Microtubules regulate pancreatic β cell heterogeneity via spatiotemporal control of insulin secretion hot spots

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Abbreviations:
- Glucose-stimulated insulin secretion (GSIS)
- Microtubule (MT)
- Nocodazole (Noc)
- Generalized Linear Model (GLiM)
- Extracellular matrix (ECM)
- Total Internal Reflection Fluorescence (TIRF)
- Kolmogorov–Smirnov (KS)

Abstract
Heterogeneity of glucose-stimulated insulin secretion (GSIS) in pancreatic islets is physiologically important but poorly understood. Here, we utilize mouse islets to determine how microtubules affect secretion toward the vascular extracellular matrix at single cell and subcellular levels. Our data indicate that microtubule stability in the β-cell population is heterogenous, and that GSIS is suppressed in cells with highly stable microtubules. Consistently, microtubule hyper-stabilization prevents, and microtubule depolymerization promotes capacity of single β-cell for GSIS. Analysis of spatiotemporal patterns of secretion
events shows that microtubule depolymerization activates otherwise dormant β-cells via
initiation of secretion clusters (hot spots). Microtubule depolymerization also enhances secretion
from individual cells, introducing both additional clusters and scattered events. Interestingly,
without microtubules, the timing of clustered secretion is dysregulated, extending the first phase
of GSIS and causing oversecretion. In contrast, glucose-induced Ca\(^{2+}\) influx was not affected by
microtubule depolymerization yet required for secretion under these conditions, indicating that
microtubule-dependent regulation of secretion hot spots acts in parallel with Ca\(^{2+}\) signaling. Our
findings uncover a novel microtubule function in tuning insulin secretion hot spots, which leads
to accurately measured and timed response to glucose stimuli and promotes functional β-cell
heterogeneity.

Introduction

Insulin secretion in pancreatic β-cells is tightly regulated and highly heterogeneous. The major
stimulator for secretion is glucose, with other nutritional and neuronal signals modulating the
response. The prevailing view is that glucose influx results in increased glucose metabolism,
higher levels of the cytoplasmic ATP/ADP ratio, and increased levels of metabolite
intermediates. The increased ATP/ADP ratio results in the closure of K\(_{ATP}\) channels, β-cell
depolarization, and Ca\(^{2+}\) influx that triggers vesicular-cell membrane fusion. Metabolite
intermediates promote secretion via known (e.g. microtubule-dependent vesicular biogenesis,
movement, and docking) and unknown mechanisms. Intriguingly, β-cells secrete on the order of
tens of granules per cell in response to each round of high-glucose stimulus, which last for
hours, despite having thousands of available granules (Dean 1973, Olofsson, Gopel et al. 2002,
Rorsman and Renstrom 2003). Glucose-stimulated insulin secretion (GSIS) has a characteristic
bi-phasic kinetics with a rapid, high peak within minutes after the stimulation (first phase)
followed by a sharp decrease and a slow, lower secreting second phase. The mechanisms
underlying this timing are believed to be associated with the availability of secretion-ready
insulin vesicle, the pool size of which does not depend on changes in Ca\(^{2+}\) levels (Gaisano
2017).

An intriguing property of β-cells is their heterogeneity in morphology, biochemical features, and
function ([Miranda, Macias-Velasco et al. 2021] and references therein). Accordingly, β-cells
were found to display different metabolic properties (Vandewinkel and Pipeleers 1983, Kieken,
Tveld et al. 1992, Vanschraendijk, Kieken et al. 1992, Giordano, Cirulli et al. 1993), Ca\(^{2+}\) influx
kinetics (Zhang, Goforth et al. 2003), proliferation rate (Bader, Migliorini et al. 2016), stress
2018, Lei, Kellard et al. 2018), expression of some key function- (Jetton and Magnuson 1992) or
marker-genes (Dorrell, Schug et al. 2016), and insulin secretion (Giordano, Bosco et al. 1991,
properties were considered important, because different β-cell subsets could contribute to
different physiological needs (Hoang Do and Thorn 2015).

Several mechanisms have been proposed to explain β-cell heterogeneity. For example, it has
been proposed that β-cell heterogeneity can result from differences in age, disease state, and
location within the islet (Dean and Matthews 1970, Stefan, Meda et al. 1987, Ballian and
Pipeleers, De Mesmaeker et al. 2017). Specifically, β-cell age/disease states can affect gene
expression, vesicle maturity, and glucose metabolic flux (Blum, Hrvatin et al. 2012, Aguayo-
Specific locations in islets can result in differential crosstalk with other islet cell subtypes (Efendic and Luft 1975, Pipeleers, in’t Veld et al. 1982, Wojtusciszyn, Armanet et al. 2008, van der Meulen, Donaldson et al. 2015) and to vasculature (Ballian and Brunicardi 2007, Low, Zavortink et al. 2014). However, heterogeneous GSIS was maintained in dissociated single cells (ref of Wojtusciszyn et al., 2008) even in adults when most β-cells are mature (Li, Chen et al. 2011, Hoang Do and Thorn 2015, Dwulet, Briggs et al. 2021). In this regard, approximately 25% adult β-cells remain unresponsive regardless of the glucose concentration (Hoang Do and Thorn 2015). More importantly, islet β-cells showed the near uniform glucose- or depolarization-induced Ca\(^{2+}\) influx, despite of the lack of uniform insulin secretion (Li, Chen et al. 2011, Dwulet, Briggs et al. 2021). These findings suggest that the currently proposed mechanisms cannot fully explain β-cell heterogeneity and are consistent with a possibility that different β-cells may have different numbers of Ca\(^{2+}\)-responsive insulin granules, contributing to their unique GSIS responses.

In addition to secretory heterogeneity in the β-cell population, uneven distribution of secretion was also observed at subcellular levels. Designation of insulin secretion to specialized loci at the cell membrane was shown to be essential for the pathophysiology of type 2 diabetes (Fu, Githaka et al. 2019). The sites of preferential insulin secretion depend on the cellular location of L-type voltage-dependent Ca\(^{2+}\) channels (VDCCs) in combination with molecular tethers and other exocytotic proteins (Bokvist, Eliasson et al. 1995, Ohara-Imaizumi, Aoyagi et al. 2019). This molecular machinery resembles the composition of “active zones” or areas of high exocytosis in neurons (Garner, Kindler et al. 2000) and is thought to underlie the hot spots of secretion at the plasma membrane (Landis, Hall et al. 1988). Interestingly, some major components of the hot spot machinery (e.g. ELKS) are assembled at the membrane in response to integrin activation by vascular extracellular matrix (ECM) proteins such as laminin (Hotta, Kawakatsu et al. 2010, Nishimune 2012), and in islets they are preferentially found at sites of β-cell contact with the vasculature (Ohara-Imaizumi, Ohtsuka et al. 2005, Low, Zavortink et al. 2014). However, as most β-cells in an in-situ islet have discrete points of contact with the capillaries (Low, Zavortink et al. 2014), it is clear that positioning and ECM-dependent activation of secretion hot spots is insufficient for the differences in secretion activity of individual cells. Thus, despite a significant progress toward the understanding of β-cell heterogeneity, some key cellular mechanisms underlying their functional differences in islets are still unknown.

Previously, we have shown that the secretion capacity of β-cells is regulated by the microtubule (MT) cytoskeleton, which is uniquely structured to help tune β-cell function (Zhu, Hu et al. 2015). Unlike in many other cell types, MTs in β-cells do not radiate from a central point in the cell, and instead form a dense mesh-like network (Zhu, Hu et al. 2015, Trogden, Zhu et al. 2019, Bracey, Ho et al. 2020). This network configuration is, to a large extent, due to the fact that most MTs in β-cells originate at the Golgi apparatus (Zhu, Hu et al. 2015, Trogden, Zhu et al. 2019). The large surface area of the Golgi acts as a MT organizing center (MTOC), leading to an unusual non-radial MT network appearance. Intriguingly, optimal β-cell function depends on a finely tuned balance of MT assembly and disassembly, which can both promote and restrict secretion capacity of a cell. On the one hand, Golgi-derived MTs function aids the budding of new insulin granules from the Golgi (Trogden, Zhu et al. 2019). High glucose stimuli lead to an increase in Golgi-derived MT nucleation, which is necessary to replenish insulin granule content after a secretion pulse (Zhu, Hu et al. 2015, Trogden, Zhu et al. 2019). Long-term loss of this MT subpopulation causes degranulation of the β-cell as vesicle budding is less efficient (Trogden,
Thus, glucose-dependent MT nucleation is a critical factor supporting the
capacity of β-cells to secrete. On the other hand, MTs act to prevent over-secretion in functional
β-cells, which contain excessive amounts of insulin granules (Zhu, Hu et al. 2015). This function
relies on the configuration of MT networks in β-cells, where the non-radial mesh in the cell
interior prevents directional granule movement, and the extremely stable MT bundles extending
along the plasma membrane serve as tracks for granule withdrawal from docking sites (Bracey,
Ho et al. 2020). Upon glucose stimulation, these pre-existing MTs are destabilized and partially
depolymerized (Ho, Yang et al. 2020), allowing for release of a subset of granules. This
regulated dynamicity of the MT network is vital for the dosage of GSIS at each stimulus: loss of
all MTs acutely leads to over-secretion, while hyper-stabilization of MTs greatly suppresses it
These effects are only seen after GSIS stimulation but not at basal glucose conditions,
indicating the leading role for other mechanisms in secretion triggering (Zhu, Hu et al. 2015,
Trogden, Zhu et al. 2019). In functional β-cells, which are able to secrete in response to a
glucose stimulus, the effects of MT destabilization are finely tuned because simultaneous
increase in MT nucleation promptly replaces depolymerizing MTs (Zhu, Hu et al. 2015). The
intriguing combination of positive (long-term) and negative (short-term) MT regulation of
secretion may be responsible for the early controversy on the role of MTs in insulin secretion
(Lacy, Walker et al. 1968, Lacy, Walker et al. 1972, Malaise, Van Obbergen et al. 1974,
Howell, Hii et al. 1982, Hill and Rhoden 1983, Mourad, Nenquin et al. 2011). It also makes the
MT cytoskeleton a candidate for differential control of secretory activity, which acts in concert
with other regulatory mechanisms to underlie β-cell functional heterogeneity.

In this paper we investigate the role of the MT cytoskeleton in β-cells as a factor in the
differential secretory response of β-cells to glucose stimulation. Our computational analysis of
insulin secretion at the single-cell level toward the vascular ECM in whole islets shows that this
regulation occurs via secretion hot spot activity. Our findings indicate that the presence of stable
MTs attenuates initiation of secretion hot spots in both otherwise dormant and already active β-
cells, thus contributing to functional heterogeneity of insulin secretion. We also show that MTs
regulate the timing of insulin secretion by restricting hot spot activity to the first phase of GSIS.

Results

Microtubule stability in pancreatic islet β-cells is heterogeneous

We have previously found that MTs serve as a critical regulator of both phases of β-cell glucose
response and insulin secretion (Zhu, Hu et al. 2015, Trogden, Zhu et al. 2019). To test whether
MT regulation plays a role in the functional heterogeneity of β-cell population, we analyzed MT
stability in mildly disseminated mouse islet β-cells plated on vascular ECM.

First, MT stability was evaluated utilizing immunostaining for Glu-tubulin (also known as
detyrosinated tubulin), a marker of long-lived or stable MTs (Gundersen, Khawaja et al. 1987,
Weiland and Weber 1987) (Figure 1 A-F, β-cells outlined in yellow). β-cells were identified by
expression of nuclear-localized mApple marker (see materials and methods). Consistent with
our previous findings, Glu-tubulin content was high in β-cells at basal glucose conditions, but
decreased following a high glucose stimulus (Zhu, Hu et al. 2015, Ho, Yang et al. 2020) (Figure
1G, H). There was no detectable difference in overall polymerized tubulin content upon glucose
stimulus (Figure 1- figure supplement 1A), likely due to glucose-stimulated nucleation of new
MTs (Trogden, Zhu et al. 2019), which replace destabilized MTs (Zhu, Hu et al. 2015, Trogden,
Zhu et al. 2019). Accordingly, the ratio of Glu-tubulin to tubulin per cell, which reflects the
proportion of stable MTs within the MT network, decreased after high glucose stimulation (Figure 1J, K).

Interestingly, the density of the MT cytoskeleton is heterogeneous within the β-cell population. While not changed by glucose stimulation, the overall tubulin polymer content was variable in the β-cell population as indicated by the intensity distributions (Figure 1- figure supplement 1B and C) and coefficients of variation (58.72% in low glucose and 60.42% in high glucose). MT stability was also highly variable, as evident from the Glu-tubulin staining, which ranged from barely detectable levels to highlighting essentially the whole MT cytoskeleton (Figure 1A, D, compare cells with red arrows). While the amount of Glu-tubulin decreased after glucose stimulation, shifting the histogram of Glu-tubulin intensities per cell to the left (Figure 1H), the degree of variation amongst the β cells was retained as indicated by the distributions of Glu-tubulin intensity normalized to the mean for each condition (Figure 1I), and by high coefficients of variation (48.56% in low glucose and 63.46% in high glucose). The proportion of stable MTs within the MT network (Glu-tubulin to tubulin) also remained variable in high glucose (Figure 1 K, L; coefficient of variation: 78.15% in low glucose and 64.39% in high glucose).

As a second measure of MT stability in β-cells, we subjected the cells to ice treatment for 30 minutes. Since MTs are temperature-sensitive and only stable MTs will remain after ice treatment, this well-established assay is used to directly assess MT stability. After extraction of free tubulin, fixation and immunostaining, the tubulin content per cell was measured to evaluate the amount of cold-resistant MTs remaining (Figure 1M-P). In basal glucose conditions, many cells contained MTs stable enough to be retained after ice treatment (Figure 1M, N). Following high glucose stimulation, the amount of cold-stable MTs significantly decreased (Figure 1Q, R), consistent with our previous findings and data described above (Zhu, Hu et al. 2015). Most of the remaining MTs were positive for Glu-tubulin, as expected for long-lived stable MTs (Figure 1- figure supplement 1D-K). Importantly, the variation in cold-resistant MTs within the β-cell population was high both in low and high glucose (Figure 1S), confirming that MT stability is highly variable in β-cell population.

As a third test of MT stability, we utilized a method where live cells were permeabilized prior to fixation to release all free tubulin, which acutely decreases tubulin concentration in the cell (Khawaja, Gundersen et al. 1988). This prevents tubulin polymerization so that dynamic MTs are lost through depolymerization without being replaced, leaving only stable MTs in a cell (Khawaja, Gundersen et al. 1988). Immunofluorescent detection of MTs after this treatment resulted in a similar variation in MTs patterns retained in cells and Glu-tubulin staining as the cold treatment (Figure 1- figure supplement 1L-N).

These three assays indicated that, similar to insulin secretion, MT stability is heterogeneous between different β-cells. While some cells have highly stable MT networks, neighboring cells can have less stable networks. This phenomenon is even more obvious after high glucose stimulation when MT stability is decreased in the entire population, but remains highly variable.

**MT stability affects insulin secretion heterogeneity**

To assess whether MT regulation affects the functional heterogeneity of β-cells, we next analyzed insulin secretion from distinct cells within the β-cell population. To detect single-cell insulin secretion in real time we use a cell impermeant zinc dye FluoZin-3 that becomes fluorescent upon zinc binding, and at each exocytic event, highlights the release of zinc co-packaged with insulin in granules (Gee, Zhou et al. 2002, Zhu, Hu et al. 2015). Intact islets were
attached to a vascular ECM, a condition that imitates secretion towards vasculature (Bonner-Weir 1988, Low, Zavortink et al. 2014, Gan, Do et al. 2018) and preserves ability of islets to efficiently respond to glucose stimulus (Patterson, Knobel et al. 2000, Zhu, Hu et al. 2015). Total internal reflection fluorescent (TIRF) microscopy was used to analyze secretion from islet β cells at the attached islet side (See materials and methods, Figure 2- figure supplement 1A). The advantage of this model is that it allows for detecting secretion distribution within 2 dimensions of plasma membrane contacting vascular ECM (a model of cell area contacting capillaries). Individual secretion events appear as flashes of bright fluorescence, the intensity of this fluorescence over time resembles a Gaussian curve with only background signal, then an initially tight circle of signal that dissipates after vesicle exocytosis (Figure 2A, A'). For quantification, image sequences were processed for better signal/noise ratio, β-cells were identified by the presence of red fluorescence in the nuclei, and cell outlines were detected by bright-field imaging.

To address heterogeneity of β-cell activity in our experimental setup, we first measured the percentage of β-cells secreting within 10 minutes of stimulation. In control islets treated with high glucose, the number of active cells increased as expected (Figure 2B, E, Figure 2- figure supplement 1B and Figure 2-Video 1 (Low et al., 2013). However, a subset of β-cells remained inactive even after stimulation (Figure 2E, 60% of cells), resembling the fraction of cells with a high content of stable MTs (59% of cells with >300 average intensity of glu-tubulin, Figure 1).

To assess the role of MTs in the distribution of secretion activity in the β-cell population, we pre-incubated islets in the presence of either the MT depolymerizing drug nocodazole, which completely eliminates cellular MTs (Figure 2- figure supplement 1I), or the MT-stabilizing drug taxol (Figure 2- figure supplement 1J), which hyper-stabilizes MT networks in cells, dramatically increasing their Glu-tubulin content (Gundersen, Khawaja et al. 1987, Wehland and Weber 1987, Townley, Zheng et al. 2015). As we have previously observed (Zhu, Hu et al. 2015), nocodazole increased insulin secretion specifically in high glucose-stimulated islets (Figure 2C, E, Figure 2- figure supplement 1C and Figure 2-Video 2). Strikingly, we also observed that a larger sub-population of β-cells (on average, 66% of cells) was activated, compared to only 42% in control islets (Figure 2E, compare Figure 2B and C). Addition of taxol also affected insulin secretion as we have previously observed (Zhu, Hu et al. 2015), blunting it in high glucose (Figure 2D, E, Figure 2- figure supplement 1D, Figure 2-Video 3). The percent of secreting cells in taxol was only 27%, similar to the percentage observed in low glucose (Figure 2D, E, Figure 2- figure supplement 1D), where MTs are intrinsically stable (Figure 1).

These results indicate that the modulation of MT stability can change the insulin secretion activity of β-cells within the population. Since in control islets cells have variable MT stability levels (Figure 1), we explored a potential connection between MT stability in individual cells and their ability to secrete. This connection was assessed by a correlative microscopy approach using whole-mount attached islets (Figure 2F-H) as well as slightly disseminated islets (Figure 2- figure supplement 2). FluoZin-3-detected secretion pattern was correlated with post-assay Glu-tubulin immunostaining. As expected, secretion levels were extremely low in cells with a high content of Glu-tubulin (Glu-tubulin intensity above islet average), indicating that cells with overly stable MTs are not capable of secretion (Figure 2H, Figure 2- figure supplement 2A-C).

Interestingly, in cells with Glu-tubulin content below the islet average, secretion levels very highly variable, indicating that in those cells other, non-MT-dependent, GSIS-regulating mechanisms significantly modulate secretion levels.
We next tested whether MT presence introduces differences between actively secreting β-cells by assessing how modulation of MTs influences the number of secretion events per cell, a criterion critical for GSIS efficiency (Low, Mitchell et al. 2013). We found that high glucose caused an increase in the number of events per cell in both control and nocodazole-treated islets, but not taxol-treated islets (Figure 2I, I', J, J'). Importantly, the number of events per cell and the fraction of highly-secreting cells in high glucose were significantly increased by nocodazole as compared to control, suggesting that MT presence attenuates glucose-stimulated secretion in individual cells (Figure 2I, I', J, J'). Consistent with this result, under all conditions of high MT stability (low glucose in all conditions and high glucose in taxol) secretion activity is distributed similarly throughout the cell population (Figure 2I', J'). Taken together, these data indicate that insulin secretion capacity on both the cellular and population levels is decreased by MT presence, and even more so, by MT stability. Furthermore, in terms of β-cell activation, MTs provide a mechanism that supports β-cell heterogeneity. While normally the glucose-stimulated β-cell population is highly heterogeneous, changing MT stability in either direction leads to a more homogenous population with either most cells secreting (nocodazole, no MTs) or most cells not secreting (taxol, hyper-stabilized MTs). It is also evident that modulation of MT dynamics decreases but does not completely eliminate variability in β-cell activity, which is in an agreement with the existence of other, MT-independent, mechanisms of heterogeneity (reviewed in Gutierrez, Gromada et al. 2017, Pipeleers, De Mesmaeker et al. 2017).

MT stability regulates β-cell activation GSIS by suppressing insulin secretion “hot spots”

To better understand MT-dependent regulation of secretion in individual cells versus a cell population, we analyzed their spatial and temporal secretion patterns. In agreement with the existing evidence that insulin secretion occurs in hot spots, defined by patches of VDCCs and other active zone proteins on the membrane (Ohara-Imaizumi, Ohtsuka et al. 2005, Yuan, Lu et al. 2015, Gandasi, Yin et al. 2017, Ohara-Imaizumi, Aoyagi et al. 2019), we observed a distinct distribution of secretion events to certain preferred areas of a cell (Figure 2B-D, Figure 2-Videos 1-3). Analyzing the nearest neighbor distances between secretion events within cells, we detected a bias towards smaller distances, with over 50% of events in all conditions occurring within 1.5 µm of each other (Figure 3A). We then performed a density-based scanning algorithm to identify clustering, defined as secretion events occurring within 1.5 µm diameter areas (Figure 3B-D, Figure 3- figure supplement 1B-D). To determine the expected frequency of clustered events occurring by chance (i.e. multiple unrelated events occurring close to each other by coincidence), we computationally simulated random secretion events in in silico cells. Results (Figure 3- figure supplement 1A) show that in cells secreting within the observed levels, clusters of two events within 1.5 µm of each other would be relatively prevalent (one or more per cell). In contrast, the likelihood of observing a cluster of three or more event due to random chance is very low. Thus, only cluster sizes of three and more events were included in the data presented below.

Our analysis (Figure 3E, F) revealed that the number of cells with secretion clusters was small under conditions with low secretion, including all low-glucose treatments and in high glucose after taxol pre-incubation. In contrast, the number of cells with clusters increased from an average 2% to 10% of the cell population by high glucose stimulation in control. Interestingly, nocodazole pre-treatment increased the percentage of cells with clusters in high glucose 2.4 fold (Figure 3E). The nocodazole-induced increase in the number of cells secreting in a non-
clustered manner was more modest (1.3 fold). This indicates that in the absence of MTs, a higher proportion of active cells had clustered secretion. Indeed, when only secreting cells were considered, the fraction of cells with clusters increased from on average 18% in control to 33% in nocodazole (Figure 3F). These data suggest that MT depolymerization specifically initiates secretion hot spots to activate dormant β-cells.

Interestingly, our correlative microscopy analyses of Glu-tubulin content in β cells after secretion (as in Figure 2F,G) demonstrated that islet β cells with high Glu-tubulin content (above the islet average) almost never had secretion clusters (Figure 3 G-J), indicating that stable MTs in cells suppress hotspot-associated secretion.

MTs regulate individual cell secretion via both clustered and non-clustered secretion

While our data indicated that the MT-dependent increase in secretion strongly relies on the activation of additional β-cells, we also detected that secretion activity per cell depended on MT presence and stability (Figure 2I, J). Exploring the secretion patterns in individual cells, striking differences between high-glucose stimulated control (Figure 4A, Figure 4-Video 1) and nocodazole-treated (Figure 4B, Figure 4-Video 2) cells are observed. We have found that, in addition to the significant increase in cluster-containing cells, MT depolymerization caused an increase in the number of secretion clusters per cell (Figure 4C, D), which, according to the computational simulation, cannot be accounted for by the increase in secretion per cell (Figure 3- figure supplement 1A). Interestingly, in a vast majority of cells with clustered secretion in control, the number of clusters per cell was restricted to one, while in nocodazole many cells had additional clusters activated (Figure 4E).

In addition, while our data shows that a significant number of exocytic events were specifically concentrated in secretion hot spots, many events were still found randomly scattered across the cell membrane. We tested how the number of non-clustered versus clustered events were changed upon glucose stimulation under conditions of MT depolymerization or stabilization. The number of clustered secretion events per cell increased between low and high glucose, and the loss of MTs resulted in an even higher increase (Figure 4F), reflecting the activation of additional hot spots as indicated above (Figure 4C-E). Interestingly, the non-clustered event number per secreting cell was not significantly increased by high glucose in control, indicating that GSIS is predominantly driven by clustered secretion. At the same time, glucose induced a consistent and significant rise in non-clustered secretion in nocodazole, indicating that without MTs, glucose-stimulated secretion can occur at random, non-hot spot sites (Figure 4F').

Individual events within clusters are independent of each other

To better assess how MT stability influences secretion dynamics within hotspots, we analyzed clustered secretion dynamics in comparison to non-clustered secretion. Our analysis detected a slight, statistically insignificant difference in the distribution of cluster sizes (the number of secretion events within a cluster) between conditions (Figure 5A, A'), suggesting that the level of secretion activity within these hot spots is to a large extent MT-independent.

We next assessed whether clustered and non-clustered secretion events are independent of each other. That is, does one secretion event influence the timing of the next (dependent) or not (independent). To test this, we analyzed the time between events, which if events are independent, should follow an exponential distribution. Non-clustered events are well fit by an exponential distribution (Fig. 5B), suggesting that non-clustered events do not affect each other,
as would be expected. A non-parametric Kolmogorov–Smirnov (KS)-test does indicate that, in
control and nocodazole treated islets stimulated with high glucose, the distribution does
statistically deviate from exponential. However, this is likely due to the clear time dependence of
secretion after glucose stimulation in these conditions (Fig. 6A, further discussion below).

By comparison, the time-between-events distribution for clustered events clearly deviates from
exponential (Fig. 5C). This is particularly obvious under conditions of high glucose stimulation
in control and nocodazole, where a high number of clustered events allows for constructing a
smooth distribution. Specifically, there is an enrichment of short wait times between events that
cannot be captured by the exponential. However, this analysis considers time-between-events
within all clusters combined into a single distribution. In this case, the observed deviation may
be caused by the presence of clusters with different secretion dynamics within analyzed cluster
populations.

To test whether individual clusters exhibit distinct secretion dynamics, we next analyzed event
dynamics separately in different cluster sizes. In control and nocodazole treated islets in high
sucrose (Fig. 5D), the average time between events was lower for larger clusters. Individual
analyses of the waiting time distributions for clusters of each size in each of the conditions (Fig.
5E) indicate that those distributions are well fit by exponential distributions. Thus, for each
condition, we constructed a generalized linear model (GLiM) with an exponential linking function
(since the data are exponentially distributed) where the rate of secretion depends on cluster size
according to \( rate = \alpha + \beta \times Size \). This model was fit to the data for each of the six conditions
separately using a Bayesian parameter estimation approach.

Our goal is to determine 1) whether \( \beta > 0 \) since this would indicate that there is a size
dependence of cluster secretion rates and 2) whether the model parameters differ across
conditions.

Results of these model fits are presented using 95% Bayesian credible intervals (Kruschke and
Liddell 2018) (Fig. 5F). In brief, a 95% credible interval depicts the range of values that the
parameter values will fall into with 95% certainty (note this is technically different than
confidence intervals that are often reported in frequentist statistics). Results show that the
credible interval for \( \beta \) is strictly greater than 0 in control and nocodazole treated islets in high
sucrose. Thus we can say that larger clusters do indeed secrete insulin at higher rates with 95%
certainty (this is the definition of a credible interval, see methods for further technical
discussion). This is confirmed by model comparison where this size-dependent model is
compared to a “null” model where the secretion rate is fixed across all sizes (model comparison
results are reported using the Watanabe–Akaike information criterion – WAIC, which is a
Bayesian companion of the common frequentist AIC). Results are inconclusive in the other four
conditions due to the relative scarcity of clustering data leading to more widely distributed
credible intervals that overlap with \( \beta = 0 \). Comparing model results across conditions shows the
credible intervals for both parameters (\( \alpha \) not shown) overlap across all six conditions. Thus,
there is no discernable difference in the rate of within-cluster secretion, consistent with our
results in Fig. 5A.

In conclusion, both clustered and non-clustered events appear to occur independently of each
other: one secretion event does not influence the timing of the next. Neither cluster size nor
secretion dynamics within clusters differ significantly between any of the examined experimental
conditions. However, larger clusters are secreting insulin at faster rates.
**MT stability affects timing of glucose-stimulated insulin secretion**

As insulin secretion is known to be tightly regulated in time, we next looked to see if MT stability had any effect on the timing of secretion events. Since the data outlined above show that MT destabilization leads to initiation of extra secretion hot spots, we also analyzed the kinetics of secretion for clustered and non-clustered events separately.

Events in low glucose occurred at random through time with no distinguishable pattern, indicating that basal secretion was random (Figure 6A). Control high glucose showed a more noticeable pattern with the first peak at 2-4 minutes and the second peak at 9-10 minutes after high glucose addition (Figure 6A). Such kinetics correspond well with the known timing of the two waves of the bi-phasic insulin secretion (Curry, Bennett et al. 1975). Interestingly, we found that clustered secretion at hot spots had a stronger impact on the first phase of secretion, while non-clustered events contributed stronger to the second phase (Figure 6B, Figure 6- figure supplement 1A). In nocodazole-treated islets, high glucose induced a much broader peak of secretion with the maximum at 4-5 minutes, which extended into the timing of the second phase without a defined minimum (Figure 6A). Interestingly, the distribution of non-clustered events maintained a slightly bi-phasic appearance, while clustered secretion appeared as a single wide peak in the time frame of the experiment (Figure 6B, Figure 4- figure supplement 1A). This indicates that timing of clustered secretion was dysregulated.

To test whether the noted distribution is a manifestation of a change in the timing of triggering and/or silencing of secretion hot spots, we analyzed the distribution of the first and last events within each cluster over time (Figure 6C, D). This analysis showed that in control, clusters were initiated mostly in the first 5 minutes after stimulation (first phase), while in the absence of MTs clusters were initiated throughout most of the imaging sequence, suggesting that MTs serve to restrict cluster initiation to the first phase of GSIS (Figure 6C, D).

Furthermore, in control glucose-stimulated islets, over 50% of clustered secretion ceased close to the end of the first phase, while the rest persisted through the second half of the movie. In the absence of MTs, in contrast, a noticeable proportion of clustered secretion lasted through to the end of the recorded sequence, and/or was still active during the last frame (Figure 6C). This indicates that while no difference in the cluster size between control and nocodazole (in any other conditions) was detected, timely cluster inactivation was disturbed in the absence of MTs, and secretion might continue at the same location for a longer period (not recorded due to the fluorescent dye background buildup).

These data are consistent with the model where MTs regulate the timely response of secretion hotspots to the stimulus, including both their initiation and silencing. Lack of such regulation led to “smearing” of the bi-phasic GSIS response, so that the decrease in secretion after the first phase was not evident.

**Microtubules control insulin secretion in addition to, rather than via, Ca$^{2+}$ signaling**

Since MTs regulate secretion hot spots, which have been previously shown to be defined by Ca$^{2+}$ channel location at the plasma membrane (Bokvist, Eliasson et al. 1995, Satin 2000), we have tested whether overall GSIS under conditions of MT disruption is still dependent on Ca$^{2+}$ influx. GSIS ELISA in intact islets has revealed that blocking Ca$^{2+}$ influx by diazoxide inhibits GSIS both in the presence and absence of nocodazole and eliminates secretion enhancement.
caused my MT disruption (Figure 7A). This indicates that Ca\(^{2+}\)-dependent pathway is essential for secretion stimulation regardless of MT presence/stability.

There is still a possibility that MT regulation may act upstream of Ca\(^{2+}\) influx induction. To address this, we have tested whether pre-treatment of islets with nocodazole influences glucose-dependent Ca\(^{2+}\) influx in individual β cells. We have utilized a fluorescent Ca\(^{2+}\) reporter Calbryte 520 to follow single-cell response to glucose stimulation in attached islets in real time by confocal microscopy (Figure 7 B,C, cyan, Figure 7-Videos 1,2). As above, β-cell identity was determined by the presence of nuclear mApple reporter (Figure 7 B,C, red). No increase in Ca\(^{2+}\) influx levels between control and nocodazole-treated cells was detected, arguing against the hypothesis of MT-dependent regulation of Ca\(^{2+}\) influx (Fig. 7D,E). While the response was expectedly variable between individuale islets (Figure 7 – supplement Figure 1), single cell Ca\(^{2+}\) fluctuations in nocodazole treated cells (Figure 7 – supplement Figure 1B) show no detectable defect in timing and/or synchronon as compared to control, suggesting that both hub cell firing and cell connectivity was not affected by MT disruption to a detectable extent.

Interestingly, while no difference in summarized Ca\(^{2+}\) influx over time has been detected (Fig 7E), our data indicate a slight decrease in the fluctuation amplitude (Fig 7D), suggesting that the absence of MTs might influence functionality of Ca\(^{2+}\) influx machinery in some minor way. Nevertheless, if such a defect exists, it must have a suppressing effect on insulin secretion, rather than underlie the observed GSIS enhancement.

Furthermore, we have explored whether single-cell secretion efficiency and heterogeneity is modulated by MTs under conditions of Ca\(^{2+}\) influx enforced by 25mM KCl (membrane depolarization). We have performed FluoZin-3 assays during KCl-induced insulin secretion. Because KCl causes immediate membrane depolarization, in these assays we had to apply stimulation during TIRF-imaging recording in order to register early secretion events. In the time frame of the assay, disruption of MTs by nocodazole pretreatment facilitate GSIS but did not affect secretion triggered by KCl alone (Figure 8 – Figure supplement 1A). However, synergistic secretion triggered by a combination of glucose and KCl was further enhanced under conditions of MT disruption. This effect was likely due to increased number of secretion hot-spots (clusters, Figure 8 – Figure supplement 1B), while the cluster size (number of events per cluster) remained constant throughout all conditions (Figure 8 – Figure supplement 1C).

At the same time, in the presence of KCl, secretion events within clusters occurred more rapidly (Figure 8 – Figure supplement 1D), and secretion concentrated within the first minutes after stimulation (Figure 8 – Figure supplement 1E), indicating that KCl-induced acute Ca\(^{2+}\) influx acted predominantly in a short time frame. To analyse the role of MTs in secretion caused by KCl-dependent Ca\(^{2+}\) influx, we have analysed FluoZin-3 assay outcomes exclusively within the active period of 2.5 minutes (Figure 8). Strickingly, none of the components of secretion caused by KCl alone was facilitated by MT disruption (Figure 8E-L, Figure 8-Video 1). However, secretion activity was significantly increased by nocodazole when glucose was used as a trigger in combination with KCl (Figure 8E, Figure 8-Videos 2, 3). The number of cells with clusters (Figure 8F,G) as well as the number of clusters per cell (Figure 8I,J) significantly increased upon nocodazole pre-treatment, indicting an increased number of secretion hot-spots. Accordingly, while the cluster size (number of events per cluster) remained constant (Figure 8H), the number of clustered events per cell was boosted by nocodazole (Figure 8K,L). The number of non-clustered events per cell was only slightly affected by nocodazole pre-treatment.
(Figure 8K, L'), in agreement with our above conclusion that MTs predominantly regulate hop-
spot associated secretion.

Together, these data indicate that MT-dependent regulation of secretion hot spots acts in
parallel to Ca\textsuperscript{2+}-dependent mechanisms and involves other pathways downstream of glucose
metabolism.

Discussion

It has been known for over 6 decades that islet β-cells, from both rodent models and human
donor islets, can be divided into different subpopulations with different gene expression,
morphology, and GSIS activities (Miranda, Macias-Velasco et al. 2021). The functional β-cell
heterogeneity, established via hemolytic plaque assays (Salomon and Meda 1986, Bosco,
Meda et al. 1995, Katsuta, Aguayo-Mazzucato et al. 2012) or real-time secreton assays (Li,
Chen et al. 2011, Hoang Do and Thorn 2015) is particularly intriguing because it was proposed
to be important for an adaptable, efficient response to various changes in physiological
conditions and is one of the parameters that dramatically changes in diabetes (Aguayo-
Mazzucato, van Haaren et al. 2017, Gutierrez, Gromada et al. 2017). Mechanism-wise, it has
been proposed that β-cell heterogeneity can result from differences in β-cell age, disease state,
β-cell maturity, and location within the islet or association with other islet cell types (Dean and
1987, Ballian and Brunicardi 2007, Wojtusciszyn, Armanet et al. 2008, van der Meulen,
Donaldson et al. 2015, Aguayo-Mazzucato, van Haaren et al. 2017, Gutierrez, Gromada et al.
2017, Pipeleers, De Mesmaeker et al. 2017) and to vasculature (Ballian and Brunicardi 2007,
Low, Zavortink et al. 2014). Yet these studies do not fully explain why similar levels of Ca\textsuperscript{2+}
influx cannot induce similar insulin secretion from different β cells.

In this study, we have evaluated the role of the MT cytoskeleton in influencing the spatial
distribution and heterogeneity of insulin secretion events both at the level of pancreatic β-cell
populations and sub-plasma membrane regions. Our data indicate that MT stability in the β-cell
population is heterogenous and that this heterogeneity is a contributing factor to the previously
established heterogeneity of insulin response. We also show that the loss of MTs causes
initiation of additional insulin secretion: 1) activation of hot spots in a higher fraction of cells, 2)
increase in the number of hotspots in active cells, and 3) broadens the timing of secretion from
the hot spots in the first phase of GSIS. Stabilizing MTs prevents GSIS so that all parameters
are indistinguishable from basal secretion levels. Importantly, MT-dependent regulation of hot-
spots acts in parallel to glucose-induced Ca\textsuperscript{2+} influx, rather than in the same pathway.

There are a number of advantages to the approach utilized in this paper. It is important to
measure the spatio-temporal distribution of secretion from individual β-cells in its natural
environment in the whole islet with intact cell-to-cell contacts and a proper basement
membrane. High-resolution detection of secretion event distribution over the surface of the
plasma membrane is a challenging task. While secretion detection in whole islets have been
successfully achieved by two-photon microscopy (Takahashi, Kishimoto et al. 2002, Low,
Zavortink et al. 2014), this method does not allow for high time resolution and for registration of
secretion hot spots over the the plasma membrane due to its complex topography. Furthermore,
it is very labor-intensive and the number of examined samples is very limited. In contrast, TIRF
microscopy with β-cells secreting towards the glass coverslip (Nagamatsu and Ohara-Imaizumi 2008, Loder, Tsuboi et al. 2013) allows for rapid imaging in the plasma membrane focal plane.

The major challenge is the rapidly compromised responsiveness to glucose characteristic for whole mount islets plated on glass. To our advantage, it has become clear that ECM signaling through integrin-dependent Ca$^{2+}$ channel activity is critical for preservation of correctly patterned secretion (Gan, Do et al. 2018, Ohara-Imaizumi, Aoyagi et al. 2019). Our approach of culturing whole-mount islets on vascular ECM preserves the functionality of β-cells (Patterson, Knobel et al. 2000, Zhu, Hu et al. 2015), supporting the idea that the signals downstream of integrins are vital to preserving β-cell identity (Gan, Do et al. 2018). TIRF microscopy in this system allows us to register single secretion events associated with their physiological organizer - the vascular ECM. Thus, this experimental model allows for the evaluation of the secretion patterning arranged by vascular cues, and at the same time, analysis of individual β-cell response as they maintain their connections with each other and other islet cell types. An additional advantage of our approach is that utilization of FluoZin-3 dye provides direct information of precise insulin secretion time and location without the need for genetically encoded markers of insulin.

One important conclusion from our study is that MT stability varies in the β-cell population. The mechanistic basis of the differences in MT stability between β-cells is yet unclear. For example, since MTs are sensitive to Ca$^{2+}$ (Hepler 2016), MT stability might be modified by the Ca$^{2+}$ influx wave, which is thought to contribute to spatial and temporal differences in β-cell response to stimulation (Benninger, Hutchens et al. 2014). However, we have recently shown that blocking Ca$^{2+}$ influx by verapamil does not prevent glucose-induced changes in MT dynamics (Ho, Yang et al. 2020). Keeping also in mind the evidence that Ca$^{2+}$ influx is insufficient to provide for secretion heterogeneity (Li, Chen et al. 2011), we suggest that MT differences are likely triggered by another mechanism. MT heterogeneity might be promoted by such factors involved in β-cell variability as cell maturity (Aguayo-Mazzucato, van Haaren et al. 2017, Gutierrez, Gromada et al. 2017, Pipeleers, De Mesmaeker et al. 2017), islet microenvironment (Trimble and Renold 1981, Trimble, Halban et al. 1982, Brereton, Vergari et al. 2015), and/or paracrine signaling from other islet cell types (Efendic and Luft 1975, Pipeleers, in't Veld et al. 1982, Wojtusciszyn, Armanet et al. 2008, van der Meulen, Donaldson et al. 2015). Interestingly, we have shown that glucokinase, an enzyme, which expression/activity is involved in promoting β-cell variability (Jetton and Magnuson 1992, Heinberg, De Vos et al. 1993), is important for MT network remodeling downstream of glucose (Ho, Yang et al. 2020). Thus, glucokinase variability could lead to heterogeneous MT stability in β-cells.

One possible important molecular player in this pathway could be the MT stabilizer tau, which we have recently found to be a critical component of glucose- and glucokinase-dependent MT remodeling, leading to efficient insulin release from β-cells (Ho, Yang et al. 2020). Other MT-stabilizing proteins found in β-cells, such as MAP2 and doublecortin, may also be involved (Krueger, Bhatt et al. 1997, Jiang, Brackeva et al. 2013). It is clear from our data that regardless of the source of MT heterogeneity, it is functioning to enhance the variability of β-cell secretion activity: pushing MTs toward uniform stability or uniform depolymerization makes β-cell secretion response more uniform in opposite directions. This role of MT heterogeneity in the regulation of variable β-cell response to stimulation is not entirely unexpected. The effect of heterogeneity of MT stability in cell populations has been described in other cell types and is implicated in the variability of cell function. For example, motile cells that have directionally stable MT arrays have an increased ability for migration in wound healing assays (Sugioka and Sawa 2012). Heterogeneity of MT dynamics in neurons has been found to underlie the
distinction between the axon and dendrites (Conde and Caceres 2009), neurite capacity for
cargo movement (Franker and Hoogenraad 2013), and modulation of local signaling and
rearrangements in neuronal connectivity (Hoogenraad and Bradke 2009). Now, our data add β-
cells to the list of cell types where the differential MT stability plays an important role in cell
physiology.

How do differential MT dynamics promote differential secretion? The first possibility is the direct
MT regulation of insulin secretion, when differently organized MT networks differentially
transport insulin granules within individual cells. This aligns well with the model supported by
our previous findings (Zhu, Hu et al. 2015, Bracey, Ho et al. 2020) where stable MTs act as
tracks for the withdrawal of insulin granules from the plasma membrane, restricting secretion.
Lack of this regulated withdrawal should allow for the increased secretion at random locations.
Indeed, this is what we observed: the increase of glucose-stimulated non-clustered events when
MTs are absent (nocodazole treatment). However, we found that MTs most prominently
regulate heterogeneity of β-cells responsiveness to glucose specifically via activation of
secretion clusters, or hot spots. Such specific restriction of clustered secretion would be
achieved by MT-dependent withdrawal if secretion-competent granules are specifically
accumulated near the hot spots prior to the secretion stimulus by a additional mechanism.
Alternatively, specific MT withdrawal from the secretion hotspots could result from local
molecular motor activation and/or local organization of MT tracks at these cellular locations. In
this regard it is interesting that ELKS, a tether molecule involved in MT anchoring at the cell
cortex in some cell types (Lansbergen, Grigoriev et al. 2006), is specifically concentrated at
secretion hotspots. While ELKS is likely involved in regulation of insulin exocytosis (Ohara-
Imaizumi, Ohtsuka et al. 2005, Ohara-Imaizumi, Aoyagi et al. 2019), it might also be a
regulation of local MT anchoring at those sites.

It is important to integrate our understanding of MT function with prior accumulated knowledge on
secretion hotspots regulation. Existing evidence indicates that loci of repeated clustered
secretion involve membrane accumulation of K+ channels (Fu, Githaka et al. 2019), VDCCs
(Bokvist, Eliasson et al. 1995), as well as tethering and membrane fusion machinery (Ohara-
Imaizumi, Nishiwaki et al. 2004, Ohara-Imaizumi, Fujiwara et al. 2007). Accordingly, it is thought
that functioning of a hotspot includes insulin granule docking in preparation for secretion, ion
channels to promote Ca\(^{2+}\) influx, and Ca\(^{2+}\)-dependent membrane fusion. Our data indicate that
MT depolymerization does not affect Ca\(^{2+}\) influx, and that acute Ca\(^{2+}\) influx stimulated secretion
of readily releasable granules at hotspots regardless of MT presence, consistent with previous
data (Mourad, Nenquin et al. 2011). Thus, Ca\(^{2+}\)-dependent steps of secretion are not MT-
dependent. In contrast, our previous findings show that insulin granule docking at the plasma
membrane is regulated by MTs, because MT-dependent transport withdraws granules from the
docking sites (Bracey, Ho et al. 2020). We propose a model (Figure 9A) where glucose
signaling stimulates both insulin granule docking and Ca\(^{2+}\) influx, which are thought to be
triggered via independent pathways (Gaisano 2017). MT-dependent transport negatively
regulates the process of docking, restricting the number of readily-releasable granules. Ca\(^{2+}\)
influx, in turn, promotes secretion of docked granules, in a MT-independent manner. In the
experimental setup when secretion is stimulated by KCl, only pre-docked granules are released,
and MT do not have a significant effect (Figure 9B).

An additional potential scenario is that dynamic MTs facilitate secretion at hotspots by coupling
insulin exocytosis with compensatory endocytic events as was shown for ELKS/LL5β patches in
cultured Ins-1 cells (Yuan, Liu et al. 2015). Another possibility is that MTs act indirectly by 
modulating one of the prominent mechanisms involved in β-cell heterogeneity regulation.
Interestingly, such master regulators of heterogeneous β-cell response such as gap junctions
and ion channels have been shown to be modulated by MTs in other cell types (Shaw, Fay et al.

Regardless of the mechanism, the important conclusion from our data is that it is the activation
of hot spots in additional cells that makes the β-cell population response more uniform in the
absence of MTs. Since Ca^{2+} is essential but likely insufficient for β-cell heterogeneity (Benninger
and Hodson 2018), it is possible that suppression of hot spot activity by stable MTs provides a
required layer of control restricting secretion in a subpopulation of cells in the presence of Ca^{2+}
 influx. Furthermore, our finding that larger clusters secrete insulin at higher rates suggests a
potential enhancement of secretion via a chemical (e.g., insulin signaling or ATP signals) or
mechanical (e.g., in terms of membrane tension) feedback mechanism (Rodriguez-Iturbe,

Another important take-home message from our analyses is that MTs regulate the timing of the
first phase of GSIS via control of clustered secretion. We show that initiation of secretion hot
spots that is normally rapidly triggered by the metabolic signal is dysregulated when MTs are
lost, possibly due to the presence of excessive, unregulated readily-releasable granules. In the
absence of MT regulation, new secretion sites continue to appear even at the time points when
the first phase of secretion should normally be fading. This suggests a potential role for MTs in
the decay of the first phase of GSIS, which could be a significant part of prevention of over
secretion under physiological conditions. At the same time, MT-dependent regulation of random
secretion applies to both stages of the bi-phasic GSIS. An extended first phase of secretion and
enhanced secretion levels in both phases are consistent with our previous findings (Zhu, Hu et
al. 2015).

At the same time, we do not find a significant role of MTs in the secretion dynamics within
individual clusters. Our data clearly indicate that MTs do not affect the frequency of secretion
events within clusters, which is at odds with a previous finding in KCl-stimulated Ins-1 cells
plated on glass, where a decreased frequency of events within a cluster was observed in the
absence of MTs (Yuan, Lu et al. 2015). It is unclear at this point whether this discrepancy is due
to the dramatic differences in the experimental models used in the two studies, but it is not
surprising that the fine regulation detected here was not observed in the absence of islet cell
interactions and vascular ECM signals.

To conclude, this study highlights a novel role of MT network in promoting β cell heterogeneity
in islets by restricting secretion hot spot activity.

Materials and Methods

Key Resources Table
<table>
<thead>
<tr>
<th>Reagent type (species) or resource</th>
<th>Designation</th>
<th>Source or reference</th>
<th>Identifiers</th>
<th>Additional information</th>
</tr>
</thead>
<tbody>
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<td>(Stancill, Osipovich et al. 2019)</td>
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<td>Histone 2B-mApple knocked into the Ins2 locus</td>
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<td>human ECM</td>
<td>Corning, cat#: 354237</td>
<td></td>
<td>Placenta-derived vascular ECM</td>
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</table>

**Mouse utilization**
Ins-Apl mice with Histone 2B-mApple knocked into the Ins2 locus (Stancill, Osipovich et al. 2019) were used for all experiments. Males and females between 2-6 months were used. Data was separated by sex to determine statistical differences before being combined. Mice utilization was supervised by the Vanderbilt Institutional Animal Care and Use Committee (IACUC).

**Cell lines and maintenance**
RPE1-hTert (ATCC) cells were maintained in DMEM/F12 with 10% FBS and antibiotic at 37 °C with 5% CO2 and were periodically tested for mycoplasma.

**Islet picking, attachment and dissemination**
Mouse pancreatic islets were hand-picked following in situ collagenase perfusion and digestion. Islets were allowed to recover for at least 1 hour in RPMI 1640 Media (Life Technologies, Frederick, MD) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1cmg/mL streptomycin in 5% CO₂ at 37°C. All coverslips and dishes were plasma cleaned and coated in placenta-dereived human ECM (Corning, cat#: 354237) which is comprised of laminin, collagen IV and heparan sulfate proteoglycan, which serves as a reconstitution of vasculature ECM, for 30 minutes at 37°C.

For experiments utilizing attached intact islets, 5-8 islets per 10 mm glass bottomed dish (Mattek) were placed in the center of the glass in 100 ul RPMI 1640 and allowed to settle for 1.5-2 hours before being transferred to 5% CO₂ at 37°C. The following day, 900 ul of RPMI 1640 was added. Islet media was changed every 2-3 days for up to 9 days within which the experiment was performed. It has been previously shown that islets attached to vascular ECM preserve normal ability to secrete in response to glucose for up to 14 days (Patterson, Knobel et al. 2000, Zhu, Hu et al. 2015). For experiments utilizing disseminated islets, 30-50 islets per coverslip or dish were used. Islets recovered for 4-24 hours following picking. To disseminate, all islets were collected into a 15 ml tube on ice and allowed to settle for 5 minutes. Most of the media was removed and islets were resuspended in 900 ul room temperature versene and mixed by pipetting up and down several times, and 100 ul warm 0.05% trypsin-EDTA was added. The mixture was pipetted up and down 25-30 times with a p1000 tip to allow for dissemination but keep clumps of islet cells present. Disseminated islets were then centrifuged at 300 x G for 2 minutes at room temperature. The versene/trypsin solution was removed and cells were resuspended in 100 µl RPMI 1640 media per 30-50 islets. The cell mixture was added to coverslips or dishes.

For correlative microscopy after FluoZin-3 assays, dishes were scratched before plasma cleaning with a diamond pen in a pattern to aid in finding the same islets/cells after live cell imaging and immunostaining.

FluoZin-3 assay

FluoZin-3 assay was preformed 4-9 days after picking to allow for robust attachment of the islets and the day following dissemination for disseminated islets. 16-20 hours prior, RPE1-hTert cells expressing GFP were plated with islets at <5% confluency. The green signal in these cells is diffuse outside of the centrosome signal. These cells allow for more accurate determination of focus and TIRF angle for the assay as the green FluoZin-3 signal only appears once it is added to the dish.

On the day of the assay, islets were incubated at 37°C in low glucose (2.8 mM glucose) KRB (110 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM KH2PO4, 1 mM HEPES, 2.5 mM, CaCl2 and 1 mg/mL BSA) for 1.5-2 hours with a change of buffer after 1 hour. For nocodazole (Sigma-Aldrich Cat#: M1404) treatment, stock solution was added to a final 5 µM concentration to treat islets for at least 4 hours before imaging. For taxol treatment (Sigma-Aldrich Cat#: PHL89806), islets were treated for two hours before imaging with 5 µM taxol. Immediately before imaging the buffer was replaced with 100 ul fresh buffer with the same treatments as before to reduce background.

Dishes were placed on the TIRF microscope and allowed to equilibrate for 10 minutes. An islet was identified by eye. A nearby Centrin-GFP RPE cell was used to focus the microscope to the bottom of the dish and set the TIRF angle of the green laser. A 10 µm stack of 0.2 µm slices was recorded of the islet before addition of the dye using both transmitted light and the 568nm laser. Stacks were started below the islet to ensure the bottom of the cells were imaged. 50 µl of KRB buffer with the cell-impermeant FluoZin-3 dye (Thermo-Fisher, Cat#: F24194) to final concentration of 20 µM was added. For high glucose treatment glucose to a final concentration...
of 20 μM was added together with the dye. For KCl treatment, KCl to a final concentration of 25mM was added together with the dye.

For 10-minute assays, focus and TIRF angle were refined after dye addition and the recording (60ms exposure, no delay) started within 2 minutes after glucose stimulation to register active GSIS. For the short-term assays (2.5-minute), the stimulation and dye addition were performed during the recording (60ms exposure, 100ms/frame) to register rapid secretion in response to KCl. The maximal recording time was restricted to 8/10 minutes to avoid photo-toxicity.

**Processing of FluoZin-3 movies**

Each image was subtracted from the previous image, briefly the first and last frame from two different copies of the file were removed and subtracted using the Image Calculator tool in ImageJ with the 32-bit (float) result box checked. For 10-minutes secretion analysis, every 5 images from this subtracted image were grouped as a max projection through time using the Grouped Z-project function in ImageJ and analyzed at 300ms time resolution. For 2.5 image analyses, sequences were analyzed at 100ms time resolution.

**Analysis of FluoZin-3 movies**

**Identifying secretion events**

Individual secretion events or "flashes" were identified using ImageJ software (Schindelin, Arganda-Carreras et al. 2012). FluoZin-3 movies were blinded then analyzed manually. A macro was written that identifies the centroid of local maximal signals after the users uses the point function identify a bright signal. Each event was identified as a flash of signal that was present in one frame only, with a noise level of 5000 and a search range of 6 pixels. To make sure that each single event was accounted for, an event was assigned to each evenly round spread of fluorescence. Rare non-round events were noted during 300ms/frame analysis and re-analyzed in the 60ms/frame sequence, where each secretion event is detected in more than one frame (Fig. 2A). This analysis design supposingly resolved a vast majority of secretion events: it is highly improbable that subsequent events within a cluster are not resolved from each other, considering time resolution at 60 or 100ms/frame, XY spatial resolution at ~200nm, and insulin granule size at ~300nm in diameter. The outcome of analysis as the centroid coordinates were exported and used for analysis. On occasion bright spots in the center of the event caused the formation of donuts preventing accurate identification of the centroid. These rare events were also noted during analysis and the centroid was identified manually and added to the data.

**Identifying cell borders**

Transmitted light and 568nm stacks were recorded before dye addition to identify individual β-cell borders. All cells with a red nucleus (Ins-Apl signal) were outlined by hand in ImageJ. If the nuclei could not be seen (above the image stack range, signal diminished because of light dispersal or out of the frame) or was Ins-Apl negative, the cell was assumed to be a non-β-cell and discarded from analysis. Each Ins-Apl positive cell outline was saved as an individual ROI in ImageJ and coordinates were exported.

**Identifying secretion events/cell and clusters**

A Matlab script (see supplemental annotated scripts) was used to compare the location of each secretion event (identified above) in relation to each cell (borders identified by ROI), outputting the number of secretion events in each cell. Some secretion events fell outside the boundaries of all β-cells identified and were discarded from analysis. Within the same annotated Matlab script clustering analysis was performed. Density based scanning was used with a neighborhood search radius of 1.5 μm and a minimum number of neighbors of 3. The script outputs (1) the movie frame (time), centroid, cell and cluster of each identified secretion event,
(2) the cell number, secretion events and clusters within each identified cell, and (3) the size of each cluster.

**GSIS assay**

Isolated mouse islets were incubated in Krebs-Ringer bicarbonate buffer (KRB, 111 mM NaCl, 4.8 mM KCl, 25 mM NaHCO₃, 2.3 mM CaCl₂, 1.2 mM MgSO₄, 0.15 mM Na₂HPO₄, 1.2 mM KH₂PO₄, 10 mM HEPES, 0.2% BSA) containing 2.8 mM glucose for 2 hour with 0.05% DMSO, 5µg/mL Nocodazole, or 5µM Diazoxide. Islets were then transferred to fresh KRB with the corresponding chemicals plus 2.8 mM or 20 mM glucose with or without 40 mM KCl and incubated for 30 min. The supernatant was collected and the insulin content was determined using the Mouse Ultrasensitive Insulin ELISA kit (ALPCO, Salem, NH. Cat#: 80-INSMSU-E01).

One islet equivalent (IEQ) is defined as a spherical islet with a diameter of 150 µm and is equal to 1767146 µm³.

**Calbryte Ca²⁺ influx assay and analysis**

Mouse Islets (from Ins-Apl mice) were harvested and allowed to attach to human ECM (see above). On day 3 post-isolation (day of experiment) islets were pre-incubated for 2 hours in KRB with 2.8mM glucose. 20mM Calbryte 520 dye (AAT Bioquest Cat#: 20650) was added 30 minutes prior to imaging to the final concentration 10µM. For nocodazole treatment, the islets were pre-incubated with Nocodazole for 4 hours prior to imaging. The islets were imaged by spinning disk confocal microscopy at a single imaging plane at 50ms exposure continuous imaging. Glucose was added to 20µM final concentration as the onset of recording. For analysis, β cells were identified by red nuclear marker. Calbryte intensity was measured within an ROI located inside individual cell borders excluding nucleus, and cells with average cytoplasmic intensity of at least 10 AU counts over background were analysed. Because the individual cellular uptake of Calbryte dye varies, fold increase of intensity as compared to the time of stimulation was used for each cell.

**Immunofluorescence**

*Intact attached and disseminate islets following FluoZin-3 assay:* Dishes were removed from the microscope stage and washed 5 times in PBS to remove the FluoZin-3 dye. Islets were fixed in 4% paraformaldehyde in PBS with 0.1% Triton-X 100 and 0.25% Glutaraldehyde for one hour (intact islets) or ten minutes (disseminated islets) at room temperature. Following fixation, islets were washed 5 times in PBS with 0.1% Triton-X 100 at room temperature.

*Disseminated islets for tubulin and Glu-tubulin intensity measurement:* Dishes or coverslips were pre-treated with low glucose (2.8 mM) KRB at 37°C for 1.5-2 hours with a change in buffer after an hour. High glucose dishes or coverslips were then treated with high glucose (20 mM) KRB for 10 minutes. For ice treatment, the dishes or coverslips were placed on ice for 30 minutes before fixation. For cytosolic pre-extraction (dilution), cells were place in 0.1% Triton in PEM buffer (0.1 M PIPES (pH 6.95), 2 mM EGTA, 1 mM MgSO₄) for one minute, then Triton was washed out and islets were kept at 37°C in PEM buffer for 20 minutes before fixation. Cells were then fixed in 4% paraformaldehyde in PBS with 0.1% Triton-X 100 and 0.25% Glutaraldehyde. Following fixation, islets were washed 5 times in PBS with 0.1% Triton-X 100.

Primary and secondary antibodies were incubated for 24 hours (disseminated islets) or 48 hours (intact islets, antibody change after 24 hours). Samples were washed three times in PBS + 0.1% Triton-X 100 after primary and secondary antibody incubations. Following the final wash post-fixing in 4% paraformaldehyde was performed (30 minutes for intact islets and 10 minutes for disseminated islets) and one more round of washing was performed before mounting coverslips.
Primary antibodies used are mouse anti-α-tubulin, DM1A clone (1:500, dilution, Sigma-Aldrich, Cat#: T9026), rabbit anti-detyrosinated tubulin (1:500, dilution, Millipore, cat#: AB3201). Alexa488- and Alexa647-conjugated highly cross-absorbed secondary antibodies were obtained from Invitrogen. Coverslips were mounted in Vectashield Mounting Medium (Vector Labs Cat#: H-1000).

Microscopes

Fixed samples were imaged on a laser scanning confocal microscope Nikon A1r based on a TiE Motorized Inverted Microscope using a 100X lens, NA 1.49, run by NIS Elements C software. Cells were imaged in 2 µm slices through the whole cell for disseminated islets. Intact islets were imaged through 20 µm. Images in Figure 1 are single slices from the bottom of the cells. Image in Figure 1- figure supplement 1 are maximum intensity projections across three slices from the bottom of the islet to better show the MT cytoskeleton.

FluoZin-3 assays for secretion analysis were imaged on a Nikon TE2000E microscope equipped with a Nikon TIRF2 System for TE2000 using a TIRF 100× 1.49 NA oil-immersion lens and an Andor iXon EMCCD camera run by NIS Elements C software.

Analysis of average intensity of Tubulin and Glu-tubulin

β-cells were identified using red nuclei and outlined with each β-cell being assigned an ROI. The bottom of the cells were used for analysis as cell boarders were clearly visible and secretion in this location was measured in FluoZin-3 assays. Using ImageJ, the mean intensity of each cell in one slice at the bottom of the stack was measured. Both the Glu-tubulin and Tubulin channels were measured. Bright primary cilia signal was removed through thresholding of bright signals, assigning an ROI and deleting the signal from both channels. A small box was drawn outside of the cell and measured in both channels for each image as the background. Background was then subtracted from the mean intensity within the cell.

Statistical modeling methods

Given the distributional nature of this secretion event data, we use statistical modeling to both assess the properties of the process giving rise to the observed data and determine how those properties change under different conditions. A natural hypothesis to test is that secretion events occur independently of each other. If events are indeed independent, then the time between successive events within a cell or cluster should be exponentially distributed. To test this, we fit each data set using an exponential distribution using python's SciPy package. For non-cluster data, all events occurring within a cell were grouped in ascending order of occurrence time to produce a time-between-event distribution that was fit to an exponential distribution. For analysis of all cluster data within each condition (e.g. high glucose Taxol treated), events occurring within each cluster were grouped to compute the time between successive events within the same cluster. These wait times for all clusters were then grouped together to form a time-between-event distribution that was fit. This analysis however grouped clusters of different sizes together. We thus separately analyzed clusters of different sizes (measured as secretion events per cluster). For this, we collected the within cluster time between events for clusters of each size separately. We then grouped all computed waiting times for clusters of a given size (e.g. 4 secretion events) and fit the resulting distribution for each size separately to an exponential.

To assess whether clusters of different sizes secrete insulin at different rates, we constructed a generalized linear model (GLiM) where the secretion rate is linearly dependent on cluster size. Since the secretion time data for each cluster size is well approximated by an exponential distribution (as verified by the previously described analysis), we use an exponential linking function. Note that due to the non-normal nature of this data and the relatively small sample
sizes, this GLM approach is more appropriate than a more common ANOVA or similar approach. This GLM was fit to the data for each condition separately using Bayesian parameter estimation with the python PyMC package. Both the parameters ($\alpha$, $\beta$) were assigned half-normal priors with a standard deviation of 0.1, which are weakly informative priors. Four MCMC chains were used with 4,000 samples each using the built in NUTS sampler.

We briefly discuss the interpretation of the Bayesian “credible interval” approach we used for hypothesis testing on the parameter $\beta$. A typical frequentist approach to this would be to determine a “point estimate” for the value of an unobserved parameter and construct a “confidence interval”. Bayesian estimation instead produces a “posterior density” (in contrast to a point estimate) describing the probability of every possible value of that unobserved parameter conditioned on the data. From this posterior, a “credible interval” (Bayesian analogue to a confidence interval) can be constructed. We specifically construct a 95% Highest Density Posterior Interval (HDPI), which is an interval within which 95% of the posterior density resides. This HDPI has a simple interpretation: given the observed data, there is a 95% probability that the parameter value falls within this interval. So if the 95% HDPI for $\beta$ does no overlap 0, we can be 95% certain that it is different from 0. Note that there are subtle but important differences between credible intervals (Bayesian) and confidence intervals (frequentist) that are beyond the scope of this article. For further discussion, see (Kruschke and Liddell 2018).

Experimental Design:
Glut-tubulin and tubulin intensity experiments were replicated at least three times. At least 20 images per experiment were obtained, random fields containing $\beta$-cells (identified by red nuclei) were imaged. All images were cell borders could be identified from the bottom of the stack where intensities were measured were analyzed.

Due to the large expected variation in insulin secretion from each $\beta$ cell (0 to >100 vesicles) reported by others, we aim to detect a 20% difference in means with a power of 0.95. This needs >327 samples (cells). FluoZin-3 assay experiments were repeated on at least seven different days, using islets from independent isolations/mice. For all data sets, islets from the same animal/isolation were compared between two or more conditions. If at least one secretion event was identified, the movie was analyzed. Any movies without a secretion event were excluded as the technical difficulty of the assay caused an inability to identify a reason for lack of secretion. Analysis of secretion events was performed blind.

Image Processing
In order to make small structures visible, adjustments were made to brightness, contrast and gamma settings of all fluorescent images presented here.

Statistics
For data sets where the distribution was appropriate (normal distribution as determined by the D’Agostino & Pearson omnibus normality test), statistics were calculated by one-way ANOVA with Tukey’s multiple comparisons test. When the data distribution was non-normal, a Kruskal-Wallis test non-parametric and multiple comparison was used (>2 data sets). For two data set comparisons, Mann-Whitney non-parametric comparison test was used. GraphPad Prism was used for statistical analyses and graphical representations. Significance was defined at $p \leq 0.05$.

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**Declaration of Interests**

The authors declare no competing interests.

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**Figure Legends**

**Figure 1. MT stability decreases in high glucose but remains heterogeneous.**

A-F) Disseminated islets treated with low (A-C) and high (D-F) glucose stained for Glu-tubulin (A,D) and tubulin (B,E). β-cells (dashed yellow line) were identified using Ins-Apl red nuclei (yellow, C,F). Red arrows point to differences between cells. Merge (C,F) shows Glu-tubulin (cyan), tubulin (magenta) and red nuclear expression of Ins-Apl (yellow). Single slice from the bottom of the cells. Scale bars 10 µm.

G) Scatterplot of Glu-tubulin average intensity for each cell. Mean, red bar. Students’ t-test, p<0.0001. n=101 cells per condition.

H) Histogram of Glu-tubulin average intensity in low (black) and high (red) glucose. Bin=100. n=101 cells per condition.

I) Histogram of Glu-tubulin average intensity normalized to the mean of each low (black) and high (glucose). Bin=0.2. Coefficient of variation= standard deviation/mean. n=101 cells per condition.

J) Scatterplot of Glu-tubulin to tubulin ratio of average intensity for each cell. Mean, red bar. Students’ t-test, p<0.0001. n=101 cells per condition.

K) Histogram of Glu-tubulin to tubulin ratio of average intensity in low (black) and high (red) glucose. Bin=0.5, overflow bin of >6. n=101 cells per condition.

L) Histogram of Glu-tubulin to tubulin ratio of average intensity normalized to the mean of each low (black) and high (glucose). Bin=0.2. Coefficient of variation= standard deviation/mean. n=101 cells per condition.

M-O) Disseminated islets placed on ice for 30 minutes in low (M-N) and high glucose (O-P) and stained for tubulin (M,O). β-cells (dashed yellow line) were identified using red nuclear expression of Ins-Apl (yellow, N,P), merged with tubulin (magenta N,P). Red arrows point to differences between cells. Single slice from the bottom of the cells. Scale bar 10 µm.

Q) Scatterplot of tubulin average intensity for each cell after 30 minutes in high glucose. Mean, red bar. Students’ t-test, p<0.0001. n=100-101 cells per condition.

H) Histogram of tubulin average intensity in low (black) and high (red) glucose after 30 min on ice. Bin=50. n=100-101 cells per condition.

I) Histogram of tubulin average intensity normalized to the mean of each low (black) and high (glucose) after 30 min on ice. Bin=0.2. Coefficient of variation= standard deviation/mean. n=100-101 cells per condition.

**Figure 2. MT instability increases β-cell activation and insulin secretion.**

A) Example images of a single secretion event pre-processing. Secretion signal starts at 65ms and dissipates out.

A') Graph of the average intensities of a circular ROI from images in panel D.

B-D) Time projections of islets from Supplemental Movies 1-3 inverted. Flouzin-3 flashes are represented as black areas. Cell borders identified via pre-assay imaging (see materials and methods) overlaid in red. Islets were pre-incubated in DMSO (control, A), nocodazole (B) or taxol (C) and stimulated with 20mM glucose. Scale bars, 100 µm.

E) Graph of the percentage of cells in each field of view with at least one secretion event. Red bars, mean. One-way ANOVA and multiple comparison tests, p value as indicated. N= 16-19 islets. Here and below, islets derived from 3 or more independent isolations per each condition.
F) Glucose-stimulated secretion as detected by FluoZin-3 assay in islets. A representative output image from Matlab script (see materials and methods) shows cell outlines (black lines) and secretion events (dots). Black dots are non-clustered secretion events, colored dots are clustered secretion events.

G) The same islet as in F fixed after the assay and stained for Glu-tubulin (grayscale). Cell outlines within which Glu-tubulin intensity was measured are shown in red. Numbers correspond to cells in (F) with the same number. The outline "bkg" indicates background measurement area. Maximum intensity projection over 1.2 µm at the bottom of the islet. Bar, 10µm.

H) Correlative analysis between data as in (F) and (G). The number of secretion events per cell with Glu-tubulin intensity below islet average and those above islet average is compared in the graph. Mann-Whitney non-parametric comparison test p-value is shown. N=98 cells from 5 islets. The same data set as in Figure 2 – Supplemental Figure 2A.

I) Graph of secretion events per cell detected by FluoZin-3 assay. All cells in a field of view are analyzed, whether activated during the movie or not. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 495-637 cells from 16-19 islets.

I') Cells from panel H, graphed as a stacked histogram of the percentage of total cells per condition that had each number of secretion events.

J) Graph of secretion events per cell only including cells with at least one event during the duration of the movie. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 88-407 cells from 16-19 islets.

J') Cells from panel I, graphed as a stacked histogram of the percentage of cells that had each number of secretion events.

Figure 3. MT stability suppresses formation of insulin secretion hot spots.

A) Histogram of nearest neighbor distances obtained by measuring the distance between secretion events in cells with more than one secretion event during the movie. Graphed as percentage of cells within each bin per condition. Bin=0.5 µm. N=191-3255 distances from 16-19 islets.

B-D) Representative images of output from Matlab script (see materials and methods) showing cell outlines (black lines) and secretion events (dots). Black dots are non-clustered secretion events, colored dots are clustered secretion events, each different color denotes a different cluster. Clusters were defined as 3+ secretion events occurring within 9 pixels (1.44 µm) by density-based scanning. Islets were pre-treated with DMSO (control, B), nocodazole (C) or taxol (D) and were stimulated with 20 um glucose.

E) Graph of the percentage of cells in each field of view with at least one cluster. Red bars, mean. One-way ANOVA and multiple comparison tests, p value as indicated. N= 16-19 islets.

F) Graph of the percentage of cells in each field of view with at least one cluster out of cells with at least one secretion event. Red bars, mean. One-way ANOVA and multiple comparison tests, p value as indicated. N= 16-19 islets.

G) Correlation of Glu-tubulin intensity (normalized to islet average) to the number of secretion clusters per cell in whole islets. Grey field, intensity below islet average (<1). Yellow field, intensity above islet average (>1). The same data set as in Figure 2F-H.

H) The number of clusters in cells with Glu-tubulin intensity below islet average and those above islet average is compared in the graph. Mann-Whitney non-parametric comparison test p-value is shown. N=98 cells from 5 islets. The same data set as in Figure 2F-H.

I) Correlation of Glu-tubulin intensity (normalized to islet average) to the number of clustered secretion events per cell in whole islets. Grey field, intensity below islet average (<1). Yellow field, intensity above islet average (>1). The same data set as in Figure 2F-H.
J) The number of clustered events in cells with Glu-tubulin intensity below islet average and those above islet average is compared in the graph. Mann-Whitney non-parametric comparison test p-value is shown. N=98 cells from 5 islets. The same data set as in Figure 2F-H.

**Figure 4. MT-disruption increases the number of hot spots per cell, increasing clustered secretion.**

A) Representative images of clusters from one cell in a control islet stimulated with 20 mM glucose. Clusters were identified by Matlab script (see materials and methods). First image is time projection through all clusters in the cell 15.925-194.675 s of the movie, clusters are identified by red text. Time in seconds of each event in the cluster above, red arrowheads identify the secretion event. Scale bar 5 µm.

B) Representative images of clusters from one cell in a nocodazole pre-treated islet stimulated with 20 mM glucose. Clusters were identified by Matlab script (see materials and methods). First image is time projection through all clusters in the cell 54.65-407.875 s of the movie, clusters are identified by red text. Time in seconds of each event in each cluster above, red arrowheads identify the secretion event. Scale bar 5 µm.

C) Graph of clusters per cell in the field of view, with all cells whether activated during the movie or not. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 495-637 cells from 16-19 islets.

C') Cells from panel C, graphed as a stacked histogram of the percentage of total cells per condition that had each number of clusters.

D) Graph of clusters per cell only including cells with at least one secretion event during the duration of the movie. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, N= 88-407 cells from 16-19 islets.

D') Cells from panel D, graphed as a stacked histogram of the percentage of cell with secretion events per condition that had each number of clusters.

E) Graph of clusters per cell only cells with at least cluster during the duration of the movie were included. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 13-143 cells from 16-19 islets.

E') Cells from panel E, graphed as a stacked histogram of the percentage of cell with clusters per condition that had each number of clusters.

F) Graph of events per cell with at least one secretion event during the movie that were in a cluster. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, N= 88-407 cells from 16-19 islets.

F') Graph of events per cell with at least one secretion event during the movie that were not in a cluster. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, N= 88-407 cells from 16-19 islets.

**Figure 5. Increased secretion from clusters leads to faster secretion at that site.**

A) Graph of events per cluster, Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests found no statistical differences between conditions N= 14-290 clusters from 16-19 islets.

A') Clusters from panel F, graphed as a stacked histogram of the percentage of clusters per condition with each number of events.

B) Histogram of the time between successively (in time) occurring non-clustered events with the best fit exponential overlaid (KS-statistic is provided for quality of fit).

C) Histogram of the time between successively occurring clustered events with the best fit exponential overlaid (KS-statistic is provided for quality of fit).

D) Graph of time (seconds) between successive events distribution for clusters of different sizes (Red bar = mean). Some conditions lack clusters of particular size (e.g. no clusters with 6 secretion events in Ctrl low), as indicated by no data.
E) Each distribution in (D) is fit separately to an exponential distribution and quality of fit is assessed with a KS-test (as in panels B, C). The resulting p-value for every test is plotted, with the black line indicating p=0.05.

F) Results of fitting a general linear model to the data from (D) (see Methods for further details) with the assumption that “secretion rate = $\alpha + \beta \times$ Cluster Size”. Bayesian credible intervals for $\beta$ are plotted for each condition. This model is also compared to a null model where “secretion rate = $\alpha$” (i.e. lacking size dependence), with model comparison results reported as the difference of WAIC scores (positive indicates the full model provides a better accounting of the data).

Figure 6. MTs restrict secretion from hot spots to the first phase of secretion, loss of MTs lengthen this phase.

A) Histogram of secretion events over time. Graphed as percentage of events within each bin per condition. Time (seconds) since dye and either high (20 mM) or low (2.8 mM) glucose addition. Bin=30 seconds. N= 16-19 islets.

B) Histogram of secretion events over time separated into secretion events not in clusters (black) and in clusters (red). Time (seconds) since dye and either high (20 mM) or low (2.8 mM) glucose addition. Bin=30 seconds. N= 16-19 islets.

C) Histogram of the first event in a cluster (black) and last event in a cluster (red) over. Time (seconds) since dye and either high (20 mM) or low (2.8 mM) glucose addition. Bin=30 seconds. N= 16-19 islets.

D) Scatterplot of each cluster in each condition with the timing of the first event in a cluster on the x-axis and the timing of the last event in a cluster on the y-axis. Time (seconds) since dye and either high (20 mM) or low (2.8 mM) glucose addition. Bin=30 seconds. N= 16-19 islets.

Figure 7. Calcium signaling is essential for secretion regardless of MT presence.

A) GSIS as detected by ELISA. Secretion over 30 minutes upon 20mM glucose stimulation is shown for DMSO control and nocodazole pre-treated cells in the presence and absence of 5µM Diazoxide. Tukey’s multiple comparisons test p values shown.

B-C) Time frames from attached islets treated with DMSO (B) and nocodazole (C) and incubated with Calbryte 520 (cyan). Time after 20MM glucose stimulation, seconds. Red, mApple (β-cell marker). Single plane spinning disk confocal microscopy images. Dotted line outlines indicate representative β cells with detectable concentration of Calbryte 520 used for analyses. Scale bar, 10 µm.

D) Graph of highest amplitudes of Calbryte 520 intensity fluctuation per cell, measured in data as in Figure 7 – Figure Supplement 1. Mann-Whittney non-parametric comparison test p-value is shown. N= 78-102 cells from 8-12 islets.

E) Summarized increase of Calbryte 520 intensity over the first minute of glucose stimulation per cell, measured in data as in Figure 7 – Figure Supplement 1. Mann-Whittney non-parametric comparison test p-value is shown. N= 78-102 cells from 8-12 islets.

Figure 8. MTs regulate glucose-stimulated secretion in addition to calcium-dependent mechanisms.

A-D) KCl- (A, B) and KCl-Glucose-stimulated (C, D) secretion as detected by FluoZin-3 assay in DMSO- (A, C) and nocodazole (B, D) pre-treated islets. Representative output images from Matlab script (see materials and methods) shows cell outlines (black lines) and secretion events (dots) over 2.5 minutes after stimulation. Black dots are non-clustered secretion events, colored dots are clustered secretion events.

E) Graph of KCl-induced secretion events per cell over 2.5 minutes detected by FluoZin-3 assay (data as in (A-D)). Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 306-708 cells from 15-17 islets.
F) Graph of the percentage of cells in each field of view with at least one cluster in KCl-induced secretion over 2.5 minutes. Red bar, mean. One-way ANOVA and multiple comparison tests, p value as indicated, N= 15-17 islets.

G) Graph of the percentage of cells in each field of view with at least one cluster out of cells with at least one secretion event. KCl-induced secretion over 2.5 minutes. Red bar, mean. One-way ANOVA and multiple comparison tests, p value as indicated, N= 15-17 islets.

H) Number of secretion events per cluster. KCl-induced secretion over 2.5 minutes. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, no significant difference, N= 109-269 clusters from 306-708 cells/15-17 islets.

I) Graph of clusters per cell in the field of view. KCl-induced secretion over 2.5 minutes. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 306-708 cells from 15-17 islets.

J) Cells from panel I, graphed as a stacked histogram of the percentage of total cells per condition that had each number of clusters.

J') Cells from panel J, graphed as a stacked histogram of the percentage of cell with secretion events per condition that had each number of clusters.

K) Graph of clustered events per cell. KCl-induced secretion over 2.5 minutes. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p values as indicated, 306-708 cells from 15-17 islets.

K') Graph of non-clustered events per cell. KCl-induced secretion over 2.5 minutes. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p values as indicated, 306-708 cells from 15-17 islets.

L) Graph of clustered events per cell out of cells with at least one secretion event. KCl-induced secretion over 2.5 minutes. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p values as indicated, 218-334 cells from 15-17 islets.

L') Graph of non-clustered events per cell out of cells with at least one secretion event. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p values as indicated, N= 218-334 cells from 15-17 islets.

Figure 1- Supplemental Figure 1. MT stability is regulated by glucose stimulation.

A) Scatterplot of tubulin average intensity for each cell. Mean, red bar. n=101 cells per condition.

B) Histogram of tubulin average intensity in low (black) and high (red) glucose. Bin=100. n=101 cells per condition.

C) Histogram of tubulin average intensity normalized to the mean of each low (black) and high (glucose). Bin=0.2. Coefficient of variation= standard deviation/mean. n=101 cells per condition.

D-E) Glu-tubulin staining in disseminated islets after 30 min on ice (corresponds to Figure 1M-O). β-cells outlined in dashed yellow lines. Single slice from the bottom of the cell. Scale bar 10 μm.

F) Scatterplot of Glu-tubulin average intensity for each cell after 30 minutes in high glucose. Mean, red bar. Students' t-test , p=0.0001. n=100-101 cells per condition.

G) Histogram of Glu-tubulin average intensity in low (black) and high (red) glucose after 30 min on ice. Bin=100. n=100-101 cells per condition.

H) Histogram of Glu-tubulin average intensity normalized to the mean of each low (black) and high (glucose) after 30 min on ice. Bin=0.2. Coefficient of variation= standard deviation/mean. n=100-101 cells per condition.

I) Scatterplot of Glu-tubulin to tubulin ratio of average intensity for each cell after 30 min on ice. Mean, red bar. Students' t-test , p=0.135. n=100-101 cells per condition.
J) Histogram of Glu-tubulin to tubulin ratio of average intensity in low (black) and high (red) glucose after 30 min on ice. Bin=1.0. n=100-101 cells per condition

K) Histogram of Glu-tubulin to tubulin ratio of average intensity normalized to the mean of each low (black) and high (glucose) after 30 min on ice. Bin=0.2. Coefficient of variation= standard deviation/mean. n=100-101 cells per condition

L-N) Disseminated islets placed extracted for one minute and placed in buffer for 20 minutes. Stained for Glu-tubulin (L) and tubulin (M). β-cells were identified by red nuclear Ins-Apl expression (N, yellow), merged with Glu-tubulin (cyan) and tubulin (magenta). Red arrows pointing to differences between cells. Single slice from the bottom of the cells. Scale bar 10 µm.

Figure 2 - Supplemental Figure 1. Assay protocol and basal glucose conditions are not affected by MT stability.

A) Overview of Fluozin-3 assays. For more information see materials and methods.

B-D) Time projections of islets from Supplemental Movies 4-6 inverted. Fluozin-3 flashes are represented as black areas. Cell borders overlaid in red. Islets were pre-incubated in DMSO (control, B), nocodazole (C) or taxol (D) and incubated in 2.8 mM glucose. Scale bars 100 µm.

E-G) Representative images of tubulin following Fluozin-3 imaging as shown in A, islets were pre-incubated in DMSO (control, E), nocodazole (F) or taxol (G) and stimulated with 20 mM glucose. 3-image max projection of the bottom of the islet. Scale bars, 10 µm.

Figure 2 - Supplemental Figure 2. Correlation of insulin secretion and MT stability.

A) Correlation of Glu-tubulin intensity (normalized to islet average) to the number of secretion events per cell in whole islets. Grey field, intensity below islet average (<1). Yellow field, intensity above islet average (>1). The same data set as in Figure 2F-H.

B) Correlation of Glu-tubulin intensity (normalized to the field of view average) to the number of secretion events per cell in disseminated islets. Correlative analysis between data as in (D) and (E). Grey field, intensity below field average (<1). Yellow field, intensity above field average (>1).

C) The number of secretion events per cell in disseminated islets with Glu-tubulin intensity below field average and those above field average is compared in the graph. The same data set as in (B). Mann-Whitney non-parametric comparison test p-value is shown. N= 124 cells.

D) Cell outlines (white line) and secretion events (red circles) from a disseminated islet after 10 minutes in 20 mM glucose and Fluozin-3 dye. Scale bar 10 µm.

E-F) Disseminated islet from E post-fixed following TIRF imaging for Glu-tubulin (E) and tubulin (F). Cells (yellow dashed lines) correspond to cells in E with the same number. Single slice from the bottom of the cells. Scale bar 10 µm.

Figure 3 - Supplemental Figure 1. 3+ event clusters are not spurious and a very rare in basal glucose conditions.

A) Computationally simulated random secretion events in in silico cells using the mean area of the cells analyzed.

B-D) Representative images of output from Matlab script (see materials and methods) showing cell outlines (black lines) and secretion events (dots). Black dots are non-clustered secretion events, colored dots are clustered secretion events, each different color denotes a different cluster. Clusters were defined as 3+ secretion events occurring with 9 pixels (1.44 µm) by density-based scanning. Islets were pre-treated with DMSO (control, B), nocodazole (C) or taxol (D) and were incubated in 2.8 µM glucose.

Figure 5 - Supplemental Figure 1. Clustered secretion is mostly restricted to the first phase of secretion in control islets.
A) Histogram of secretion events over time separated into secretion events not in clusters (black) and in clusters (red). Graphed as percentage of cells within each bin per condition. Time (seconds) since dye and either high (20 mM) or low (2.8 mM) glucose. Bin=30 seconds. N= 16-19 islets.

Figure 7 - Supplement Figure 1. Calcium influx over time in glucose-stimulated islets.
A-B) Analysis of a representative experiment (one islet isolation) summarized in Figure 7D,E. Each curve represents Calbryte 520 intensity dynamics of a single β cell, 1 minute before and 1 minute after the fluctuations start. Each graph combines cells from one islet, either control DMSO- (A) or nocodazole-pretreated (B). Fold increase over mean intensity at the time of stimulation is shown. Time in min after stimulation is shown. Time, minutes.

Figure 8 - Supplemental Figure 1. Rapid secretion induction by KCL as compared to glucose.
A) Graph of glucose, KCl- and KCl+Glucose-stimulated secretion events per cell over 8 minutes in DMSO- and nocodazole pre-treated islets. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 306-708 cells from 13-17 islets.
B) Graph of clusters per cell in the field of view shown as a stacked histogram of the percentage of total cells per condition that had each number of clusters. Secretion over 8 minutes. N= 306-708 cells from 13-17 islets.
C) Number of secretion events per cluster over 8 minutes. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, no significant difference, N= 72-342 clusters from 306-708 cells/13-17 islets.
D) Graph of time between successive events in clusters over 8 minutes (Red bar = mean). Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 72-342 clusters from 306-708 cells/13-17 islets.
E) Histogram of secretion events over time (8 minutes). Graphed as percentage of cells within each bin per condition. Time (seconds) after stimulation (20mM glucose, 25mM KCl, or combination of both, as indicated). Bin=30 seconds. N= 13-17 islets.

Figure 9. MT regulation of secretion at a hot spot
A) Glucose signaling stimulates both insulin granule docking and calcium influx; calcium influx, in turn, promotes secretion of docked graules.MT-dependent transport negatively regulates the process of docking, restricting the number of readily-releasable granules and secretion outcome.
B) KCl treatment causes extreme calcium influx, which in turn facilitates secretion of pre-docked granules. No additional docking occurs in the absence of glucose stimulation, and MT regulation does not influence the number of pre-docked granules and secretion levels.

Source Data
Figure 1- source data 1- Data for graphs depicted in Figure 1 G, H, I, J, K, L, Q, R, S and Figure supplement 1 A, B, C, F, G, H, I, J, K. Each data set is a separate sheet.
Figure 2-source data 1- Data for graphs depicted in Figure 2 E, H, I, I', J, J' and Figure 2 supplement 2 A, B, C. Each data set is a separate sheet.
Figure 2-source data 2- Matlab script to identify secretion events and clusters. Script is annotated.
Figure 2-source data 3- Matlab script to identify secretion events and clusters for correlative microscopy. Script is annotated.
Figure 3-source data 1- Data for graphs depicted in Figure 3 A, E, F, G, H, I, J. Each data set is a separate sheet.

Figure 4-source data 1- Data for graphs depicted in Figure 4 C, C', D, D', E, E', F, F'. Each data set is a separate sheet.

Figure 5-source data 1- Data for graph depicted in Figure 5A.

Figure 5-source data 2- Python scripts that produce the statistical analysis and plots in Figure 5 B, C, D, E, F.

Figure 6-source data 1- Data for graphs depicted in Figure 6 A, B, C, D and Figure Supplement 1 A. Each data set is a separate sheet.

Figure 7-source data 1- Data for graphs depicted in Figure 7 A, D, E and Figure 7 Supplement 1 A, B. Each data set is a separate sheet.

Figure 8-source data 1- Data for graphs depicted in Figure 8 E, F, G, H, I, I', J, J', K, K', L, L'.

Figure 8-source data 2 - Data for graphs depicted in Figure 8 Supplement 1 A, B, C, D, E. Each data set is a separate sheet.

Video Legends

Figure 2-Video 1. Control islet insulin secretion in low and high glucose.
DMSO treated islets in low glucose (left) and high glucose (right). Five frame projection through time, each slice is 325 ms over about 8.5 minutes. FluoZin-3 dye creates flashes upon zinc binding, representing a single insulin secretion event.

Figure 2-Video 2. Nocodazole islet insulin secretion in low and high glucose.
Nocodazole treated islets in low glucose (left) and high glucose (right). Five frame projection through time, each slice is 325 ms over about 8.5 minutes. FluoZin-3 dye creates flashes upon zinc binding, representing a single insulin secretion event.

Figure 2-Video 3. Taxol islet insulin secretion in low and high glucose. Related to Figure 2.
Taxol treated islets in low glucose (left) and high glucose (right). Five frame projection through time, each slice is 325 ms over about 8.5 minutes. FluoZin-3 dye creates flashes upon zinc binding, representing a single insulin secretion event.

Figure 4-Video 1. Clusters in a single cell in control islet in high glucose.
A single cell from Figure 2-Video 1 high glucose. Projection through time of 5 frames, each slice is 325 ms. Movie is 15.925- 194.675 s of the movie.

Figure 4-Video 2. Clusters in a single cell in nocodazole treated islet in high glucose.
A single cell from Figure 2-Video 2 high glucose. Projection through time of 5 frames, each slice is 325 ms. Movie is 54.65- 407.875 s of the movie.

Figure 7-Video 1. Glucose-dependent Ca\(^{2+}\) influx in control detected by Calbryte 520.
An edge of DMSO-treated islet, preincubated with Calbryte 520 dye. Single-plane spinning disk confocal imaging with each 10 time frames averaged. The sequence within 1 min 33 sec – 2 min 22 sec after 20mM glucose stimulation shows the onset of Ca\(^{2+}\) oscillations. Cyan, Calbryte 520. Red, mApple nuclei to mark β-cells.

Figure 7-Video 2. Glucose-dependent Ca\(^{2+}\) influx in the absence of MTs detected by Calbryte 520.
An edge of nocodazole-treated islet, preincubated with Calbryte 520 dye. Single-plane spinning disk confocal imaging with each 10 time frames averaged. The sequence within 1 min 23 sec – 2 min 12 sec after 20mM glucose stimulation shows the onset of Ca^{2+} oscillations. Cyan, Calbryte 520. Red, mApple nuclei to mark β-cells.

**Figure 8-Video 1. KCl-induced insulin secretion in DMSO and nocodazole.**
DMSO-treated (left) and nocodazole-treated (right) islets stimulated by 10mM KCl. Five-frame projection through time, each slice is 500 ms over 2.5 minutes after stimulation. Fluozin-3 dye creates flashes upon zinc binding, representing a single insulin secretion event.

**Figure 8-Video 2. KCl- and glucose-induced insulin secretion in DMSO and nocodazole.**
Examples of big DMSO-treated (left) and nocodazole-treated (right) islets stimulated by a mixture of KCl and glucose. Five-frame projection through time, each slice is 500 ms over 2.5 minutes after stimulation. Fluozin-3 dye creates flashes upon zinc binding, representing a single insulin secretion event.

**Figure 8-Video 3. KCl- and glucose-induced insulin secretion in DMSO and nocodazole.**
Examples of small DMSO-treated (left) and nocodazole-treated (right) islets stimulated by a mixture of KCl and glucose. Five-frame projection through time, each slice is 500 ms over 2.5 minutes after stimulation. Fluozin-3 dye creates flashes upon zinc binding, representing a single insulin secretion event.

**Bibliography**


Glucose

1416 Lasting Secretion Patterns of Individual Rat Pancreatic B


1418 pancreatic beta


Figure 1. MT stability decreases in high glucose but remains heterogenous.

A-F) Disseminated islets treated with low (A-C) and high (D-F) glucose stained for Glu-tubulin (A,D) and tubulin (B,E). β-cells (dashed yellow line) were identified using Ins-Apl red nuclei (yellow, C,F). Red arrows point to differences between cells. Merge (C,F) shows Glu-tubulin (cyan), tubulin (magenta) and red nuclear expression of Ins-Apl (yellow). Single slice from the bottom of the cells. Scale bars 10 µm.

G) Scatterplot of Glu-tubulin average intensity for each cell. Mean, red bar. Students’ t-test, p<0.0001. n=101 cells per condition.

H) Histogram of Glu-tubulin average intensity in low (black) and high (red) glucose. Bin=100. n=101 cells per condition.

I) Histogram of Glu-tubulin average intensity normalized to the mean of each low (black) and high (glucose). Bin=0.2. Coefficient of variation= standard deviation/mean. n=101 cells per condition.

J) Scatterplot of Glu-tubulin to tubulin ratio of average intensity for each cell. Mean, red bar. Students’ t-test, p<0.0001. n=101 cells per condition.

K) Histogram of Glu-tubulin to tubulin ratio of average intensity in low (black) and high (red) glucose. Bin=0.5, overflow bin of >6. n=101 cells per condition.

M-P) Disseminated islets placed on ice for 30 minutes in low (M-N) and high glucose (O-P) and stained for tubulin (M,O). β-cells (dashed yellow line) were identified using red nuclear expression of Ins-Apl (yellow, N,P), merged with tubulin (magenta N,P). Red arrows point to differences between cells. Single slice from the bottom of the cells. Scale bar 10 µm.

Q) Scatterplot of tubulin average intensity for each cell after 30 minutes in high glucose. Mean, red bar. Students’ t-test, p<0.0001. n=100-101 cells per condition

R) Histogram of tubulin average intensity in low (black) and high (red) glucose after 30 min on ice. Bin=50. n=100-101 cells per condition

S) Histogram of tubulin average intensity normalized to the mean of each low (black) and high (glucose) after 30 min on ice. Bin=0.2. Coefficient of variation= standard deviation/mean. n=100-101 cells per condition.
Figure 1- figure supplement 1. MT stability is regulated by glucose stimulation.

A) Scatterplot of tubulin average intensity for each cell. Mean, red bar. n=101 cells per condition.

B) Histogram of tubulin average intensity in low (black) and high (red) glucose. Bin=100. n=101 cells per condition, Students’ t-test found no statistical significance.

C) Histogram of tubulin average intensity normalized to the mean of each low (black) and high (glucose). Bin=0.2. Coefficient of variation= standard deviation/mean. n=101 cells per condition.

D-E) Glu-tubulin staining in disseminated islets after 30 min on ice (corresponds to Figure 1M-O). β-cells outlined in dashed yellow lines. Single slice from the bottom of the cell. Scale bar 10 µm.

F) Scatterplot of Glu-tubulin average intensity for each cell after 30 minutes in high glucose. Mean, red bar. Students’ t-test , p<0.0001. n=100-101 cells per condition.

G) Histogram of Glu-tubulin average intensity in low (black) and high (red) glucose after 30 min on ice. Bin=100. n=100-101 cells per condition.

H) Histogram of Glu-tubulin average intensity normalized to the mean of each low (black) and high (glucose) after 30 min on ice. Bin=0.2. Coefficient of variation= standard deviation/mean. n=100-101 cells per condition.

I) Scatterplot of Glu-tubulin to tubulin ratio of average intensity for each cell after 30 min on ice. Mean, red bar. Students’ t-test , p=0.135. n=100-101 cells per condition.

J) Histogram of Glu-tubulin to tubulin ratio of average intensity in low (black) and high (red) glucose after 30 min on ice. Bin=1.0. n=100-101 cells per condition.

K) Histogram of Glu-tubulin to tubulin ratio of average intensity normalized to the mean of each low (black) and high (glucose) after 30 min on ice. Bin=0.2. Coefficient of variation= standard deviation/mean. n=100-101 cells per condition.

L-N) Disseminated islets placed extracted for one minute and placed in buffer for 20 minutes. Stained for Glu-tubulin (L) and tubulin (M). β-cells were identified by red nuclear Ins-Apl expression (N, yellow), merged with Glu-tubulin (cyan) and tubulin (magenta). Red arrows pointing to differences between cells. Single slice from the bottom of the cells. Scale bar 10 µm.
A. 0 ms  65 ms  130 ms
   195 ms  260 ms  325 ms

A'.

B. Ctrl High
C. Noc High
D. Taxol High

E.

F. Secretion Events
   bkg

G. Glu-tubulin Post-fixed

H. Events/Cell (whole islets)

I. Events/Cell- All Cells

J. Events/Cell- Only Secreting Cells
Figure 2. MT instability increases β-cell activation and insulin secretion.
A) Example images of a single secretion event pre-processing. Secretion signal starts at 65ms and dissipates out.
A’) Graph of the average intensities of a circular ROI from images in panel D.
B-D) Time projections of islets from Supplemental Movies 1-3 inverted. FluoZin-3 flashes are represented as black areas. Cell borders identified via pre-assay imaging (see materials and methods) overlaid in red. Islets were pre-incubated in DMSO (control, A), nocodazole (B) or taxol (C) and stimulated with 20mM glucose. Scale bars, 100 µm.
E) Graph of the percentage of cells in each field of view with at least one secretion event. Red bars, mean. One-way ANOVA and multiple comparison tests, p value as indicated. N= 16-19 islets. Here and below, islets derived from 3 or more independent isolations per each condition.
F) Glucose-stimulated secretion as detected by FluoZin-3 assay in islets. A representative output image from Matlab script (see materials and methods) shows cell outlines (black lines) and secretion events (dots). Black dots are non-clustered secretion events, colored dots are clustered secretion events.
G) The same islet as in F fixed after the assay and stained for Glu-tubulin (grayscale). Cell outlines within which Glu-tubulin intensity was measured are shown in red. Numbers correspond to cells in (F) with the same number. The outline “bkg” indicates background measurement area. Maximum intensity projection over 1.2 µm at the bottom of the islet. Bar, 10µm.
H) Correlative analysis between data as in (F) and (G). The number of secretion events per cell with Glu-tubulin intensity below islet average and those above islet average is compared in the graph. Mann-Whittney non-parametric comparison test p-value is shown. N=98 cells from 5 islets. The same data set as in Figure 2 – Supplemental Figure 2A.
I) Graph of secretion events per cell detected by FluoZin-3 assay. All cells in a field of view are analyzed, whether activated during the movie or not. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 495-637 cells from 16-19 islets.
I’) Cells from panel H, graphed as a stacked histogram of the percentage of total cells per condition that had each number of secretion events.
J) Graph of secretion events per cell only including cells with at least one event during the duration of the movie. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 88-407 cells from 16-19 islets.
J’) Cells from panel I, graphed as a stacked histogram of the percentage of cells that had each number of secretion events.
**A** Islet Preparation

Vascular ECM

Flattened Mouse Islet

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**B** Ctrl Low

**C** Noc Low

**D** Taxol Low

---

**Pre-dye imaging**

visualize cell borders
determine β-cells

**TIRF Imaging**

No delay, 60ms exposure
10 minutes

**Image Processing**

Remove first ~2.5 minutes
Image subtraction
Grouped t-projection

**Data Processing**

Identify secretion events
Overlay with cell borders
Matlab script to process data

---

**E** Ctrl

**F** Noc

**G** Taxol
Figure 2 - Supplemental Figure 1. Assay protocol and basal glucose conditions are not affected by MT stability.

A) Overview of Fluozin-3 assays. For more information see materials and methods.

B-D) Time projections of islets from Supplemental Movies 4-6 inverted. Fluozin-3 flashes are represented as black areas. Cell borders overlaid in red. Islets were pre-incubated in DMSO (control, B), nocodazole (C) or taxol (D) and incubated in 2.8 mM glucose. Scale bars 100 µm.

E-G) Representative images of tubulin following Fluozin-3 imaging as shown in A, islets were pre-incubated in DMSO (control, E), nocodazole (F) or taxol (G) and stimulated with 20 mM glucose. 3-image max projection of the bottom of the islet. Scale bars, 10 µm.
A  Event/Cell vs Glu-tubulin (whole islets)

B  Event/Cell vs Glu-tubulin (disseminated islets)

C  Event/Cell (disseminated islets)

D  Secretion Events

E  Glu-tubulin Post-fixed

F  Tubulin Post-fixed

Glu-tubulin Post-fixed

Secretion Events

Glu-tubulin Post-fixed

Tubulin Post-fixed

0.0026
Figure 2 - Supplemental Figure 2. Correlation of insulin secretion and MT stability.

A) Correlation of Glu-tubulin intensity (normalized to islet average) to the number of secretion events per cell in whole islets. Grey field, intensity below islet average (<1). Yellow field, intensity above islet average (>1). The same data set as in Figure 2F-H.

B) Correlation of Glu-tubulin intensity (normalized to the field of view average) to the number of secretion events per cell in disseminated islets. Correlative analysis between data as in (D) and (E). Grey field, intensity below field average (<1). Yellow field, intensity above field average (>1).

C) The number of secretion events per cell in disseminated islets with Glu-tubulin intensity below field average and those above field average is compared in the graph. The same data set as in (B). Mann-Whitney non-parametric comparison test p-value is shown. N= 124 cells.

D) Cell outlines (white line) and secretion events (red circles) from a disseminated islet after 10 minutes in 20 mM glucose and Fluozin-3 dye. Scale bar 10 µm.

E-F) Disseminated islet from E post-fixed following TIRF imaging for Glu-tubulin (E) and tubulin (F). Cells (yellow dashed lines) correspond to cells in E with the same number. Single slice from the bottom of the cells. Scale bar 10 µm.
Figure 3. MT stability suppresses formation of insulin secretion hot spots.
A) Histogram of nearest neighbor distances obtained by measuring the distance between secretion events in cells with more than one secretion event during the movie. Graphed as percentage of cells within each bin per condition. Bin=0.5 μm. N=191-3255 distances from 16-19 islets.
B-D) Representative images of output from Matlab script (see materials and methods) showing cell outlines (black lines) and secretion events (dots). Black dots are non-clustered secretion events, colored dots are clustered secretion events, each different color denotes a different cluster. Clusters were defined as 3+ secretion events occurring within 9 pixels (1.44 μm) by density-based scanning. Islets were pre-treated with DMSO (control, B), nocodazole (C) or taxol (D) and were stimulated with 20 um glucose.
E) Graph of the percentage of cells in each field of view with at least one cluster. Red bars, mean. One-way ANOVA and multiple comparison tests, p value as indicated. N= 16-19 islets.
F) Graph of the percentage of cells in each field of view with at least one cluster out of cells with with at least one secretion event. Red bars, mean. One-way ANOVA and multiple comparison tests, p value as indicated. N= 16-19 islets.
G) Correlation of Glu-tubulin intensity (normalized to islet average) to the number of secretion clusters per cell in whole islets. Grey field, intensity below islet average (<1). Yellow field, intensity above islet average (>1). The same data set as in Figure 2F-H.
H) The number of clusters in cells with Glu-tubulin intensity below islet average and those above islet average is compared in the graph. Mann-Whitney non-parametric comparison test p-value is shown. N=98 cells from 5 islets. The same data set as in Figure 2F-H.
I) Correlation of Glu-tubulin intensity (normalized to islet average) to the number of clustered secretion events per cell in whole islets. Grey field, intensity below islet average (<1). Yellow field, intensity above islet average (>1). The same data set as in Figure 2F-H.
J) The number of clustered events in cells with Glu-tubulin intensity below islet average and those above islet average is compared in the graph. Mann-Whitney non-parametric comparison test p-value is shown. N=98 cells from 5 islets. The same data set as in Figure 2F-H.
A

Size 2
Size 3
Size 4
Size 5

B

Ctrl Low

C

Noc Low

D

Taxol Low
Figure 3- figure supplement 1. 3+ event clusters are not spurious and a very rare in basal glucose conditions. Corresponds to Figure 3.

A) Computationally simulated random secretion events in *in silico* cells using the mean area of the cells analyzed.

B-D) Representative images of output from Matlab script (see materials and methods) showing cell outlines (black lines) and secretion events (dots). Black dots are non-clustered secretion events, colored dots are clustered secretion events, each different color denotes a different cluster. Clusters were defined as 3+ secretion events occurring with 9 pixels (1.44 µm) by density-based scanning. Islets were pre-treated with DMSO (control, B), nocodazole (C) or taxol (D) and were incubated in 2.8 um glucose.
Figure 4. MT-disruption increases the number of hot spots per cell, increasing clustered secretion.

A) Representative images of clusters from one cell in a control islet stimulated with 20 mM glucose. Clusters were identified by Matlab script (see materials and methods). First image is time projection through all clusters in the cell 15.925- 194.675 s of the movie, clusters are identified by red text. Time in seconds of each event in the cluster above, red arrowheads identify the secretion event. Scale bar 5 µm.

B) Representative images of clusters from one cell in a nocodazole pre-treated islet stimulated with 20 mM glucose. Clusters were identified by Matlab script (see materials and methods). First image is time projection through all clusters in the cell 54.65- 407.875 s of the movie, clusters are identified by red text. Time in seconds of each event in each cluster above, red arrowheads identify the secretion event. Scale bar 5 µm.

C) Graph of clusters per cell in the field of view, with all cells whether activated during the movie or not. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 495-637 cells from 16-19 islets.

C') Cells from panel C, graphed as a stacked histogram of the percentage of total cells per condition that had each number of clusters.

D) Graph of clusters per cell only including cells with at least one secretion event during the duration of the movie. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 88-407 cells from 16-19 islets.

D') Cells from panel E, graphed as a stacked histogram of the percentage of cell with secretion events per condition that had each number of clusters.

E) Graph of clusters per cell only cells with at least cluster during the duration of the movie were included. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 13-143 cells from 16-19 islets.

E') Cells from panel D, graphed as a stacked histogram of the percentage of cell with clusters per condition that had each number of clusters.

F) Graph of events per cell with at least one secretion event during the movie that were not in a cluster. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, N= 88-407 cells from 16-19 islets.

F') Graph of events per cell with at least one secretion event during the movie that were in a cluster. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, N= 88-407 cells from 16-19 islets.
Figure 5. Increased secretion from clusters leads to faster secretion at that site.

A) Graph of events per cluster, Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests found no statistical differences between conditions N= 14-290 clusters from 16-19 islets.

A') Clusters from panel F, graphed as a stacked histogram of the percentage of clusters per condition with each number of events.

B) Histogram of the time between successively (in time) occurring non-clustered events with the best fit exponential overlaid (KS-statistic is provided for quality of fit).

C) Histogram of the time between successively occurring clustered events with the best fit exponential overlaid (KS-statistic is provided for quality of fit).

D) Graph of time between successive events distribution for clusters of different sizes (Red bar = mean). Some conditions lack clusters of particular size (e.g. no clusters with 6 secretion events in Ctrl low), as indicated by no data.

E) Each distribution in (D) is fit separately to an exponential distribution and quality of fit is assessed with a KS-test (as in panels B, C). The resulting p-value for every test is plotted, with the black line indicating p=0.05, below which the lack of fit is significant.

F) Results of fitting a generalized linear model to the data from (D) (see Methods for further details) with the assumption that “secretion rate = \( \alpha + \beta \times \text{Cluster Size} \)”. Bayesian credible intervals for \( \beta \) are plotted for each condition. This model is also compared to a null model where “secretion rate = \( \alpha \)” (i.e. lacking size dependence), with model comparison results reported as the difference of WAIC scores (\( \Delta w \), positive indicates the full model provides a better accounting of the data).
Figure 6. MTs restrict secretion from hot spots to the first phase of secretion, loss of MTs lengthen this phase.

A) Histogram of secretion events over time. Graphed as percentage of cells within each bin per condition. Time (seconds) since dye and either high (20 mM) or low (2.8 mM) glucose addition. Bin=30 seconds. N= 16-19 islets.

B) Histogram of secretion events over time separated into secretion events not in clusters (black) and in clusters (red). Time (seconds) since dye and either high (20 mM) or low (2.8 mM) glucose addition. Bin=30 seconds. N= 16-19 islets.

C) Histogram of the first event in a cluster (black) and last event in a cluster (red) over. Time (seconds) since dye and either high (20 mM) or low (2.8 mM) glucose addition. Bin=30 seconds. N= 16-19 islets.

D) Scatterplot of each cluster in each condition with the timing of the first event in a cluster on the x-axis and the timing of the last event in a cluster on the y-axis. Time (seconds) since dye and either high (20 mM) or low (2.8 mM) glucose addition. Bin=30 seconds. N= 16-19 islets.
Figure 6- figure supplement 1. Clustered secretion is mostly restricted to the first phase of secretion in control islets. Corresponds to Figure 5.

A) Histogram of secretion events over time separated into secretion events not in clusters (black) and in clusters (red). Graphed as percentage of cells within each bin per condition. Time (seconds) since dye and either high (20 mM) or low (2.8 mM) glucose. Bin=30 seconds. N= 16-19 islets.
Figure 7. Calcium signaling is essential for secretion regardless of MT presence.
A) GSIS as detected by ELISA. Secretion over 30 minutes upon 20mM glucose stimulation is shown for DMSO control and nocodazole pre-treated cells in the presence and absence of 5µM Diazoxide. Tukey's multiple comparisons test p values shown.

B-C) Time frames from attached islets treated with DMSO (B) and nocodazole (C) and incubated with Calbryte 520 (cyan). Time after 20MM glucose stimulation, seconds. Red, mApple (β-cell marker). Single plane spinning disk confocal microscopy images. Dotted line outlines indicate representative β cells with detectable concentration of Calbryte 520 used for analyses. Scale bar, 10 µm.

D) Graph of highest amplitudes of Calbryte 520 intensity fluctuation per cell, measured in data as in Figure 7 – Figure Supplement 1. Mann-Whitney non-parametric comparison test p-value is shown. N= 78-102 cells from 8-12 islets.

E) Summarized increase of Calbryte 520 intensity over the first minute of glucose stimulation per cell, measured in data as in Figure 7 – Figure Supplement 1. Mann-Whitney non-parametric comparison test p-value is shown. N= 83-102 cells from 9-12 islets.
Figure 7 - Supplement Figure 1. Calcium influx over time in glucose-stimulated islets.

A-B) Analysis of a representative experiment (one islet isolation) summarized in Figure 7D,E. Each curve represents Calbryte 520 intensity dynamics of a single β cell, 1 minute before and 1 minute after the fluctuations start. Each graph combines cells from one islet, either control DMSO- (A) or nocodazole-pretreated (B). Fold increase over mean intensity at the time of stimulation is shown. Time in min after stimulation is shown. Time, minutes.
Figure 8. MTs regulate glucose-stimulated secretion in addition to calcium-dependent mechanisms.

A-D) KCl- (A, B) and KCl+Glucose-stimulated (C, D) secretion as detected by FluoZin-3 assay in DMSO- (A, C) and nocodazole (B, D) pre-treated islets. Representative output images from Matlab script (see materials and methods) shows cell outlines (black lines) and secretion events (dots) over 2.5 minutes after stimulation. Black dots are non-clustered secretion events, colored dots are clustered secretion events.

E) Graph of KCl-induced secretion events per cell over 2.5 minutes detected by FluoZin-3 assay (data as in (A-D)). Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 306-708 cells from 15-17 islets.

F) Graph of the percentage of cells in each field of view with at least one cluster in KCl-induced secretion over 2.5 minutes. Red bar, mean. One-way ANOVA and multiple comparison tests, p value as indicated, N= 15-17 islets.

G) Graph of the percentage of cells in each field of view with at least one cluster out of cells with at least one secretion event. KCl-induced secretion over 2.5 minutes. Red bar, mean. One-way ANOVA and multiple comparison tests, p value as indicated, N= 15-17 islets.

H) Number of secretion events per cluster. KCl-induced secretion over 2.5 minutes. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, no significant difference, N= 109-269 clusters from 306-708 cells from 15-17 islets.

I) Graph of clusters per cell in the field of view. KCl-induced secretion over 2.5 minutes. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 306-708 cells from 15-17 islets.

I’) Cells from panel I, graphed as a stacked histogram of the percentage of total cells per condition that had each number of clusters.

J) Graph of clusters per cell only including cells with at least one secretion event. KCl-induced secretion over 2.5 minutes. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p values as indicated, 218-334 cells from 15-17 islets.

J’) Cells from panel J, graphed as a stacked histogram of the percentage of cell with secretion events per condition that had each number of clusters.

K) Graph of clustered events per cell. KCl-induced secretion over 2.5 minutes. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p values as indicated, 306-708 cells from 15-17 islets.

K’) Graph of non-clustered events per cell. KCl-induced secretion over 2.5 minutes. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p values as indicated, 306-708 cells from 15-17 islets.

L) Graph of clustered events per cell out of cells with at least one secretion event. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p values as indicated, 218-334 cells from 15-17 islets.

L’) Graph of non-clustered events per cell out of cells with at least one secretion event. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p values as indicated, N= 218-334 cells from 15-17 islets.
Figure 8 - Supplemental Figure 1. Rapid secretion induction by KCL as compared to glucose.

A) Graph of glucose, KCl- and KCl+Glucose-stimulated secretion events per cell over 8 minutes in DMSO- and nocodazole pre-treated islets. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 306-708 cells from 13-17 islets.

B) Graph of clusters per cell in the field of view shown as a stacked histogram of the percentage of total cells per condition that had each number of clusters. Secretion over 8 minutes. N= 306-708 cells from 13-17 islets.

C) Number of secretion events per cluster over 8 minutes. Red bar, mean. Kruskal-Wallis test non-parametric and multiple comparison tests, no significant difference, N= 72-342 clusters from 306-708 cells/13-17 islets.

D) Graph of time between successive events in clusters over 8 minutes (Red bar = mean). Kruskal-Wallis test non-parametric and multiple comparison tests, p value as indicated, N= 72-342 clusters from 306-708 cells/13-17 islets.

E) Histogram of secretion events over time (8 minutes). Graphed as percentage of cells within each bin per condition. Time (seconds) after stimulation (20mM glucose, 25mM KCl, or combination of both, as indicated). Bin=30 seconds. N= 13-17 islets.
Figure 9. MT regulation of secretion at a hot-spot.
A) Glucose signaling stimulates both insulin granule docking and calcium influx; calcium influx, in turn, promotes secretion of docked granules. MT-dependent transport negatively regulates the process of docking, restricting the number of readily-releasable granules and secretion outcome.
B) KCl treatment causes extreme calcium influx, which in turn facilitates secretion of pre-docked granules. No additional docking occurs in the absence of glucose stimulation, and MT regulation does not influence the number of pre-docked granules and secretion levels.