islet cells clearly demonstrated the initial expression of immature dsRed-E5 as a distinct green-positive, red-negative granule population at 24 h (green gate). Over 72 h, a gradual shift to red-positive mature dsRed-E5 expression (red gate) is observed, while non-transduced control islet cells displayed no fluorescent granules (Figure 3B). By 48 h, numbers of younger green-positive granules appear to be stable, while older red-positive granules begin to increase in number from 24 h onwards (Figure 3C). Syncollin-dsRedE5TIMER behavior and subsequent FAOS profiles were similar in transduced dispersed human islet cells (Figure 3D). We subsequently expressed these data as a ratio of the old/young granule population percentages, highlighting the increase of the ratio over time (Figure 3E).

Using back-gating analysis, we demonstrated comparable size and scatter properties of both young green-positive granules and aged red-positive granules from primary mouse islet cells 72 h after transduction (Supplementary Figure 2A). Furthermore, both these populations also came from gates of comparable size and scatter characteristics as fluorescent particles from INS-1 832/13 GRINCH cells [36] which express hPro-CpepsfGFP within insulin SG but not control INS-1 832/13 cells (Supplementary Figure 2B and C), as well as intracellularly-fluorescently stained insulin particles in MIN6 cells (Supplementary Figure 2D), further establishing their status as genuine insulin SGs.

**Preferential secretion of younger insulin SGs is selective for glucose-stimulation**

To address the preferential secretion model, we utilised a glucose-stimulated insulin secretion assay immediately followed by FAOS to measure relative populations of young and old SGs within the cell (Figure 4A). 72 h post-transduction with syncollin-dsRedE5TIMER, dispersed mouse islet cells were subjected to 1 h of resting condition in KRBH buffer containing 2.8 mM glucose (basal), then stimulated with KRBH containing either 2.8 mM glucose or 16.7 mM glucose. Immediately after stimulation, cells were lysed in 0.3 M sucrose in HEPES-EGTA buffer and processed for FAOS to analyse syncollin-dsRedE5TIMER-positive insulin SG populations. Paired analysis of treatments across 8 separate experiments found a significantly increased ratio of old to young SGs remaining in
cells post-stimulation with 16.7 mM glucose compared to 2.8 mM glucose (Figure 4B and 4C). FAOS analysis after high glucose stimulation in human donor islets from one experiment also showed a preference for younger granule secretion (Figure 4D).

Using live cell confocal imaging, preferential secretion of young SGs was also visualised in MIN6 cells. After 1 h incubation at 2.8 mM glucose KRBH, cells were imaged 10 min after the addition of either 2.8 mM or 16.7 mM glucose KRBH (Figure 4E). Regions of interest containing 3 – 8 cells were averaged across 5 experiments to obtain intensity values corresponding to the 505 – 550 nm and 590 – 700 nm emission spectra of young and old granules respectively (Figure 4F). As was observed with FAOS analysis of dispersed islets, 16.7 mM glucose stimulation resulted in an increased ratio of old to young granules retained in MIN6 cells – indicative of selective secretion biased towards younger granules. In live cell imaging of syncollin-dsRedE5TIMER-transduced INS1 cells, selective young granules secretion was similarly observed. INS1 cells stimulated with 16.7 mM glucose and the addition of a “hyperstimulation cocktail” of glyburide, forskolin and IBMX, showed increased visible red fluorescence after 10 min of stimulation. More interestingly, 60 min post-stimulation, reappearance of green fluorescence was observed, indicating replenishment of the young ISG pool (Figure 4G).

These data together readily confirm [12-16] the occurrence of preferential secretion of younger insulin SG in both MIN6 and INS1 beta-cells and dispersed mouse and human islet cells. Moreover, they highlight the phenomenon as glucose-regulated.

**The beta-cell environment alters the dynamics of young and old insulin SG populations**

We next examined the relationship between SG age and the beta-cell environment. Though there is evidence that newly synthesised insulin SGs are preferentially secreted upon stimulation under physiological conditions, it is not known how the changing environment of beta-cells under metabolic stress affects the turnover and dynamics of age distinct SG pools.

Islets were isolated from C57BL/6J wildtype mice and dispersed prior to transduction with syncollin-dsRedE5TIMER. After 24 h, culture media was replaced with 2.8 mM, 8.3 mM or 16.7 mM glucose RPMI to simulate a hypoglycaemic, euglycaemic or hyperglycaemic environment respectively. After an additional 48 h culture period, islet cells were lysed for FAOS analysis. Healthy wildtype islets demonstrated a strikingly consistent phenotype in granule population age, with a significantly increased younger granule population observed in hyperglycaemic conditions, and concomitantly, significantly reduced younger granules observed in hypoglycaemic conditions (Figure 5A and 5B). At 16.7 mM glucose, the older granule population pool also increased. This consequently resulted in a higher old/young granule population ratio at both hypoglycaemic and hyperglycaemic conditions (Figure 5C).

Next we employed the leptin receptor-deficient db/db mouse model to assess granule behavior under conditions of beta-cell dysfunction. Db/db mice display severe obesity, glucose intolerance, hyperinsulinemia, and eventually hyperglycemia [37, 38]. Prior to in vitro experiments, fasting glucose was obtained and intraperitoneal glucose tolerance tests performed to verify mutant obese db/db mice as diabetic (Figure 5D and 5E). Dispersed islet cells obtained from db/db mutant mice and their littermate controls, were transduced with syncollin-dsRedE5TIMER, and then lysed for FAOS analysis after 72 h. Across three litters, db/db mice exhibited higher percentages of younger granule populations compared to wildtype controls (Figure 5F and 5G), subsequently resulting in a significantly reduced
old/young population ratio (Figure 5H), and echoing the behaviour previously observed in wildtype islets under chronic in vitro high glucose environment. Together these data demonstrate that the beta-cell, under conditions of metabolic stress, is capable of highly subtle modulation of the intracellular SG population by age.

Discussion

Insulin SGs exist in distinctly behaving pools; some of which exhibit higher motility [14, 39] or enhanced membrane docking properties [8] and others that are more prone to fusion with the plasma membrane [40, 41] or appear to be preferentially degraded [14, 42]. By adding a temporal layer to these behaviors, we and others [14] have demonstrated that granule age is a key determinant of secretory preference.

By exploiting syncollin-dsRedE5TIMER’s ability to traffic as an insulin SG cargo protein, we demonstrate that granule preference can occur in SGs in as young as 24 h old (as syncollin-dsRedE5TIMER takes approximately 18 h to mature from green to red). These data are congruous with those previously described using the SNAP tag reporter system [15], which defined younger granules as less than 5 h old, and older granules between 5 – 30 h in age, and have linked SG “youth” to increased motility, as well as secretory competency [43].

In the db/db mouse, increased immature SG biogenesis has previously been described as a result of beta-cell compensatory responses [26]. However, concomitant loss of functional secretory capacity is also observed [41] with a heightened beta-cell workload resulting in increased ISG/proinsulin secretion as insulin granule maturation processes become are unable to keep up with secretory demand. We similarly describe a significant increase in young or immature insulin SGs in our model of FAOS, validating the capacity of syncollin-dsRedE5TIMER to measure heightened insulin SG biogenesis in a model of beta-cell dysfunction. It is important to note that the db/db mouse undergoes a significant period of beta-cell compensation prior to beta-cell failure, and it is possible that the elevated younger SG population observed in our db/db islets are a result of this compensatory response in the remaining beta-cell. This is further evidenced by the fact that the C57BL6/J islet cells also display this significantly increased population. Accordingly, this compensatory behaviour is reflected as high insulin turnover, which has previously been observed in ex vivo rat islets under hyperglycaemic conditions of 16.7 mM glucose, which demonstrated increased insulin secretion coupled with significantly higher biosynthesis [16].

In the db/db mice, we also observed a smaller, but still evident, increase in the mature SG population. Similarly, we are also able to elicit an almost identical in vitro chronic hyperglycaemic response from cultured wildtype islets – an increase in an aged granule population at 16.7 mM glucose. This mature SG persistence is likely a result of decreased intracellular insulin degradation when insulin secretion is high [21, 44], and suggests that this compensatory mechanism is driven primarily by the hyperglycaemic environment, as it appears conserved in both ex vivo models. Hypoglycaemia also selectively reduced the young insulin SG population in our FAOS model, but did not affect the older SG population. This is consistent with the observation that SG content is downregulated in chronic starvation in vivo [45], while SG degradation pathways remain unaffected [16]. Altogether these data reinforce the dogma that beta-cells exhibit functional adaptive flexibility to maintain homeostasis with environments of metabolic stress [45, 46], and suggests that insulin SG population age correlates with this flexibility.

We presently show that syncollin-dsRedE5TIMER is a valuable tool for this field,
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capable of distinguishing subtle granule dynamics within not just beta-cell culture, but also the primary beta-cell under both genetic and environmental perturbations. The application of FAOS with a fluorescent timer construct allows quantitative assessment of SGs population age dynamics, as well as the potential isolation of these populations for downstream transcript, proteomic or lipidomics analyses. We note the temporal resolution of our model, which identifies older SGs as between 24 and 72 h old. At 24 h, insulin SGs are arguably still relatively young, but by 72 h are appreciably older than those previously investigated [15]. Use of other fluorescent timers with different temporal qualities in this way will allow further interrogation of the properties behind insulin SG age – and the metaphorical “switch” between youth and old age. There is evidence that younger SG motility mechanistically drives preferential secretion [14], and it could be speculated that the mobilisation of these younger SGs may be driven by differential responses to intracellular calcium [39], but the reasons for granule selection bias remains unknown. Uncovering the necessity for this behaviour within beta-cells – how and why a beta-cell determines insulin granule priority – may be key to developing therapies to rescue conditions of degranulation or beta-cell dysfunction.

Experimental procedures

Cell culture

MIN6 cells were purchased from AddexBio (sourced from New Zealand), with passages less than 30 used in this study. GRINCH cells [36] were a gift from Professor Peter Arvan, University of Michigan. MIN6, INS1 and GRINCH were grown in DMEM and RPMI medium respectively, supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 20mM HEPES, pH 7.4, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were incubated at 37 ºC, in humidified 95 % air and 5 % CO2 and passaged every 3 – 4 days using TrypLE Express for 3 min at 37 ºC. All cells were tested mycoplasma negative, except GRINCH cells used in preliminary data. All cell culture reagents were from Life Technologies.

Adenovirus syncollin-dsRedE5TIMER infection

Syncollin-dsRedE5TIMER was commercially amplified and characterised by ViraQuest, Inc. (Iowa, USA). Cells or dispersed islets were infected with 50 - 100 MOI of Ad-syncollin-dsRedE5TIMER respectively in RPMI supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. After 24 h of transduction, cells were washed thrice and then cultured in RPMI containing 10 % fetal bovine serum, 100U/mL penicillin and 100 µg/mL streptomycin (Islet Media). Fluorescence of granular structures at 490 nm excitation were typically visible from 12 h post-transduction.

Mice

Male C57BL/6J mice of 10 – 12 weeks were purchased from Australian BioResearch (Moss Vale, NSW, Australia). Mice were housed on a 12 h light/dark cycle in standard home cages in groups of up to 6 mice, with food and water given ad libitum. All procedures performed were in compliance with the National Health and Medical Research Council guidelines for animal research and approved by the University of Sydney Animal Ethics Committees. BKS.Cg-Dock7m+/+Lepr<sup>db/db</sup> mice were obtained from Garvan Institute breeding colonies (Australian BioResources, Moss Vale, NSW, Australia). Three litters of mice were used containing 7 (2 controls, 5 mutant), 6 (2 controls, 4 mutant) and 8 (5 controls, 2 mutant) mice respectively. Littermates were used in each experiment as controls. Mouse procedures were approved by the Garvan Institute/St. Vincent’s Hospital Animal Experimentation Ethics Committee.
Human islets

Human islets were sourced from both Faculty of Medicine and Health, The University of Sydney, Sydney, NSW, Australia; National Pancreas and Islet Transplant Unit (NPITU), Westmead Hospital, Sydney, NSW, Australia and Islet Transplant Unit, St Vincent’s Hospital, Melbourne, Australia.

Approval for research use of human islets was obtained from the University of Sydney Human Research Ethics Committees [HREC 2017-042]. All studies involving human samples abide by the Declaration of Helsinki principles.

Human pancreata were obtained, with informed consent from next of kin, from heart-beating, brain-dead donors. Human islets were purified by intraductal perfusion and digestion of the pancreas with collagenase followed by purification using Ficoll density gradients [47]. Purified islets were cultured in Connaught Medical Research Laboratories (CMRL) 1066 medium (Invitrogen) supplemented with 4% human serum albumin, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine (complete CMRL), in a 37 °C, 5 % CO₂ humidified incubator.

Mouse islets

Islets were isolated as described previously [30]. Briefly, a 2 mL volume of 0.25 mg/mL Liberase (Roche) in HBSS (Life Technologies) with 20 mM HEPES was injected into the common bile duct. The inflated pancreas was extracted, incubated at 37 °C for 13 min, and washed twice before passing through a 1000 mm mesh filter. The resulting tissue was centrifuged with no break at 1 k x g in a Histopaque 1119 and 1077 (Life Technologies) gradient for 20 min, before islets were hand-picked in Hank’s Buffered Saline Solution (Life Technologies).

For infection of C57Bl/6K and db/db mouse islets after isolation, islets were recovered in Islet Media for 1 h, before infection with Syncollin-dsRedE5TIMER for 24 h as stated above, then washed thrice and incubated in Islet Media for another 48 h prior to analysis.

Fluorescence-assisted organelle sorting (FAOS)

Fluorescence-assisted organelle sorting protocols were adapted from previous studies [32, 33] for beta-cells. Islets were first dispersed by incubating in 100 uL of TrypLE Express (Thermo Fisher Scientific, Massachusetts, USA) for 3 min at 37 °C, pipetting up and down, before resuspension in Islet Media for plating onto tissue culture dishes. Dispersed islets were infected with Syncollin-dsRedE5TIMER as stated above and incubated for indicated times. Cells were then scraped into 0.5 mL PBS containing 0.3 M sucrose, 10 mM HEPES and 0.25 mM EDTA (HEPES-EGTA buffer), then passed 15 times each through a 21 G and 25 G gauge needle. The resulting homogenate was centrifuged at 1,000 x g for 5 min to remove intact cells, and the supernatant diluted into 1.5 mL PBS for flow cytometry sorting on an Influx 10 laser cell sorter (BD Biosciences, New Jersey, USA) with a 70 µm filter, using 488 nm and 562 nm lasers. Calibration size reference beads (BD Biosciences) of 100 nm and 500 nm were used to determine size gate for insulin secretory granules, and 1 x 10⁵ events within this gate was collected per sample, with percentage of fluorescent particles normalised to total subcellular events from single particle gated populations. Flow data was analyzed using FlowJo 10 software (BD Biosciences).

Glucose-stimulated insulin secretion (GSIS) assay

Syncollin-dsRedE5TIMER - transduced cells were incubated for 1 h in basal conditions of Krebs-Ringer bicarbonate HEPES (KRBBH buffer; 20 mM HEPES, pH 7.4; 119 mM NaCl; 4.75 mM KCl; 2.54 mM CaCl₂; 1.2 mM MgSO₄; 1.18 mM KH₂PO₄; 5 mM NaHCO₃) containing...
2.8 mM glucose, before stimulation for 10 min in KBRH containing either 2.8 mM (low) glucose or 16.7 mM (high) glucose. Supernate was removed, cells washed once in PBS before homogenisation and analysis with FAOS. For measurements of secreted protein, GSIS was performed with 1 h basal as above, with 2.8 mM or 16.7 mM glucose stimulation for 2 h prior to the collection of supernate from cells. Supernate was measured for insulin secretion by HTRF assay (Cisbio) or centrifuged at 1,000 x g to remove cellular debris, then precipitated with trichloroacetic acid (6% final concentration) in the presence of 0.02% sodium deoxycholate. Precipitate was washed in acetone, then resuspended into reducing Laemmli buffer for SDS-PAGE.

**Glucose/Glyburide/Forskolin/IBMX experiment**

INS-1 cells were grown on coverslips and transduced with Syncollin-dsRedE5TIMER for 48 h in 5.6 mM glucose. At the time of infection cells were approximately 30% confluent. At the end of 48 h, cells were incubated for 1 h in basal conditions of KRBH containing 2.8 mM glucose, before stimulation for 10 min in KBRH containing either 2.8 mM (low) glucose or hyperstimulation cocktail (cocktail) of 16.7 mM glucose + 5 µM glyburide + 10 µM forskolin + 1mM 3-isobutyl-1-methylxanthine (IBMX). Cells were fixed in 4% paraformaldehyde for 10 min and then mounted with Prolong Diamond containing DAPI and visualized by confocal microscopy.

**Taxol and Brefeldin-A experiments**

INS-1 cells were grown on coverslips and transduced with Syncollin-dsRedE5TIMER for 48 h in 11 mM glucose. At the time of infection cells were approximately 30% confluent. At the end of 48 h, cells were then incubated in KRBH containing 11 mM glucose plus 5 µM Taxol (Paclitaxel – Sigma) or 10 µg/ml Brefeldin-A (Sigma) for 3 h. Cells were fixed in 4% paraformaldehyde for 15 min and then mounted with Prolong Diamond containing DAPI and visualized by confocal microscopy.

**Subcellular fractionation and differential centrifugation**

A continuous sucrose gradient was made from 2 M, 1.85 M, 1.6 M, 1.45 M, 1.2 M, 1.05 M, 0.9 M, 0.75 M, 0.6 M and 0.45 M sucrose in HEPES-EGTA buffer (10 mM HEPES, 0.25 mM EGTA) and left to equilibrate at 4°C overnight. Syncollin-dsRedE5TIMER-transduced cells were lysed via needle pulse (10 x 19G needle, 10 x 25G needle) in 0.3 M sucrose, 10mM HEPES, 0.25mM EGTA. The resulting homogenate was centrifuged for 5 min at 1,000 x g, the supernate loaded onto the top of the continuous sucrose gradient, and centrifuged for 50,000 x g for 18 h at 4°C. 13 fractions were collected, and diluted 1:4 into reducing 4X Laemmli buffer for SDS-PAGE.

**Immunofluorescent staining**

MIN6 cells were seeded onto 1.5 mm coverslips within 6-well plates 24 h prior to syncollin-dsRedE5TIMER transduction. Cells were fixed in 4% paraformaldehyde and washed twice with PBS before antigen-retrieval was performed with 0.1 % SDS for 5 min at room temperature. After washing twice with PBS containing 0.1 % BSA and 0.01 % Triton-X, cells were blocked for 1 h at RT with Protein Block (Agilent DAKO, Santa Clara, USA), then incubated in a humidified chamber overnight with guinea pig anti-insulin antibody at 1:1000 dilution (DAKO). The following day, cells were washed twice with PBS containing 0.1 % BSA and 0.01 % Triton-X, incubated for 1 h at RT with anti-guinea pig secondary, washed 3 times, and mounted onto microscope slides with Prolong Diamond containing DAPI.

**Microscopy**
Fixed slides and live cell dishes were both imaged using a Leica LCS SP8 confocal microscope (Wetzlar, Germany), using a white light laser and 100 X magnification oil lens. Images were collected using Leica LAS X software and analysed using FIJI Image J software [48].

Fixed slides were imaged for TIRF microscopy using a Nikon H-TIRF module with an argon laser (488 nm, 561 nm) and 60 X magnification oil lens. Illumination angle was set to ~110 nm. Images were collected using NIS Elements software.

**Statistical analysis**

Data analyses were performed using GraphPad Prism 7 and 8 software. Statistical significance was set at p < 0.05. p values were calculated using student’s t-test, Tukey’s multiple comparisons post-test in one-way ANOVA, or Sidak’s multiple comparison post-test in two-way ANOVA, where indicated. Data are expressed as mean ± S.E.M.
Data Availability Statement
All data is contained within the manuscript or available upon request of the corresponding author M.A.K.

Acknowledgments
We thank Professor David E James and his research group, University of Sydney for insightful discussions. We thank all organ donors and their families for their generosity and for enabling this work. Thanks to the staff of St Vincent’s Institute, Melbourne and Westmead Hospital, Sydney involved in the islet isolation program and Donatelife for obtaining research consent and providing the human pancreata. Human tissue retrieval was supported by the Operational Infrastructure Support Scheme of the Government of Victoria. The authors acknowledge the facilities, and the scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at Sydney Microscopy and Microanalysis, University of Sydney, and Sydney Cytometry Facility, University of Sydney and Centenary Institute. The work was supported by the National Health and Medical Research Council (NHMRC) of Australia grant ID GNT1139828. M. A. K is supported by a Jennie Mackenzie Philanthropic Fellowship, University of Sydney.

Competing interests
The authors declare no competing financial or non-financial interests.

Author contribution
M.A.K conceived and designed the study. B.Y performed the majority of the experiments. C.J.R and L.H made the construct, performed experiments in Figure 1I, 1J and 4G. H.E.T, W.J.H and L.W prepared Human Islets. P.T, D.R.L and C. L provided or isolated islets from the db/db mice. All authors performed data analysis and statistical analysis for the respective experiments they performed. B.Y and M.A.K wrote the paper with help from all authors. All authors reviewed and edited the manuscript and approved the final version of the manuscript.
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Figure 1. Characterisation of syncollin-dsRedE5TIMER behaviour in MIN6 beta-cells and primary mouse islets. (A) Lambda excitation peak spectra obtained from a 10 nm step acquisition of primary mouse islet cells, 48 h post-transduction with syncollin-dsRedE5TIMER. Green and red coloured bars represent emission detection ranges for young and old granule populations respectively. (B) Confocal immunofluorescence imaging and (C) co-localisation analysis of young and old granule populations, and anti-insulin stained granules, in mouse MIN6 cells, at 24, 48 and 72 h post-transduction with syncollin-dsRedE5TIMER. (D) Representative western blot and (E) representative densitometry analysis of syncollin, insulin granule-associated proteins, and beta-actin expression after subcellular sucrose fractionation of MIN6 cells, 72 h post-transduction with syncollin-dsRedE5TIMER. (F) Western blot of syncollin and prohormone convertase PC2 in MIN6 cells, after 1 h 2.8mM glucose basal and 1 h 2.8mM or 16.7mM glucose stimulation assay. Media was precipitated with trifluoroacetic acid protein precipitation. (G) HTRF assay of secreted insulin from dispersed primary mouse islets normalised to DNA content after glucose-stimulated secretion assay, after 48 h culture with or without transduction of syncollin-dsRedE5TIMER. (H) Confocal fluorescence imaging of primary mouse and human islet cells after 18, 24, 48 and 72 h post-transduction with syncollin-dsRedE5TIMER.
Figure 2. Syncollin-dsRedE5TIMER behaviour with Brefeldin A and Taxol treatment in INS1 beta-cells. Confocal fluorescence imaging of INS1 cells 48 h post-transduction with syncollin-dsRedE5TIMER, after treatment with 10 µg/mL Brefeldin-A (A) or 5 µM Taxol (B) for 3 h at 5.6 mM glucose. Quantification of green and red fluorescence intensity ratio in Brefeldin-A (C) or Taxol (D) treatments normalised to DMSO control, calculated from average of 20 – 30 cells per experiment in 3 separate experiments.
Figure 3. Flow cytometry-assisted organelle sorting of young and old insulin granules in primary mouse and human islets. (A) Representative gating strategy for syncollin-dsRedE5TIMER-positive insulin granules. First panel and second; BD Biosciences size beads for sub-micron particle size reference, gated for particles between 100 nm and 500 nm and applied to processed islet cell lysate. Third and fourth panel; trigger pulse width and side scatter width gating to observe single particles of similar scatter properties. (B) FAOS gating strategy applied to dispersed mouse islets and (C) human islets, then gated for 488 nm and 562 nm fluorescence at 24, 48 and 72 h post-transduction with syncollin-dsRedE5TIMER. Arbitrary “Young” and “Old” gates defined with first presence of fluorescent particles at 24 h, or with total fluorescent population divided along diagonal. All gates are consistent throughout samples for each FAOS experiment. (D) Number of fluorescent particles in defined young and old gates expressed as a percentage of total gated subcellular particles in dispersed mouse islets at 24, 48 and 72 h post-transduction with syncollin-dsRedE5TIMER. (E) Ratio of the percentage of old subcellular particles over percentage of young subcellular particles in dispersed mouse and human islets at 24, 48 and 72 h post-transduction with syncollin-dsRedE5TIMER.
Figure 4. Flow cytometry-assisted organelle sorting analysis of glucose-stimulated insulin secretion in primary mouse and human islets. (A) FAOS analysis applied to dispersed mouse islets, 72 h post-transduction with syncollin-dsRedE5TIMER. Islet cells were pre-treated at 2.8 mM glucose for 1 h, then treated for 10 min with 2.8 mM glucose (LG) or 16.7 mM glucose (HG). (B) Percentage of total young and old subcellular particles expressed as fold change over 2.8 mM glucose (basal) after HG stimulation of dispersed mouse islets, 72 h post-transduction with syncollin-dsRedE5TIMER. (C) Ratio of the percentage of old subcellular particles over young subcellular particles in LG and HG conditions after GSIS assay of dispersed mouse islets and (D) human islets, 72 h post-transduction with syncollin-dsRedE5TIMER. *p < 0.05, one-way ANOVA with Tukey’s multiple comparisons post-test. (E) Live cell confocal imaging and (F) fluorescence intensity analysis of fluorescing particles in MIN6 cells after 10 min GSIS assay, 72 h post-transduction with syncollin-dsRedE5TIMER. (G) Live cell confocal imaging of syncollin-dsRedE5TIMER-transduced INS1 cells, pre-treated at 5.6 mM glucose for 1 h, then stimulated with HG and 5µM glyburide, 10µM forskolin and 1mM IBMX at 10 and 60 min. *p = 0.02, paired t-test.
Figure 5. Young and old granule populations are modulated by the glucose environment in INS1 beta-cells and primary mouse islets. (A) Young and old fluorescing particles defined as a percentage of total subcellular events were obtained from dispersed mouse islets. Islet cells were transduced with syncollin-dsRedE5TIMER for 24 h in 11 mM glucose RPMI, then placed in 2.8, 8.3 and 16.7 mM glucose RPMI for 48 h prior to processing for FAOS analysis. (B) Representative FAOS gating for young and old particles of dispersed islet cells at 2.8, 8.3 and 16.7 mM glucose conditions. (C) Ratio of the percentage of total old subcellular particles over the percentage of total young subcellular particles at 2.8, 8.3 and 16.7 mM glucose conditions. ***p < 0.001, ****p < 0.0001 compared to 8.3 mM glucose condition, two-way ANOVA with Sidak’s multiple comparisons tests. (D) Blood glucose measurements in littermate control (Ctrl) and db/db mice after 5 h fast. Pooled data from two litters. ***p < 0.001, unpaired student’s t-test. (E) Blood glucose measurements during an intraperitoneal glucose tolerance test in one litter of db/db and littermate controls after 5 h fast. (F) Representative FAOS gating for dispersed littermate ctrl and db/db mice, 72 h post-transduction with syncollin-dsRedE5TIMER. (G) Percentage of total young and old subcellular particles and (H) Ratio of percentage of total old over percentage of total young subcellular particles in littermate control and db/db mice, 72 h post-transduction with syncollin-dsRedE5TIMER. Control and db/db mouse islets were recovered and dispersed into single islet cells in supplemented RPMI culture (11 mM glucose, “Islet Media”) conditions, with no glucose stimulation prior to FAOS analysis. *p < 0.05, two-way ANOVA with Sidak’s multiple comparisons test, **p < 0.01, unpaired t-test.
