1   Running title: Loss of synchronized intra-islet Ca^{2+} oscillations *in vivo* in *Robo*-deficient β cells

2

3   **Synchronized β cell response to glucose is lost concomitant with loss of islet architecture in**

4   **Robo deficient islets of Langerhans *in vivo***

5

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23   **Abstract**

24   The spatial architecture of the islets of Langerhans is hypothesized to facilitate synchronized

25   insulin secretion between β cells, yet testing this *in vivo* in the intact pancreas is challenging.

26   *Robo βKO* mice, in which the genes *Robo1* and *Robo2* are deleted selectively in β cells, provide

27   a unique model of altered islet spatial architecture without loss of β cell differentiation or islet

28   damage from diabetes. Combining *Robo βKO* mice with intravital microscopy, we show here

29   that *Robo βKO* islets lose synchronized intra-islet Ca^{2+} oscillations between β cells *in vivo*. We

30   provide evidence that this loss is not due to a β cell-intrinsic function of Robo, loss of

31   Connexin36 gap junctions, or changes in islet vascularization, suggesting that the islet

32   architecture itself is required for synchronized Ca^{2+} oscillations. These results have implications

33   for understanding structure-function relationships in the islets during progression to diabetes

34   as well as engineering islets from stem cells.

Introduction

35

36   The islets of Langerhans, which comprise the endocrine pancreas, are highly organized

37   micro-organs responsible for maintaining blood glucose homeostasis. Islets are composed of

38   five endocrine cell types (α, β, δ, PP, and ε) which, in rodents, are arranged such that the β cells

39   cluster in the core of the islet, while other non-β endocrine cells populate the islet mantle, so

40   that most β cells are in direct contact preferentially with β cells (homotypic interactions)[1].

41   Human islet architecture is more complex and, though its exact organization pattern is still

42   debated, the prevailing idea is that it still follows a non-random distribution of the different

43   endocrine cell types[2-6]. In agreement with this notion, computational analysis of human islet

44   architecture found lower probability of heterotypic interactions and a higher probability of

45   homotypic interactions between the various endocrine cell types than would be expected if the

46   islet displayed random distribution of endocrine cells[7,8]. Thus, in both rodent and human islets,

47   respective stereotypical islet architectures prioritize homotypic over heterotypic interactions

48   between endocrine cell types[7]. The biological reason for preferential homotypic interactions

49   between endocrine cells is not completely clear, but it has been suggested to be important for

50   dictating the level of Connexin36 (Cx36)-mediated electrical β cell-β cell coupling, thus allowing

51   for synchronization of glucose-stimulated insulin secretion (GSIS) between neighboring β

52   cells[9,10].

53   Activation of insulin secretion in the β cell is triggered when glucose from the blood

54   enters the β cells through glucose transporters. As this glucose is metabolized, the ratio of

55   intracellular ATP/ADP in the cells increases. This rise in ATP causes ATP sensitive K^+ channels to

56   close, resulting in membrane depolarization. The resultant depolarization causes voltage-gated

57   Ca^{2+} channels to open, triggering an influx of Ca^{2+} into the cell, which in turn promotes

58   exocytosis of insulin granules[11-13]. This chain of events is cyclical and thus results in oscillations

59   of membrane potential, cytosolic Ca^{2+} levels, and insulin secretion in response to glucose[14].

60   Because β cells within an islet are gap-junctionally coupled, and thus electrically coupled, these

61   oscillations are synchronous across all islet β cells[15]. It is thus hypothesized that preferential β

62   cell homotypic contact allows for the necessary amount of gap junctions to form between

63   neighboring β cells in order to synchronize the oscillations in an entire islet, facilitating pulsatile

64   insulin secretion[10,16]. Indeed, modeling experiments in which the number of homotypic β cell-β

65   cell nearest neighbor connections is lowered within an islet result in predicted perturbation of

66   synchronous Ca^{2+} oscillations[10]. If this *in silico* prediction is correct, then disrupting spatial

67   organization of the different endocrine cell types within the islet alone, without affecting any

68   other property of the cells, would be sufficient to disturb synchronized insulin secretion

69   between β cells. However, direct empirical evidence for this hypothesis is lacking.

70   Most genetic mouse models that show disruption of islet architecture also display

71   defects in glucose homeostasis[17]. However, in many of these models, the disrupted islet

72   architecture phenotype is linked to either developmental defects in β cell differentiation or

73   maturation[18-29] or to pathologies related to β cell damage in diabetes[30-35]. This introduces a

74   strong confounding factor for studying the role of islet architecture on β cell function.

75   Therefore, current mouse models of disrupted islet architecture are not suitable for directly

76   testing the hypothesis that preferential homotypic β cell-β cell interactions, dictated by

77   canonical islet architecture, regulate synchronized insulin secretion between β cells within the

78   same islet.

4

79   Recently, we have described a mouse model in which the cell-surface receptors *Robo1*

80   and *Robo2* are deleted specifically in β cells (*Robo βKO*), resulting in disruption of canonical

81   endocrine cell type sorting within the islets[36]. Unlike other models of disrupted islet

82   architecture, the β cells in the islets of *Robo βKO* express normal levels of markers for β cell

83   differentiation and functional maturity. We reasoned that this model would allow for direct

84   testing of the role of islet architecture on synchronous islet oscillations between β cells in a fully

85   differentiated, non-diabetic islet setting.

86

87   **Results**

88   ***Robo βKO* islets have fewer homotypic β cell-β cell contacts than control islets**

89   *In silico* simulations where the degree of β cell-β cell coupling is changed through a

90   decrease in homotypic nearest neighbors predict that disruption in islet architecture will disrupt

91   synchronous intra-islet Ca^{2+} oscillations and hormone secretion pulses[7,10,37]. To test whether β

92   cells in *Robo βKO* islets have a decreased ratio of homotypic β cell neighbors on average than

93   control islets, we performed nearest neighbor analysis on islets from pancreatic sections of

94   *Robo βKO* and control mice (**Figure 1**). We found that *Robo βKO* islets possess significantly

95   fewer β cell-β cell contacts (*n*=9-11 islets for 3 mice from each genotype; control 75.35%±4.1,

96   *Robo βKO* 50.37%±4.1, *p*=0.01), and homotypic contacts in general when compared to control

97   islets (*n*=9-11 islets for 3 mice from each genotype; control 83.7%±1.7, *Robo βKO* 64.43%±1.2,

98   *p*=0.0008). We also found that *Robo βKO* islets possess significantly more β cell-α cell contacts

99   (*n*=9-11 islets for 3 mice from each genotype; control 11.21%±2.7, *Robo βKO* 25.99%2.9,

100   *p*=0.02), and heterotypic contacts in general when compared to control islets (*n*=9-11 islets for

101   3 mice from each genotype; control 16.3%,±1.7 *Robo βKO* 35.57%±1.2, *p*=0.0008). Together,

102   this suggests that *Robo βKO* islets make fewer homotypic β cell-β cell connections compared to

103   control islets.

104   We have previously shown that genetic deletion of *Robo1* and *Robo2* selectively in β

105   cells using either *Ins1-Cre; Robo1$^{Δ/Δ}$2$^{flx/flx}$* or *Ucn3-Cre; Robo1$^{Δ/Δ}$2$^{flx/flx}$* mice (*Robo βKO*) results in

106   disrupted islet architecture and endocrine cell type sorting without affecting β cell death or the

107   expression of the β cell maturation markers MafA and Ucn3[36]. To verify that β cells in *Robo βKO*

108   islet are truly mature, we expanded the analysis to look at transcript levels of 15 additional

109   maturity markers. RNA sequencing and differential gene expression analysis on FACS-purified β

110   cells from both *Robo βKO* and control islets revealed no change in transcript levels of any

111   hallmark β cell maturity or differentiation genes (*n*=2 mice of each genotype; **Supplemental**

112   **Figure 1**). Thus, unlike other mouse models with disrupted islet architecture, β cells in *Robo*

113   *βKO* islets maintain maturity and differentiation despite loss of normal islet architecture.

114   We reasoned that the altered degree of homotypic β cell-β cell interaction in *Robo βKO*

115   islets together with the seemingly retained β cell maturity provide a unique model by which to

116   test the hypothesis that endocrine cell type organization affects synchronous insulin secretion

117   in the islet.

118

119   ***Robo βKO* islets display unsynchronized Ca^{2+} oscillations *in vivo***

120   We set out to investigate how the reduced homotypic β cell-β cell connections in *Robo*

121   *βKO* islets affects dynamic insulin secretion in the islet by measuring dynamic Ca^{2+} signaling.

122   *Robo βKO* islets spontaneously dissociate during isolation and culture[36], making them

123   unsuitable for *in vitro* analyses of whole-islet Ca^{2+} oscillations. To overcome this limitation, we

124   adopted a novel intravital Ca^{2+} imaging method which enables imaging of islet Ca^{2+} dynamics *in*

125   *situ* within the intact pancreas[38]. In brief, this method employs an intravital microscopy (IVM)

126   platform and adeno-associated viral (AAV) delivery of insulin promoter-driven GCaMP6s, a

127   fluorescent Ca^{2+} biosensor, to quantitate β cell Ca^{2+} dynamics *in vivo* in both *Robo βKO* and

128   control islets. This method also allows for retention of the islet's *in vivo* microenvironment,

129   blood flow, and innervation, and provides more realistic conditions than *in vitro* approaches

130   allow for.

131   We verified that synchronous Ca^{2+} oscillations are maintained *in vivo* in islets by

132   measuring GCaMP6s intensity of β cells within AAV8-RIP-GCaMP6 infected islets of control

133   (*Robo WT*) mice (**Figure 2**). As expected, control mice displayed whole islet synchronous Ca^{2+}

134   oscillations when imaged at 0.03, 0.1, and 0.2Hz for at least 10 minutes after glucose elevation

135   (*n*=8 islets from 3 mice; **Figure 2** and **Supplemental Video 1 and 2**). We quantified the degree to

136   which these oscillations are synchronous within the islet by analyzing the amount of correlation

137   between GCaMP6s active areas within individual islets. While oscillations vary in frequency

138   between islets, the degree of correlation between β cells within any one islet is very high,

139   confirming that control islets possess highly synchronous intra-islet Ca^{2+} oscillation *in vivo*

140   (fraction of GCAMP6s active islet area with correlated Ca^{2+} oscillations=0.90±0.04, *n*=8 islets

141   from 3 mice; see **Figure 4A**).

142   Conversely, we found that most *Robo βKO* islets display asynchronous intra-islet Ca^{2+}

143   oscillations *in vivo* when imaged at 0.03, 0.1, and 0.2Hz (**Figure 3, Supplemental Figure 2,**

144   **Supplemental Videos 3, 4, and 5**). Quantification of this asynchronous behavior through

145   correlation analysis of GCaMP6s activity within individual *Robo βKO* islets revealed significant

146   reduction in intra-islet correlated oscillation areas compared to controls (fraction of GCAMP6s

147   activity with correlated Ca^{2+} oscillations=0.58±0.10, *n*= 11 islets from 5 mice, *p*<0.01; **Figure 4A**).

148   Further, asynchronous *Robo βKO* islets showed spatially distinct areas within the islet that

149   oscillated synchronously with immediate β cell neighbors but not with more distant regions

150   within the same islet (**Figure 3C-D, Supplemental Figure 2, Supplemental Videos 3, 4, and 5**).

151   This was not due to differences in the proportion of GCaMP6s positive cells showing elevated

152   Ca^{2+} activity within *Robo βKO* islets compared to controls (control islets 0.97±0.01 fraction

153   active, *n*=8 islets from 3 mouse; *Robo βKO* islets 0.97±0.02 fraction active, *n*=8 islets from 4

154   mice, *p*=0.79; **Figure 4B**).

155

156   ***Unsynchronized Ca^{2+} oscillations in Robo βKO islets are not due to β cell intrinsic defects in***

157   ***glucose stimulated Ca^{2+} oscillations***

158   *In vitro* experiments have shown that Robo receptors play a role in β cell biology and are

159   involved in the stimulus secretion cascade linking glucose to insulin secretion[39]. Thus it is

160   possible that defects in synchronous Ca^{2+} oscillations observed in *Robo βKO* islets are due to a

161   Robo-mediated, β cell-intrinsic effect on dynamic Ca^{2+} signaling in response to stimuli.

162   However, 4 out of the 11 *Robo βKO* islets imaged showed synchronous Ca^{2+} activity in greater

163   than 90% of GCaMP6s positive areas (**Figure 4A, Supplemental Figure 3 and Supplemental**

164   **video 6**). The existence of this highly synchronous population of *Robo βKO* islets suggests that

165   the ability of individual β cells to oscillate intracellular Ca^{2+} levels in response to stimuli is

166   unaffected by deletion of *Robo*. Instead, it suggests that islet architecture itself is responsible

167   for controlling synchronized Ca^{2+} oscillations, and that some *Robo βKO* islets may escape this

168   defect due to less severe architectural disruption. With this is mind, we wanted to test the

169   extent to which the unsynchronized Ca^{2+} oscillations observed in *Robo βKO* islets *in vivo* are due

170   to β cell-intrinsic deletion of *Robo per se*.

171   To test whether *Robo βKO* β cells are able to undergo Ca^{2+} oscillations in response to

172   stimuli, we performed *in vitro* Ca^{2+} imaging on single β cells from dissociated *Robo βKO* and

173   control islets, exposed to glucose followed by KCL (**Figure 5**). We found no difference in the

174   proportion of β cells that undergo Ca^{2+}oscillations in response to 10mM glucose between

175   control and *Robo βKO* β cells (Control: 70.24%±3.6, *n*=13-32 β cells per mouse for 4 mice, *Robo*

176   *βKO*: 61.61%±5.6, *n*=6-24 β cells per mouse for 4 mice, *p*=0.24) (**Figure 5 A-C**). We also saw no

177   significant difference in area under the curve (AUC) of calcium traces in response to 10mM

178   glucose (Control: 2.9±0.17, *n*=13-32 β cells per mouse for 4 mice, *Robo βKO:* 3.0±0.21, *n*=6-24 β

179   cells per mouse for 4 mice, *p*=0.39)(**Figure 5D**), or peak height of Ca^{2+} corresponding to first

180   phase insulin secretion (Control: 0.34±0.02, *n*=13-32 β cells per mouse for 4 mice, *Robo βKO*:

181   0.41±0.04, *n*=6-24 β cells per mouse for 4 mice**, *p*=0.08**) (**Figure 5E**) in *Robo βKO* β cells

182   compared to controls. There was, however, a significant increase in AUC of Ca^{2+} signal in

183   response to KCL in *Robo βKO* β cells compared to controls (Control: 1.67±0.07, *n*=13-32 β cells

184   per mouse for 4 mice, *Robo βKO*: 2.12±0.12, *n*=6-24 β cells per mouse for 4 mice**, *p*=0.002**)

185   (**Figure 5F**), indicating that *Robo βKO* islets may actually have an increase in magnitude of Ca^{2+}

186   response to stimuli. Together, this demonstrates that *Robo βKO* β cells show no defects in their

187   ability to undergo Ca^{2+} oscillations in response to glucose, and in fact may have an improved

188   stimulus secretion response compared to controls. This is in support of a scenario in which the

9

189   arrangement of β cells within the islet, rather than a β cell-intrinsic function of Robo, dictates

190   whole-islet synchronous Ca^{2+} oscillations.

191

192   **_Robo βKO_ islets retain the ability to form gap junctions and have similar levels of**

193   **vascularization**

194   Besides a decrease in β cell-β cell homotypic contacts within the islet, a possible

195   explanation for the loss of synchronized whole islet Ca^{2+} oscillations in _Robo βKO_ is that their β

196   cells no longer possess the gap junctions necessary for adequate electrical coupling. Indeed, the

197   phenotype described above is reminiscent of that observed in mice heterozygous for a _Cx36_

198   null allele[15,40]. To test whether _Robo βKO_ mice form fewer gap junctions between β cells, we

199   measured the area of Cx36 protein immunofluorescence normalized to islet area in _Robo βKO_

200   and control islets (**Figure 6**). We found no difference in Cx36 immunofluorescence between

201   _Robo βKO_ islets and controls, but significant differences in these two groups compared to _Cx36_

202   _KO_ mice (control islets: 0.46%±0.06 Cx36 signal/μm^2, _Robo βKO_ islets: 0.69%±0.11 Cx36

203   signal/μm^2, _n_=10-15 islets from 4 mice each, p=0.11, _Cx36 KO_ islets: 0.13%±0.04, n=15-20 islets

204   from 2 mice) (**Figure 6B**). Furthermore, we found normal co-localization of Cx36 to β cell

205   borders in both control and _Robo βKO_ islets (**Figure 6C**) Overall, this suggests that loss of

206   synchronous intra-islet Ca^{2+} oscillations is not due to failure of gap junction formation in β cells.

207   Another possible explanation for the observed uncoupling of intra-islet Ca^{2+} oscillations

208   in _Robo βKO_ islets is that β cell-β cell contacts are disrupted due to physical blocking by non-

209   endocrine tissue. To determine if other non-endocrine architectural changes within the islet

210   occur in _Robo βKO_ mice we quantified the amount of matrix components secreted by vessels as

10

211   a surrogate for vasculature (laminin, and collagen IV) in *Robo βKO* and control islets. In both

212   cases we found no significant difference in area of vessel matrix components (**Figure 7**)

213   between *Robo βKO* and control islets, suggesting that interfering blood vessels are likely not the

214   cause of loss of whole islet synchronous Ca^{2+} oscillations (normalized laminin β1 area: control

215   0.31±0.05 μm^2, *n*=10-12 islets from 8 mice; *Robo βKO* 0.38±0.05 μm^2, *n*=10-12 islets from 8

216   mice; *p*= 0.39; normalized Col IV area: control 0.18±0.01 μm^2, *n*=10-12 islets from 4 mice; *Robo*

217   *βKO* 0.16±0.02 μm^2, *n*=10-12 islets from 6 mice, *p*=0.35).

218

219   **Discussion**

220   In this study, we provide evidence for the importance of islet architecture for proper

221   islet function *in vivo*. When islet architecture is disrupted while β cell maturity is retained in

222   *Robo βKO* mice, synchronized Ca^{2+} oscillations in the islet *in vivo* are perturbed. This is not due

223   to a Robo-mediated β cell-intrinsic defect in glucose-stimulated Ca^{2+} signaling, loss of Cx36, or

224   change in amount of islet vascularization. *Robo βKO* β cells possess a smaller fraction of

225   homotypic nearest neighbors than controls, suggesting a limited capacity to electrically couple

226   β cells across the islet. Taken together, these data suggest that islet architecture itself,

227   uncoupled from β cell maturity, Robo-mediated β cell-intrinsic defects in Ca^{2+} signaling, or

228   availability of gap junction machinery, is important for coordinated insulin secretion between β

229   cells.

230   Robo, and its ligand Slit, have been previously shown to affect Ca^{2+} oscillations in β cells

231   *in vitro*[39]. However, Robo-mediated β cell-intrinsic effects are likely not the cause of

232   asynchronous *in vivo* Ca^{2+} oscillations in *Robo βKO* islets. This is supported by two independent

233   observations: 1) single cell Ca^{2+} oscillations in dissociated β cells triggered by glucose stimulus *in*

234   *vitro* are similar between *Robo βKO* and control, and 2) a subset of *Robo βKO* islets analyzed *in*

235   *vivo* still show synchronized whole-islet Ca^{2+} oscillations despite the absence of Robo. Further,

236   this phenotypic heterogeneity is likely not due to an incomplete deletion of *Robo* because all

237   islets were detected during the Ca^{2+} imaging experiment by the fluorescent labeling of β cells

238   with the H2B-mCherry lineage-tracing reporter[36] which uses the same Cre deriver that is used

239   to delete *Robo* in those β cells. Thus high expression of H2B-mCherry suggests efficient

240   recombination of the *Robo* floxed allele. All this together supports the idea that *Robo* deletion

241   in β cells causes disruption of islet architecture, and that this architectural disruption itself

242   causes loss of synchronized Ca^{2+} oscillations. Further, the unsynchronized phenotype in *Robo*

243   *βKO* islets is clear from the videos we captured at 0.2Hz, 0.1Hz, and 0.03Hz; however we are

244   aware that even higher imaging frequency may be needed to see asynchronous behavior in

245   *Robo WT* islets. That said, we note that while faster acquisition would be beneficial to show

246   more rapid dynamics, we believe it important to capture the dynamics in 3D, in order to ensure

247   we are not biased by examining certain 'synchronized regions' that would not be representative

248   of the islet dynamics as a whole. We would further argue that the oscillations we observe are

249   on a 3-5 minute time scale, and therefore to sample these oscillations even at a frequency of 1

250   frame per 10s of seconds is sufficient. Indeed if we were to image more rapidly and capture

251   other non-synchronized dynamics in the *Robo βKO* islets, this would reduce the measured

252   coordination even further. Thus the low sample frequency provides an upper limit to the

253   coordination.

254   It remains possible that other components of islet architecture besides endocrine cell

255   type sorting contribute to disruption in Ca^{2+} oscillations found in *Robo βKO* islets. While we

256   have shown that the amount of vascularization between *Robo βKO* islets and controls is similar,

257   we cannot draw conclusions on whether the pattern of vessels is unchanged. Further, it is

258   possible that the amount and patterning of innervation may vary between *Robo βKO* and

259   controls. These are particularly of interest because Robo receptors have known roles in

260   angiogenesis and axon guidance, and thus could affect precisely how the islet is innervated and

261   vascularized[41]. Robo also has known roles in controlling cell polarity, and thus it is possible that

262   this process is disrupted in *Robo βKO* islets as well. Gap junctions are known to be localized to

263   the junctional membranes between β cells, which themselves are defined by β cell polarity

264   within the islet[40,42]. Thus if β cell polarity is disrupted in *Robo βKO* islets, this could possibly

265   contribute to the asynchronous Ca^{2+} oscillations observed.

266   Finally, while we propose that defects in synchronous Ca^{2+} oscillation in response to

267   glucose in *Robo βKO* islets are due to inefficient electrical β cell coupling as a result of

268   decreased homotypic β cell interactions in these islets[43], we cannot rule out that this effect is

269   instead due to changes in diffusible paracrine factors. Under this hypothesis, the intermixed

270   islet architecture that results from deletion of *Robo* in β cells would change the amount and

271   type of diffusible paracrine signals that endocrine cells within the islet are exposed to. This

272   change in diffusible factors could affect glucose stimulated insulin secretion and thus could be

273   contributing to the *in vivo* asynchronous Ca^{2+} oscillation phenotype that we observe[44].

Acknowledgments

Author Contribution

Conceptualization, B.B. and M.T.A; Methodology, B.B., M.T.A., C.A.R, and J.M.D.; Investigation, M.T.A., C.A.R, J.M.S, M.R.L, S.M.S, , E.J, and S.D.N; Formal Analysis, M.T.A., C.A.R, M.R.L., E.J, and J.M.D.; Resources, S.M.P, R.G.M, A.K.L, M.J.M. and R.K.P.B.; Writing Original Draft, B.B and M.T.A.; Writing, Review and Editing, all authors; Funding Acquisition, B.B., S.M.P, M.J.M, R.K.P.B., R.G.M., and A.K.L; Supervision, B.B.

Materials and Methods

Animals

The experimental protocol for animal usage was reviewed and approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC) under Protocol #M005221 and Protocol #M005333, and all animal experiments were conducted in accordance with the University of Wisconsin-Madison IACUC guidelines under the approved protocol. *Robo1$^\Delta$,2^{flx45}*, *Ins1-Cre*[46], *Urocortin3-Cre*[47] and *Rosa26-Lox-Stop-Lox-H2BmCherry*[48] mice were previously described. All mouse strains were maintained on a mixed genetic background. Control colony mates in all analyses were *Robo$^{+/+}$* with the either *Ins1-Cre or Ucn3-Cre*.

Immunofluorescence

Pancreata were fixed with 4% PFA at 4°C for 3h, embedded in 30% sucrose and frozen in OCT (Tissue-Tek). Pancreatic sections (10 µm) were stained using a standard protocol. The following primary antibodies and dilutions were used: guinea pig anti-Insulin (1:6, Dako, IR00261-2), mouse anti-Glucagon (1:500, Sigma G2654), rabbit anti-Glucagon (1:200, Cell Signaling 2760S), rabbit anti-Somatostatin (1:1000, Phoenix G-060-03), rabbit anti-Connexin36 (1:80, Invitrogen 36-4600), rabbit anti-Col IV (1:300, Abcam Ab656), rat anti-Laminin β1 (1:500, Invitrogen MA5-14657). The following secondary antibodies were used at 1:500: Donkey anti-Guinea Pig 594 (Jackson), Donkey anti-Guinea Pig 647 (Jackson), Donkey anti-Rabbit 488 (Invitrogen), Donkey anti-Rabbit 594 (Invitrogen), Donkey anti-goat 647 (Invitrogen), and Donkey anti-rat 488 (Invitrogen). Slides were imaged using a Leica SP8 Scanning Confocal microscope or a Zeiss Axio Observer.Z1 microscope.

316

RNA sequencing

318   RNA was isolated from FACS sorted lineage-traced β cells[36] from control and *Robo βKO* mice

319   using phenol chloroform extraction (TRIzol). DNA libraries were generated using Takara's

320   SMART-Seq v4 Low Input RNA Kit for Sequencing (Takara, Mountain View, California, USA) for

321   cDNA synthesis and the Illumina NexteraXT DNA Library Preparation (Illumina, San Diego, CA,

322   USA) kit for cDNA dual indexing. Full length cDNA fragments were generated from 1-10ng total

323   RNA by SMART (Switching Mechanism at 5' End of RNA Template) technology. cDNA fragments

324   were fragmented and dual indexed in a single step using the Nextera kit's simultaneous

325   transposon and tagmentation step. Quality and quantity of completed libraries were assessed

326   using Agilent DNA series chip assay (Agilent Technologies, Santa Clara, CA) and Invitrogen Qubit

327   ds DNA HS Kit (Invitrogen, Carlsbad, California, USA), respectively. Each library was standardized

328   to 2nM. Cluster generation was performed on Illumina cBot, with libraries multiplexed for

329   1x100bp sequencing using TruSeq 100bp SBS kit (v4) on an Illumina HiSeq2500. Images were

330   analyzed using standard Illumina Pipeline, version 1.8.2.

331

Intravital imaging

333   Mouse pancreata were exposed in anesthetized mice by making a small incision on the right

334   side of the mouse, and externalizing the tip of the pancreas. A glass dish was placed over the

335   exposed pancreas and the mouse was placed on a microscope stage with isoflurane anesthesia

336   for the remainder of imaging. Islets were identified on the surface of the pancreas by detecting

337   Histone H2B-mCherry fluorescent nuclei labeled by β cell-specific lineage-tracing reporter[36].

16

338   Once islets were identified, mice were given injections of 1g/kg body weight glucose (30% in

339   saline) intraperitoneally. Blood glucose levels were monitored through tail vein bleeds. Once

340   the blood glucose reached ~300 mg/dL, GCaMP6s activity was identified using the microscope

341   eye piece. When imaging a time course of GCaMP6s intensity, a z-stack was set to 3, 8 or 12

342   slices each 8μm apart. Images were captured at 0.2Hz, 0.1Hz, or 0.03Hz respectively over at

343   least 10 minutes at a resolution of 512x512 pixels. After time courses were recorded, high

344   resolution image z-stacks were taken with 60 z planes taken 1μm apart or 8 z-planes taken 8μm

345   apart at 1024x1024 pixel resolution. For some images, rhodamine-dextran was injected retro-

346   orbitally to mark the vasculature of the islets *in vivo.*

347

348   **Gap junction and vasculature quantification**

349   Cx36 levels were quantified from images of islets co-stained with rabbit anti-Cx36 (Invitrogen)

350   and Guinea Pig anti-insulin (Dako) antibody. Vasculature levels were quantified from images co-

351   stained with rat anti-Laminin β1 (Invitrogen) or rabbit anti-col IV and Guinea Pig anti-insulin

352   (Dako). 8 Z-planes were taken 1μm apart on a Leica SP8 Scanning Confocal microscope using a

353   40x oil immersion objective (Cx36) or 20X (vasculature). Threshold masks were made of both

354   channels for each islets, and the area of each staining was measured using FIJI's analyze

355   particles functions. The area of gap junctions or blood vessels (marked by their respective

356   antibody) was divided by the area of DAPI or insulin respectively for each islet. 10-14 islets were

357   analyzed for *n*=3-8 mice for each genotype. Student's T-test was performed to obtain *P* values.

358

359   **Nearest neighbor analysis**

17

360   β cells were identified using the lineage tracer *Rosa26-Lox-Stop-Lox-H2BmCherry* crossed to

361   *Ucn3-Cre* and tissue sections were stained with antibodies against glucagon and somatostatin

362   to identify α and δ cells respectively. The 3D Tissue Spatial Analysis Toolbox for Fiji[49] was used

363   to identify specific cell types using the above markers and to calculate the number of cell type

364   specific nearest neighbors from all identified endocrine cells. Analysis was performed on 9-11

365   islets from *n*=3 mice from each genotype.

366

367   **Time course image analysis**

368   All images were analyzed using previously published methods[50] with custom Matlab

369   (Mathworks) scripts. For activity analysis, images were smoothed using a 5x5 pixel averaging

370   filter. Areas without significant fluorescence were removed. Saturated areas were also removed

371   by limiting the area to intensity below the maximum value. Photobleaching was adjusted for by

372   removing any linear trend. Any islets with significant motion artifacts were removed or time

373   courses were shortened to the time over which no significant movement occurred

374   (displacement of <0.5 cell width). For the time course of each pixel in the image with significant

375   fluorescence, a peak detection algorithm was used to determine if the areas had peak

376   amplitudes significantly above background. A region was considered "active" if the

377   corresponding time course for each pixel had a peak amplitude >2.4x background. The fraction

378   of active area was calculated as the number of pixels detected as "active" across all z-planes,

379   normalized to the total number of pixels that showed significant fluorescence across all z-

380   planes that were not saturated. Islets with significant background fluorescence from spectral

381   overlap of channels were excluded from activity analysis because "inactive" cells were

18

382   indistinguishable from background and therefore total islet area could not be accurately

383   calculated. Coordination was determined based on coincident timing of identified peaks, where

384   areas were segmented by identified peaks occurring at similar time points. The cross

385   correlation of the time courses for two 5x5 pixel subregion was taken. If the correlation

386   coefficient was >0.75, then the two subregions were considered highly coordinated and merged

387   into a larger region. The coordinated area was calculated as the number of pixels in the largest

388   area of coordination across all z-planes normalized to the total number of pixels of the islet that

389   were determined to be 'active' for all planes. This analysis is based on previous analysis[50], but

390   adjusted for 3-dimensional data. All statistical analysis was performed in Prism (Graphpad) or

391   Matlab. First a F-test was used to determine if variances were equal then a Student's t-test or

392   Welch t-test (for unequal variance) were utilized for determining whether activity,

393   coordination, phase lag and speed were significantly different. $p<0.05$ was considered

394   significant.

395

396   ***In vitro* single cell Ca^{2+} imaging**

397   Islets were isolated according to standard protocol from 3-6 month old *Robo βKO* and control

398   mice. For islet dispersion, 12mm round No. 1.5 coverslips contained in a 24-well plate were pre-

399   coated overnight with 50μL 1:15000 PEI (Sigma P3143) overnight. Groups of 100 mouse islets

400   were dispersed into single cells in 3mL Accutase (Thermo Fisher A1110501) at 37°C for 10 min.

401   During the incubation, PEI was replaced with 100μL Geltrex (Thermo Fisher A1413302) and

402   centrifuged at 500*g* for 5 min at 4°C, followed by removal of excess Geltrex. The cells were

403   washed once with islet culture medium (RPMI1640 supplemented with 10% FBS (v/v), 100

19

404   units/mL penicillin, and 100ug/mL streptomycin (Invitrogen)) and resuspended in 1mL medium

405   before plating 500μL per coverslip. The plate was centrifuged for 5 minutes at 500g and

406   cultured overnight before imaging. For measurements of cytosolic Ca^{2+}, dispersed islet cells

407   were pre-incubated in 5μM Fura2-AM (Thermofisher F1201) in islet media containing 11.1mM

408   glucose for 45 min at 37°C, followed by 15 min incubation in islet media containing 2.7mM

409   glucose. Coverslips were transferred to a RC-48LP imaging chamber (Warner Instruments)

410   mounted on a Nikon Ti-Eclipse inverted microscope equipped with a 20X/0.75N.A. SuperFluor

411   objective and PerfectFocus (Nikon Instruments). The chamber was perfused with a standard

412   external solution containing 135mM NaCl, 4.8mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgCl_2$, 20mM

413   HEPES, and glucose as indicated (pH 7.35). The flow rate was set to 0.4mL/min (Fluigent MCFS-

414   EZ) and temperature was maintained at 33°C using solution and chamber heaters (Warner

415   Instruments). Excitation was provided by a SOLA SE II 365 (Lumencor) set to 10% output and an

416   inline neutral density filter (Nikon ND4). Fluorescence emission was collected with a

417   Hamamatsu ORCA-Flash4.0 V2 Digital CMOS camera at 0.1Hz. Excitation (x) and emission (m)

418   filters were used in combination with a ET FURA2/GFP C164605 dichroic (Chroma): Fura2,

419   ET365/20x, ET535/30m; mCherry ET572/35x and ET632/60m. β cells were identified by the

420   expression of mCherry. Baseline-normalized cytosolic calcium was quantified using Nikon

421   Elements and GraphPad Prism software.

Figure Legends

Figure 1: *Robo βKO* islets have a smaller fraction of homotypic nearest neighbors than controls (A) Immunofluorescence images (left and middle panels) and cell connectivity maps generated by nearest neighbor analysis (right panels) of control and *Robo βKO* islets. β cells (red), α cells (green), and δ cells (blue) are denoted by nodes on the connectivity maps. A line the same color as both nodes it connects denotes a homotypic interaction of that corresponding cell type. A white line connecting two nodes denotes a heterotypic interaction between cell types. (B) Probability of β cell-β cell contacts in *Robo βKO* islets *vs*. controls (*n*=9-11 islets for 3 mice from each genotype; control 75.35%±4.1, *Robo βKO* 50.37%4.1, *p*=0.01). (C) Probability of any homotypic cell-cell contact in *Robo βKO* islets vs controls (*n*=9-11 islets for 3 mice from each genotype; control 83.7%±1.7, *Robo βKO* 64.43%±1.2, *p* 0.0008). (D Probability of β cell- α cell contacts in *Robo βKO* islets vs controls (*n*=9-11 islets for 3 mice from each genotype; control 11.21%±2.7, *Robo βKO* 25.99%±3.0, *p*=0.02). (E) Probability of any heterotypic cell-cell contact in *Robo βKO* islets *vs*. controls (*n*= 9-11 islets for 3 mice from each genotype; control 16.3%±1.7, *Robo βKO* 35.57%1.2, *p*=0.0008). (B-E Similar shaded points in graphs indicate islets from the same mouse).

Figure 2: Control islets show highly synchronized whole islet Ca^{2+} oscillations (A) High resolution maximum intensity projection of a control islet *in vivo* in an *AAV8-RIP-GCaMP6s-*injected mouse showing GCaMP6s in green, nuclear mCherry β cell lineage-tracing in red, and collagen (second-harmonic fluorescence) in blue. (B) Stills over one oscillation period from control islet in supplementary video 1, starting after blood glucose level reached ~300mg/dL

21

444   from IP glucose injection. Video was recorded for 10 minutes with an acquisition speed of

445   0.1Hz. (C) Representative time courses of Ca^{2+} activity in 4 individual areas from control islet in

446   supplementary video 1 showing correlation over 98% of the active islet area. Time courses are

447   normalized to average fluorescence of individual area over time. Similar color indicates that the

448   time courses have a Pearson's correlation coefficient of ≥0.75 and matches the region of

449   coordination that is seen in D. (D) False color map of top five largest coordinated areas across z-

450   stack of control islet from analysis in C. Areas in grey are not coordinated. The color represents

451   a region of coordination with Pearson's Correlation Coefficient ≥0.75 of GCaMP6s activity. Cells

452   used in time courses in C are labeled.

453

454   **Figure 3: *Robo βKO* islets show uncoordinated whole islet Ca^{2+} oscillations** (A) High resolution

455   maximum intensity projection of a *Robo βKO* islet *in vivo* in an *AAV8-RIP-GCaMP6s*-injected

456   mouse showing GCaMP6s in green, nuclear mCherry β cell lineage-tracing in red, and collagen

457   (second-harmonic fluorescence) in blue. (B) Stills over one oscillation period from *Robo βKO*

458   islet in supplemental video 4, starting after blood glucose level reached ~300mg/dL from IP

459   glucose injection. Video was recorded for 10 minutes with an acquisition speed of 0.1Hz. (C)

460   Representative time courses of Ca^{2+} activity in 4 individual areas from *Robo βKO* islet in

461   supplementary video 4, showing correlation of 50% of the active islet area. Time courses are

462   normalized to average fluorescence of individual area over time. Similar color indicates that the

463   time courses. Similar color indicates that the time courses have a Pearson's correlation

464   coefficient of ≥0.75 and matches the region of coordination that is seen in D. (D) False color

465   map of top five largest coordinated areas across z-stack of *Robo βKO* islet from analysis in C.

466   Areas in grey are not coordinated. The color represents a region of coordination with Pearson's

467   correlation coefficient of ≥0.75 of GCaMP6s activity.

468

469   **Figure 4: Quantification of *Robo βKO* Ca^{2+} oscillation phenotype** (A) Largest fraction of area in

470   islet exhibiting coordinated Ca^{2+} oscillations for control and *Robo βKO* islets. (B) Fraction of

471   active islet area showing elevated Ca^{2+} activity for control and *Robo βKO* islets.

472

473   **Figure 5*: Dissociated *Robo βKO* β cells show no difference in glucose stimulated Ca^{2+}**

474   **oscillations** (A) Representative Ca^{2+} trace (Fura2) of a single dispersed β cell from a control islet.

475   10G line marks the addition of 10mM glucose. (B) Representative Ca^{2+} trace (Fura2) of a

476   dispersed β cell from a *Robo βKO* islet. 10G line marks the addition of 10mM glucose. (C) Graph

477   showing the proportion of Ca^{2+} responsive β cells in *Robo βKO* compared to controls (D) Graph

478   showing peak height of Ca^{2+} oscillation corresponding to first phase insulin secretion from

479   control and *Robo βKO* single dispersed β cells in response to 10mM glucose. (E) Graph showing

480   area under the curve (AUC) of Ca^{2+} oscillations (Fura2) from control and *Robo βKO* single

481   dispersed β cells in response to 10mM glucose. (F) Graph showing area under the curve (AUC)

482   of Ca^{2+} oscillations (Fura2) from control and *Robo βKO* single dispersed β cells in response to

483   KCL.

484

485   **Figure 6: Amount of Cx36 gap junctions remains unchanged in *Robo βKO*** (A)

486   Immunofluorescent images showing Cx36 (gray or green) and insulin (red) in Control, *Robo*

487   *βKO*, and *Cx36 KO* islets. (B) Quantification of area of Cx36 staining normalized to islet area in

23

488   *Robo βKO* islets and controls showing no significant difference (*n*=10-20 islets from 2-4 mice per

489   group, *p values shown*). similar colored dots represent islet from the same mouse (C)

490   Immunofluorescent images showing histone H2B-mCherry β cell lineage trace in red, F-Actin

491   (phalloidin) in blue, and Cx36 in yellow demonstrating normal localization of Cx36 to plasma

492   membrane (visible as white dots) of β cells in both control and *Robo βKO* islets.

493

494   **Figure 7: Amount of vascularization remains unchanged in *Robo βKO* islets** (A) Representative

495   immunofluorescent staining of Collagen IV marking vasculature showing similar amounts in

496   *Robo βKO* and control islets (B) Quantification of area of Collagen IV staining normalized to islet

497   area showing no difference in amounts of basement membrane marking blood vessels in *Robo*

498   *βKO* compared to control islets. (C) Representative immunofluorescent staining of laminin

499   marking vasculature showing similar amounts in *Robo βKO* and control islets (D) Quantification

500   of area of laminin staining normalized to islet area showing no difference in amounts of

501   basement membrane marking blood vessels in *Robo βKO* compared to control islets.

502 **Supplemental Figures**

503 **Supplemental Video 1: Control islets show highly synchronized Ca^{2+} oscillations**. Intravital time

504 course video of an islet within the *in vivo* pancreas of a control β cell lineage traced mouse

505 infected with *AAV8-Ins1-GCaMP6s*. Lineage traced β cells are marked by mCherry in red and

506 GCaMP6s is shown in green. Mouse was injected IP with glucose, and video was recorded once

507 blood glucose levels reached ~300mg/dL. Z-stack of 8 slices each 8μm apart were recorded at

508 0.1Hz over 10 minutes. Scale bar is 100μm. Time stamp shown in in upper left corner shows

509 time of image in min:sec.

510

511 **Supplemental Video 2: Control islets show highly synchronized Ca^{2+} oscillations**. Intravital time

512 course video of an islet within the *in vivo* pancreas of a control β cell lineage traced mouse

513 infected with *AAV8-Ins1-GCaMP6s*. Lineage traced β cells are marked by mCherry in red and

514 GCaMP6s is shown in green. Mouse was injected IP with glucose, and video was recorded once

515 blood glucose levels reached ~300mg/dL. Z-stack of 3 slices each 8μm apart were recorded at

516 0.2Hz over 12 minutes. Scale bar is 100μm. Time stamp shown in in upper left corner shows

517 time of image in min:sec.

518

519 **Supplemental Video 3: *Robo βKO* islets show unsynchronized Ca^{2+} oscillations.** Intravital time

520 course video of an islet within the *in vivo* pancreas of a *Robo βKO* β cell lineage traced mouse

521 infected with *AAV8-Ins1-GCaMP6s*, and retro-orbitally injected with rhodamine-dextran to

522 mark vasculature. Lineage traced β cells are marked by mCherry in red and GCaMP6s is shown

523 in green, and vasculature is shown in yellow. Mouse was injected IP with glucose, and video

25

524 was recorded once blood glucose levels reached ~300mg/dL. Z-stack of 12 slices each 8μm

525 apart were recorded at 0.03Hz over 10 minutes. Scale bar is 100μm. Time stamp shown in in

526 upper left corner shows time of image in min:sec.

527

528 **Supplemental Video 4: *Robo βKO* islets show unsynchronized Ca^{2+} oscillations.** Intravital time

529 course video of an islet within the *in vivo* pancreas of a *Robo βKO* β cell lineage traced mouse

530 infected with *AAV8-Ins1-GCaMP6s*. Lineage traced β cells are marked by mCherry in red and

531 GCaMP6s is shown in green. Mouse was injected IP with glucose, and video was recorded once

532 blood glucose levels reached ~300mg/dL. Z-stack of 8 slices each 8μm apart were recorded at

533 0.1Hz over 10 minutes. Scale bar is 100μm. Time stamp shown in upper left corner shows time

534 of image in min:sec.

535

536 **Supplemental Video 5: *Robo βKO* islets show unsynchronized Ca^{2+} oscillations.** Intravital time

537 course video of an islet within the *in vivo* pancreas of a *Robo βKO* β cell lineage traced mouse

538 infected with *AAV8-Ins1-GCaMP6s*. Lineage traced β cells are marked by mCherry in red and

539 GCaMP6s is shown in green. Mouse was injected IP with glucose, and video was recorded once

540 blood glucose levels reached ~300mg/dL. Z-stack of 3 slices each 8μm apart were recorded at

541 0.2Hz over 10 minutes. Scale bar is 100μm. Time stamp shown in upper left corner shows time

542 of image in min:sec.

543

544 **Supplemental Video 6: A subset of *Robo βKO* islets retain synchronized Ca^{2+} oscillations.**

545 Intravital time course video of an islet within the *in vivo* pancreas of a *Robo βKO* β cell lineage

546   traced mouse infected with AAV8-Ins1-GCaMP6s. Lineage traced β cells are marked by mCherry

547   in red and GCaMP6s is shown in green. Mouse was injected IP with glucose, and video was

548   recorded once blood glucose levels reached ~300mg/dL. Z-stack of 8 slices each 8μm apart

549   were recorded at 0.1Hz over 10 minutes. Scale bar is 100μm. Time stamp shown in in upper left

550   corner shows time of image in min:sec.

551

552   **Supplemental Figure 1: *Robo βKO* islets retain β cell differentiation and maturity markers**

553   Volcano plot of differential gene expression from bulk RNA sequencing on lineage traced FACS

554   sorted β cells from *Robo βKO* and control mice showing no significant differential gene

555   expression of markers (*n*=2 mice from each group) Red lines denote a fold change of 1.5 and

556   blue line denotes a p value of 0.05.

557

558   **Supplemental Figure 2: *Robo βKO* islets show uncoordinated whole islet Ca^{2+} oscillations** (A)

559   High resolution maximum intensity projection of a *Robo βKO* islet *in vivo* in an *AAV8-RIP-*

560   *GCaMP6s*-injected mouse showing GCaMP6s in green, nuclear mCherry β cell lineage tracing in

561   red, and collagen in blue. (B) Stills over one oscillation period from *Robo βKO* islet in

562   supplementary video 3, starting after blood glucose level reached ~300mg/dL from IP glucose

563   injection. Video was recorded for 10 minutes with an acquisition speed of 0.03Hz. (C)

564   Representative time courses of Ca^{2+} activity in 4 individual areas from *Robo βKO* islet in

565   supplementary video 3, showing correlation of 43.6% of the active islet area. Time courses are

566   normalized to average fluorescence of individual area over time. Similar color indicates that the

567   time courses have a Pearson's correlation coefficient of ≥0.75 and matches the region of

568   coordination that is seen in D. (D) False color map of top five largest coordinated areas across z-

569   stack of *Robo βKO* islet from analysis in C. Areas in grey are not coordinated. The color

570   represents a region of coordination with Pearson's Correlation Coefficient ≥0.75 of GCaMP6s
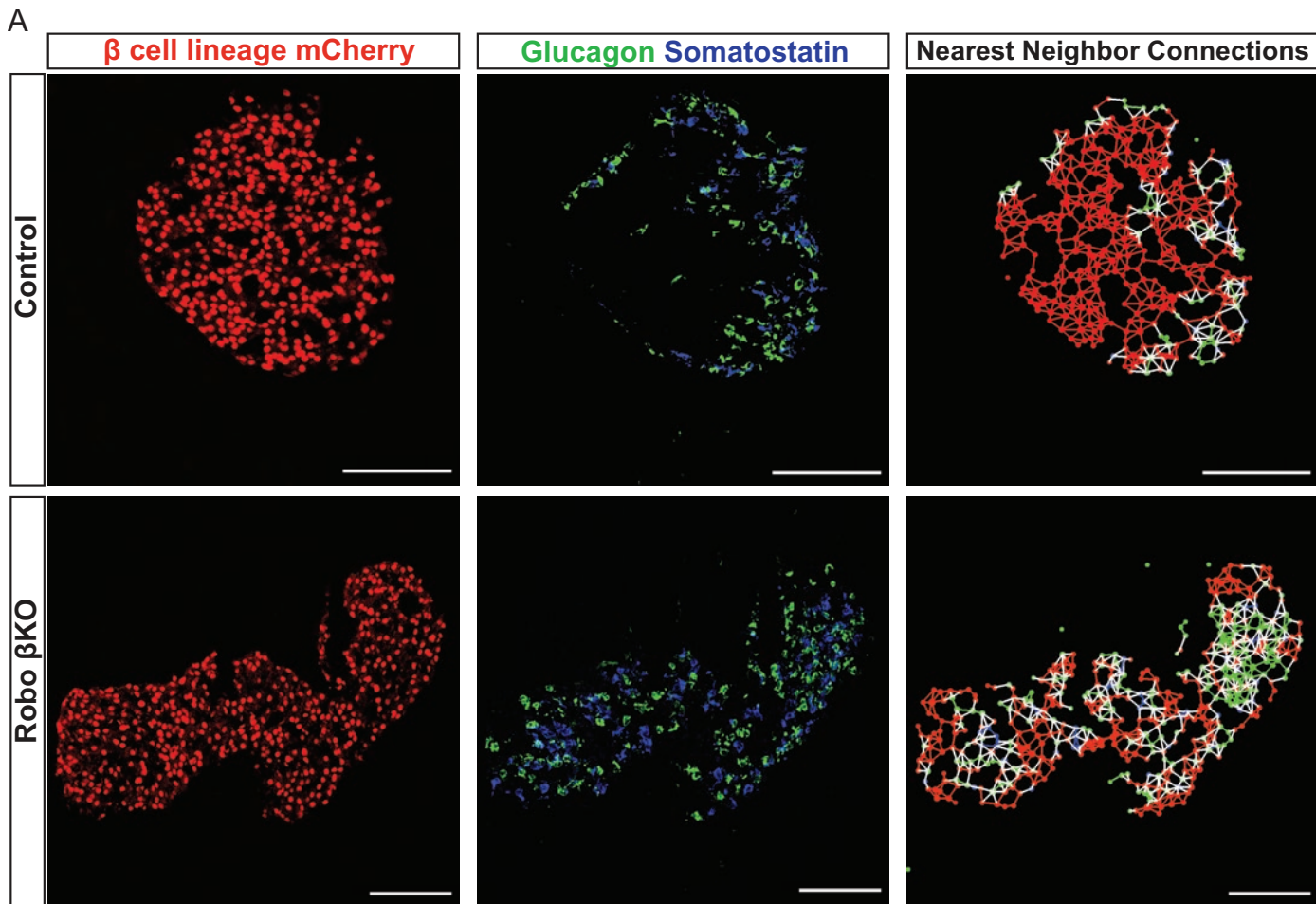
571   activity. Cells used in time courses in C are labeled.

572

573   **Supplemental Figure 3: A subset of *Robo βKO* islets show coordinated whole islet Ca^{2+}**

574   **oscillations** (A) High resolution maximum intensity projection of a *Robo βKO* islet *in vivo* in an

575   *AAV8-RIP-GCaMP6s*-injected mouse showing GCaMP6s in green, nuclear mCherry β cell lineage

576   tracing in red, and collagen in blue. (B) Stills over one oscillation period from *Robo βKO* islet in

577   supplementary video 6, starting after blood glucose level reached ~300mg/dL from IP glucose

578   injection. Video was recorded for 10 minutes with an acquisition speed of 0.1Hz. (C)

579   Representative time courses of Ca^{2+} activity in 4 individual areas from *Robo βKO* islet in

580   supplementary video 6, showing correlation of 98% of the active islet area. Time courses are

581   normalized to average fluorescence of individual area over time. Similar color indicates that the

582   time courses have a Pearson's correlation coefficient of ≥0.75 and matches the region of

583   coordination that is seen in D. (D) False color map of top five largest coordinated areas across z-

584   stack of *Robo βKO* islet from analysis in C. Areas in grey are not coordinated. The color

585   represents a region of coordination with Pearson's correlation coefficient of ≥0.75 of GCaMP6s

586   activity.

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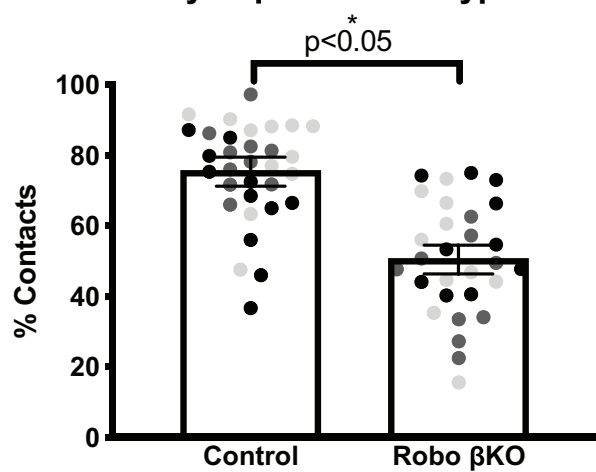676   *Diabetes* **27**, 1-7 (1978).

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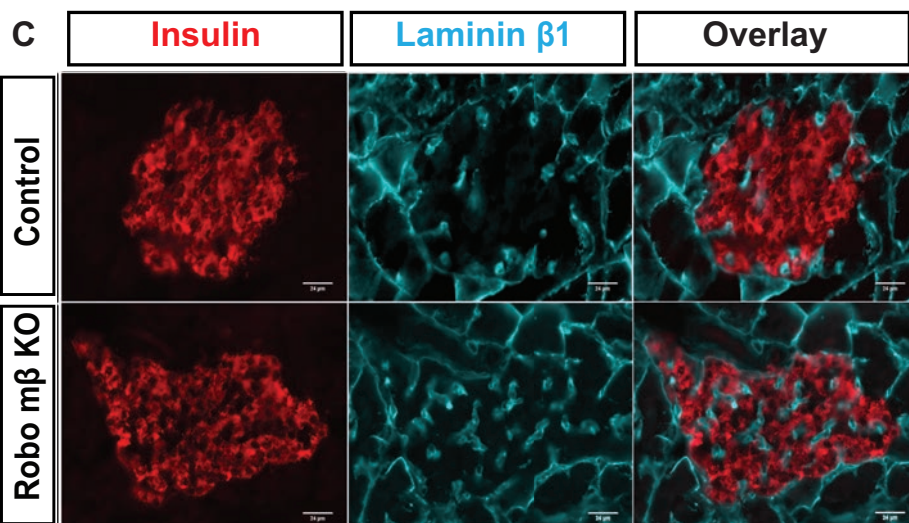729   Secretory Dynamics in Human Pancreatic Islets. *Diabetes* **66**, 2436-2445,
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Figure 1
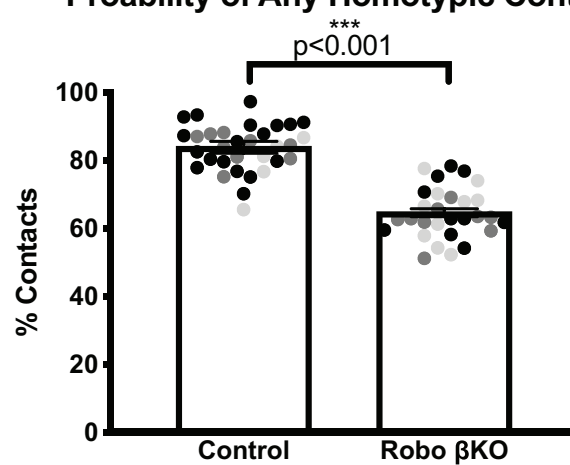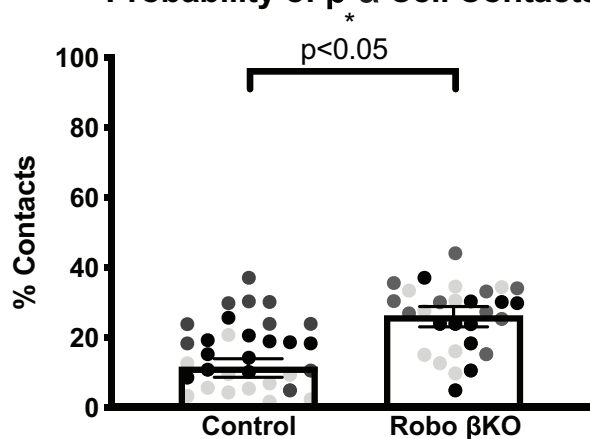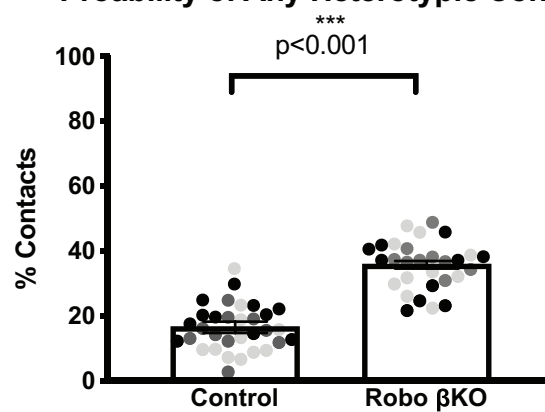
Figure 2

Figure 3

A

B

C

D

Figure 4

A **Fraction of Correlated GCaMP6s Activity Across Islet**

B **Fraction of Active GCaMP6s Area Across Islet**

Figure 5

A Representative Ca^{2+} trace from control single β cell

B Representative Ca^{2+} trace from Robo βKO single β cell

C Percent mCherry+ cells with Ca^{2+} response to 10mM glucose

D Ca^{2+} peak height of first phase insulin secretion in single β cells

E AUC of β cell Ca^{2+} response from exposure to 10mM glucose

AUC of β cell Ca^{2+} response from exposure to KCL
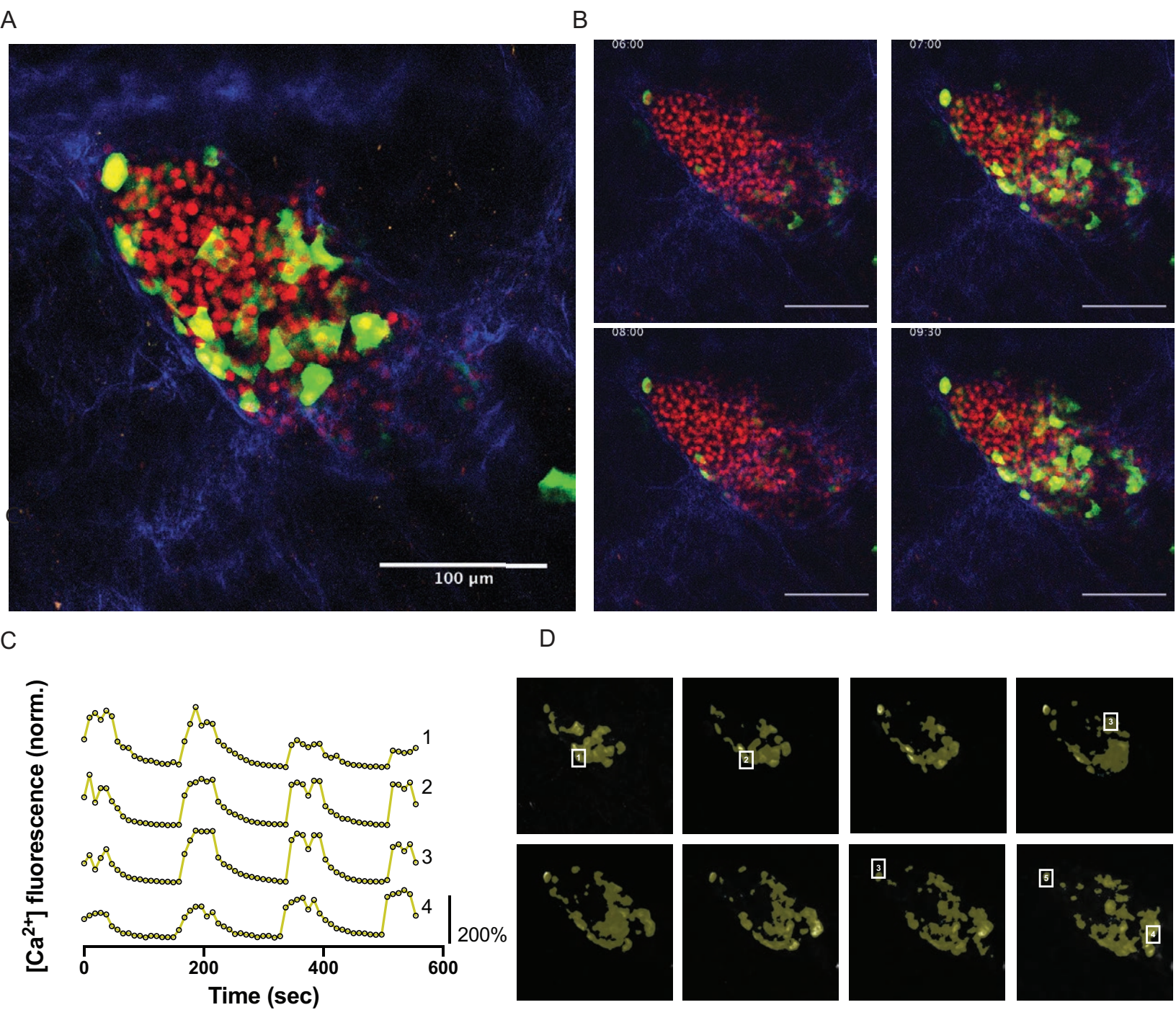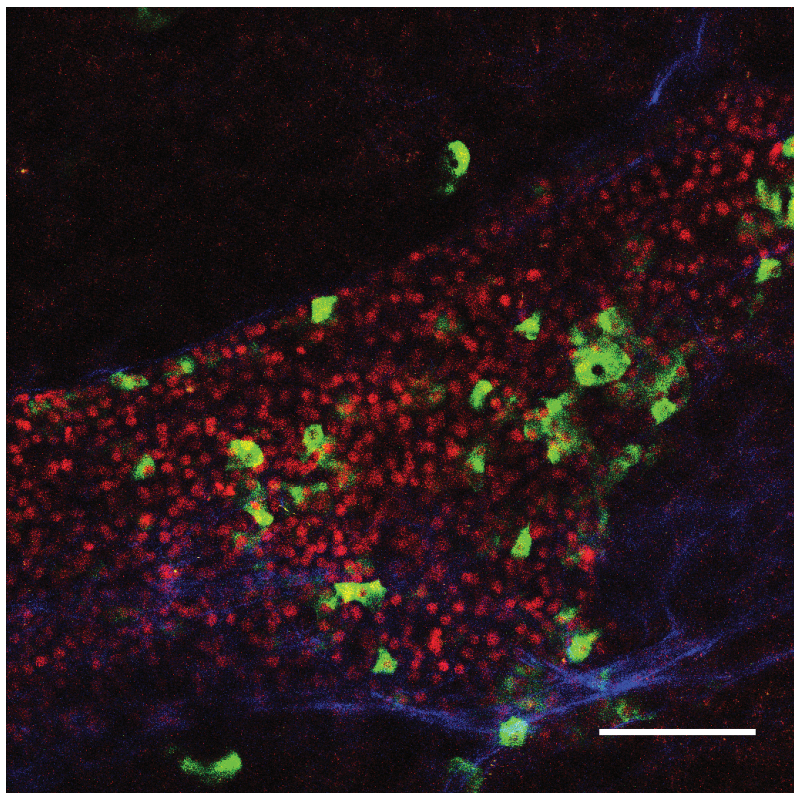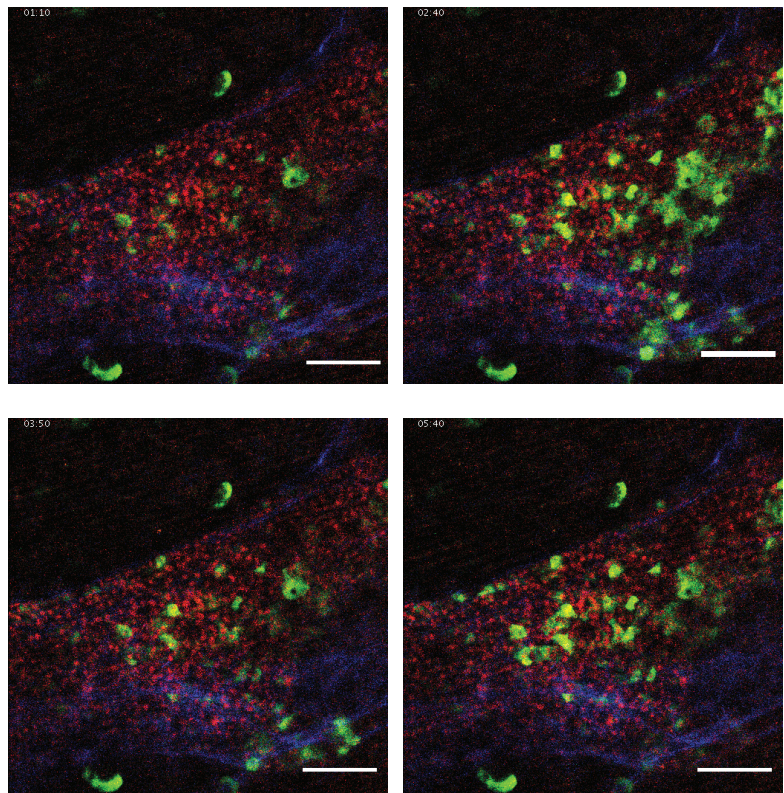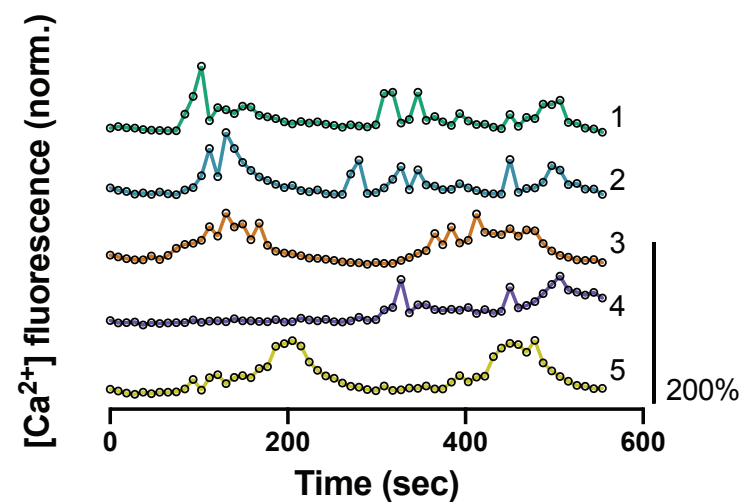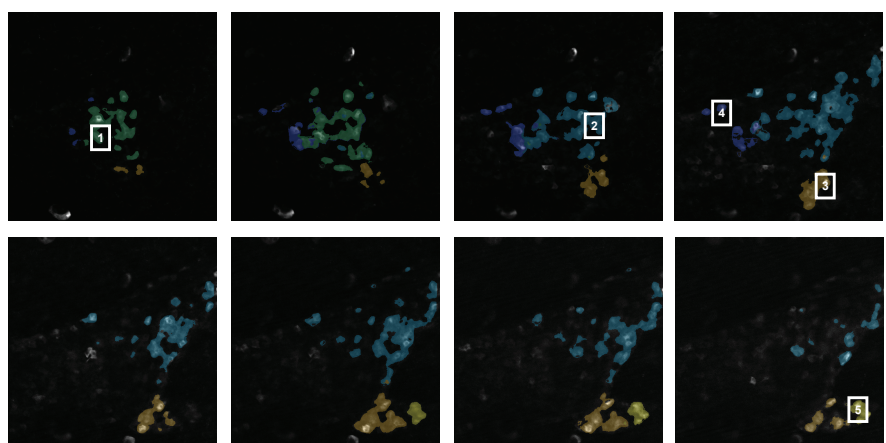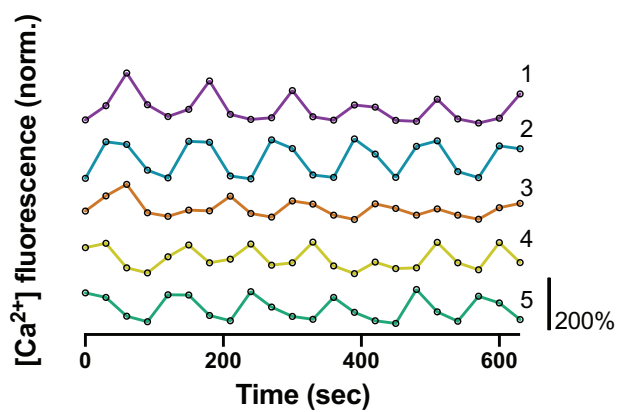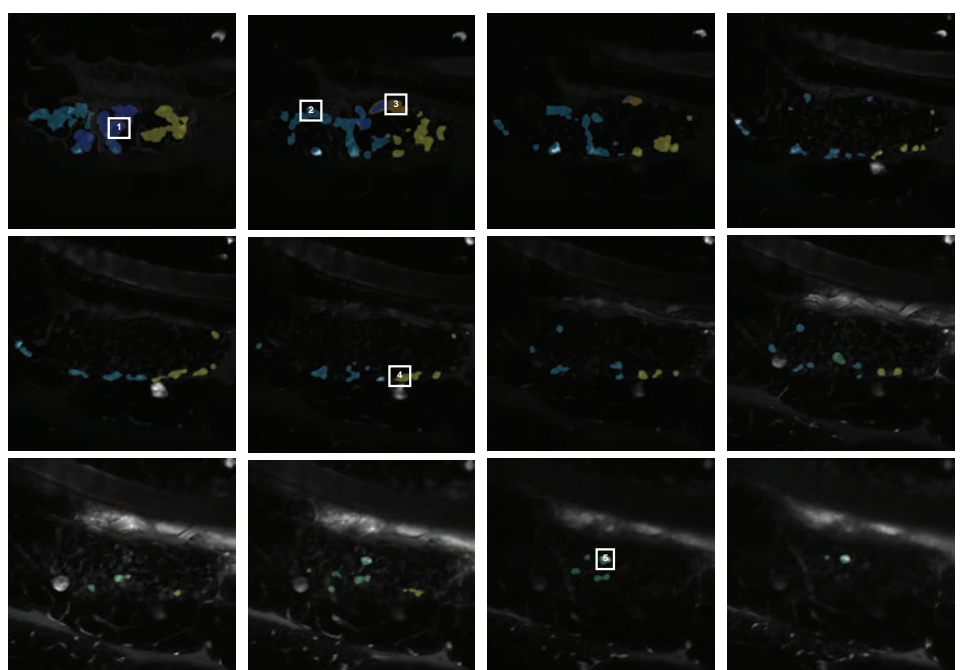
Figure 6

Figure 7

Supplemental Figure 1

Supplemental Figure 2

Supplemental Figure 3