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RESEARCH ARTICLE

Figures and figure supplements

Age-related islet inflammation marks the proliferative decline of pancreatic beta-cells in zebrafish

Sharan Janjuha *et al*

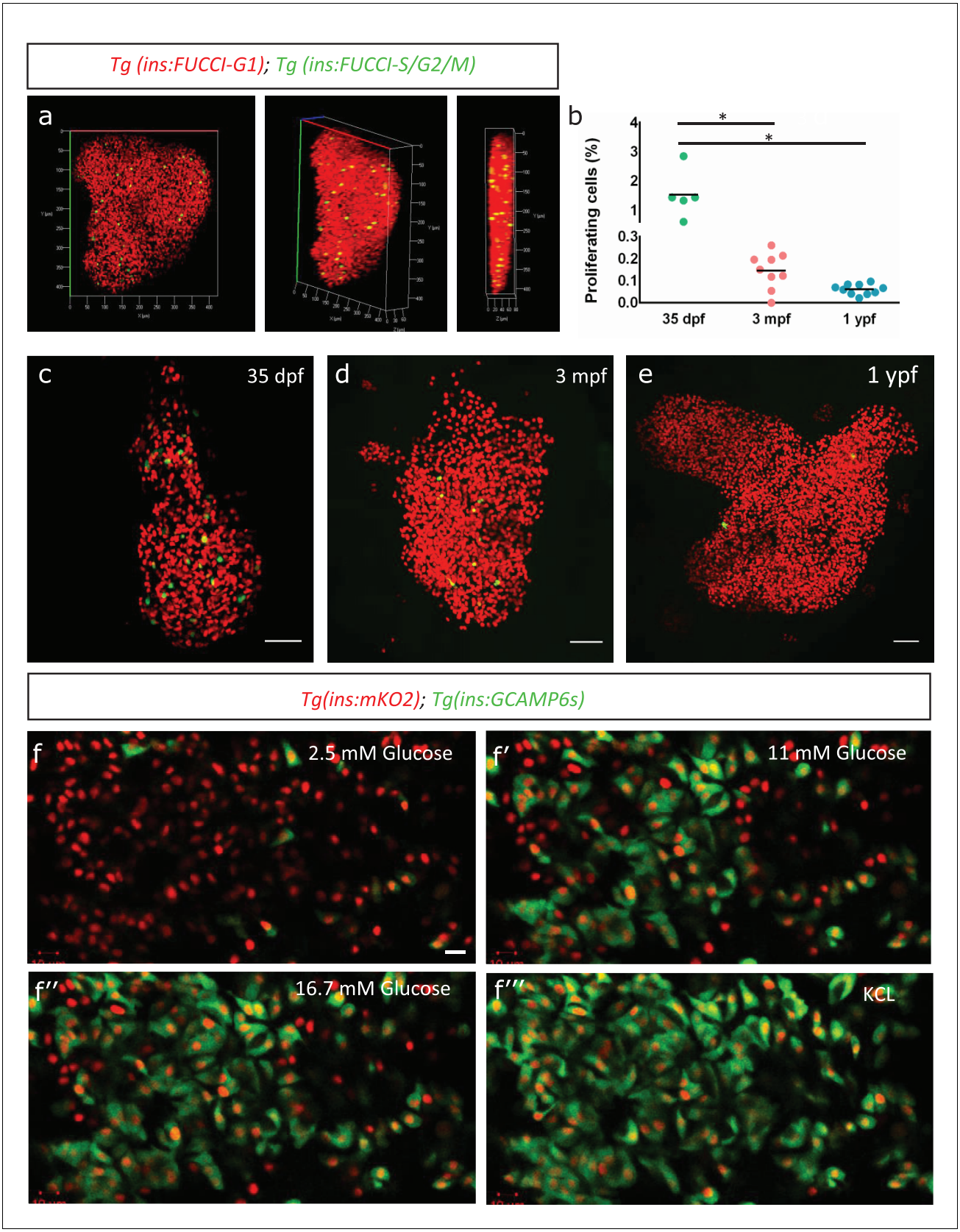

Figure 1. Beta-cell proliferation declines with age. (**a**) 3D-rendering of a primary islet from *Tg(ins:Fucci-G1);Tg(ins:Fucci-S/G2/M)* animals at 3 mpf showing nuclear *Tg(ins:Fucci-G1)* (red) and *Tg(ins:Fucci-S/G2/M)* (green) expression. (**b**) Quantification of percentage of *Tg(ins:Fucci-S/G2/M)*-positive

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Figure 1 continued

and *Tg(ins:Fucci-G1)*-negative (green-only) beta-cells at 35 dpf (n = 5), 3 mpf (n = 9) and 1 ypf (n = 10) animals. Each dot represents one animal. Horizontal bars represent mean values (one-way ANOVA, *p<0.05). (**c, d, e**) Confocal projection of whole-mount islets from *Tg(ins:Fucci-G1);Tg(ins:Fucci-S/G2/M)* animals at 35 dpf, 3 mpf and 1 ypf. Anterior to the top. Scale bar 50 μm. (**f**) Ex vivo live-imaging of beta-cells from *Tg(ins:nlsRenilla-mKO2)*;Tg(ins:GCaMP6s) animals at 3 mpf. Beta-cells (red) were stimulated with 2.5 (basal) mM D-Glucose, (**f'**) 11 mM D-glucose, (**f''**) 16.7 mM D-glucose and (**f'''**) depolarized using 30 mM KCl while monitoring GCAMP6s-fluorescence (green). Scale bar 10 μm.

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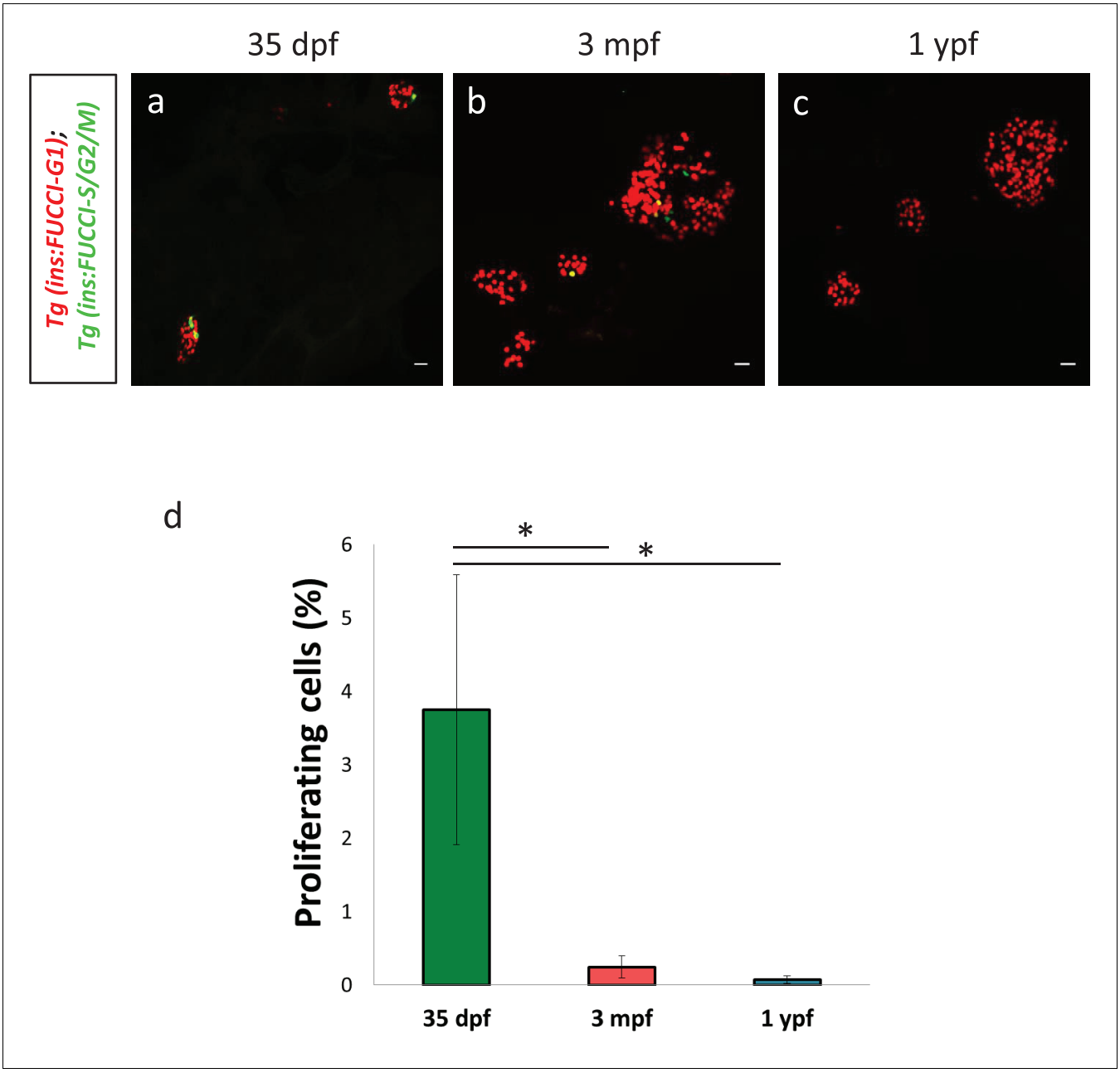

Figure 1—figure supplement 1. Beta-cell proliferation declines with age in secondary islets. (**a,b**) Confocal projection of whole-mount secondary islets from *Tg(ins:Fucci-G1);Tg(ins:Fucci-S/G2/M)* animals at 35 dpf, 3 mpf and 1 ypf. Scale bar 20 μm. (**c**) Quantification of percentage of *Tg(ins:Fucci-S/G2/M)*-positive and *Tg(ins:Fucci-G1)*-negative (green-only) beta-cells from 35 dpf (n = 8 fish, secondary islets = 16), 3 mpf (n = 8 fish, secondary islets = 70) and 1 ypf (n = 8 fish, secondary islets = 76) animals. Error bars show s.e.m. (one-way ANOVA, *p<0.05).

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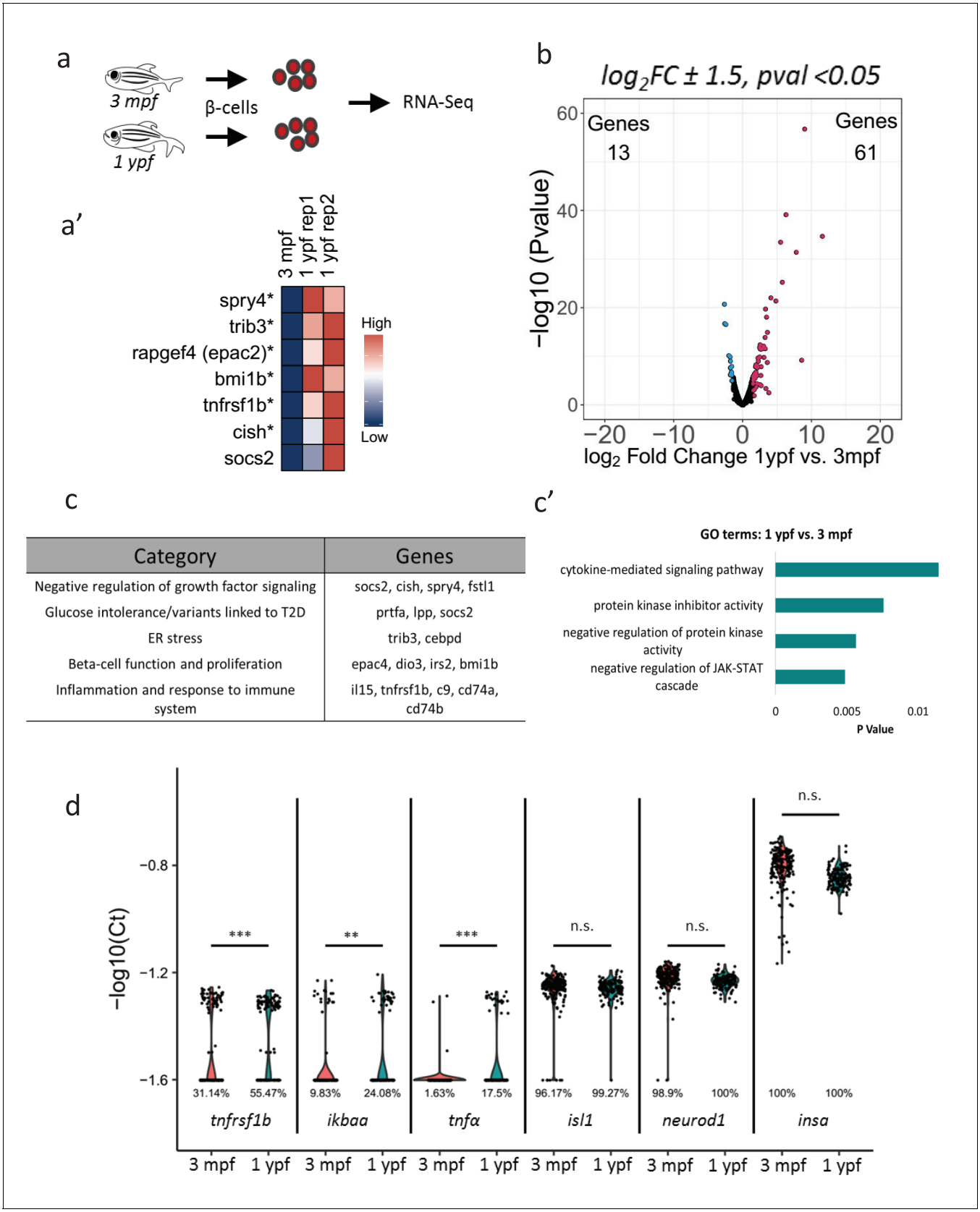

Category	Genes
Negative regulation of growth factor signaling	socs2, cish, spry4, fstl1
Glucose intolerance/variants linked to T2D	prtfa, lpp, socs2
ER stress	trib3, cebpd
Beta-cell function and proliferation	epac4, dio3, irs2, bmi1b
Inflammation and response to immune system	il15, tnfrsf1b, c9, cd74a, cd74b

Figure 2. Transcriptome profiling of younger and older beta-cells. (**a**) Schematic showing isolation and FAC-sorting of beta-cells from *Tg(ins:nlsRenilla-mKO2)* animals at 3 mpf and 1 ypf followed by high-throughput mRNA-Sequencing. (**a'**) Heatmap depicting differentially regulated genes among the

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beta-cells at 1 ypf and 3 mpf involved in beta-cell proliferation, function and inflammation (asterisks denote genes vaildated by single-cell RT-qPCR). (**b**) Volcano plot representing the distribution of genes that were differentially regulated in beta-cells from 1 ypf and 3 mpf (1.5-log_2fold change, $p<0.05$). (**c**) The biological categories of enriched genes in beta-cells at one ypf (1.5-log_2fold change, $p<0.05$) based on literature survey. (**c'**) Unbiased gene-ontology analysis using DAVID of genes enriched in beta-cells at 1 ypf ($p<0.05$). (**d**) Gene expression analysis was carried out using single-cell RT-qPCR. Violin plots denote expression distribution of the candidate genes. The Y-axis shows -log_{10}(Ct) values of transcript levels in single beta-cells. The X-axis shows gene names and the respective developmental stages. The percentage values under each violin plot denote the proportion of beta-cells with detectable transcript levels. The cycle threshold for detectable gene expression was set as Ct = 40. The value −1.6 (-log_{10}(40)) on the Y-axis represents undetectable expression as measured by single-cell RT-qPCR (see Materials and methods). Each dot represents one beta-cell. Significance testing for differences in proportion of cells with detectable gene expression at each stage was performed using Pearson's Chi-Square test (**$p<0.01$, ***$p<0.001$).
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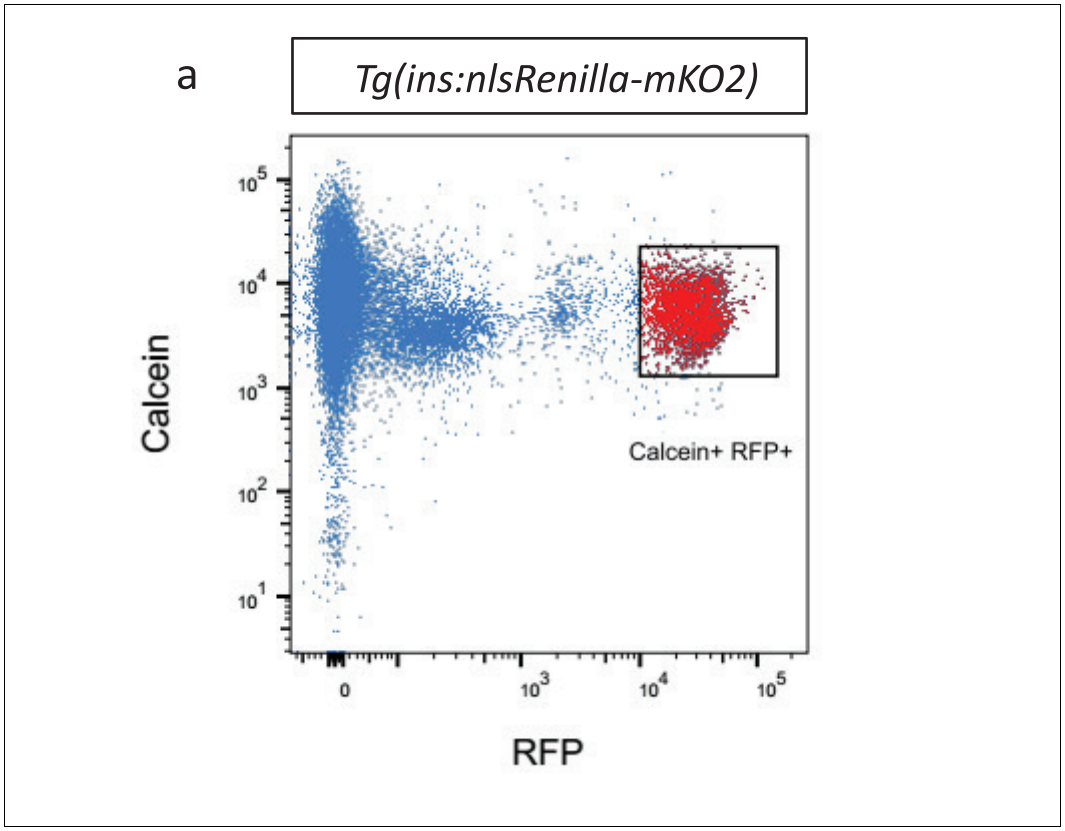

Figure 2—figure supplement 1. Fluorescent activated cell sorting of beta-cells. (**a**) Fluorescent activated cell sorting (FACS) of RFP-positive and calcein-positive beta-cells from *Tg(ins:nlsRenilla-mKO2)* animals.
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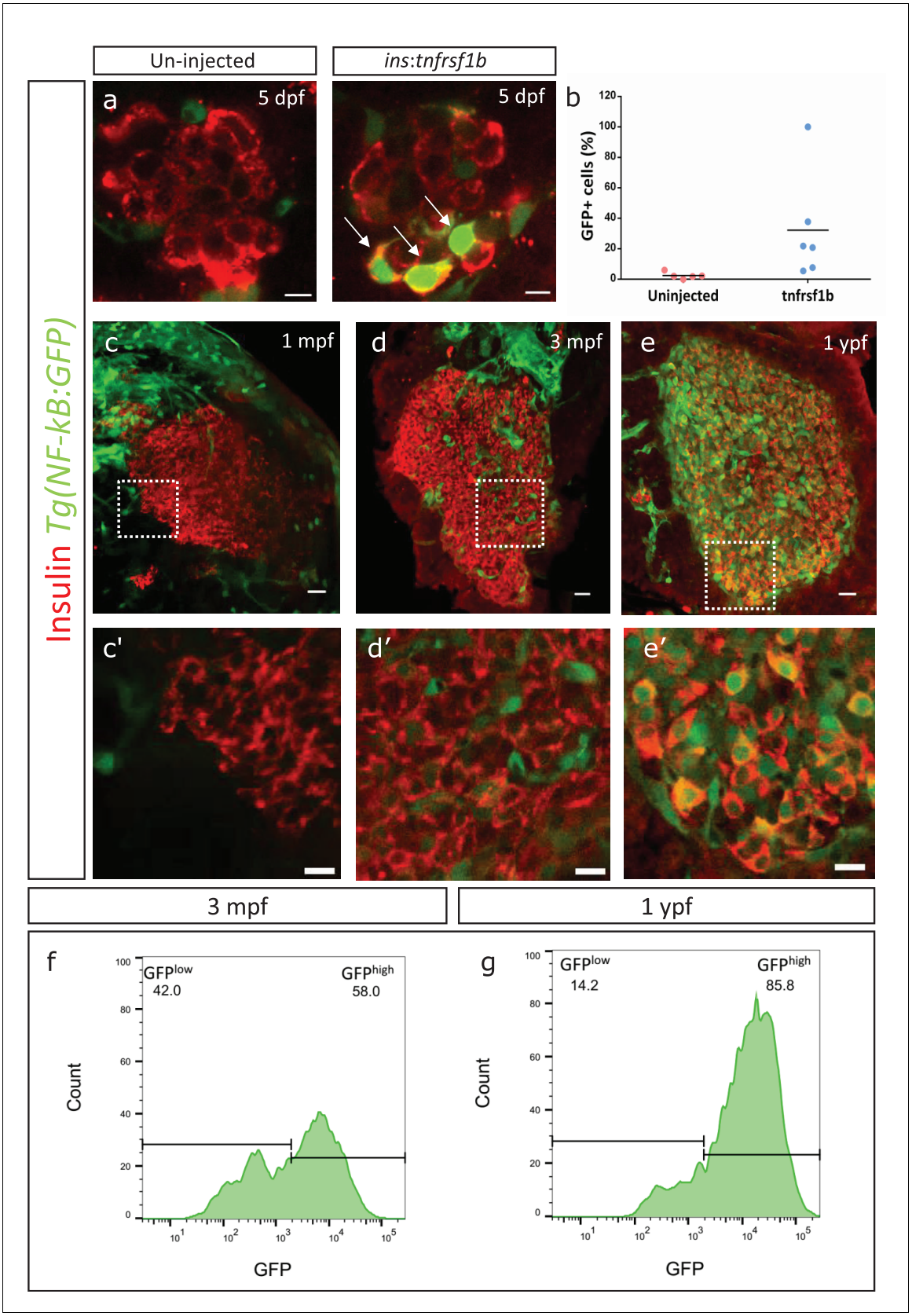

Figure 3. An inflammation reporter reveals heterogeneous activation of NF-kB signaling in beta-cells with age. (**a**) The images show single confocal planes from islets of 5 dpf larvae. The *tnfrsf1b* coding sequence was expressed under the control of the insulin promoter. The plasmid was injected in

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Tg(NF-kB:GFP) embryos at the one-cell-stage, leading to mosaic and stochastic expression of the construct in beta-cells. The *Tg(NF-kB:GFP)* reporter expresses GFP (green) under the control of six tandem repeats of NF-kB DNA-binding sites. Beta-cells were labelled using an insulin antibody (red). Arrows indicate GFP-positive beta-cells. Scale bar 5 μm. (**b**) The graph shows the percentage of GFP-positive and insulin-positive cells in uninjected controls (n = 5) and *tnfrsf1b* injected animals (n = 6) at 5 dpf. Horizontal bars represent mean values. (**c–e**) Confocal stack of islets from *Tg(NF-kB:GFP)* animals at 1 mpf, 3 mpf and 1 ypf. Beta-cells were labeled using an insulin antibody (red). *NF-kB*:GFP reporter expression is shown in green. Scale bars 20 μm. (**c'–e'**) Insets show high magnification single planes of the confocal stacks (corresponding to the regions shown using white dotted-lines in the top panels). Scale bar 10 μm. (**f–g**) Beta-cells from 3 mpf *Tg(NF-kB:GFP)* animals were labeled with TSQ (Zn2+ labeling dye) and analyzed using FACS. The graph shows GFP intensity (along the X-axis) and the distribution of beta-cells at 3 mpf and 1 ypf. Horizontal lines indicate the division point between GFP^{low} and GFP^{high} levels. Percentage values represent proportion of cells with GFP^{low} or GFP^{high} expression.

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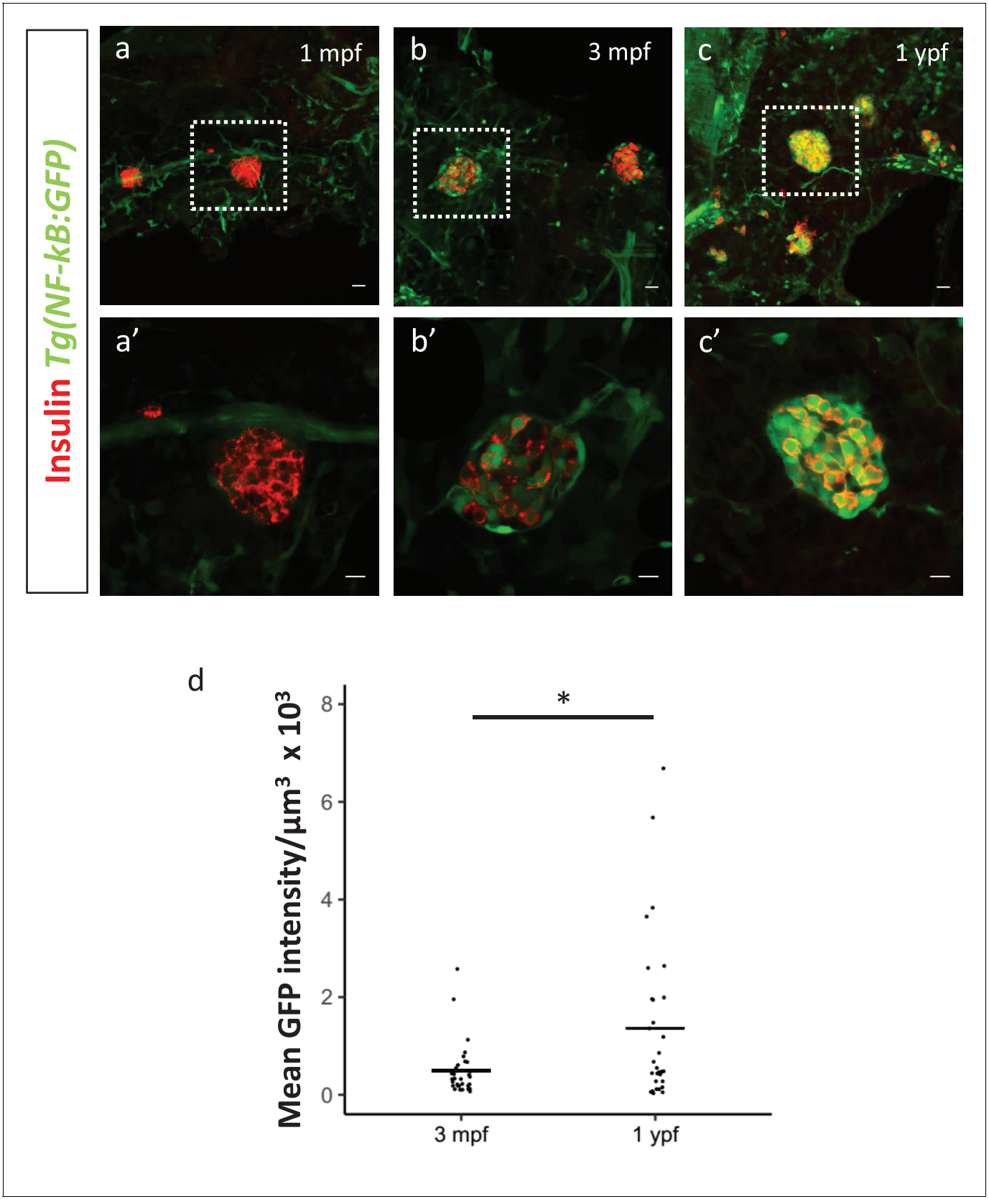

Figure 3—figure supplement 1. Activation of NF-kB signaling in beta-cells of the secondary islets with age. (**a,b,c**) Confocal stack of secondary islets from *Tg(NF-kB:GFP)* animals at 1 mpf, 3 mpf and 1 ypf. Beta-cells were labeled using an insulin antibody (red). *NF-kB*:GFP reporter expression is shown

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in green. Scale bars 20 μm. (**a',b',c'**) Insets show high-magnification single planes of the confocal stacks corresponding to the regions outlined using white dotted-lines in the top panels. Scale bar 10 μm. (**d**) Graph showing the total normalized GFP fluorescence intensity of the secondary islets from 3 mpf (n = 9 fish, secondary islets = 32) and 1 ypf (n = 8, secondary islets = 30) animals. Each dot represents one islet (two-tailed t-test, *p<0.05).
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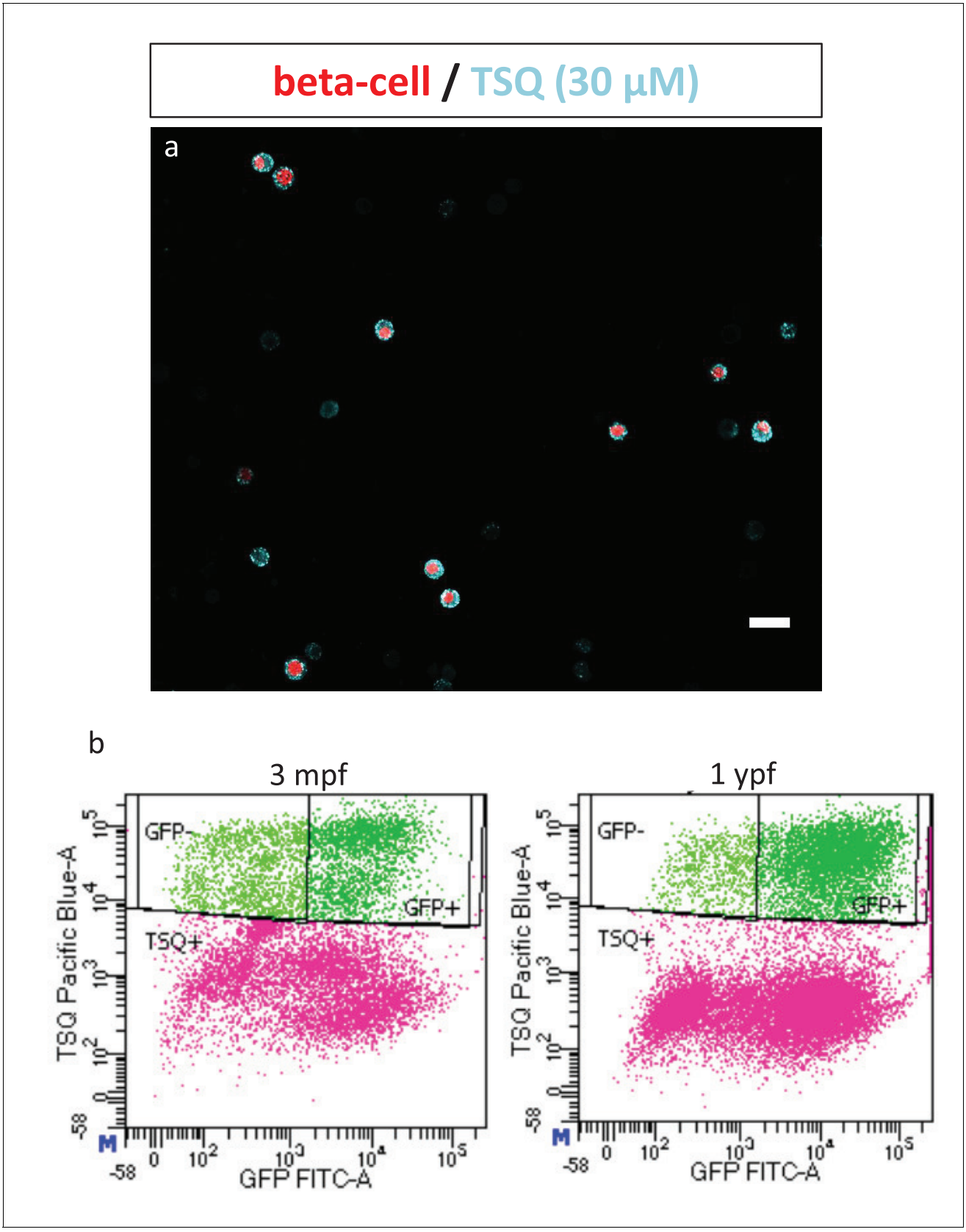

Figure 3—figure supplement 2. Fluorescent activated cell sorting of *NF-kB*:GFP^{high} and *NF-kB*:GFP^{low} beta-cells. (**a**) Confocal image of dissociated cells from dissected islets of 3 mpf *Tg(ins:nlsRenilla-mKO2)* animals that were labeled with TSQ (Zn^{2+} labeling dye). Islets were incubated with TSQ after

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dissociation and imaged using a confocal microscope. Beta-cells show RFP expression (red) while cells rich in Zn^{2+} are labeled with TSQ (cyan). TSQ strongly labels all beta-cells and weakly labels some unknown endocrine cells. (**b**) Fluorescent activated cell sorting (FACS) of live TSQ-positive GFP^{high} and GFP^{low} cells from *Tg(NF-kB:GFP)* animals at 3 mpf and 1 ypf. Dead cells were labelled using far-red stain DRAQ7.

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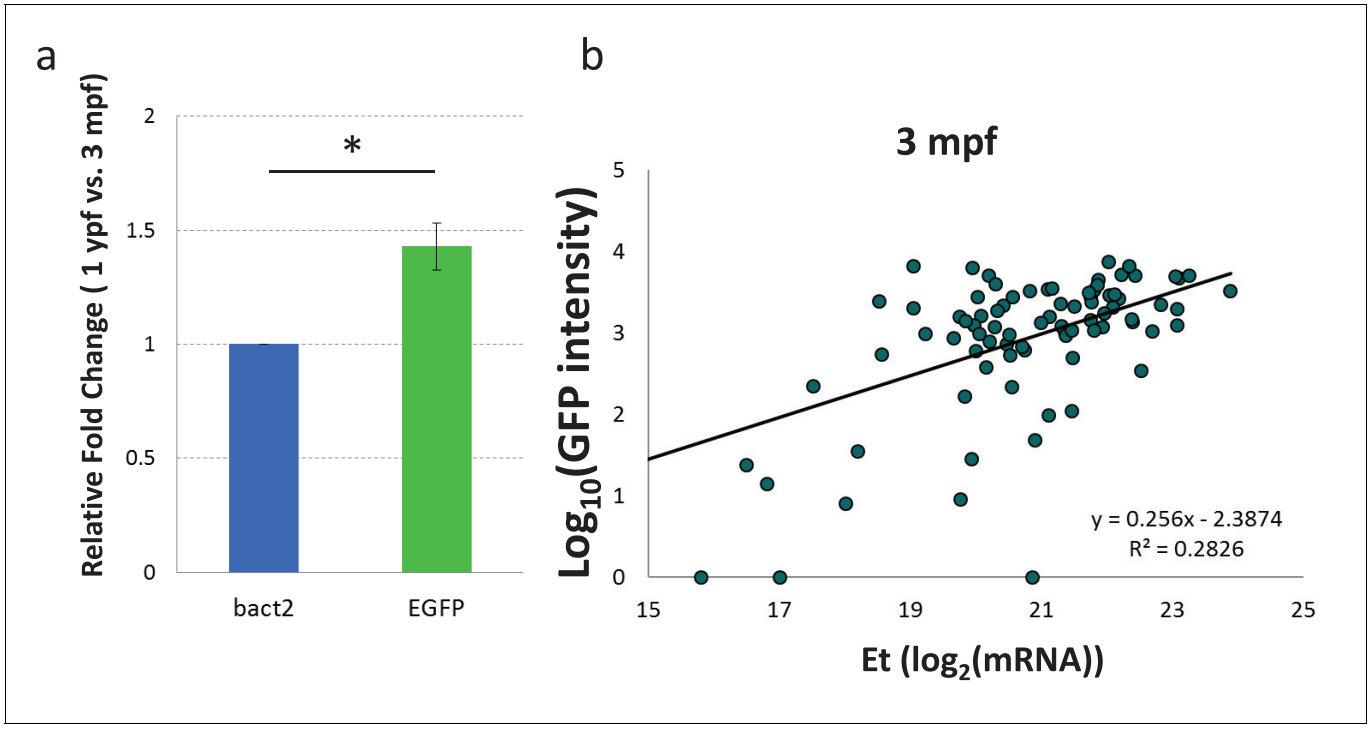

Figure 3—figure supplement 3. *NF-kB:EGFP* mRNA levels in beta-cells increase with age. (**a**) Graph showing the relative fold change increase in EGFP mRNA levels in beta-cells from 1 ypf compared to 3 mpf animals, as measured using RT-qPCR (n=5 biological replicates from three fish each, 1000 cells for each condition). Error bars show SD (two-tailed paired t-test, *p<0.05). (**b**) The graph shows the fluorescence levels of individual FAC-sorted beta-cells from *Tg(nF-kB:GFP);Tg(ins:mCherry)* animals as $\log_{10}$(GFP intensity) (along the Y-axis) and Expression threshold (Et) values of GFP mRNA (along X-axis) measured using single-cell RT-qPCR. Line indicates the correlation between GFP fluorescence intensity and GFP-mRNA expression levels in single cells. Each dot represents one beta-cell. $R^2 = 0.28$.

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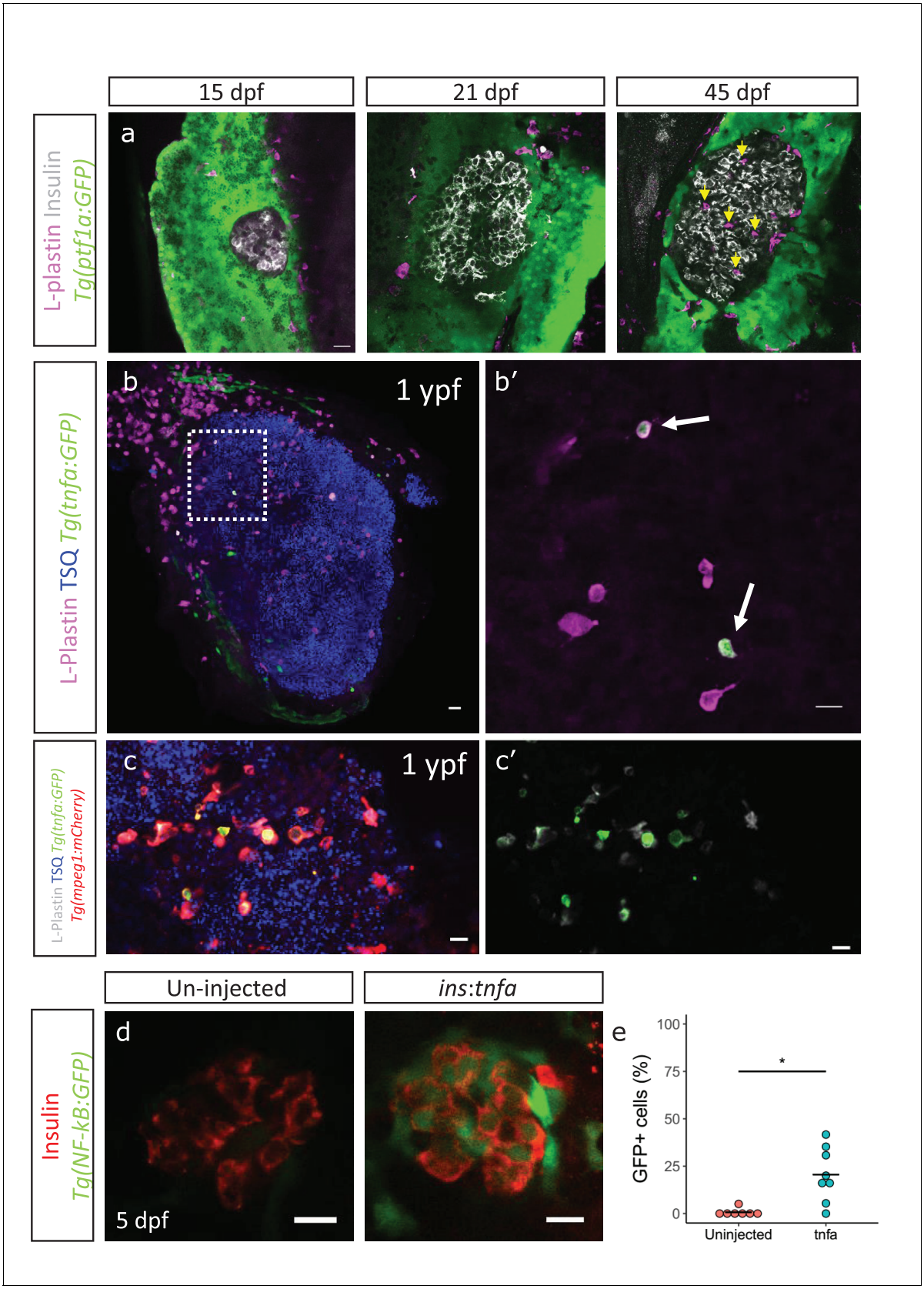

Figure 4. Immune cells infiltrate the islet during development and persist throughout adult life. (**a**) Confocal images of pancreata from 15, 21 and 45 dpf animals. Beta-cells were labeled using an insulin antibody (grey), leukocytes were labeled using an L-plastin antibody (magenta) and *Tg*(*ptf1a:*GFP)

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marks the acinar cells (green). Immune cells are present within the islet at 45 dpf (arrows). (**b**) Confocal images of whole islets from *Tg(tnfα:GFP)* animals at 1 ypf. Islets were labeled using TSQ (Zn^{2+} labeling dye) (blue), leukocytes were labelled with an L-plastin antibody (magenta) and *Tg*(*tnfα*:GFP) marks cells expressing *tnfα* (green). Scale bars 20 μm. (**b'**) Insets show high-magnification single planes from the confocal stacks (corresponding to the area marked using a white dotted-line in b). Scale bar 10 μm. (**c–c'**) Confocal image of a one ypf islet showing a single plane. The *TgBAC(tnfα:GFP)* line marks the *tnfα*-positive cells (green), whereas *Tg(mpeg1:mCherry)* marks the macrophages (red). The L-plastin antibody marks all leukocytes (grey) and TSQ (Zn2 +labeling dye) was used to mark the islet (n = 5). Scale bar, 10 μm. (**d**) Confocal images showing islets at five dpf. The *tnfα* cDNA was expressed under the insulin promoter. The plasmid was injected in *Tg(NF-kB:GFP)* embryos at the one-cell-stage and the islets were analyzed at 5 dpf. Beta-cells were labeled with an insulin antibody (red). *Tg(NF-kB*:GFP) reporter expression is shown in green. (**e**) The graph shows the percentage of GFP and insulin double-positive cells in un-injected controls (n = 7) and *ins:tnfα* injected animals (n = 8) at five dpf. Horizontal bars represent mean values (two-tailed t-test, *p<0.05).

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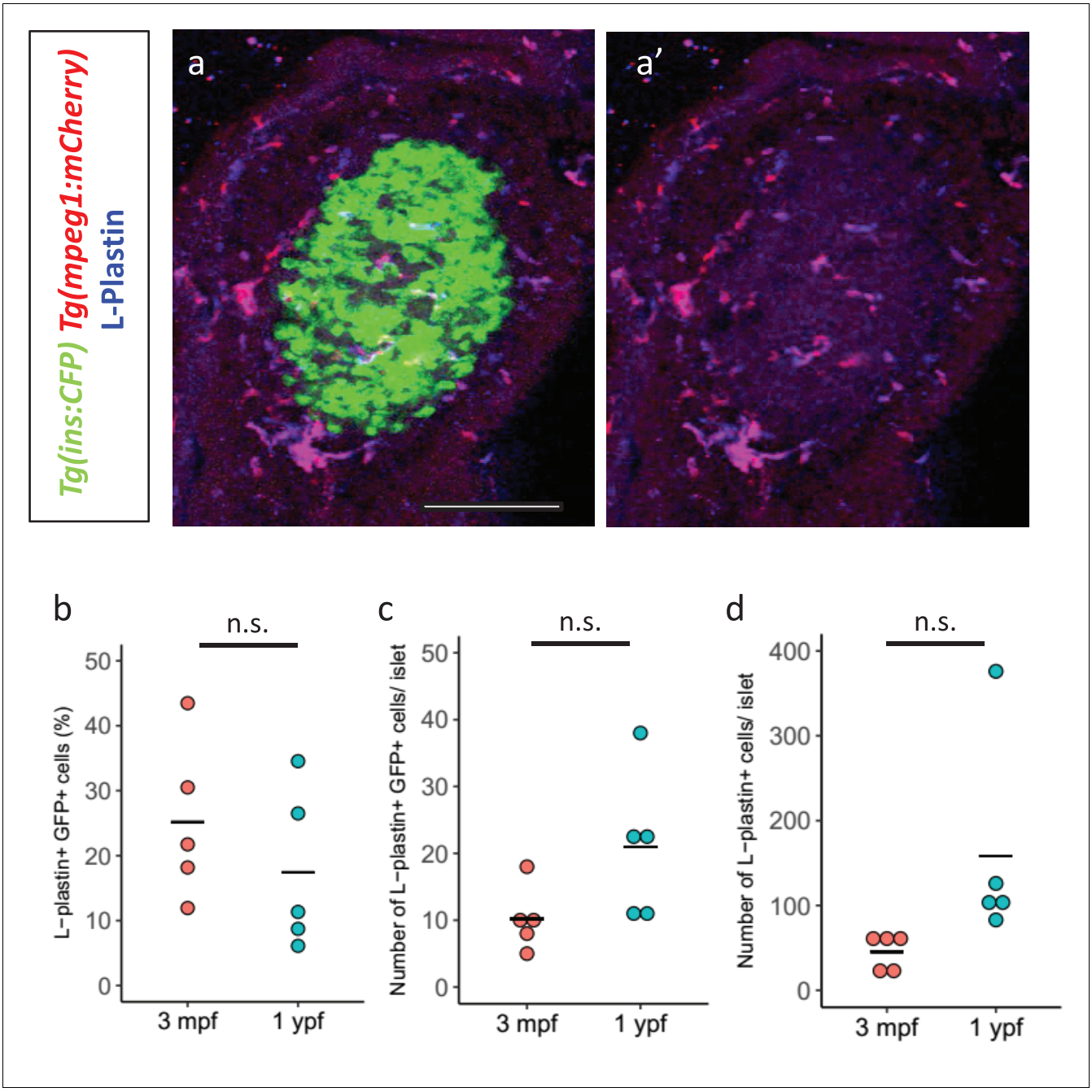

Figure 4—figure supplement 1. Immune cells infiltrate the islet during development. (**a,a'**) Confocal image of a juvenile islet. *Tg(ins:CFP-NTR)* line marks the beta-cells with CFP (green), leukocytes were labelled with the L-plastin antibody (blue) and *Tg(mpeg1:mCherry)* marks the macrophages (red). (**b**) Quantification of the percentage of *TgBAC(tnfa*:GFP) and L-plastin double-positive cells over the total number of L-plastin-positive cells in the islets of *TgBAC(tnfa:GFP)* animals at 3 mpf (n = 5) and 1 ypf (n = 5). Horizontal bars represent mean values (two-tailed t-test, p>0.05). (**c**) Quantification of the total number of *TgBAC(tnfα*:GFP)-positive and L-plastin double-positive cells at 3 mpf (n = 5) and 1 ypf (n = 5). Horizontal bars represent mean values (two-tailed t-test, p>0.05). (**d**) Quantification of the total number of L-plastin positivecells at 3 mpf (n = 5) and 1 ypf (n = 5). Horizontal bars represent mean values (two-tailed t-test, p>0.05).

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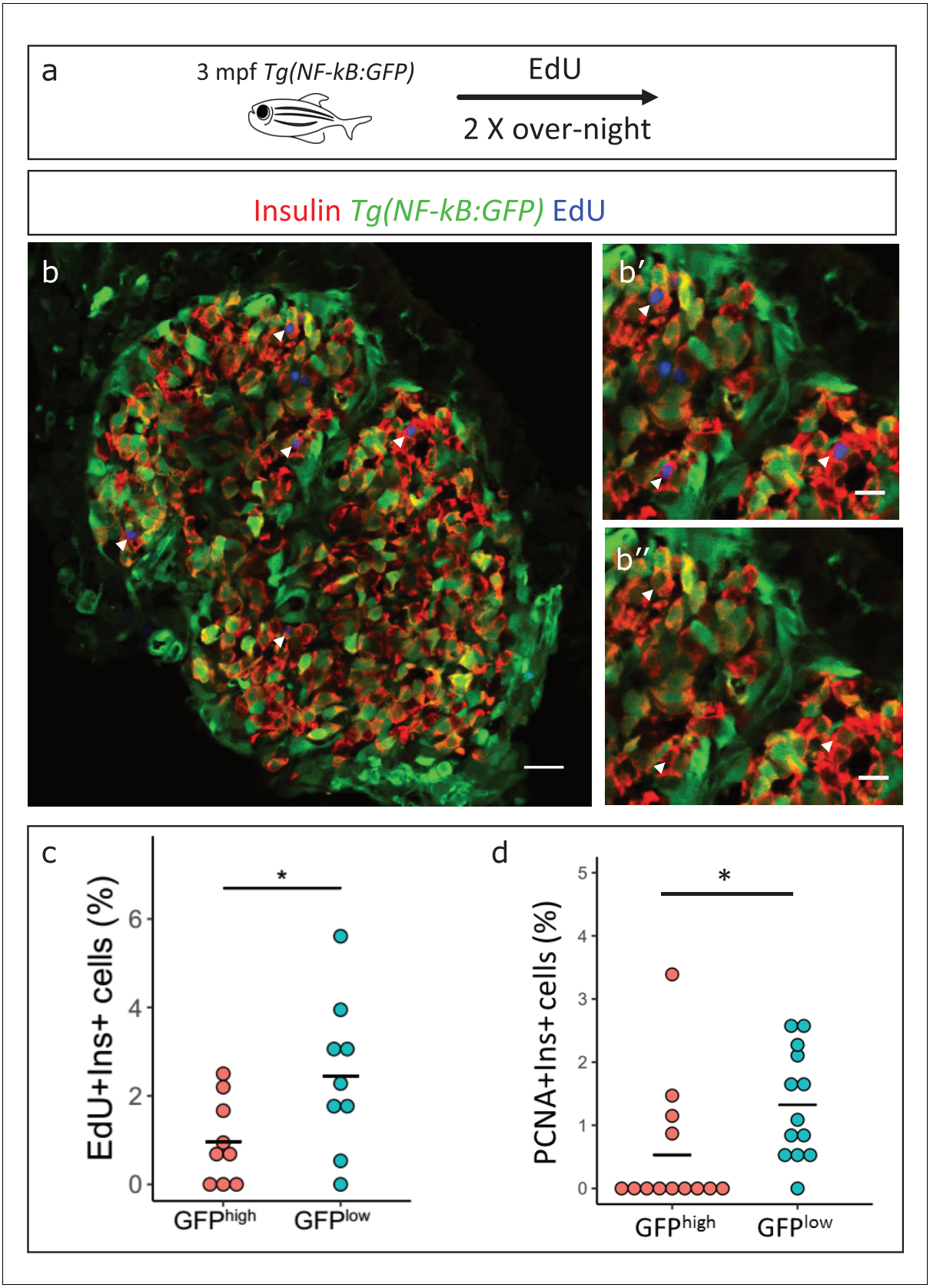

Figure 5. *NF-kB*:GFP^{high} beta-cells proliferate less than their neighbors. (**a**) Schematic showing the EdU (5-ethynyl-2′-deoxyuridine) incorporation assay. *Tg(NF-kB:GFP)* animals were incubated in EdU at 3 mpf for two consecutive nights and fed during each day. (**b**) EdU incorporation assay was performed

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Figure 5 continued

to mark the proliferating beta-cells in *Tg(NF-kB:GFP)* animals at 3 mpf. The confocal image (single plane) shows an overview of a section through the islet. Beta-cells were labeled with an insulin antibody (red), a GFP antibody (green) and EdU (blue). Arrowheads point to EdU-positive beta-cells. (**b'–b''**) The insets show higher magnification images with and without the EdU channel. EdU incorporation can be observed in some of the GFP^{low} cells (white arrow-heads). (**c**) An insulin-positive cell was first located by going through individual sections in the confocal z-stack. The optical section containing the largest area of the nucleus was chosen as the center of the cell. A region-of-interest (ROI) was drawn around the nucleus and the fluorescence intensities of the GFP and DAPI channels were recorded. The normalized GFP intensity was calculated as a ratio of mean GFP intensity and mean DAPI intensity for each ROI. The average total normalized GFP-intensity of each islet was set as a threshold for dividing the cells into GFP^{high} and GFP^{low} populations. The graph shows the percentage of EdU and insulin double-positive cells among the GFP^{high} and GFP^{low} populations. Each dot represents one islet (n = 9). Horizontal bars represent mean values (two-tailed t-test, *p<0.05). (**d**) The graph shows the percentage of PCNA and insulin double-positive cells among the GFP^{high} and GFP^{low} populations. Each dot represents one islet (n = 13). Horizontal bars represent mean values (two-tailed t-test, *p<0.05). See also ***Figure 5—figure supplement 2*** for representative PCNA antibody staining.

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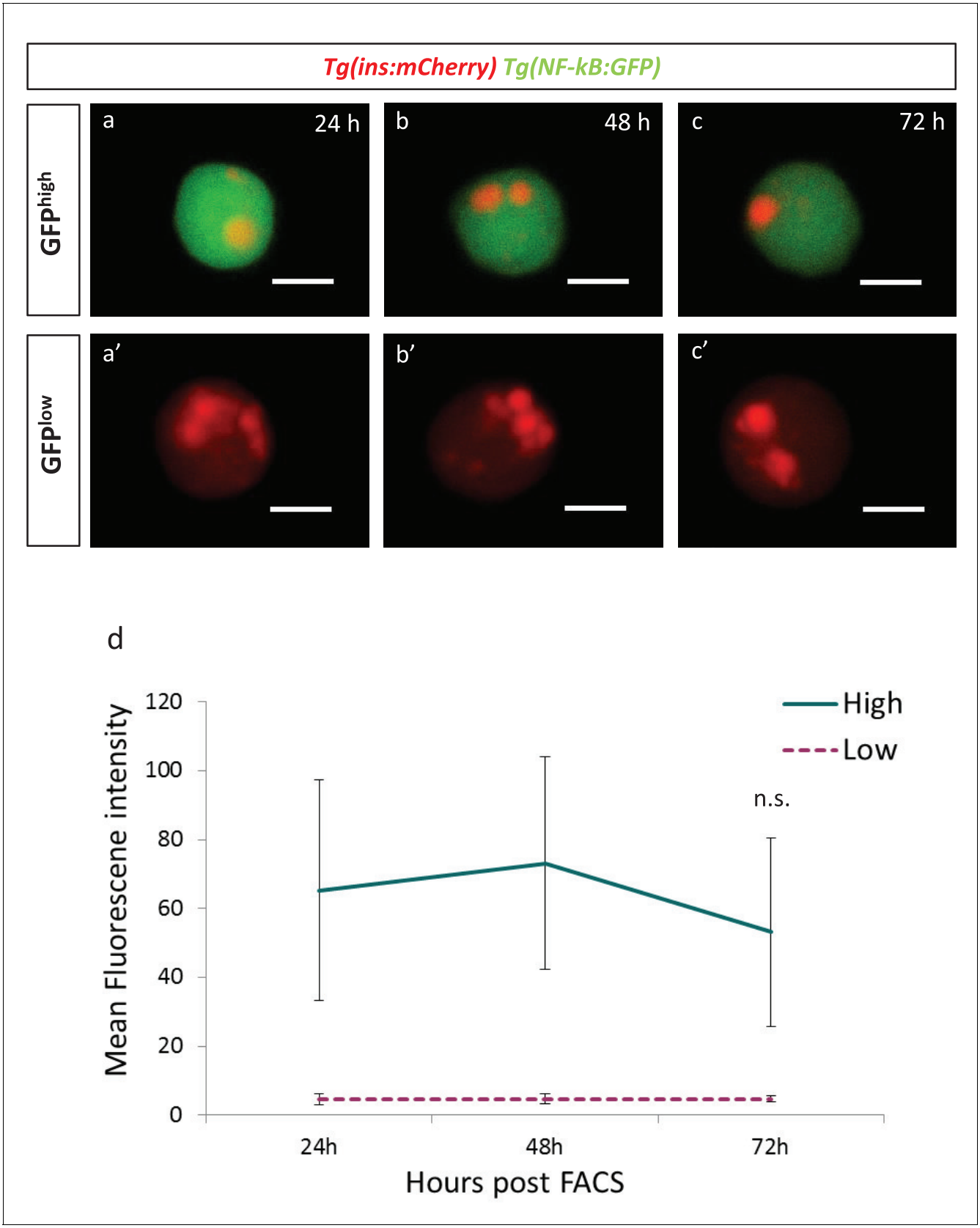

Figure 5—figure supplement 1. The GFP fluorescence of the transgenic reporter*Tg(NF-kB:GFP)*remains stable for 72 hours in beta-cells. (**a**,**b**,**c**) Beta-cells from *Tg(NF-kB:GFP);Tg(ins:mCherry)* animals at 3 mpf were FAC-sorted as single cells in 384-well plates and followed over 72 hr. *NF-kB*:GFP^{high}

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cells at 24, 48 and 72 hr post-FAC-sorting. Scale bar 5 μm. (**a'**,**b'**,**c'**) *NF-kB*:GFP^{low} cells at 24, 48 and 72 hr post-FAC-sorting. Scale bar 5 μm. (**d**) Quantification showing the mean GFP fluorescence intensity of GFP^{high} and GFP^{low} cells (n = 7 GFP^{high} cells and n = 5 GFP^{low} cells).

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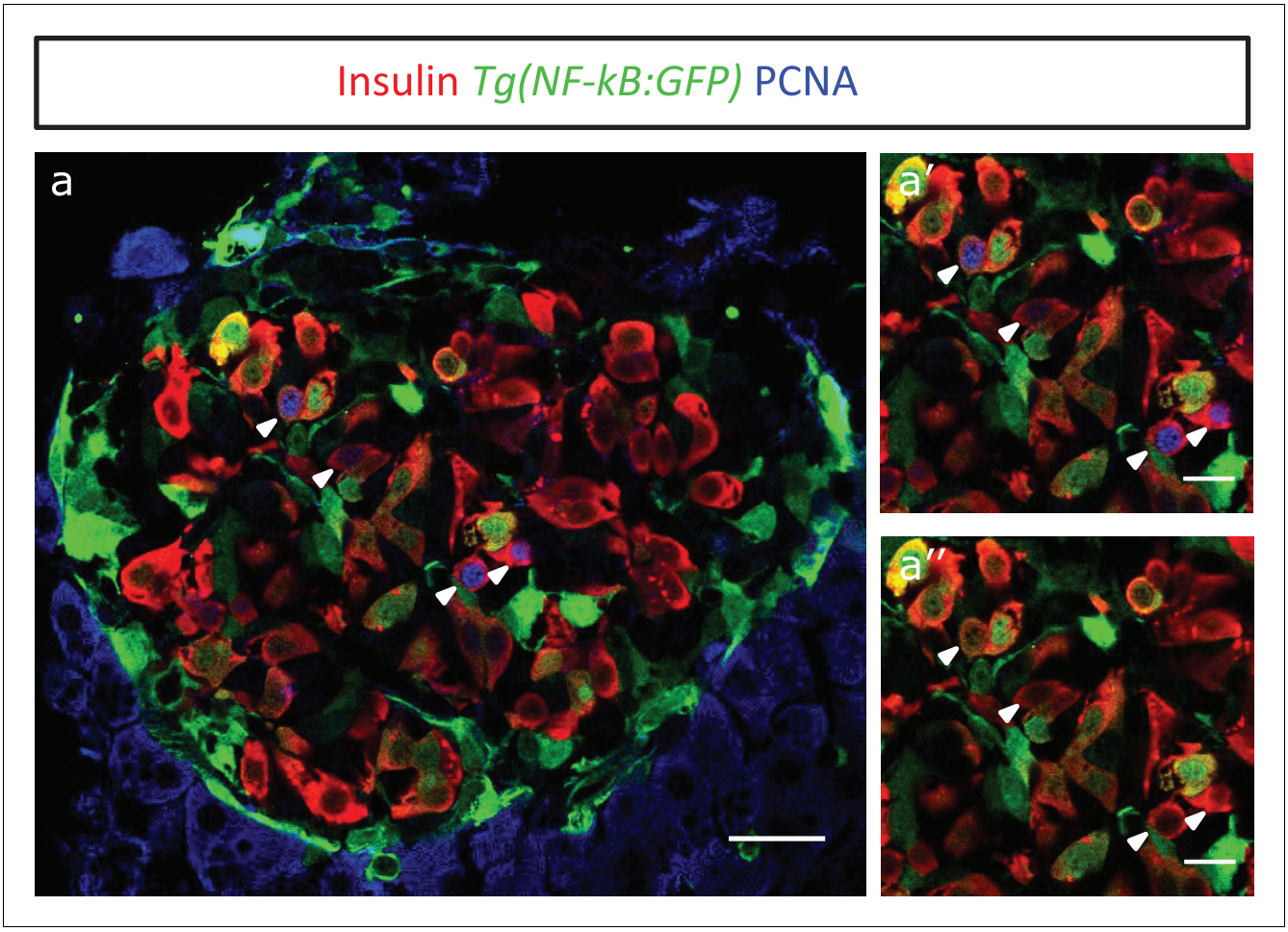

Figure 5—figure supplement 2. Proliferating cell nuclear antigen (PCNA) antibody staining shows that *NF-kB*:GF Phighbeta-cells proliferate less than NF-kB:GFP low beta-cells. (**a**) Islets were stained for PCNA to mark the proliferating beta-cells in *Tg(NF-kB:GFP)* animals at 3 mpf. The confocal image (single plane) shows an overview of a section through the islet. The sections were stained with an insulin antibody (red), a GFP antibody (green) and PCNA (blue). (**a'–a''**) The insets show higher magnification images. PCNA can be observed in some of the GFP^{low} cells (white arrow-heads).
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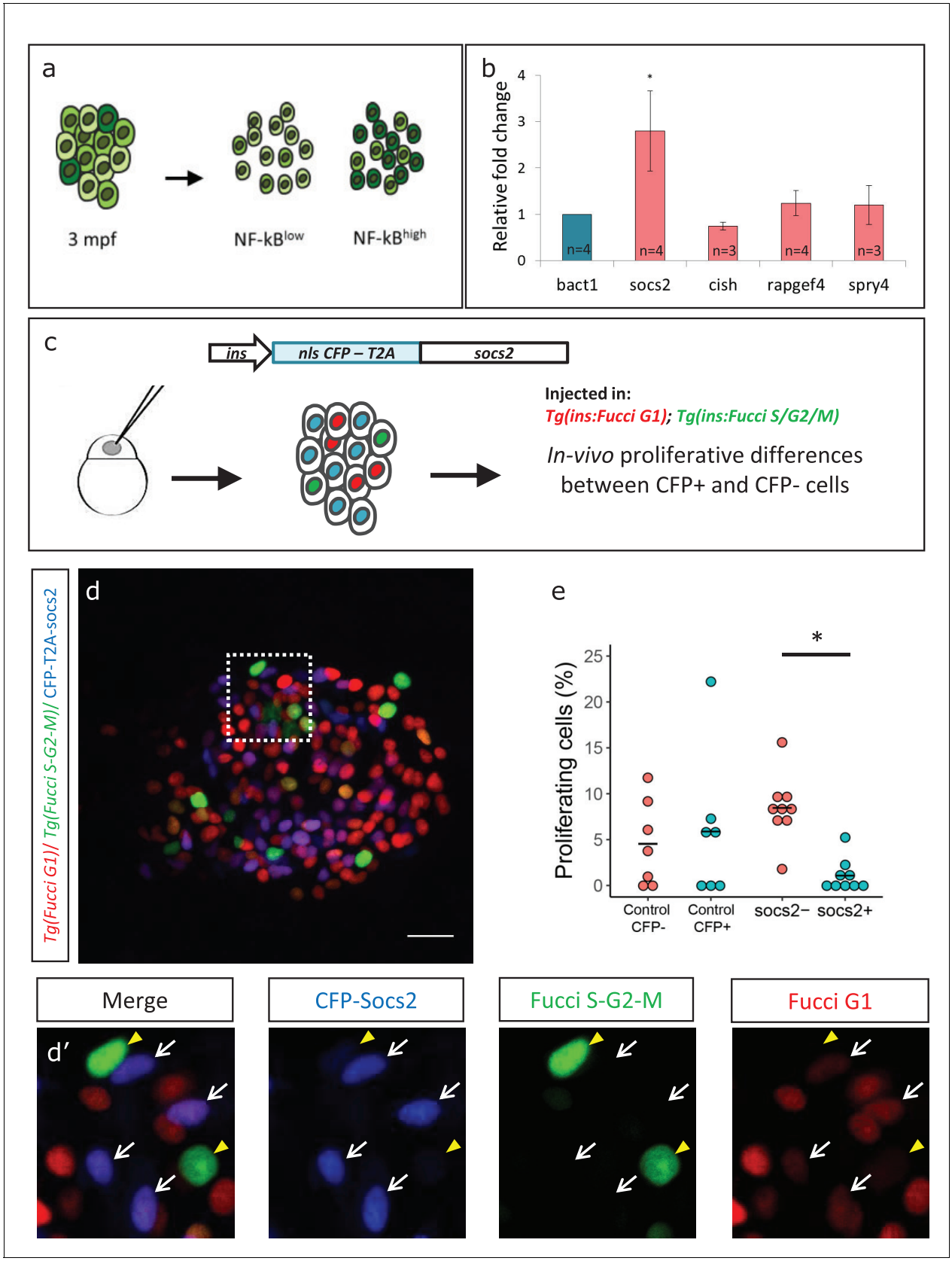

Figure 6. *Socs2* is enriched in *NF-kB*:GFP^{high} cells and inhibits beta-cell proliferation in a cell-autonomous manner. (**a**) Schematic showing the sorting of beta-cells from the double transgenic line *Tg(ins:mCherry);Tg(NF-kB:GFP)* at 3 mpf into GFP^{high} and GFP^{low} cells using FACS. (**b**) Bulk RT-qPCR was

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Figure 6 continued

performed on the GFP^{high} and GFP^{low} beta-cells (n = 3 to 4 biological replicates, n = 3 animals per replicate, 1000 cells per condition). Candidate genes significantly enriched in beta-cells at 1 ypf were chosen to be compared between the GFP^{high} and GFP^{low} populations at 3 mpf. The graph shows relative fold-change between GFP^{high} and GFP^{low} cells. The expression of all genes was normalized to β-actin expression before calculating fold-change. *socs2* shows higher expression in the GFP^{high} cells. Error bars, SD (two-tailed paired t-test, *p<0.05). (**c**) Schematic showing the method for mosaic overexpression of candidate genes in beta-cells. The *socs2* coding sequence is linked to nuclear-CFP using a T2A sequence. The entire construct was expressed under the insulin promoter. This construct was injected in one-cell-stage-embryos from *Tg(ins:Fucci-G1);Tg(ins:Fucci-S/G2/M)* animals leading to mosaic and stochastic expression of *socs2* in beta-cells during islet development. Control animals were injected with plasmid containing only nuclear-CFP sequence (See ***Figure 6—figure supplement 2***). (**d**) Confocal projections showing mosaic expression of *socs2-T2A-CFP (blue)* at 23 dpf (blue). Proliferating beta-cells are marked by *Tg(ins:Fucci-S/G2/M)* expression (green) and absence of *Tg(ins:Fucci-G1)* expression (red). Anterior to the left. Scale bar 10 μm. (**d'**) Insets show higher magnification single planes from the confocal stacks (white dotted-line in d) with separate channels. The proliferating beta-cells are CFP-negative (yellow arrowheads), whereas some of the non-proliferating cells are CFP-positive (white arrowheads) (**e**) Quantification of the percentage of *Tg(ins:FUCCI-S/G2/M)*-positive and *Tg(ins:FUCCI-G1)*-negative (green only) beta-cells. The *socs2* expressing β-cells exhibit reduced cell-cycle progression compared to wild-type neighbors (n = 9). Horizontal bars represent mean values (two-tailed t-test, *p<0.05).

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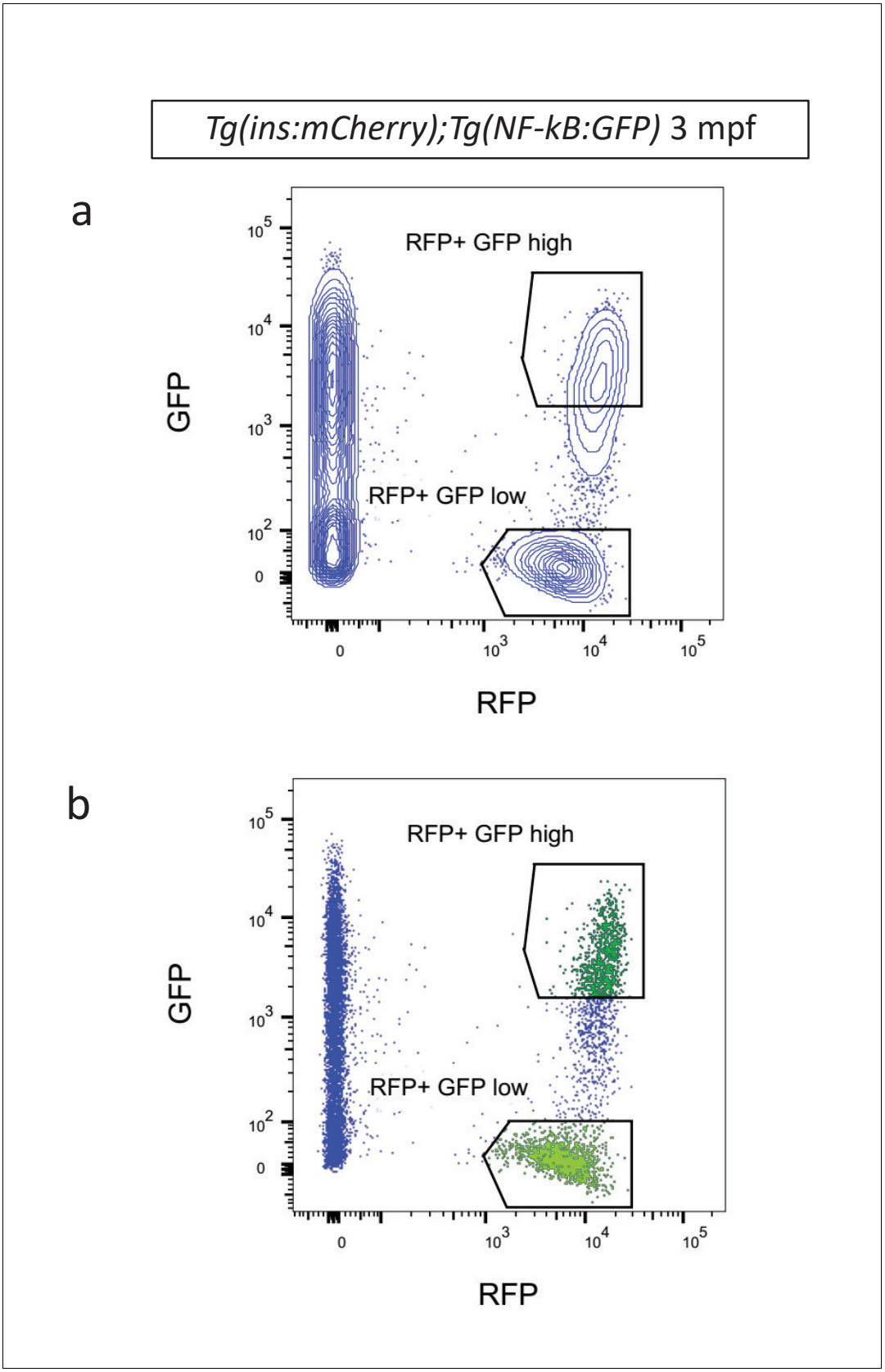
Tg(ins:mCherry);Tg(NF-kB:GFP) 3 mpf
a
RFP+ GFP high
RFP+ GFP low
GFP
RFP
b
RFP+ GFP high
RFP+ GFP low
GFP
RFP

Figure 6—figure supplement 1. Fluorescent activated cell sorting of *NF-kB*:GFP^{high} and *NF-kB*:GFP^{low}beta-cells. (**a**) Contour plot showing FACS of live RFP-positive GFP^{high} and GFP^{low} cells from *Tg(NF-kB:GFP);Tg(ins:mCherry)* animals at 3 mpf. Live cells were labeled with calcein. (**b**) Dot plot shows FACS of live RFP-positive GFP^{high} and GFP^{low} cells from *Tg(NF-kB:GFP);Tg(ins:mCherry)* animals at 3 mpf.
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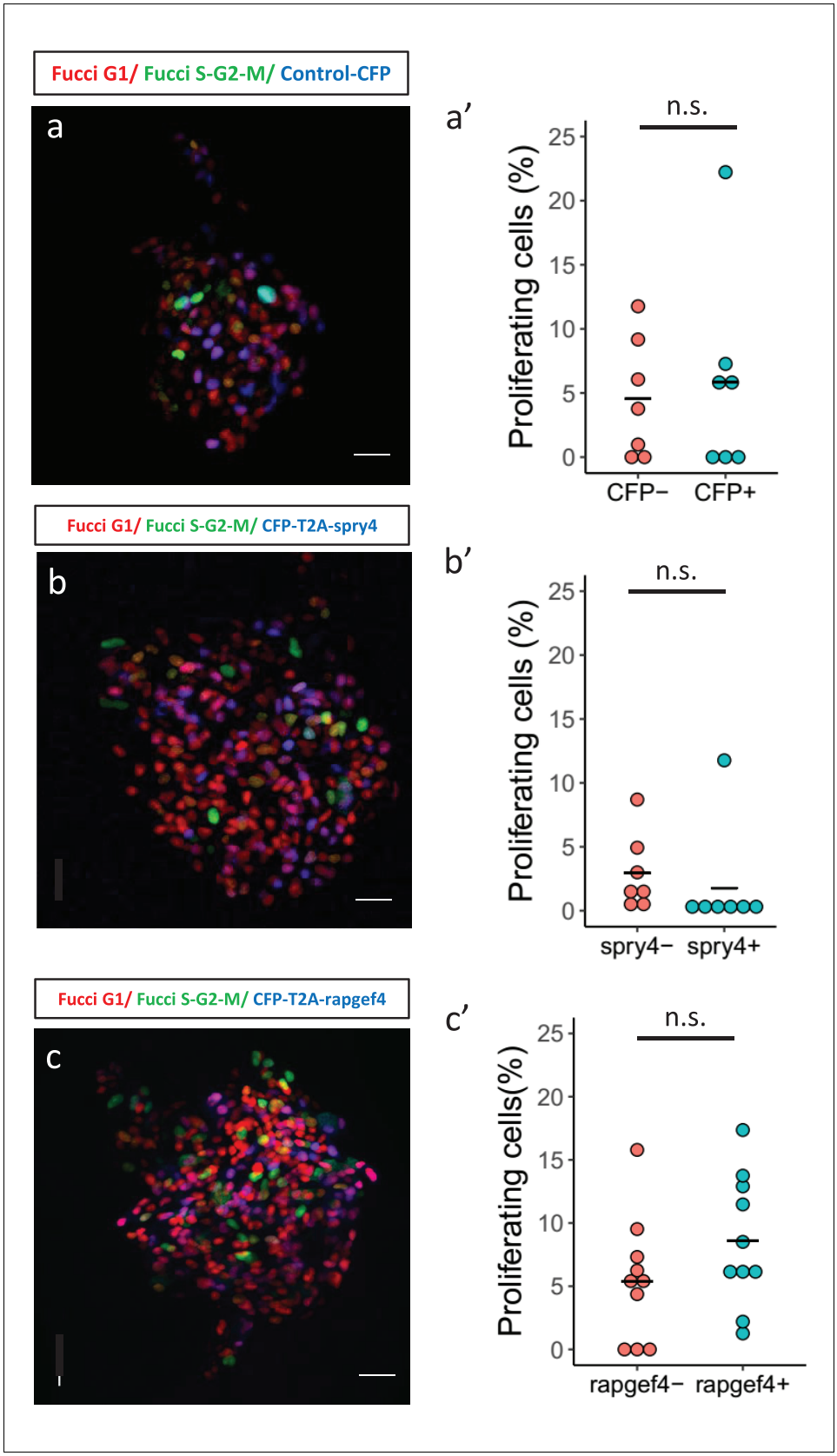

Figure 6—figure supplement 2. Mosaic expression of candidate genes in beta-cells to study their effect on proliferation. (**a,b,c**) Confocal images showing mosaic expression of nuclear-CFP (**a**), CFP-T2A-*spry4* (**b**) and CFP-

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Figure 6—figure supplement 2 continued

T2A-*rapgef4* (**c**) at 23 dpf. *Tg(ins:Fucci-G1)* expression is shown in red, *Tg(ins:Fucci-S/G2/M)* expression in green and CFP expression in blue. Anterior to the top. Scale bar 20 μm. (**a'**,**b'**,**c'**) Quantification of the percentage of *Tg(ins:FUCCI-S/G2/M)*-positive and *Tg(ins:FUCCI-G1)*-negative (green only) beta-cells among the CFP-positive and CFP-negative sub-populations for each experiment shown in the left-hand panels. Horizontal bars represent mean values (two-tailed t-test, $p>0.05$).

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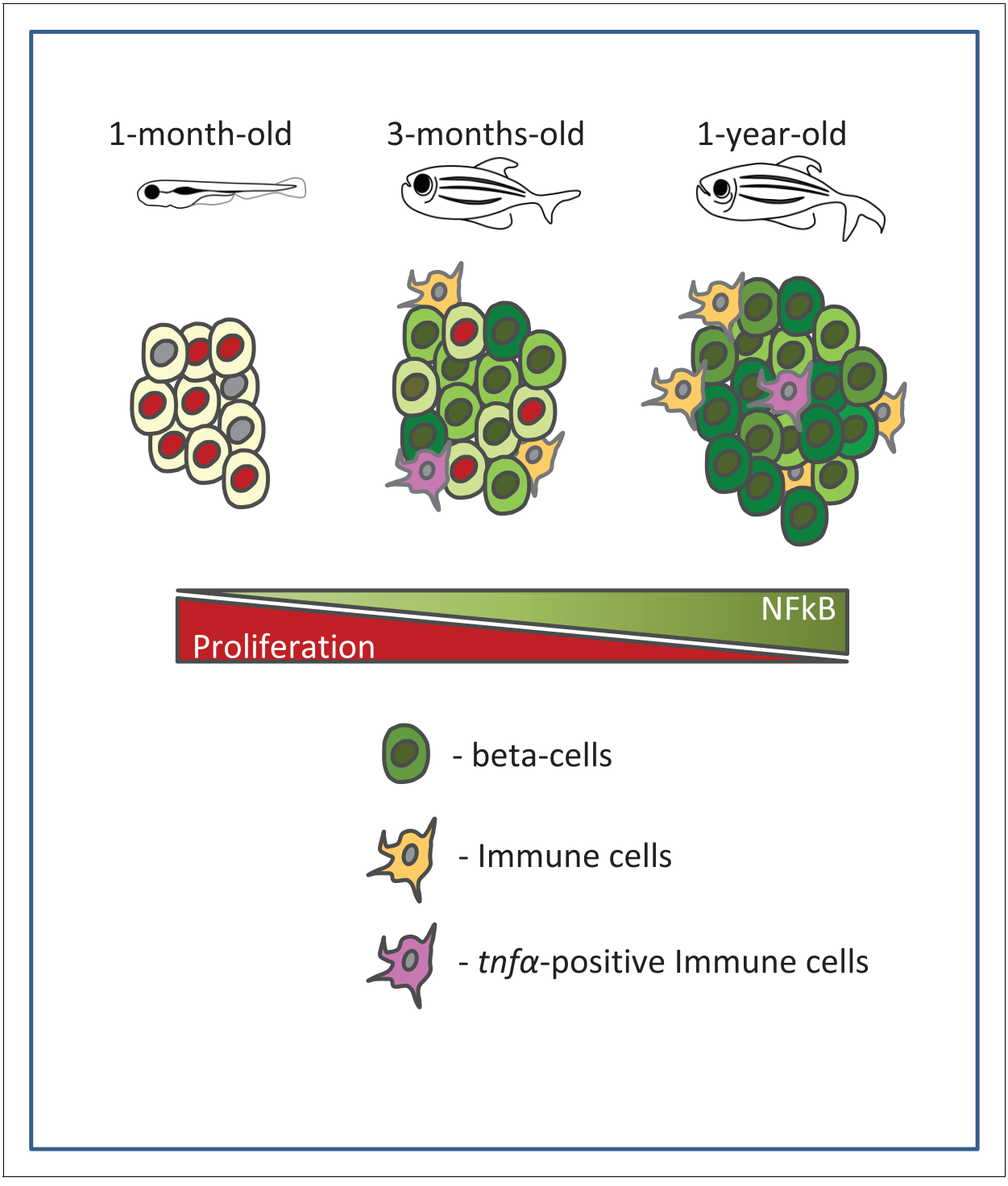

Figure 7. A schematic summarizing our model. Beta-cell proliferation declines with age together with a concurrent increase in NF-kB signaling. The activation of NF-kB signaling is heterogeneous among beta-cells and correlates with their proliferative heterogeneity. In particular, beta-cells with higher NF-kB activity proliferate less compared to neighbors with lower activity, and express higher levels of *socs2*, which can inhibit beta-cell proliferation. Furthermore, the crosstalk with *tnfα*-positive immune cells in the islet provides a potential source of inflammation and NF-kB activation in beta-cells.

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