

## **Beta Cell Hubs Dictate Pancreatic Islet Responses to Glucose.**

Johnston, NR; Mitchell, RK; Haythorne, E; Pessoa, MP; Semplici, F; Ferrer, J; Piemonti, L; Marchetti, P; Bugliani, M; Bosco, D; Berishvili, E; Duncanson, P; Watkinson, M; Broichhagen, J; Trauner, D; Rutter, GA; Hodson, DJ

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1 **Title:** Beta cell hubs dictate pancreatic islet responses to glucose

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3 **Authors:** Natalie R. Johnston<sup>1</sup>, Ryan K. Mitchell<sup>1</sup>, Elizabeth Haythorne<sup>1</sup>, Maria Paiva Pessoa<sup>1</sup>,  
4 Francesca Semplici<sup>1</sup>, Jorge Ferrer<sup>2</sup>, Lorenzo Piemonti<sup>3</sup>, Piero Marchetti<sup>4</sup>, Marco Bugliani<sup>4</sup>,  
5 Domenico Bosco<sup>5</sup>, Ekaterine Berishvili<sup>5</sup>, Philip Duncanson<sup>6</sup>, Michael Watkinson<sup>6</sup>, Johannes  
6 Broichhagen<sup>7</sup>, Dirk Trauner<sup>7</sup>, Guy A. Rutter<sup>1\*</sup> and David J. Hodson<sup>1,8,9\*</sup>

7  
8 **Affiliations:** <sup>1</sup>Section of Cell Biology and Functional Genomics, Division of Diabetes,  
9 Endocrinology and Metabolism, Department of Medicine, Imperial College London, London  
10 W12 0NN, UK. <sup>2</sup>Beta Cell Genome Regulation Lab, Department of Medicine, Imperial College  
11 London, London W12 0NN, UK. <sup>3</sup>Diabetes Research Institute (HSR-DRI), San Raffaele  
12 Scientific Institute, Via Olgettina 60, 20132 Milan, Italy. <sup>4</sup>Department of Clinical and  
13 Experimental Medicine, Islet Cell Laboratory, University of Pisa, Pisa, Italy. <sup>5</sup>Cell Isolation and  
14 Transplantation Center, Department of Surgery, Geneva University Hospitals and University of  
15 Geneva, Geneva, Switzerland. <sup>6</sup>School of Biological and Chemical Sciences, Queen Mary  
16 University of London, Mile End Road, London, E1 4NS, UK. <sup>7</sup>Department of Chemistry,  
17 Ludwig-Maximilians-Universität München, and Munich Center for Integrated Protein Science,  
18 Butenandtstrasse 5-13, 81377 München, Germany. <sup>8</sup>Institute of Metabolism and Systems  
19 Research (IMSR), University of Birmingham, Edgbaston, B15 2TT, UK. <sup>9</sup>Centre for  
20 Endocrinology, Diabetes and Metabolism, Birmingham Health Partners, Birmingham, B15 2TH,  
21 UK.

22  
23  
24 \*Correspondence to:

25 [d.hodson@imperial.ac.uk](mailto:d.hodson@imperial.ac.uk)

26 [d.hodson@bham.ac.uk](mailto:d.hodson@bham.ac.uk)

27 [g.rutter@imperial.ac.uk](mailto:g.rutter@imperial.ac.uk)

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1 **HIGHLIGHTS**

- 2 • Optogenetic and photopharmacological targeting reveals a pacemaker-like beta cell  
3 subpopulation  
4 • These cells, termed hubs, are required for normal insulin release  
5 • Photopainting demonstrates that hubs are highly metabolic and transcriptionally immature  
6 • Hubs are targeted by a diabetic milieu to induce islet failure  
7

8 **eTOC Blurb**

9 Using advanced imaging approaches, Johnston et al show that a few (1-10%) beta cells exert  
10 disproportionate control over islet responses to glucose. These specialized cells, termed hubs, are  
11 partly immature and highly metabolic. Their failure during type 2 diabetes mellitus may lead to  
12 reduced insulin secretion and impaired glucose homeostasis.  
13

14 **SUMMARY**

15 The arrangement of beta cells within islets of Langerhans is critical for insulin release through the  
16 generation of rhythmic activity. A privileged role for individual beta cells in orchestrating these  
17 responses has long-been suspected, but not directly demonstrated. We show here that the beta cell  
18 population *in situ* is operationally heterogeneous. Mapping of islet functional architecture  
19 revealed the presence of hub cells with pacemaker properties, which remain stable over recording  
20 periods of 2-3 hours. Using a dual optogenetic/photopharmacological strategy, silencing of hubs  
21 abolished coordinated islet responses to glucose, whereas specific stimulation restored  
22 communication patterns. Hubs were metabolically-adapted and targeted by both pro-  
23 inflammatory and glucolipotoxic insults to induce widespread beta cell dysfunction. Thus, the  
24 islet is wired by hubs, whose failure may contribute to type 2 diabetes mellitus.  
25

1 **INTRODUCTION**

2 The release of insulin from pancreatic beta cells is necessary for proper glucose homeostasis in  
3 mammals. Beta cells respond to glucose with increased oxidative metabolism, elevations in  
4 cytosolic ATP/ADP ratio and closure of ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels (Rutter et al., 2015).  
5 The consequent plasma membrane depolarization activates voltage-dependent  $Ca^{2+}$  channels  
6 (VDCC), leading to  $Ca^{2+}$  influx and exocytosis of secretory granules (Rutter et al., 2015).

7 The three-dimensional organization of beta cells is also important for the normal  
8 regulation of insulin secretion. Thus, beta cells throughout the islet microorgan display rhythmic  
9 activity patterns in the presence of high glucose (Benninger et al., 2008; Santos et al., 1991). A  
10 role for specialized beta cells in orchestrating these dynamics has long been postulated, including  
11 the presence of putative ‘pacemakers’ (Ämmälä et al., 1991; Benninger et al., 2014; Squires et al.,  
12 2002). Indeed, isolated beta cells possess discrete metabolic characteristics and secretory profiles  
13 (Katsuta et al., 2012; Kiekens et al., 1992; Salomon and Meda, 1986), and phase lags in the onset  
14 of electrical activity can be detected between distant islet regions (Benninger et al., 2008; Meda et  
15 al., 1984; Palti et al., 1996). More recent studies have revealed functional differences between  
16 hundreds of individual beta cells monitored *in situ* in the intact islet (Hodson et al., 2013; Li et  
17 al., 2011; Stozer et al., 2013). Such heterogeneity may be relevant for type 2 diabetes  
18 pathogenesis, since specific insults might target single cells or defined islet regions to induce  
19 insulin secretory failure. However, whether particular subsets of cells drive the behavior of others  
20 has so far been difficult to prove empirically.

21 Over the past decade, optogenetics has allowed reversible control of neuronal activity  
22 with light (Zhang et al., 2007). In parallel, photopharmacology has harnessed the power of  
23 azobenzene photoresponsive units to produce exogenously-applied compounds that turn ion  
24 channels and G-protein-coupled receptors into endogenous photoswitches (Broichhagen et al.,  
25 2015). As both these approaches are applicable to electrically-excitabile endocrine tissue  
26 (Broichhagen et al., 2014; Reinbothe et al., 2014), they afford the unique opportunity to precisely  
27 manipulate cell activity with high spatiotemporal fidelity. Using all-optical interrogation of  
28 individual beta cells *in situ*, we therefore set out to probe the topology that regulates population  
29 glucose responsiveness, with the aim of identifying the islet-resident pacemaker.

30  
31

## 1 RESULTS

### 2 Hubs are a feature of beta cell population dynamics

3 To visualize the large-scale organization of beta cell activity underlying calcium ( $\text{Ca}^{2+}$ )-  
4 dependent exocytosis of insulin granules, intact mouse islets were subjected to high-speed (2-8  
5 Hz) multicellular  $\text{Ca}^{2+}$ -imaging (Hodson et al., 2012). This was combined with Monte Carlo-  
6 based correlation analyses in which repeated shuffling of  $\text{Ca}^{2+}$ -events ( $> 9999$  iterations) is used  
7 to determine whether cells are coordinated due to chance or not (*i.e.* contributing to the same  
8 insulin release event). Together, these approaches allow online mapping of the islet functional  
9 circuitry. Initial experiments confirmed that beta cells form a scale-free network (Stozer et al.,  
10 2013) that supports the synchronous propagation of glucose (11 mM)-stimulated  $\text{Ca}^{2+}$  waves by  
11 efficiently connecting distant islet regions ( $R^2 = 0.72$ ; Fig. 1A). Scale-free networks are  
12 ubiquitous throughout biology, are identified by their power-law link-probability distribution  
13 (Hodson et al., 2010), and adopt a hub and spoke formation where a few cells possess the  
14 majority of connections. Accordingly, a stereotypical feature of such topology in islets was the  
15 non-random appearance of rare super-connected hubs, whose firing activity tended to repetitively  
16 precede and outlast that of the remainder of the population (*i.e.* was pacemaker-like) (Fig. 1B and  
17 C) (Movie S1).

18 Such islet architecture was dependent on information exchanges through gap junctions,  
19 since reversible blockade of connexin channels using  $18\alpha$ -glycyrrhetic acid (AGA) (Farnsworth  
20 et al., 2014) reduced the number of hubs, decreased coordinated population activity, and  
21 increased signal propagation path length (Fig. 1D-G). This may reflect the inability to identify  
22 hubs due to loss of cell-cell entrainment, as well as re-routing of information over longer  
23 distances by the remaining hubs. Notably, no differences in the amplitude of  $\text{Ca}^{2+}$  rises were seen  
24 in control and AGA-treated tissue (Fig. S1A-C), suggesting minimal impact upon VDCC activity.  
25 In all cases, parallel experiments were performed using glycyrrhizic acid (BGA), the inactive  
26 precursor of AGA that exerts similar non-specific effects (Desarmenien et al., 2013). Results  
27 could be replicated using mebeverine (Farnsworth et al., 2014) (Fig. S1D), a gap junction  
28 inhibitor with no reported effects on VDCC or  $\text{K}_{\text{ATP}}$  channel activity, as well as *Gjd2* shRNA to  
29 specifically silence connexin 36 at the islet surface (Fig. S1E-H).

### 31 Hubs are stable and present across species

32 To assess network topology stability, islets were recorded and then left on the microscope for  
33 between 30 mins and 3 hrs before re-recording. Network topology was stable both over time and  
34 in response to perturbation, as statistically assessed *versus* a third experiment subjected to either  
35 randomization (*i.e.* to re-distribute the wiring pattern) or enforced-dissimilarity (*i.e.* to form a  
36 different wiring pattern) (Fig. 1H and I). Network indices were unaffected in the presence of  
37 either a specific glucagon receptor antagonist (Fig. S1I and J) or a glucagon-neutralizing antibody  
38 (Fig. S1K and L), suggesting that any glucagon present *in vitro* is unlikely to influence hub  
39 function. Hinting at a conserved role for hub architecture, islet functional topologies were similar  
40 in glucose-stimulated mouse and human islets, as shown by the similar link-probability  
41 distributions (*i.e.* both are fitted with a power-law of near-identical exponent value). However,  
42 synchrony tended to be compartmentalized into subregions/clusters in human islets (Fig. 1J), in  
43 line with the different structural arrangement of beta- *versus* alpha-cells in this species (Bosco et  
44 al., 2010). Beta cell  $\text{Ca}^{2+}$  responses were not dependent on orientation toward the islet center or  
45 periphery ( $\Delta Y \text{ Fluo2} = 0.14 \pm 0.01$  *versus*  $0.13 \pm 0.004$  AU, periphery *versus* center,  
46 respectively), and identical results were obtained using the genetically-encoded indicator  
47 GCaMP6 (Fig. 1K and L), engineered to interfere less with intracellular  $\text{Ca}^{2+}$  levels.

### 49 A strategy for all-optical interrogation of beta cell function

50 To functionally dissect the role of hubs, an optogenetic strategy was developed and validated,  
51 enabling electrical silencing following *Ins1Cre*-directed expression of the light-gated chloride  
52 ( $\text{Cl}^-$ ) pump halorhodopsin (eNpHR3.0) (Zhang et al., 2007) in beta cells (Fig. 2A and B). This

1 approach allowed the reversible silencing of single beta cell or population  $\text{Ca}^{2+}$ -spiking activity  
2 and extracellular  $\text{Ca}^{2+}$  influx following illumination ( $\lambda = 560\text{-}590\text{ nm}$ ) (Fig. 2C-G) (Movies S2-  
3 4). Application of the depolarizing agent potassium chloride was able to overcome silencing by  
4 restoring VDCC activity (Fig. 2H). Of note, wild-type beta cells were refractory to silencing (Fig.  
5 2I and J), and eNPHR3.0-expressing beta cells under irradiation were not further hyperpolarized  
6 using diazoxide to force open  $\text{K}_{\text{ATP}}$  channels (Fig. 2K). As measured using patch-clamp  
7 electrophysiology, illumination induced photocurrents (Fig. 3A), leading to membrane  
8 hyperpolarization and electrical silencing only in eNPHR3.0-expressing beta cells (Fig. 3B-D).  
9 Thus, specific and powerful optogenetic silencing could be achieved.

10 Animals harboring a single eNpHR3.0 allele unexpectedly demonstrated improved  
11 glucose tolerance compared to wild-type littermates, despite normal insulin sensitivity (Fig. 4A-  
12 F) and body weight (Fig. 4G and H). This was probably due to enhanced *in vivo* insulin secretion  
13 (Fig. 4I), as beta cell mass was apparently normal (Fig. 4J). Activation of eNpHR3.0 on an  
14 Ins1Cre background also led to similar results, suggesting that alternation in insulin gene dosage  
15 in the context of the transgene was unlikely to be a contributing mechanism (Fig. 4K and L).  
16 Pertinent to the *in vitro* studies here, however, isolated islets responded normally to glucose in  
17 terms of ionic fluxes and insulin release (Fig. S2A-I), and eNpHR3.0 does not possess basal  
18 activity in the absence of light (Zhang et al., 2007) (also shown in Fig. 3C).

### 19 Hubs orchestrate beta cell population responses to glucose

20 By performing analysis in real-time using islets maintained on the microscope stage, hubs could  
21 be identified and subsequently manipulated (Fig. 5A-C). Silencing of individual hubs using a  
22 pinpointing laser had catastrophic consequences for coordinated islet responses to high glucose  
23 (Fig. 5D and E) (Movies S5-6), an effect reversed simply by ceasing illumination (Fig. 5F and  
24 G). The strength of inhibition following targeting of individual hubs tended to be inversely  
25 associated with the number of these cells per islet before silencing (Fig. S2J), suggesting that  
26 some redundancy is present in the system, most likely due to follower cells being controlled by  
27 more than one hub. By contrast, silencing of individual non-hub or follower cells did not  
28 significantly perturb islet dynamics (Fig. 5H), demonstrating the specificity of the approach.

29 Using a similar technique, hubs were firstly identified at high glucose, before inactivation  
30 using low glucose and stimulation with JB253, an exogenously-applied  $\text{K}_{\text{ATP}}$  channel photoswitch  
31 based on glimepiride (Broichhagen et al., 2014). Following targeted illumination of JB253-  
32 treated islets, hub connectivity could be mimicked without activation of intervening cells, as  
33 determined by the presence of glucose- and gap junction-dependent entrainment patterns in  
34 follower cells (conduction velocity =  $47.0 \pm 8.9\ \mu\text{m/s}$ ) (Fig. 5I-K). Such effects were unlikely to  
35 stem from diffusion of active JB253, since this molecule turns off within milliseconds in the dark  
36 (Broichhagen et al., 2014), and proximate cells remained unaffected by hub stimulation (Fig. S3).

### 37 Hubs are required for insulin secretion

38 We were unable to measure insulin secretion accurately from a single islet over the 5 min  
39 experimental period used here, since levels were below the detection sensitivity of current assays.  
40 Therefore, to link hub activity with hormone release, the cell-surface-attached fluorescent  $\text{Zn}^{2+}$   
41 probe JP-107 (Pancholi et al., 2014) was instead employed as a surrogate to dynamically report  
42  $\text{Zn}^{2+}$  co-released with insulin from cells at the islet surface, as previously reported with ZIMIR  
43 (Li et al., 2011). Using this approach, silencing of follower cells or wild-type islets was without  
44 effect, as evidenced by a linear increase in fluorescence due to  $\text{Zn}^{2+}$  accumulation at the probe.  
45 By contrast, hub shutdown or global illumination lowered insulin/ $\text{Zn}^{2+}$  release to below the  
46 dissolution rate of the probe (*i.e.*  $\text{Zn}^{2+}$  binding is lower than  $\text{Zn}^{2+}$  removal) (Fig. 5L).

47 While it was not technically possible to directly link hub activity with pulsatile insulin  
48 release, the acetylcholinomimetic carbachol (Zhang et al., 2008a) was able to accelerate beta cell  
49 population activity (Fig. S3E), without altering the proportion of links or hubs (Fig. S3F, G).  
50 Moreover, rapid imaging performed over dozens of minutes- *i.e.* within the range of insulin  
51 secretion.

1 pulses (Head et al., 2012)- revealed that hubs are also a feature of population behavior over  
2 longer periods (proportion hubs =  $7.1 \pm 1.3\%$ ; proportion links =  $9.7 \pm 2.0\%$ ). Since carbachol  
3 has been shown to phase-set activity between islets *in vitro* (Zhang et al., 2008a),  
4 parasympathetic neurons may plausibly target hubs *in vivo* to synchronize islet activity and  
5 generate insulin pulses.

### 7 Hubs possess a characteristic metabolic signature

8 We next sought to understand what makes a hub cell unique. Islet-wide  $\text{Ca}^{2+}$  signals were  
9 recorded before metabolic profiling of the hub population in the same islet using the  
10 mitochondrial potential dye tetramethylrhodamine ethyl ester (TMRE), which sequesters in  
11 active, hyperpolarized mitochondria. Following stimulation at high glucose, mitochondria in hubs  
12 became more hyperpolarized *versus* those in non-hubs (Fig. 6A and B), suggesting increased  
13 proton-pumping, ATP synthase activity and ATP generation (Tarasov et al., 2012). While the  
14 duty cycle (*i.e.* proportion of time the cell spends 'ON') was slightly increased in hubs compared  
15 to non-hubs (Fig. 6C), other activity parameters including  $\text{Ca}^{2+}$ -spiking amplitude and frequency  
16 were broadly similar (Fig. 6D-F). Spatially, hubs and non-hubs were intermingled, with no clear  
17 preference for the islet center or periphery detected for either population based on polar  
18 coordinates (angle and distance taken from the islet center) (Fig. 6G and H).

### 20 Hubs display features of both mature and immature beta cells

21 Using photoactivatable Tag-RFP (PA-TagRFP) to photopaint single hubs within islets using a  
22 405 nm laser (Fig. S4A and B), post-hoc immunostaining against a variety of markers of beta cell  
23 'identity' (Rutter et al., 2015) could be performed (Fig. 6I), without adversely altering  $\text{Ca}^{2+}$   
24 dynamics (Fig. S4C). These studies revealed reduced insulin content, increased glucokinase  
25 (GK/*Gck*) levels, lowered expression of pancreatic duodenum homeobox-1 (*Pdx1*), but normal  
26 levels of the mitochondrial import receptor subunit Tom20 in hubs *versus* the rest of the  
27 population (Fig. 6I and J) (Fig. S5). The transcription factor Nkx6.1, recently shown to be  
28 required for insulin biosynthesis and beta cell proliferation (Taylor et al., 2013), was almost  
29 absent from hubs (Fig. 6I and J). Suggesting that hubs are unlikely to represent a multihormonal  
30 (*e.g.* Glu+, Ins+) population, no co-localization with glucagon was detected (Fig. 6K). Likewise,  
31 neurogenin-3, a beta cell precursor marker, was undetectable at the protein level in the adult islet,  
32 implying that hubs are unlikely to be trapped in a progenitor state (Fig. 6K). Inspection of  
33 oversampled and deconvolved superresolution confocal images revealed no differences in  
34 mitochondrial distribution/shape or endoplasmic reticulum content in hubs (Fig. 6L-P), although  
35 expression of the sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$ /ATPase, SERCA2, was markedly reduced  
36 (Fig. 6O and P).

37 Suggesting a hyposecretory (or degranulated) nature, insulin granule numbers were lower  
38 in hubs *versus* non-hubs, despite a similar distribution (Fig. 6Q and R). Furthermore, the area of  
39 individual hub cells was comparable to the rest of the population (range =  $122\text{-}381 \mu\text{m}^2$  and  $194\text{-}$   
40  $355 \mu\text{m}^2$ , non-hubs *versus* hubs, respectively), and their shape appeared to be normal.  
41 Consequently, hubs constitute a metabolically-adapted, repurposed subpopulation of beta cell  
42 which displays features of immature cells.

### 44 Hubs are targeted by diabetic milieu

45 Lastly, the robustness of hubs was determined by challenging islets with cytokine cocktails (IL-  
46  $1\beta$ /IL-6, or IL- $1\beta$ /TNF- $\alpha$ ) to re-create the pro-inflammatory milieu thought to be associated with  
47 diabetes (O'Neill et al., 2013). Acutely, the application of cytokines led to a large ramp-up in  $\text{Ca}^{2+}$   
48 spiking activity in the presence of high glucose (Fig. S6). However, after only 2 h incubation, a  
49 collapse in hub cell number was apparent (Fig. 7A and B), and this could be viewed in real-time  
50 by recording the same islet left *in situ* before and during exposure to cytokine (IL- $1\beta$ /IL-6) (Fig.  
51 7C and D). The cytokine-induced disruption to hub cell function was further evidenced by a  
52 reduction in the number of cells occupying the upper or 'high connectivity' region of the link-

1 probability distribution (Fig. 7E and F), as shown by a decrease in the exponent value of the  
2 power-law fit. This resulted in a dramatic decline in correlated beta cell population function (Fig.  
3 7G) due to the presence of fewer and less well-connected hubs. The actions of cytokines were not  
4 explained by effects on cell viability, as assessed using indices of necrosis (Fig. 7H and I) and  
5 apoptosis (Fig. 7J). However, 2 h cytokine exposure decreased mRNA levels of the major islet  
6 gap junction isoform connexin 36 (*GJD2*) three-fold (Fig. 7K and L), and this was already  
7 associated with a substantial reduction in gap junction plaque number (Fig. 7M), in line with that  
8 recently reported using a similar paradigm (Farnsworth et al., 2015). Likewise, preferential hub  
9 failure was detected in both rodent and human tissue in response to gluco(lipo)toxic insults (Fig.  
10 7N and O).



## 1 DISCUSSION

2 Beta cells are a phenotypically diverse population, presenting a mosaic of metabolic and  
3 electrical activity patterns (Pralong et al., 1990), which is mirrored at the level of insulin  
4 secretory capacity (Katsuta et al., 2012; Kiekens et al., 1992; Li et al., 2011; Salomon and Meda,  
5 1986). When viewed as a population, beta cells are often termed a functional syncytium, although  
6 a role for cell heterogeneity in generating multicellular dynamics has been invoked repeatedly  
7 (Benninger and Piston, 2014; Stozer et al., 2013). Indeed, it has been shown that a subset (~10-15  
8 %) of beta cells may exert a disproportionate influence over islet dynamics (Hraha et al., 2014).  
9 By combining large-scale functional cell mapping with optogenetics and photopharmacology, we  
10 provide here a revised blueprint for islet function whereby a few pioneer hubs with reduced beta  
11 cell identity dictate emergent population behavior in response to glucose. Importantly, hub  
12 topologies are a feature of dynamical systems, including cell networks in the brain and pituitary  
13 (Bonifazi et al., 2009; Hodson et al., 2012), since they are functionally robust at a low wiring cost  
14 (Bullmore and Sporns, 2009) (*i.e. the chances of randomly hitting a hub are low*). However,  
15 should a hub be **specifically** targeted, the effects on cell population function are far-reaching, as  
16 observed in the islet during exposure to cytokine or glucolipototoxicity.

17 The present study used a single photon-based confocal system to control the activity of  
18 individual hubs or followers within isolated islets. While two photon approaches in theory  
19 increase the accuracy of cell targeting by restricting the beam to within a few microns of the focal  
20 point, there are drawbacks when used with optogenetics. Firstly, a diffraction-limited two-photon  
21 spot (*i.e. ~ 500 nm*) is insufficient to reliably activate optogenes, and the long excited state  
22 half-time can quickly saturate the rhodopsin (Rickgauer and Tank, 2009). Secondly, commercial  
23 lasers are unable to deliver the > 1100 nm excitation required for eNpHR3.0 activation without an  
24 optical-parametric oscillator (Andresen et al., 2009). By contrast, a single-photon diffraction-  
25 limited laser spot (~ 500 nm) of known absorbance cross-spectrum can be introduced to the  
26 surface of the sample, with minimal aberration and steep power drop-off as a function of  
27  $1/\text{distance}^2$ . Demonstrating the high degree of localization of the effective beam, we were clearly  
28 able to photopaint single cells within an islet, and did not see any population silencing when a  
29 follower cell was targeted.

30 Using patch clamp recordings of dissociated beta cells, eNpHR3.0 activation  
31 hyperpolarized membrane potential by -60 mV, in line with previous reports (Mattis et al., 2012).  
32 While photocurrent size may be underestimated due to the presence of an electrochemical  
33 gradient, it should be noted that halorhodopsin derives energy from photons rather than the ion  
34 gradient itself (Pfisterer et al., 2009), and the photocycle is unaffected even in the presence of  
35 high  $\text{Cl}^-$  concentration (Varo et al., 1995). In any case, it is unlikely that hyperpolarizing spread  
36 throughout the islet *per se* could account for these observations, since: 1) only 30% of voltage  
37 spreads to an immediately coupled cell and an 86 mV depolarizing step is required for activation  
38 *via* gap junctions (Zhang et al., 2008b); and 2) stimulation of follower cells was without effect.  
39 We prefer an explanation whereby large changes in conductance attributable to the hub cell or its  
40 very close neighbors are removed through eNpHR3.0-mediated silencing, leading to impaired  
41 propagation of  $\text{Ca}^{2+}$  waves (Benninger and Piston, 2014; Benninger et al., 2008; Zhang et al.,  
42 2008b). Although membrane potential was slightly more depolarized following cessation of  
43 illumination, this is also seen in neurons (Mattis et al., 2012) and may reflect the reversal  
44 potential of  $\text{Cl}^-$ . We did not notice significant effects on hub indices during the  $\text{Ca}^{2+}$  imaging  
45 studies here due to use of a 5-10 min 'rest' period to allow  $\text{Cl}^-$  re-equilibration.

46 **Experiments in which hub cells were stimulated revealed that hubs and followers are**  
47 **unlikely to form local syncytia.** While the exact mechanisms for antipodal signal propagation are  
48 difficult to determine precisely, a role for 3D chains of electrically-coupled cells is plausible,  
49 given that entrainment was markedly blunted by both gap junction blockade and perfusion with 1  
50 mM glucose. Other communication possibilities include autonomic neurons, which possess > 100  
51  $\mu\text{m}$  axonal arborizations in pancreatic slices (Rodriguez-Diaz et al., 2012), and cilia, which  
52 provide a restricted signaling corridor due to their presence in only ~ 25 % of beta cells (Gerdes

1 et al., 2014). Along these lines, the effect of hub silencing on islet function was surprisingly  
2 strong, given the relatively mild phenotype of animals deleted for the gap junction protein Cx36  
3 (Ravier et al., 2005). However, considerable redundancy exists in the latter model with Cx30.2  
4 and ephrins providing alternative signaling routes (Farnsworth and Benninger, 2014;  
5 Konstantinova et al., 2007).

6 An intriguing possibility is that hubs are related to the previously described Pdx1+,  
7 Ins(low) beta cell subpopulation (Szabat et al., 2009), albeit distinct in their low levels of both  
8 markers. Impaired identity, while conceivably restraining stimulus-induced secretion, may also  
9 limit GK-induced proliferation (Porat et al., 2011; Stolovich-Rain et al., 2015) to maintain the  
10 role of these cells as specialized pacemakers. Indeed, **high levels of GK expression may sensitize  
11 hubs cells to increases in glucose concentration, allowing these cells to respond earlier and more  
12 robustly than their neighbours.** By contrast, the failure of hubs when faced with a  
13 gluco(lipo)toxic/pro-inflammatory milieu indicates that these cells are metabolically fragile. This  
14 vulnerability might reflect high *Gck* (Roma et al., 2015) expression coupled to low Pdx1 and  
15 SERCA2 levels (Fonseca et al., 2011; Fujimoto et al., 2009), which ultimately lead to ER stress  
16 and cell dysfunction.

17 We acknowledge that the hub protein characterization performed here constitutes a biased  
18 screen, but it nonetheless provides a strong foundation for understanding the biology of these  
19 unusual cells. In the future, unbiased multiplex approaches, including massive parallel  
20 sequencing (RNASeq) and CyTOF (single cell mass cytometry) (Proserpio and Lonnberg, 2015),  
21 will help define the hub signature. Although attempts were made to obtain dissociated  
22 cells/cytoplasm for these purposes, PA-TagRFP fluorescence disappeared following dissociation  
23 of islets, possibly reflecting either the fragility of these cells, or the fluorophore itself. Similar  
24 problems were encountered with electron microscopy, where available antibodies cannot  
25 differentiate between activated and non-activated PA-TagRFP.

26 The recording approaches used to monitor hubs were technically-constrained to 2-3 h.  
27 **Indeed, such experiments necessitate leaving the islets *in situ* on the microscope and re-loading  
28 with Ca<sup>2+</sup> indicator, since the same field of view must be maintained for analysis purposes.** Thus,  
29 it cannot be excluded that hubs may represent a transitory subpopulation that drifts over dozens of  
30 hours in line with transcriptional/translational processes. Indeed, modelling studies predict that  
31 ‘pacemakers’ arise from the most excitable beta cell, which is assumed to shift due to a random  
32 distribution of excitability as K<sub>ATP</sub> channel expression levels vary (Benninger et al., 2014).  
33 However, the possibility that such cells may arise during development could not be excluded  
34 (Benninger et al., 2014), and studies in FACS-purified GFP-labelled beta cells suggest the  
35 presence of distinct transcriptional pools, with the proportions remaining similar between animals  
36 and days (Katsuta, Aguayo-Mazzucato et al. 2012). Moreover, to the best of our knowledge, there  
37 is no evidence that K<sub>ATP</sub> channel levels change over time, though the presence of a substantial  
38 proportion of channel subunits on internal membranes (Varadi et al., 2006) may complicate such  
39 measures.

40 **Lastly, it should be noted that experiments in isolated islets may not necessarily reflect  
41 the situation *in vivo*, where blood flow direction (beta cell -> alpha cell) (Nyman et al., 2008) and  
42 molecule access dynamics (Michau et al., 2015) may all affect the role of hubs in dictating  
43 population dynamics and insulin secretion. This possibility might be tested in the future using *in  
44 vivo* imaging approaches (Nyman et al., 2008; Speier et al., 2008).**

45 In summary, the present findings provide new insights into the regulation of islet function  
46 by individual beta cells, and the mechanisms that likely target and impair this during type 2  
47 diabetes pathogenesis and treatment. More generally, the paradigm developed here to study the  
48 roles of individual cells within the functioning islet may be broadly applicable to other tissues or  
49 organisms.

50

## 1 MATERIALS AND METHODS

### 2 **Animals, glucose/insulin tolerance testing and insulin measures**

3 Beta cell-specific expression of halorhodopsin, a light-activated hyperpolarising Cl<sup>-</sup> pump (Zhang  
4 et al., 2007), was achieved by crossing the *Ins1Cre* deleter strain (Thorens et al., 2014) with  
5 animals engineered to express eNpHR3.0-EYFP following excision of a *loxP*-flanked STOP  
6 cassette (B6;129S-Gt(ROSA)26Sortm39(CAG-hop/EYFP)Hze/J). For detailed information see  
7 Supplemental Experimental Procedures.

### 8 **Islet isolation**

9 Animals were euthanized in a rising concentration of CO<sub>2</sub> before laparotomy and injection of  
10 collagenase solution (1mg/ml) into the bile duct (clamped at the duodenal ampulla). Following  
11 incubation for 10 min at 37 °C, digested pancreata were subjected to density gradient purification.  
12 Islets retrieved from the interface between the 1.083 g/ml and 1.077 g/ml layers were cultured for  
13 24-72 h in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% foetal calf  
14 serum, 100 U/mL penicillin and 100 µg/mL streptomycin.

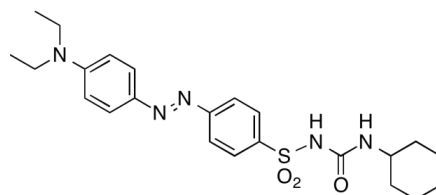
### 15 **Human islet culture**

16 Material from heart-beating donors was obtained from isolation centres in Italy (Pisa and Milan)  
17 and Switzerland (Geneva), with necessary local and national ethical permissions, including  
18 consent from the next of kin. Islets were cultured in RPMI supplemented with 10% foetal calf  
19 serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 mg/mL fungizone, and supplemented  
20 with 5.5 mM *D*-glucose. All studies involving human tissue were approved by the National  
21 Research Ethics Committee London (Fulham), REC #07/H0711/114.

### 22 **Calcium and mitochondrial potential imaging**

23 Multicellular Ca<sup>2+</sup> and mitochondrial potential imaging was performed as detailed (Hodson et al.,  
24 2013). Single cell silencing in eNpHR3.0-expressing tissue was achieved using a λ = 585 nm  
25 laser (300 mW) linked *via* a single-mode fibre optic to a custom-manufactured dichroic array,  
26 configured to deliver a diffraction-limited (~ 500 nm) spot (6.8-23.4 mW/mm<sup>2</sup>) to the focal plane  
27 (Cairn Research). Single cell stimulation was performed using the photoswitchable sulfonylurea  
28 JB253 and a diffraction-limited λ = 470 nm laser. Mitochondrial potential was monitored using  
29 TMRE. For detailed information see Supplemental Experimental Procedures.

30



*trans*-JB253

31

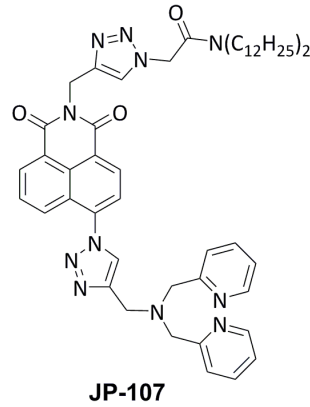
### 32 **Electrophysiology**

33 Pancreatic islets were dissociated into single β-cells and plated onto glass coverslips.  
34 Electrophysiological recordings were performed in either perforated patch-clamp or whole-cell  
35 configuration using an EPC9 patch-clamp amplifier controlled by Pulse acquisition software  
36 (HEKA). For detailed information see Supplemental Experimental Procedures.

### 37 **Dynamic insulin secretion measures**

38 Zinc (Zn<sup>2+</sup>) co-released from insulin-containing granules was measured as a proxy for insulin  
39 secretion using the chemical probe JP-107 (300 µM), as described (Pancholi et al., 2014). For  
40 detailed information see Supplemental Experimental Procedures.

41



1

2 **Generation of adenoviral PA-TagRFP and photopainting**

3 cDNA encoding the photoactivatable fluorescent protein PA-TagRFP (Subach et al., 2010) was  
 4 cloned into pShuttleCMV *via Xho I* and *Xba I* sites before recombination with pAdEasy1 and  
 5 virus production as described (Luo et al., 2007). Islets were incubated for 48 h with adenovirus  
 6 harboring PA-TagRFP at a multiplicity of infection (MOI) = 100. Photopainting was performed  
 7 *post facto* following fMCI and correlation analysis by bounding the identified cell(s) with a  
 8 region of interest (ROI) and scanning the area with a  $\lambda = 405$  nm diode laser (x20/0.8NA  
 9 objective).

10 **shRNA-silencing of connexin 36**

11 For detailed information see Supplemental Experimental Procedures.

12 **Immunohistochemistry**

13 Islets were fixed at 4 °C overnight in paraformaldehyde (4%, wt/vol) before permeabilization  
 14 (PBS + Triton 0.1%) and application of either guinea pig anti-insulin 1:200 (DAKO), mouse anti-  
 15 glucagon 1:1000 (Sigma), rabbit anti-glucokinase 1:50 (Santa-Cruz), rabbit anti-Pdx1 1:50  
 16 (Rafiq, 2000), goat anti-Pdx1 1:2500 (Abcam), mouse anti-neurogenin-3 (Ngn3) 1:1000 (DSHB  
 17 Hybridoma Product F25A1B3), rabbit anti-Tom20 1:250 (Santa Cruz Biotechnology), rabbit anti-  
 18 Nkx6.1 1:250 (Sigma), rabbit anti-SERCA2 1:250 (Alomone Labs) or rabbit anti-PDI 1:200 (Cell  
 19 Signaling Technology) antibodies for 24-48 h at 4°C. Staining was revealed following 2 h  
 20 incubation at room temperature with secondary antibodies conjugated to Alexa Fluor-488, -568  
 21 and -633 (1:500) (Invitrogen). Connexin 36 (Cx36) staining was performed as above, but  
 22 following 10 min fixation in ice-cold acetone and using rabbit anti-Cx36 1:50 (Zymed). To live  
 23 image mitochondria, islets were incubated in 100 nM MitoTracker Red FM (Invitrogen) for 30  
 24 min.

25 **Beta and alpha cell mass**

26 For detailed information see Supplemental Experimental Procedures.

27 **Necrosis and apoptosis assays**

28 For detailed information see Supplemental Experimental Procedures.

29 **Real-time PCR**

30 Relative mRNA abundance was determined on an Applied Biosystems ABI 7500 Fast Real-Time  
 31 PCR System using SYBR Green reagents and primers against connexin 36 (*Gjd2*)  
 32 (GATTGGGAGGATCCTGTTGAC and AGGGCTAGGAAGACAGTAGAG). Gene expression  
 33 was normalised to  $\beta$ -actin (CGAGTCGCGTCCACCC and CATCCATGGCGAACTGGTG) and  
 34 fold-change in mRNA expression compared to control calculated using the  $2^{-\Delta\Delta CT}$ .

35 **Correlation, similarity analyses and polar coordinates**

36 Individual EYFP-expressing beta cells were identified using an ROI to produce a mask overlay of  
 37 the imaged population. Correlation analyses were then performed in MATLAB on Hilbert-Huang  
 38 transformed  $Ca^{2+}$  signals using binarization and matrix analyses, and statistical significance

1 assigned using non-deterministic (Monte-Carlo methods), as described (Hodson et al., 2010;  
2 Hodson et al., 2012). For detailed information see Supplemental Experimental Procedures.

### 3 **Measurements of insulin secretion from isolated islets**

4 Insulin secretion was measured using static incubation of islets (batches of 6-8) for 30 min in  
5 HEPES-bicarbonate buffer at 37°C containing the indicated glucose concentrations (da Silva  
6 Xavier et al., 2009). Insulin concentration in the supernatant was determined using a proprietary  
7 Homogeneous Time Resolved Fluorescence (HTRF)-based assay (Cisbio) according to the  
8 manufacturer's instructions.

### 9 **Cytokines and glucolipotoxicity**

10 Interleukin 1 beta (IL-1 $\beta$ ), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- $\alpha$ ) (all from  
11 R&D Biosystems) were stored as stock solutions at -20 °C and used at 20 pg/ml, 40 pg/ml and 20  
12 pg/ml, respectively (Maedler et al., 2004; O'Neill et al., 2013). For gluco(lipo)toxicity studies,  
13 cells were exposed to 33mM glucose and/or 0.5 mM BSA-conjugated palmitate for 48 h.

### 14 **Statistical analyses**

15 Data normality was assessed using the D'Agostino Pearson omnibus test. Pairwise comparisons  
16 were performed using paired or unpaired Student's t-test. Interactions between multiple  
17 treatments were determined using one-way or two-way ANOVA (adjusted for repeated measures  
18 as necessary), followed by pairwise comparisons with Bonferonni's or Tukey's posthoc tests.  
19 Analyses were conducted using R (R-project), Graphpad Prism 6.0 (Graphpad Software), IgorPro  
20 (Wavemetrics) and MATLAB (Mathworks), and results deemed significant at P<0.05. Unless  
21 otherwise stated, data are presented as the mean  $\pm$  SEM.  
22

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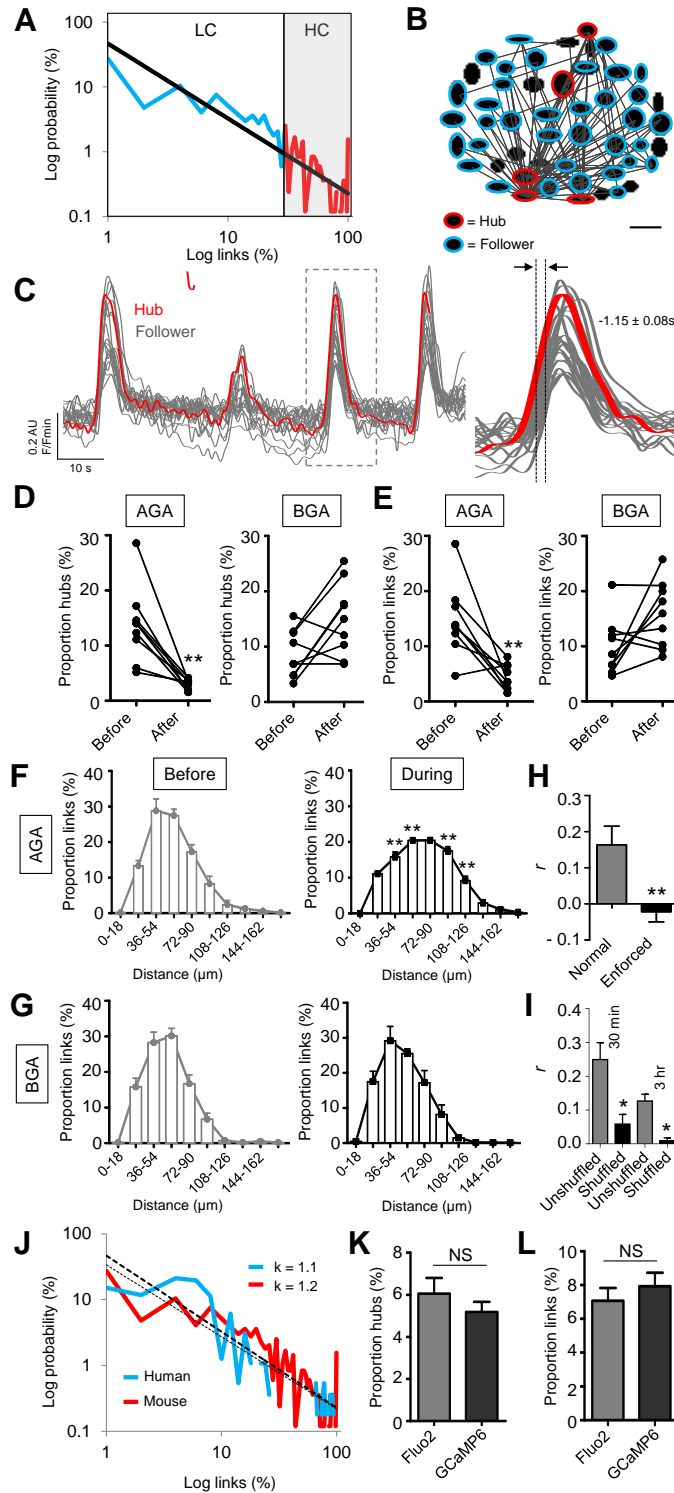
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24 to disclose.

## 25 26 **AUTHOR CONTRIBUTIONS**

27 N.R.J., G.A.R. and D.J.H. conceived and designed the experiments. N.R.J, R.K.M, E.H., M.P.P.,  
28 F.S. and D.J.H. conducted the experiments. J.F. provided reagents and intellectual input. L.P.,  
29 P.M., M.B., D.B., E.B. isolated and provided human islet of Langerhans. P.D., M.W., J.B. and  
30 D.T. designed, synthesized and provided chemical reagents. N.R.J., G.A.R. and D.J.H. wrote the  
31 paper with input from all the authors.

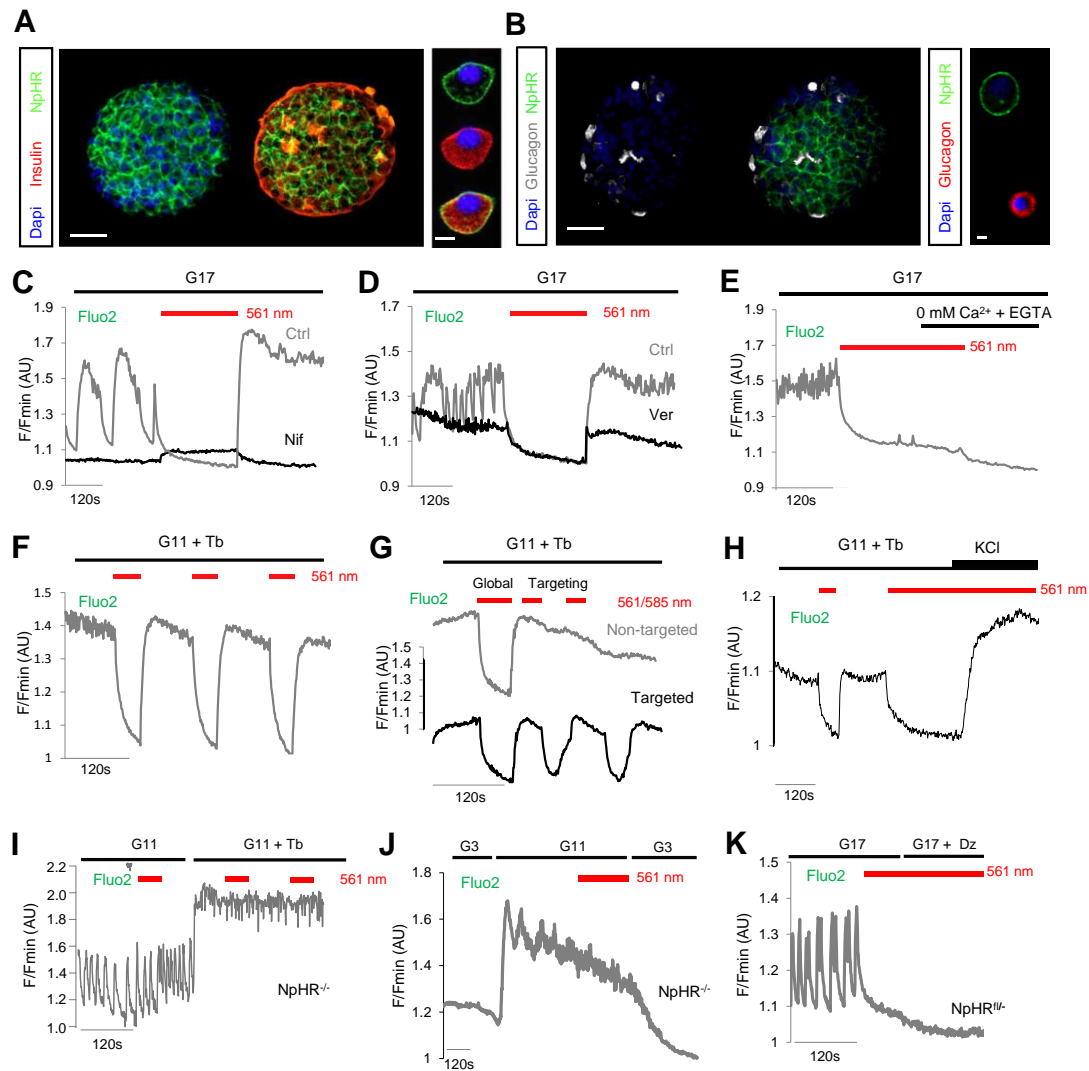


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**Fig. 1. Functional mapping of beta cell population dynamics** (A) At elevated glucose (11 mM), islets house a scale-free network where a few (< 10 %) beta cells host the majority of correlated links ( $P < 0.01$ ), as shown by the power law-fitted probability distribution (LC, low connectivity range; HC, high connectivity range) ( $R^2 = 0.72$ ) ( $n = 12$  recordings from 5 animals). **To better demonstrate the distribution, a log-log scale is used to convert the power-law into a linear relationship.** (B) Representative functional connectivity map displaying the x-y position of analyzed cells and their links (followers, blue; hubs, red) (scale bar = 20  $\mu\text{m}$ ). (C) **Representative trace showing that hub (red) activity tends to precede and outlast that of follower cells (mean lag value calculated from  $n = 5$  recordings from 3 animals).** (D) Treatment of islets with the gap junction blocker 18 $\alpha$ -glycyrrhetic acid (AGA; 20  $\mu\text{M}$ ) (left), but not its inactive analog glycyrrhizic acid (BGA; 20  $\mu\text{M}$ ) (right) reduces the proportion of hubs ( $n = 9$  recordings from 5

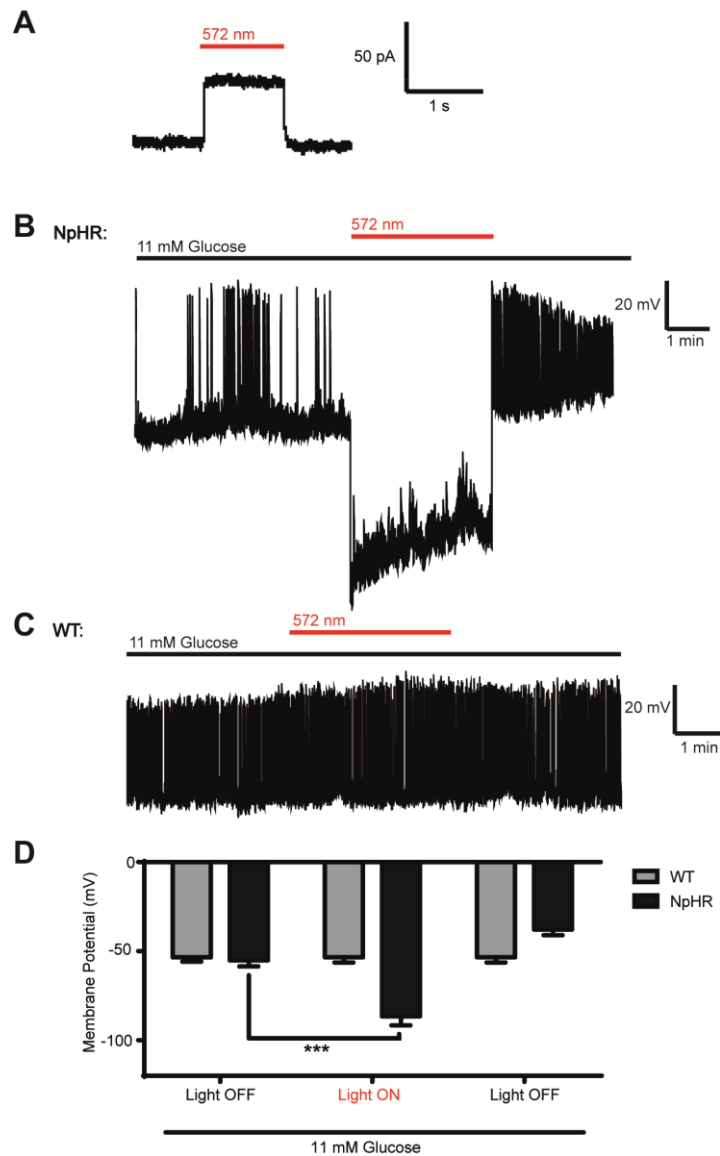
1 animals) (before, islet in control buffer; after, same islet in the presence of either AGA or BGA). (E) As  
2 for (D) but the percentage (%) of correlated links. (F and G) Gap junction blockade increases the length  
3 between correlated links ( $n = 9$  recordings from 5 animals). (H) Wiring patterns are statistically stable  
4 upon re-recording after 30 min, as determined against the same islet but with enforced dissimilarity ( $n = 8$   
5 recordings from 5 animals). (I) Wiring patterns are statistically stable upon re-recording after 30 min  
6 (Fluo2) and 3 hrs (GCaMP6), as compared to the randomly-shuffled matrix for each islet ( $n = 4-6$   
7 recordings from 2-3 animals). (J) As for (A) but showing almost identical link-probability distributions in  
8 mouse and human islets, as shown by the exponent values ( $\kappa$ ) for the fitted power laws ( $n = 8$  recordings  
9 from 3 donors). (K-L) Imaging using GCaMP6 and Fluo2 return similar hub and link proportions ( $n = 12$   
10 recordings from 4-6 animals). Data are means  $\pm$  SEM. \* $P < 0.05$  and \*\* $P < 0.01$  and NS, non-significant.

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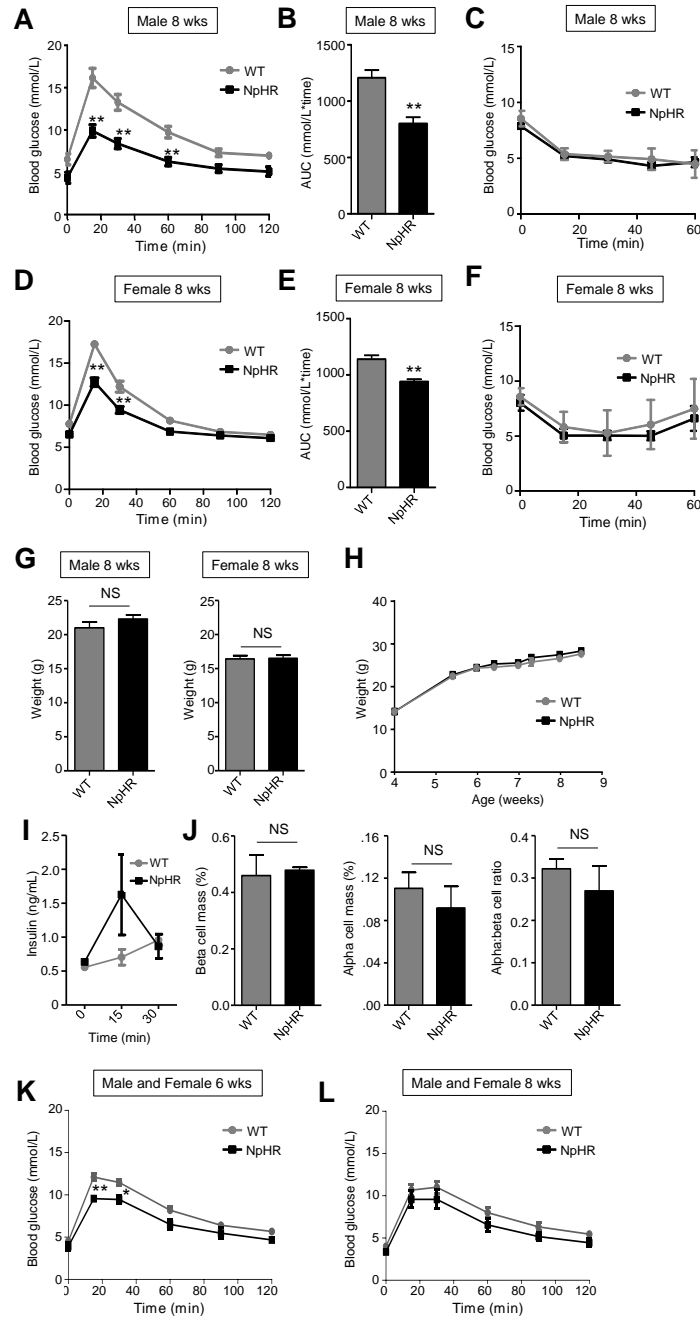
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**Fig. 2. Reversible and repeated silencing of beta cell  $\text{Ca}^{2+}$ -spiking activity.** (A) Immunostaining for insulin showing membrane-localized expression of eNpHR3.0-EYFP in beta cells (Dapi, nuclei) ( $n = 3$  preparations). (B) As for (A), but immunostaining for glucagon showing absence of eNpHR3.0-EYFP in alpha cells ( $n = 3$  preparations). Scale bar = 50  $\mu\text{m}$  (or 10  $\mu\text{m}$  for dissociated cells). (C) Reversible silencing of beta cell  $\text{Ca}^{2+}$  oscillations in eNpHR3.0-expressing islets in response to illumination with  $\lambda = 561$  nm ( $n = 5$  recordings). Treatment of islets with nifedipine 50  $\mu\text{M}$  (Nif; black trace) abolishes the rebound in  $\text{Ca}^{2+}$  upon inactivation of eNpHR3.0 ( $n = 5$  recordings) (traces are from different islets). (D) As for (C) but in the presence of verapamil 10  $\mu\text{M}$  (Ver; black trace) ( $n = 5$  recordings) (traces are from different islets). (E) Perfusion of islets with zero  $\text{Ca}^{2+}$  supplemented with EGTA was able to prevent recovery of  $[\text{Ca}^{2+}]_i$  in islets following silencing ( $n = 5$  recordings). (F) Beta cell population  $\text{Ca}^{2+}$ -spiking activity can be repeatedly silenced following exposure to  $\lambda = 561$  nm ( $n = 3$  recordings). (G) Global silencing ( $\lambda = 561$  nm) induced a decrease in intracellular  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_i$ ) throughout the islet, whereas a diffraction-limited laser ( $\lambda = 585$  nm) only silenced  $[\text{Ca}^{2+}]_i$  in the targeted area ( $n = 3$  recordings). (H) Silencing can be overcome using the depolarizing agent KCl 30 mM to re-activate VDCC ( $n = 6$  recordings). (I-J) Wild-type islets (NpHR $^{-/-}$ ) do not respond to illumination with decreases in  $[\text{Ca}^{2+}]_i$  ( $n = 5$  recordings). (K) Diazoxide (Dz) 100  $\mu\text{M}$  is unable to further suppress  $[\text{Ca}^{2+}]_i$  in eNpHR3.0-silenced islets ( $n = 5$  recordings). Where indicated, tolbutamide (Tb) 100  $\mu\text{M}$  was added to maintain a stable plateau from which to better detect silencing. G17, glucose 17 mM; G11, glucose 11 mM.; G3, glucose 3 mM.



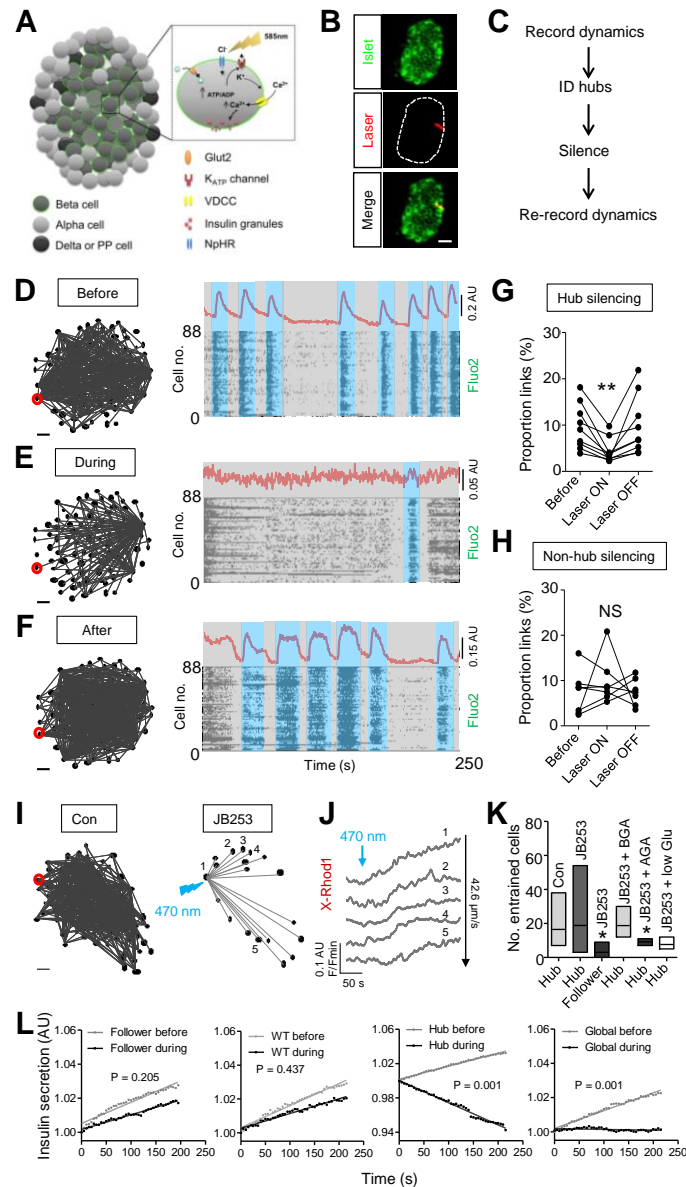
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**Fig. 3. Yellow light hyperpolarizes eNpHR3.0-expressing pancreatic beta-cells.** (A) Voltage clamp (whole cell) recording of an eNpHR3.0-expressing beta cell showing induction of photocurrents with yellow light ( $\lambda = 572$  nm). (B-D) Representative current clamp (perforated patch) recordings showing reversible membrane hyperpolarization with yellow light ( $\lambda = 572$  nm) in an eNpHR3.0-expressing, but not wild-type (WT) beta cell. In all cases,  $n = 6-11$  cells. Data are means  $\pm$  SEM. \*\*\* $P < 0.001$ .



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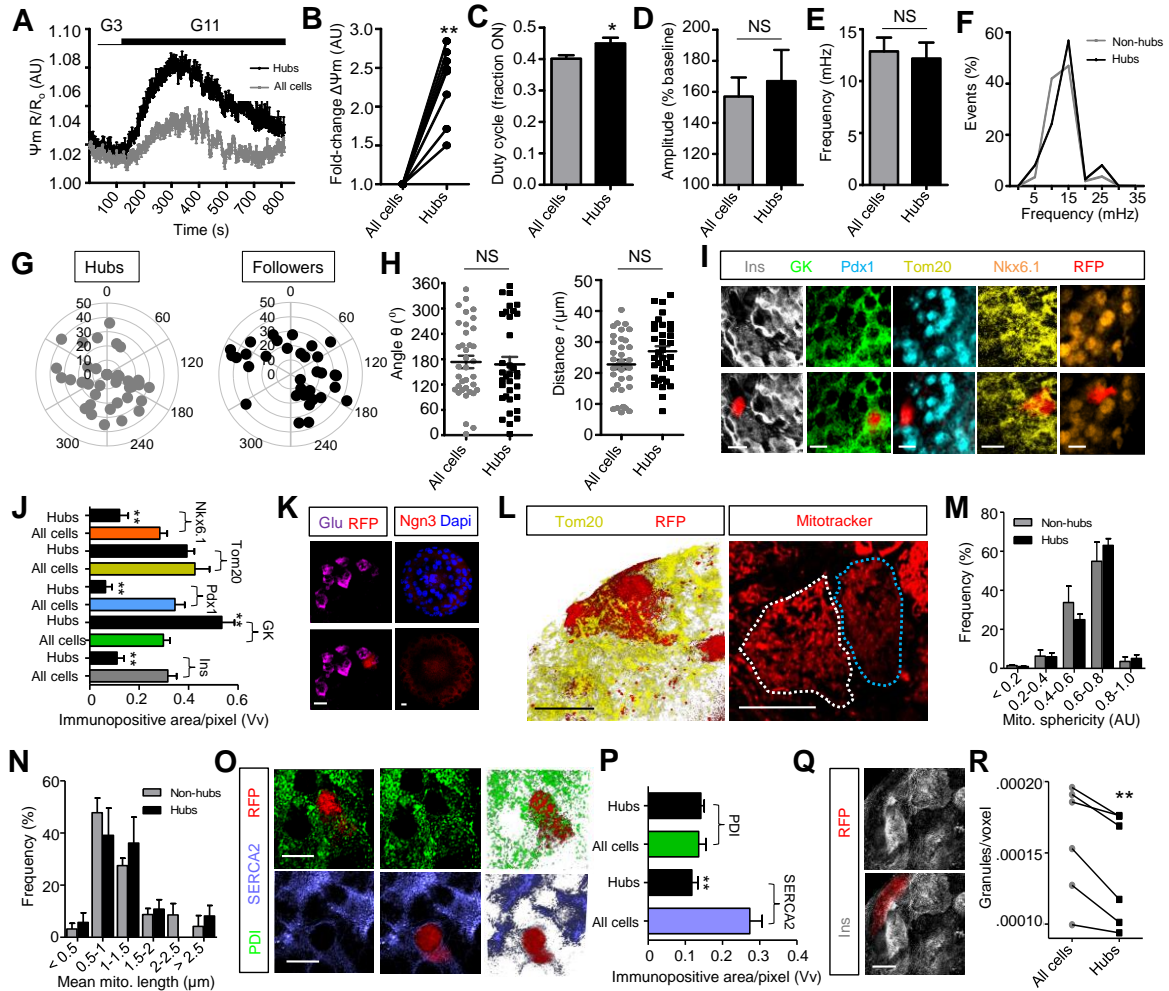
2 **Fig. 4. Glucose homeostasis in eNpHR3.0 mice.** (A-B) Glucose tolerance is improved in male 8 wk  
3  $Ins1Cre^{+/-};eNpHR3.0-EYFP^{fl/-}$  (NpHR) animals ( $n = 7$ ) compared to  $Ins1Cre^{-/-};eNpHR3.0-EYFP^{fl/-}$  (wild  
4 type, WT) littermates ( $n = 7$ ) (*i.e. activation of  $Ins1Cre$  on an eNpHR3.0-EYFP background*), as assessed  
5 using IPGTT. (C) Insulin sensitivity is similar in male NpHR mice animals and WT littermates ( $n = 6-11$ ),  
6 as determined using ITT. (D-E) As for (A-B) but female 8 wk mice ( $n = 7-9$ ). (F) As for (C) but female 8  
7 wk ( $n = 4$ ). (G-H) Fasting body weight and growth curves (non-fasted) are similar in WT and NpHR  
8 animals ( $n = 9-13$ ). (I) *In vivo* insulin release tended to be increased in NpHR compared to WT animals at  
9 15 min post-IP glucose injection ( $n = 4$ ). (J) Beta cell mass, alpha cell mass and alpha:beta cell ratio are  
10 similar in WT and NpHR animals ( $n = 3$ ). (K-L) As for (A-B) but glucose tolerance in 6 and 8 wk  
11  $Ins1Cre^{+/-};eNpHR3.0-EYFP^{fl/-}$  (NpHR) ( $n = 3-4$ ) compared to  $Ins1Cre^{+/-};eNpHR3.0-EYFP^{-/-}$  (wild type,  
12 WT) animals ( $n = 8$ ) (*i.e. activation of eNpHR3.0-EYFP on an  $Ins1Cre$ -background*). Data are means  $\pm$   
13 SEM. \*\* $P < 0.01$  and NS, non-significant.



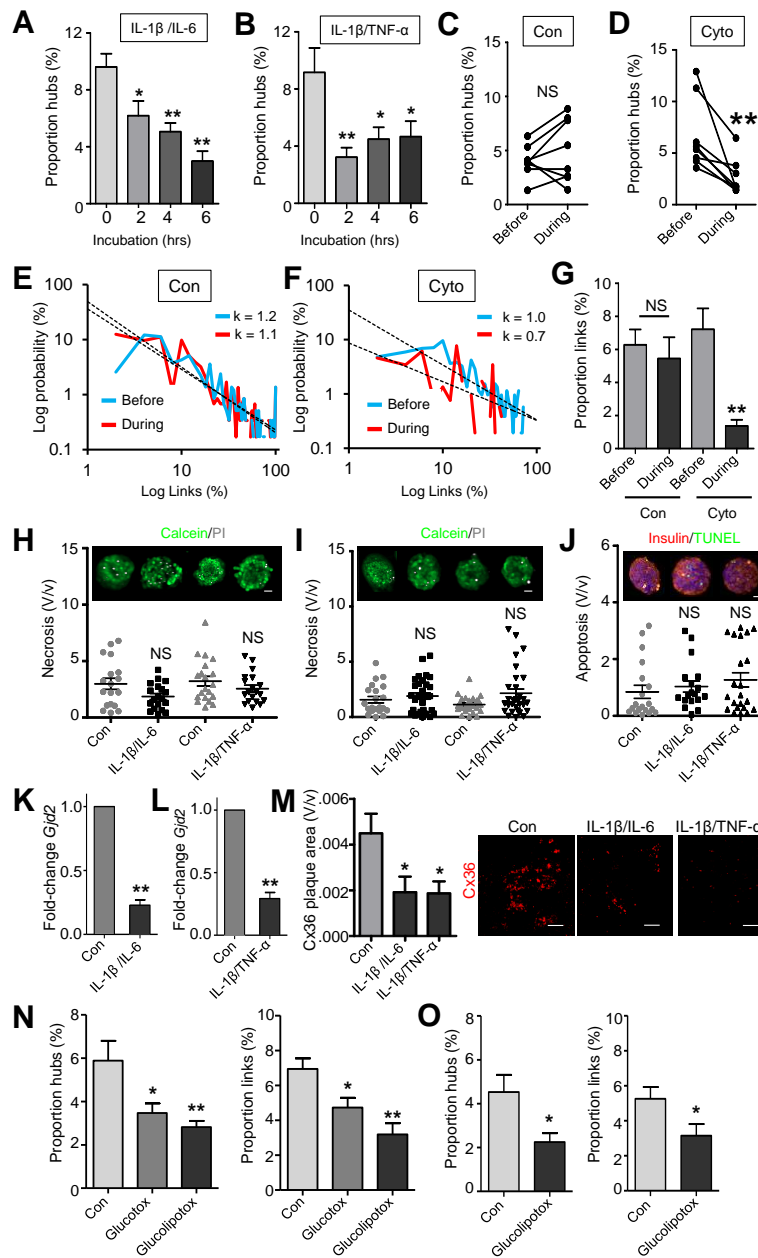
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2 **Fig. 5. Real-time analysis and targeting of beta cell hubs.** (A-B) Schematic showing the effects of  
 3 eNpHR3.0 activation upon beta cell  $Ca^{2+}$  signaling (A), and snapshot showing placement of a diffraction-  
 4 limited laser spot over a discrete islet region (B) (scale bar, 25  $\mu$ m; image cropped to display a single islet).  
 5 (C) Experimental flowchart for real-time manipulation of hub function. (D to F) Representative functional  
 6 connectivity map and activity plots at high glucose (11 mM), before (D), during (E) and after (F)  
 7 optogenetic silencing (identified hub cell; red). A representative  $Ca^{2+}$  trace is displayed above. (G-H)  
 8 Summary data showing a reversible collapse in the proportion of correlated cell links following hub (G),  
 9 but not follower (H) silencing ( $n = 7-9$  recordings from 4 animals). (I-K) Representative experiment  
 10 showing cell-cell entrainment patterns (I) and representative  $Ca^{2+}$  rises in linked cells (J) following  
 11 photopharmacological stimulation of an identified hub (red) at 3 mM glucose using JB253 (50  $\mu$ M) (low  
 12 Glu = 1 mM glucose). Box and whiskers plot shows the range and mean number of hub- or follower-  
 13 entrained cells under normal (high glucose) conditions, and following targeted stimulation using JB253  
 14 in the presence of control (Con; 3 mM glucose), BGA, AGA and low glucose (1 mM) (K) ( $n = 4-7$  recordings  
 15 from 3-4 animals). (L) Insulin secretion measured using JP-107 is unaffected following illumination of  
 16 follower cells or wild-type (WT) islets, but suppressed in response to hub or islet (global) shutdown (mean  
 17 traces shown) ( $n = 8$  islets from 4 animals). Scale bars = 20  $\mu$ m. Data are means  $\pm$  SEM. \* $P < 0.05$  and NS,  
 18 non-significant.





**Fig. 6. Phenotypic profiling of hub cell function.** (A-B) Hubs display elevated mitochondrial potential ( $\Psi_m$ ) compared to the rest of the population, as measured using TMRE to label active mitochondria ( $n = 9$  recordings from 3 animals) (G3, glucose 3 mM; G11, glucose 11 mM). (C-F) Duty cycle (*i.e.* fraction spent 'ON') (C), and  $Ca^{2+}$  oscillation amplitude (D) and frequency (E-F) are similar in hubs and followers ( $n = 8$  recordings from 4 animals). (G-H) Polar coordinates showing that hub distribution is not spatially-biased *versus* followers (angle  $\theta$  and distance  $r$  from the islet center 0,0 are shown in the bar graphs). (I-J) PA-TagRFP-identified hubs (red; RFP) express less insulin (Ins), less Pdx1, less Nkx6.1, more glucokinase (GK), and normal Tom20 compared to the rest of the population ( $n = 5-9$  hubs from 3-4 animals). (K) Hubs were not immunopositive for glucagon (Glu), and Neurogenin 3 (Ngn3) expression was largely undetectable in the adult islet. (L-N) High resolution Z-projections of Tom20- and MitoTracker-stained islets (L) reveal normal mitochondrial sphericity (M) and length (N) (white-dashed line, hub; blue-dashed line, non-hub) (3D render shown for TOM20 and Z-projection for MitoTracker) ( $n = 6$  hubs from 3 animals). (O-P) As for (L-N), but staining for PDI and SERCA2 showing normal ER abundance and lowered  $Ca^{2+}$ -ATPase content in hubs (Z-projection, left; 3D render, right) ( $n = 4-5$  hubs from 3 animals). (Q-R) High-resolution snapshot of insulin staining (Q) showing a reduction in granule content in hubs (red) (R) ( $n = 6$  hubs from 3 animals). Scale bars = 12.5  $\mu$ m. Data are means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$  and NS, non-significant.



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2 **Fig. 7. Disruption of hubs by pro-inflammatory and glucolipotoxic insults.** (A-B) IL-1 $\beta$ /IL-6 and IL-  
3 1 $\beta$ /TNF- $\alpha$  reduce hub number after 2 h ( $n = 6$  islets from 3 animals). (C-D) Cytokine (Cyto; IL-1 $\beta$ /IL-6)  
4 decreases hub number in real-time ( $n = 8$  from 4 animals). (E-F) Cytokine alters the distribution of  
5 correlated links and power-law scaling exponent ( $k$ ) value, indicating a decreased number of cells in the  
6 high connectivity (*i.e.* hub) range ( $n = 8$  from 4 animals) ( $R^2 = 0.38-0.74$ ). **The power-law was log-log**  
7 **transformed to a linear relationship to better demonstrate the distribution.** (G) Cytokine (IL-1 $\beta$ /IL-6)  
8 exposure dramatically reduces the proportion of correlated links. (H to I) Application of IL-1 $\beta$ /IL-6 or IL-  
9 1 $\beta$ /TNF- $\alpha$  for 2 h (H) or 4 h (I) is not cytotoxic ( $n = 21$  islets per condition from 6 animals) (scale bar, 25  
10  $\mu\text{m}$ ). (J) 2 h application of IL-1 $\beta$ /IL-6 or IL-1 $\beta$ /TNF- $\alpha$  does not induce apoptosis ( $n = 18-20$  islets from 5  
11 animals). (K-L) IL-1 $\beta$ /IL-6 and IL-1 $\beta$ /TNF- $\alpha$  decrease connexin 36 (*GJD2*) mRNA levels ( $n = 10$   
12 animals). (M) IL-1 $\beta$ /IL-6 and IL-1 $\beta$ /TNF- $\alpha$  reduce the number of immunostained gap junction (Cx36)  
13 plaques ( $n = 9-12$  islets from 6 animals) (scale bar, 12.5  $\mu\text{m}$ ). (N) Glucotoxicity (Glucotox) and  
14 glucolipotoxicity (Glucolipotox) reduce the proportion of hubs and correlated links in mouse islets ( $n = 6$   
15 animals). (O) As for (N) but showing effects of glucolipotoxicity-alone on human islets ( $n = 5$  donors).  
16 Data are means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$  and NS, non-significant.