

1 **T Cell Calcium Dynamics Visualized in a Ratiometric tdTomato-**  
2 **GCaMP6f Transgenic Reporter Mouse**

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16 two-photon microscopy

17

18 **Abstract**

19 Calcium is an essential cellular messenger that regulates numerous functions in living  
20 organisms. Here we describe development and characterization of “Salsa6f”, a fusion of  
21 GCaMP6f and tdTomato optimized for cell tracking while monitoring cytosolic  $\text{Ca}^{2+}$ , and  
22 a transgenic  $\text{Ca}^{2+}$  reporter mouse with Salsa6f floxed and targeted to the Rosa26 locus  
23 for expression in specific cell types. Using CD4-Cre-Salsa6f mice, we report normal  
24 development and function of T cells expressing Salsa6f and demonstrate  $\text{Ca}^{2+}$  signaling  
25 dynamics during T cell receptor engagement in naïve T cells, helper Th17 T cells and  
26 regulatory T cells. Salsa6f expression also revealed functional expression of  
27 mechanosensitive Piezo1 channels in T cells. Transgenic expression of Salsa6f enables  
28 ratiometric imaging of  $\text{Ca}^{2+}$  signals in complex tissue environments found *in vivo*. Deep  
29 tissue two-photon imaging of T cells in the steady-state lymph node revealed a highly  
30 localized  $\text{Ca}^{2+}$  signaling behavior (“sparkles”) as cells migrate.

31

32 **Introduction**

33 Calcium ( $\text{Ca}^{2+}$ ) is an essential second messenger responsible for a wide variety of  
34 cellular functions (Berridge, Lipp et al. 2000, Clapham 2007, Berridge 2012). Through  
35 the use of synthetic small molecule  $\text{Ca}^{2+}$  indicators such as fura-2 and fluo-4, imaging  
36 studies have greatly expanded our understanding of  $\text{Ca}^{2+}$  signaling dynamics (Tsien,  
37 Pozzan et al. 1982, Grynkiewicz, Poenie et al. 1985). However, such indicators cannot  
38 be targeted to specific subcellular compartments or cell populations, and are unsuitable  
39 for long-term studies due to leakage out of cells. Moreover, they often do not faithfully  
40 report pure cytosolic  $\text{Ca}^{2+}$  signals due to diffusion into other cellular compartments such  
41 as the nucleus. One alternative to overcoming these limitations is with genetically  
42 encoded  $\text{Ca}^{2+}$  indicators (GECIs), first developed two decades ago as FRET-based  
43 fluorescence probes (Miyawaki, Llopis et al. 1997, Romoser, Hinkle et al. 1997, Perez  
44 Koldenkova and Nagai 2013). Key advantages to GECIs include the capability for  
45 genetic targeting to specific cell types or subcellular organelles, measuring local  $\text{Ca}^{2+}$   
46 levels by direct fusion to a protein of interest, modulation of expression levels by  
47 inclusion of an inducible promoter, and long term studies due to continuous expression  
48 of the genetic indicator (Miyawaki, Llopis et al. 1997, Perez Koldenkova and Nagai  
49 2013). Despite these inherent advantages, the initial FRET-based GECI probes were  
50 not widely used as their performance fell far behind small molecule  $\text{Ca}^{2+}$  indicators,  
51 particularly in  $\text{Ca}^{2+}$  sensitivity, brightness, and dynamic range. Since then, successive  
52 rounds of design and contributions from multiple research groups have resulted in  
53 numerous variants of GECIs with high dynamic range and dramatically improved  
54 performance (Baird, Zacharias et al. 1999, Nakai, Ohkura et al. 2001, Tian, Hires et al.

55 2009, Zhao, Araki et al. 2011, Akerboom, Chen et al. 2012, Akerboom, Carreras  
56 Calderon et al. 2013, Chen, Wardill et al. 2013). Single fluorescent protein-based GECIs  
57 containing a circularly permuted green fluorescent protein (GFP) exhibit high  
58 brightness, fast response kinetics, and offer multiple color variants, including the GECO  
59 and the GCaMP series (Tian, Hires et al. 2009, Zhao, Araki et al. 2011, Akerboom,  
60 Chen et al. 2012, Chen, Wardill et al. 2013). FRET-based GECIs have continued to  
61 evolve as well, with sequential improvements including incorporation of circularly  
62 permuted yellow fluorescent proteins (cpYFPs) to improve dynamic range in the yellow  
63 cameleon (YC) family (Nagai, Yamada et al. 2004), use of troponin C as the  $\text{Ca}^{2+}$   
64 sensing element in the TN indicator family (Heim and Griesbeck 2004), computational  
65 redesign of the calmodulin-M13 interface to increase the range of  $\text{Ca}^{2+}$  sensitivity and  
66 reduce perturbation by native calmodulin in the DcpV family (Palmer, Giacomello et al.  
67 2006), and complete redesign of the troponin C domain to increase response kinetics  
68 and reduce buffering of cytosolic  $\text{Ca}^{2+}$  in the TN-XXL family (Mank, Reiff et al. 2006,  
69 Mank, Santos et al. 2008).

70 The latest generation of GECIs have crossed key performance thresholds  
71 previously set by small-molecule indicators, enabling GECIs to be widely applied in  
72 diverse  $\text{Ca}^{2+}$  imaging studies without sacrificing performance. Members of the GCaMP6  
73 family are capable of tracking cytosolic  $\text{Ca}^{2+}$  changes from single neuronal action  
74 potentials, with higher sensitivity than small-molecule indicators such as OGB-1 (Chen,  
75 Wardill et al. 2013). The availability of multicolored variants in the GECO family and the  
76 RCaMP series allowed for simultaneous measurement of  $\text{Ca}^{2+}$  dynamics in different cell  
77 populations in the same preparation, or in different subcellular compartments within the

78 same cell (Zhao, Araki et al. 2011, Akerboom, Carreras Calderon et al. 2013). These  
79 variants can be integrated with optogenetics to simultaneously evoke channel rhodopsin  
80 activity while monitoring localized  $\text{Ca}^{2+}$  responses in independent spectral channels  
81 (Akerboom, Carreras Calderon et al. 2013). Moreover, individual GECIs can be tagged  
82 onto membrane  $\text{Ca}^{2+}$  channels to directly measure  $\text{Ca}^{2+}$  influx through the target  
83 channel of interest, enabling optical recording of single channel activity without the need  
84 for technique-intensive patch clamping (Dynes, Amcheslavsky et al. 2016).

85 Another advantage of GECIs is the capability to be incorporated into transgenic  
86 organisms. Although several GECI-expressing transgenic mouse lines have already  
87 been reported, many of these studies used older variants of GECIs that are expressed  
88 only in selected tissues (Hasan, Friedrich et al. 2004, Ji, Feldman et al. 2004, Tallini,  
89 Ohkura et al. 2006, Heim, Garaschuk et al. 2007). The Ai38 mouse line overcomes  
90 these issues by combining GCaMP3 with a robust and flexible Cre/lox system for  
91 selective expression in specific cell populations (Zariwala, Borghuis et al. 2012). Based  
92 on a series of Cre-responder lines designed for characterization of the whole mouse  
93 brain (Madisen, Zwingman et al. 2010), the Ai38 mouse line contains GCaMP3 targeted  
94 to the Rosa26 locus but requires Cre recombinase for expression. By crossing Ai38 with  
95 various Cre mouse lines, GCaMP3 can be selectively expressed in specific cell  
96 populations. Thus, target cells may be endogenously labeled without invasive  
97 procedures, avoiding potential off-target side effects reported in GECI transgenic lines  
98 with global expression (Direnberger, Mues et al. 2012). The newly released PC::G5-tdT  
99 mouse line provides improved functionality by targeting a Cre-dependent GCaMP5G-  
100 IRES-tdTomato transgenic cassette to the *Polr2a* locus (Gee, Smith et al. 2014).

101 However, in the PC::G5-tdT mouse line, GCaMP5G and tdTomato are expressed  
102 individually, and localize to different cell compartments. Since expression of tdTomato is  
103 driven by an internal ribosomal entry site, the expression level is highly variable and  
104 weaker than GCaMP5G, limiting identification of positive cells and preventing accurate  
105 ratiometric measurements.

106 Although single fluorescent protein-based indicators have high brightness and  
107 fast response kinetics, as non-ratiometric probes they are problematic for  $\text{Ca}^{2+}$  imaging  
108 in motile cells where fluorescence changes resulting from movement are  
109 indistinguishable from actual changes in  $\text{Ca}^{2+}$  levels. Here, we introduce a novel  
110 genetically encoded  $\text{Ca}^{2+}$  indicator - that we christen 'Salsa6f' - by fusing green  
111 GCaMP6f to the  $\text{Ca}^{2+}$ -insensitive red fluorescent protein tdTomato. This probe enables  
112 true ratiometric imaging, in conjunction with the high dynamic range of GCaMP6. We  
113 further describe the generation of a transgenic mouse enabling Salsa6f expression in a  
114 tissue-specific manner, and demonstrate its utility for imaging cells of T lymphocytes in  
115 vitro and in vivo.

116

117 **Results**

118 **A novel ratiometric genetically encoded  $\text{Ca}^{2+}$  indicator, Salsa6f**

119 In order to develop a better tool to monitor  $\text{Ca}^{2+}$  signaling in T cells both *in vivo* and *in*  
120 *vitro*, we first evaluated the latest generation of genetically encoded  $\text{Ca}^{2+}$  indicators  
121 (GECIs) (Zhao, Araki et al. 2011, Chen, Wardill et al. 2013). A variety of single  
122 fluorescent protein-based GECIs were transiently expressed and screened in HEK  
123 293A cells (**Figure 1A**), and GCaMP6f was selected based on fluorescence intensity,  
124 dynamic range, and  $\text{Ca}^{2+}$  affinity suitable for detecting a spectrum of cytosolic  $\text{Ca}^{2+}$   
125 signals ( $K_d = 375 \text{ nM}$ ). To enable cell tracking even when basal  $\text{Ca}^{2+}$  levels evoke little  
126 GCaMP6f fluorescence, we fused GCaMP6f to the  $\text{Ca}^{2+}$ -insensitive red fluorescent  
127 protein tdTomato, chosen for its photostability and efficient two-photon excitation  
128 (Drobizhev, Makarov et al. 2011). A V5 epitope tag (Lobbestael E 2010) serves to link  
129 tdTomato to GCaMP6f (**Figure 1C**). The resultant ratiometric fusion indicator, coined  
130 “Salsa6f” for the combination of red tdTomato with the green GCaMP6f, was readily  
131 expressed by transfection into HEK 293A cells and human T cells. Salsa6f exhibited a  
132 ten-fold dynamic range, with a brightness comparable to GCaMP6f alone (**Figure**  
133 **1A,B**). For two-photon microscopy, both components of Salsa6f can be visualized by  
134 femtosecond excitation at 900 nm (**Figure 1D**). GCaMP6f produces increased green  
135 fluorescence during elevations in cytosolic  $\text{Ca}^{2+}$ , while tdTomato provides a stable red  
136 fluorescence that facilitates cell tracking and allows for ratiometric  $\text{Ca}^{2+}$  imaging (**Figure**  
137 **1D; Video 1**). Salsa6f is excluded from the nucleus, ensuring accurate measurement of  
138 cytosolic  $\text{Ca}^{2+}$  fluctuations (**Figure 1D,E**). When expressed by transfection in human T

139 cells, Salsa6f reported  $\text{Ca}^{2+}$  oscillations induced by immobilized  $\alpha\text{CD3}/28$  antibodies  
140 with a high signal to noise ratio and time resolution (**Figure 1E,F**).

141 **Generation of Salsa6f transgenic reporter mice and validation in immune cells**

142 Guided by the transgenic targeting strategy for the Ai38 mouse line (Zariwala, Borghuis  
143 et al. 2012), we inserted Salsa6f into a *ROSA26-pCAG-LSL-Salsa6f-WPRE-bGHpA-*  
144 *NeoR* cassette, then targeted it to the Rosa26 locus in JM8.N4 mouse embryonic stem  
145 (ES) cells (**Figure 2A**). Insertion events were selected by neomycin resistance, and  
146 correctly targeted clones were screened by Southern blot (**Figure 2B**), then injected into  
147 C57BL/6J blastocysts for implantation. Chimeric pups carrying the Salsa6f transgene  
148 were identified by PCR screening for the *Nnt* gene, as the initial JM8.N4 ES cells were  
149 *Nnt*<sup>+/+</sup> while the C57BL/6J blastocysts were *Nnt*<sup>-/-</sup> (**Figure 2C**). Positive chimeras were  
150 bred to R26 $\Phi$ C31o mice to remove the neomycin resistance gene and to produce  
151 Salsa6f<sup>LSL/-</sup> F1 founders, then further bred to generate homozygotic Salsa6f<sup>LSL/LSL</sup> mice.

152 Salsa6f<sup>LSL/LSL</sup> mice were bred to CD4-Cre<sup>+/+</sup> mice to obtain CD4-Cre<sup>+-</sup> Salsa6f<sup>+-</sup>  
153 reporter mice, designated as CD4-Salsa6f<sup>+-</sup> mice from here on, that selectively express  
154 Salsa6f in T cells (**Figure 3A**). Salsa6f was detected by tdTomato fluorescence on flow  
155 cytometry. 88% of Salsa6f<sup>+</sup> cells in thymus were double positive for CD4 and CD8  
156 (**Figure 3B**). This is due to the double-positive stage during development, in which  
157 developing thymocytes will express both CD4 and CD8 before undergoing positive and  
158 negative selection to become either mature CD4<sup>+</sup> or CD8<sup>+</sup> T cells. Salsa6f was readily  
159 detected in cells from spleen (40%), lymph node (57%), and thymus (93%) (**Figure 3C**).  
160 As expected, double positive cells were not detected in the spleen (**Figure 3D**). More  
161 than 98% of CD4<sup>+</sup> and CD8<sup>+</sup> T cells from these reporter mice were positive for Salsa6f.

162 Salsa6f was also detected in 5% of CD19<sup>+</sup> cells and 3% of CD11b<sup>+</sup> cells (**Figure 3E**). A  
163 small fraction of B cells express CD4 mRNA, which may explain the presence of  
164 Salsa6f in CD19<sup>+</sup> cells (Zhang and Henderson 1994). CD11b<sup>+</sup> cells positive for Salsa6f  
165 may be splenic resident dendritic cells that also express CD4 (Vremec, Pooley et al.  
166 2000, Turley, Fletcher et al. 2010). The total number and relative frequencies of CD4<sup>+</sup>,  
167 CD8<sup>+</sup>, CD19<sup>+</sup>, and CD11b<sup>+</sup> cells were similar to the CD4-Cre controls (**Figure 3F,G**).

168 To evaluate functional responses downstream of Ca<sup>2+</sup> signaling in Salsa6f-  
169 expressing T cells, we first purified CD4<sup>+</sup> T cells and monitored cell proliferation in vitro  
170 during TCR engagement of  $\alpha$ CD3 and co-stimulating  $\alpha$ CD28 antibodies attached to  
171 activating beads. Salsa6f-expressing CD4<sup>+</sup> T cells proliferated similar to the CD4-Cre  
172 controls (**Figure 4A,B**). To further probe functional responses, we differentiated naive  
173 CD4<sup>+</sup> T cells during polarizing cytokine stimuli to generate Th1, Th17 and induced  
174 regulatory T cells (iTregs). Salsa6f<sup>+</sup> naive CD4<sup>+</sup> T cells readily differentiated into various  
175 helper T cell subtypes similar to the CD4-Cre controls (**Figure 4C-E**). In addition, as  
176 described in the companion paper, adoptively transferred Salsa6f<sup>+</sup> cells readily homed  
177 to lymph nodes where they exhibited normal motility. In summary, our results  
178 demonstrate normal T cell function of CD4-Salsa6f<sup>+-</sup> T cells with respect to cellular  
179 phenotype, cell proliferation, differentiation, homing, and motility.

180 **Single-cell ratiometric Ca<sup>2+</sup> measurement in CD4-Salsa6f reporter mice**

181 CD4<sup>+</sup> T cells were purified from CD4-Salsa6f<sup>+-</sup> reporter mice, stimulated with plate-  
182 bound  $\alpha$ CD3/28 antibodies for two days, and imaged by confocal microscopy while still  
183 in contact with immobilized antibodies. Red and green fluorescence emitted from the  
184 cytosol of individual cells was tracked (**Figure 5A, Video 2**). Activated CD4<sup>+</sup> T cells

185 expressing Salsa6f exhibited stable red fluorescence and wide fluctuations in green  
186 fluorescence due to  $\text{Ca}^{2+}$  oscillations resulting from T cell receptor engagement (**Figure**  
187 **5B**). Despite variability in total fluorescence between cells due to individual differences  
188 in cell size, the basal and peak green/red Salsa6f ratios (referred from now on as G/R  
189 ratio for GCaMP6f/tdTomato intensity) were comparable between cells and showed up  
190 to six-fold increases during peaks in  $\text{Ca}^{2+}$  fluctuations. This level of response matches  
191 our previous experiments in activated human T cells transfected with Salsa6f (c.f.,  
192 **Figure 1E,F**), and supports the consistency in making ratiometric measurements with  
193 Salsa6f. Flow cytometric analysis of Salsa6f<sup>+/−</sup> mouse T cells revealed a thirteen-fold  
194 increase in G/R ratio, by pretreatment with ionomycin in free  $\text{Ca}^{2+}$  to deplete cytosolic  
195  $\text{Ca}^{2+}$  followed by addback of extracellular  $\text{Ca}^{2+}$ , further emphasizing the high dynamic  
196 range of Salsa6f (**Figure 5D**). Finally, to test if increasing the genetic dosage can  
197 improve the brightness of Salsa6f, we compared CD4<sup>+</sup> T cells from heterozygotic CD4-  
198 Salsa6f<sup>+/−</sup> mice and homozygotic CD4-Salsa6f<sup>+/+</sup> mice. T cells from homozygous mice  
199 with two allelic copies of the Salsa6f reporter cassette exhibited almost a two-fold  
200 increase in tdTomato fluorescence compared to heterozygous mice (**Figure 5E**),  
201 allowing for genetic control of Salsa6f expression level when brightness is an issue.

## 202 **Cytosolic localization and calibration of Salsa6f in transgenic T lymphocytes**

203 We first examined the localization of Salsa6f in naïve CD4<sup>+</sup> T cells isolated from CD4-  
204 Salsa6f<sup>+/−</sup> mice and in CD4<sup>+</sup> T cells activated for 2 days on plate-bound  $\alpha$ CD3/28. Line  
205 scans of the confocal images of cells plated on poly-L-lysine coated coverslips showed  
206 that Salsa6f is primarily localized to the cytoplasm and is excluded from the nucleus  
207 (**Figure 6A,B**). Increasing the cytosolic  $\text{Ca}^{2+}$  levels using thapsigargin (TG) in 2 mM

208  $\text{Ca}^{2+}$  Ringer's solution caused a selective increase in the GCaMP6f signal, without  
209 altering the localization of Salsa6f probe. In contrast, the chemical  $\text{Ca}^{2+}$  indicators fluo-4  
210 or fura-2 loaded into  $\text{CD4}^+$  T cells from CD4-Cre mice are localized throughout the cell,  
211 including the nucleus (**Figure 6C** and data not shown). A different transgenic mouse,  
212 CD4-Cre 5GtdT<sup>+/−</sup>, utilizes an internal ribosomal entry site to express both tdTomato and  
213 GCaMP5G as separate proteins that localize differently in cells (Gee, Smith et al. 2014),  
214 tdTomato throughout the cell including the nucleus and GCaMP5G predominantly in the  
215 cytosol (**Figure 6-figure supplement 1**). In contrast, our tandem probe, Salsa6f, results  
216 in both red and green fluorescent proteins co-localized in the cytosol, allowing true  
217 ratiometric  $\text{Ca}^{2+}$  imaging and facilitating tracking of cells.

218 To estimate the dynamic range of the probe in Salsa6f-expressing T cells, we  
219 treated the cells with ionomycin in  $\text{Ca}^{2+}$ -free and 20 mM  $\text{Ca}^{2+}$  Ringer's solution to get  
220 the minimum and maximum fluorescence signals and ratios ( $F_{\max}$ ,  $R_{\max}$ ,  $F_{\min}$  and  $R_{\min}$ ).  
221 Addition of high  $\text{Ca}^{2+}$  buffer resulted in a robust and selective increase in the GCaMP6f  
222 signal without significantly affecting the tdTomato signal (**Figure 6D**). Based on the fold  
223 change in the G/R ratio, the dynamic range of the probe was calculated to be ~ 5.5-fold.

224 GCaMP6f is reported to have a  $K_d$  of 290 nM at 25° C and 375 nM at 37° C  
225 based on in vitro measurements with purified protein (Chen, Wardill et al. 2013, Badura,  
226 Sun et al. 2014). In situ  $K_d$  values of  $\text{Ca}^{2+}$  indicators often vary significantly from the  
227 reported in vitro values (Negulescu and Machen 1990), although to our knowledge, the  
228 in situ  $K_d$  of GCaMP6f has not been determined in any specific cell type. To  
229 characterize the  $\text{Ca}^{2+}$  affinity and binding kinetics of Salsa6f in situ, we performed time-  
230 lapse imaging at 25° C on 2 day-activated  $\text{CD4}^+$  cells isolated from CD4-Salsa6f<sup>+/−</sup> mice

231 and plated on poly-L-lysine. We recorded G/R ratios in response to ionomycin and  
232 applied stepwise increases in the external  $\text{Ca}^{2+}$  concentration (**Figure 6E**). Our strategy  
233 for calibration was to compare these results with  $\text{Ca}^{2+}$  signals in fura-2-loaded  $\text{CD4}^+$  T  
234 cells from CD4-Cre mice using exactly the same protocol of cell isolation, plating, and  
235 solution application (**Figure 6E,F**), the rationale being that fura-2 has an *in situ*  $K_d$  of  
236 around 225 nM at 25° C (Lewis and Cahalan 1989), which is close to the range of *in*  
237 *vitro*  $K_d$  values reported for GCaMP6f (Chen, Wardill et al. 2013, Badura, Sun et al.  
238 2014). For meaningful comparison, the traces were normalized with  $R_{\min}=0$  and  $R_{\max}=1$   
239 for both fura-2 and Salsa6f. To our surprise, Salsa6f responded with faster rise times  
240 and at lower external  $\text{Ca}^{2+}$  concentrations than did fura-2 to progressive increases in  
241 cytosolic  $\text{Ca}^{2+}$  levels. These observations were unexpected, given that the reported *in*  
242 *situ*  $\text{Ca}^{2+}$  affinity of fura-2 is higher than the *in-vitro* affinity of GCaMP6f. Additionally, the  
243  $\text{Ca}^{2+}$  signal measured with Salsa6f decayed faster than that measured with fura-2. This  
244 effect was more prominent at lower cytosolic  $\text{Ca}^{2+}$  levels and as the  $\text{Ca}^{2+}$  levels  
245 increased, the rate of decay diminished and the overall kinetics were then similar to  
246 fura-2. The faster rise and fall times of the  $\text{Ca}^{2+}$  signals seen in Salsa6f expressing cells  
247 is not likely to be due to differential activity of  $\text{Ca}^{2+}$  influx and efflux mechanisms  
248 between CD4-Cre and CD4-Salsa6f<sup>+/−</sup> cells since fura-2 signals in Salsa6f cells also  
249 displayed kinetics very similar to that seen in WT CD4-Cre cells (data not shown).

250 Secondly, Salsa6f responses saturated at lower cytosolic  $\text{Ca}^{2+}$  levels than fura-2  
251 responses. This is not altogether surprising given that genetically encoded  $\text{Ca}^{2+}$   
252 indicators have been reported to have a steeper Hill coefficient than chemical indicators  
253 (Badura, Sun et al. 2014). Obtaining steady-state cytosolic  $\text{Ca}^{2+}$  concentrations from

254 fura-2 measurements in WT CD4-Cre cells, and assuming that CD4-Salsa6f<sup>+/−</sup> cells  
255 reach similar Ca<sup>2+</sup> levels, we plotted both the peak and the steady state Salsa6f G/R  
256 ratios against the cytosolic Ca<sup>2+</sup> concentrations obtained from the fura-2 experiment  
257 (**Figure 6G**). Using steady-state levels for Salsa6f, we calculated a  $K_d$  of ~300 nM, while  
258 using peak levels for Salsa6f probe gave a  $K_d$  of ~160 nM. Furthermore, Salsa6f was  
259 sensitive in detecting cytosolic Ca<sup>2+</sup> in the range of 100 nM - 2 μM. Based on these  
260 results, we conclude that Salsa6f probe with its high sensitivity is well suited to detect  
261 small changes in cytosolic Ca<sup>2+</sup> in response to various physiological stimuli, while its  
262 excellent dynamic range also allows us to detect larger elevations in Ca<sup>2+</sup> up to 2 μM.

263 **T cell Ca<sup>2+</sup> signaling in response to Ca<sup>2+</sup> store depletion, T cell receptor  
264 engagement, and mechanical stimulation**

265 TCR engagement activates a canonical Ca<sup>2+</sup> signaling pathway in T cells, characterized  
266 by IP<sub>3</sub>-induced Ca<sup>2+</sup> release from the endoplasmic reticulum, leading to store-operated  
267 Ca<sup>2+</sup> entry (SOCE) through Orai1 channels (Cahalan and Chandy 2009, Prakriya and  
268 Lewis 2015). Past studies on T cell Ca<sup>2+</sup> signaling have largely relied on chemical  
269 indicators like fura-2 and fluo-4 which have the drawback of being distributed into  
270 nucleus and other cellular compartments, thus confounding the measurement of pure  
271 cytoplasmic Ca<sup>2+</sup> signals, a problem particularly notable in T cells with its large nuclear  
272 to cytoplasmic volume. Salsa6f, with its large dynamic range, ratiometric readout and  
273 targeted localization in the cytosol, is thus well suited to record physiological Ca<sup>2+</sup>  
274 signals that are primarily cytosolic. To this end, we recorded Ca<sup>2+</sup> signals from 2 day-  
275 activated CD4<sup>+</sup> T cells from CD4-Salsa6f<sup>+/−</sup> mice in response to a variety of stimuli.

276 To study SOCE more directly, we depleted ER  $\text{Ca}^{2+}$  stores with TG in  $\text{Ca}^{2+}$  free  
277 solution. We observed a small but sharp initial peak indicating ER store release and a  
278 sustained  $\text{Ca}^{2+}$  signal upon restoring  $\text{Ca}^{2+}$  to the external bath suggestive of SOCE  
279 (**Figure 7A, Video 3**). Single traces revealed that almost all cells responded to this  
280 supra-physiological stimulus (**Figure 7A**, right panel).

281 In contrast, single cell analysis of cells plated on  $\alpha\text{CD3}/28$  to activate TCR-  
282 induced signaling revealed a heterogeneous pattern of activation with cells showing  
283 asynchronous  $\text{Ca}^{2+}$  oscillations of varying frequencies and time-widths with a  
284 percentage of cells not responding at all (**Figure 7B, Video 4**). This is in contrast to a  
285 single transient  $\text{Ca}^{2+}$  peak in response to soluble  $\alpha\text{CD3}/28$  (data not shown). The  
286 average elevation of cytosolic  $\text{Ca}^{2+}$  was significantly above the resting levels, but below  
287 that of TG-induced SOCE. Past studies have attributed these  $\text{Ca}^{2+}$  oscillations to SOCE  
288 from repetitive opening and closing of Orai1 channels allowing  $\text{Ca}^{2+}$  to enter T cells in a  
289 periodic and asynchronous manner (Lewis and Cahalan 1989, Dolmetsch and Lewis  
290 1994), unlike with TG treatment. Cells plated on  $\alpha\text{CD3}$  alone also showed rhythmic  
291 oscillatory  $\text{Ca}^{2+}$  signals; however, the percentage of responding cells was significantly  
292 lower than with  $\alpha\text{CD3}/28$ , resulting in a lower average signal (**Figure 7C, Video 5**).  
293 These results suggest that co-stimulatory signaling through CD28 is essential to  
294 maintain TCR signaling, in alignment with previous observations (Chen and Flies 2013).

295 Finally, we focused on a novel  $\text{Ca}^{2+}$  signaling pathway as yet unreported in T  
296 cells. The Piezo family of mechanosensitive channels plays a vital role in cell motility  
297 and development (Nourse and Pathak 2017). It is not known whether these channels  
298 are expressed, and if they play any role in immune cell function. Recently, Yoda1, a

299 selective small molecule activator of Piezo1 was identified through a drug screen  
300 (Syeda, Xu et al. 2015). We examined activation of mechanosensitive channels in cells  
301 that were plated on  $\alpha$ CD3/28 coated glass coverslips, by flowing external solution  
302 rapidly past the cells. Perfusion of media alone produced a transient rise in the cytosolic  
303  $\text{Ca}^{2+}$  signal (**Figure 7D**). We then tested responses to Yoda1 to assess whether Piezo1  
304 channels can be activated in T cells and found robust and sustained  $\text{Ca}^{2+}$  signals  
305 evoked by Yoda1.  $\text{Ca}^{2+}$  responses to TG and Yoda1 responses in naïve T cells were  
306 very similar to those observed in 2-day activated T cells (**Figure 7-figure supplement**  
307 **1**). In summary, for the first time, we show  $\text{Ca}^{2+}$  signals in T cells in response to  
308 activation of Piezo1 channels. These results illustrate the utility of Salsa6f for screening  
309 agents that modulate  $\text{Ca}^{2+}$  signaling in T cells and open the possibility for further  
310 exploration of the functional role of Piezo1 channels in T cell function.

311 **TCR-induced  $\text{Ca}^{2+}$  signaling in helper T cell subsets**

312 We also monitored  $\text{Ca}^{2+}$  signaling in response to TCR activation with  $\alpha$ CD3/28 in  
313 various subsets of T cells from CD4-Salsa6f<sup>+/−</sup> mice, including naïve T cells, Th17 cells  
314 and iTregs (**Figure 8A-C**). All subtypes of T cells responded to plate-bound stimulation  
315 of  $\alpha$ CD3/28 with oscillatory changes in their cytosolic  $\text{Ca}^{2+}$  levels, very similar to the  
316  $\text{Ca}^{2+}$  responses seen in 2 day-activated T cells shown in the previous figure.  
317 Furthermore, as with 2 day-activated T cells, responses were heterogeneous, with cells  
318 showing multiple peaks of varying width and amplitude, occasional sustained signals  
319 and a variable percentage of non-responders. While the overall average responses  
320 were not very different between the three subtypes examined here, single cell  
321 responses in Th17 cells and iTregs showed higher amplitude signals than naïve T cells,

322 but with a greater percentage of non-responding cells. Taken together, we conclude that  
323 the CD4-Salsa6f<sup>+/−</sup> mouse opens up new avenues to study the fundamental nature of  
324 Ca<sup>2+</sup> signals in T cell subsets, generated in response to variety of stimuli and to explore  
325 the relationship between types of Ca<sup>2+</sup> signals and specific downstream functions.

326 **Two-photon microscopy of CD4-Salsa6f<sup>+/−</sup> T cells in lymph node.**

327 After establishing Salsa6f as a robust reporter of cytosolic Ca<sup>2+</sup>, we imaged lymph  
328 nodes from homozygous CD4-Salsa6f<sup>+/−</sup> mice using 2-photon microscopy under steady-  
329 state conditions. Over time, sporadic T cell-sized green fluorescent signals were seen,  
330 and the pattern of fluorescence observed is consistent with the cytosolic localization of  
331 the Salsa6f indicator, which is excluded from the nucleus (**Figure 9A-C**). Numerous  
332 small, bright, and transient green fluorescent signals about 2 μm<sup>2</sup> in area were also  
333 observed (**Figure 9B,D; Video 6**). We termed these fluorescent transients “sparkles”,  
334 because during rapid playback of time-lapse image streams cells appear to sparkle  
335 (**Video 6**). The existence of sparkles was surprising, because sparkles are too small to  
336 result from cell-wide elevations of Ca<sup>2+</sup>, and T cells lack extended cellular processes,  
337 like neurons, that confine Ca<sup>2+</sup> responses.

338 Since T cells move rapidly and are not uniformly distributed in lymph nodes, we  
339 developed an image processing approach to minimize fluctuations in background  
340 fluorescence in order to sensitively identify cell-wide Ca<sup>2+</sup> signals and sparkles (**Figure**  
341 **9-figure supplement 1**). A key feature of the Salsa6f fusion protein is the one-to-one  
342 correspondence of tdTomato and GCaMP6f, and we used this to estimate and subtract  
343 out fluctuations in green background fluorescence due to cell movement and  
344 distribution. After processing, sparkles were found to occur widely across the imaging

345 field and many had similar intensities (**Figure 9E-G**). The brightness of sparkles and the  
346 uniformity of background fluorescence allowed us to use a stringent 5.4 SD threshold to  
347 systematically identify bright sparkles; hundreds (mean of 6.5 SD above background)  
348 were observed in each 25-minute imaging session (one image stack every five  
349 seconds), whereas less than one was expected to occur by chance. Sparkles were also  
350 more frequent than cell-wide transients (**Figure 9H**). Most sparkles were between the  
351 defined minimum  $1.4 \mu\text{m}^2$  and  $3 \mu\text{m}^2$  in area (median of  $1.9 \mu\text{m}^2$ , 95% CI of  $1.9\text{-}2.3 \mu\text{m}^2$ ;  
352 n=441 sparkles from 3 cells; **Figure 9I**). Sparkles were typically found in one or two  
353 consecutive frames (**Figure 9J**). Sparkle trace shape differs from that expected for  
354 autofluorescent cell processes drifting into the imaging field (**Figure 9K**). Taken  
355 together, these observations suggest that sparkles correspond to local  $\text{Ca}^{2+}$  signals  
356 restricted to small subcellular domains of T cells migrating through the lymph node.

357 To establish that T cells labeled in CD4-Salsa6f<sup>+/+</sup> mice exhibit subcellular  $\text{Ca}^{2+}$   
358 signals, we adoptively transferred CD4-Salsa6f<sup>+/+</sup> T cells into wild type recipients so  
359 these cells could be viewed in isolation. Sparkles were identified in time lapse images of  
360 lymph nodes, and when traced back they were found to originate in red fluorescent cells  
361 in most cases. Thus, sparkles correspond to restricted subcellular domains of elevated  
362  $\text{Ca}^{2+}$  in Salsa6f CD4<sup>+</sup> T cells. Cell movement was used to define the front and back of  
363 labeled T cells for mapping the subcellular location of  $\text{Ca}^{2+}$  signals. Local  $\text{Ca}^{2+}$  signals  
364 were most frequently found in the back of motile T cells (**Figure 10 A-E**). Green-red  
365 channel ratiometric images, enabled by Salsa6f labeling, confirmed cytosolic  
366 localization patterns (**Figure 10D**). Local  $\text{Ca}^{2+}$  signals were found less frequently in the  
367 front, front and back, and sides of motile T cells (**Figure 10 F-H**). In a companion paper,

368 we use Salsa6f transgenic mice to consider the relationship between  $\text{Ca}^{2+}$  signals, both  
369 cell-wide and local, and T cell motility in the lymph node.

370

371 **Discussion**

372 We introduce Salsa6f, a novel, ratiometric genetically-encoded  $\text{Ca}^{2+}$  probe. Salsa6f is a  
373 fusion of the high performing green fluorescent GECI GCaMP6f and the bright red  
374 fluorescent tdTomato. This simple modification imparts powerful capabilities, which  
375 include tracking cells in the absence of  $\text{Ca}^{2+}$  signaling, ratiometric imaging to eliminate  
376 motility artifacts, and convenient single-wavelength femtosecond excitation for two-  
377 photon microscopy. Salsa6f addresses a key weakness of single fluorescent protein-  
378 based GECIs by enabling tracking of motile cells and identification of cell morphology,  
379 even at basal  $\text{Ca}^{2+}$  levels when GCaMP6f fluorescence is very weak. We further  
380 generated a transgenic reporter mouse with Cre-dependent expression of Salsa6f,  
381 enabling  $\text{Ca}^{2+}$  signals to be imaged in specific, genetically defined cell types.  
382 Transgenic expression of Salsa6f brings the power of ratiometric chemical  $\text{Ca}^{2+}$   
383 indicators to imaging cellular  $\text{Ca}^{2+}$  signals amid the complex tissue environments found  
384 *in vivo*.

385 Salsa6f preserves the exceptional performance of GCaMP6f, which in the  
386 presence of high levels of  $\text{Ca}^{2+}$  is as bright as the standard high performing green  
387 fluorescent protein, EGFP (Chen, Wardill et al. 2013). We find that Salsa6f possesses a  
388 dynamic range similar to GCaMP6f as well, and both are superior to FRET-based  
389 GECIs (Heim, Garaschuk et al. 2007, Thestrup, Litzlbauer et al. 2014). Salsa6f's  $\text{Ca}^{2+}$   
390 affinity, 160-300 nM, is well suited to detecting a variety of cellular  $\text{Ca}^{2+}$  signals.  
391 Inclusion of tdTomato in Salsa6f enables ratiometric imaging, calibration, and  
392 measurement of  $\text{Ca}^{2+}$  concentrations within cells. Salsa6f is uniformly distributed  
393 throughout the cytosol; its exclusion from the nucleus provides reliable and selective

394 reporting of cytosolic  $\text{Ca}^{2+}$  signaling. This is in contrast to the recently developed CD4-  
395 Cre 5GtdT<sup>+/−</sup> mouse strain in which the tdTomato is found throughout the cell but the  
396 separately expressed GCaMP5G is excluded from the nucleus (Gee, Smith et al. 2014).  
397 Finally, Salsa6f expression is non-perturbing; we saw no effects of Salsa6f expression  
398 in CD4<sup>+</sup> immune cells with respect to cellular phenotype, cell proliferation,  
399 differentiation, and, in our companion paper, homing and T cell motility.

400 We have created a transgenic mouse strain in which Salsa6f is expressed under  
401 genetic control using the Rosa26-Cre recombinase system, and we have used this  
402 system to label immune cells that express CD4. Salsa6f labeling enables readout of  
403 cytosolic  $\text{Ca}^{2+}$  dynamics in T cells in vitro with high dynamic range without the handling  
404 and potential toxicity associated with loading of chemical  $\text{Ca}^{2+}$  indicators. Salsa6f was  
405 used to detect  $\text{Ca}^{2+}$  influx due to direct activation of SOCE, TCR stimulation, and Piezo1  
406 channel opening, detected in T cells for the first time to our knowledge. We also  
407 detected differences in patterns of  $\text{Ca}^{2+}$  signaling between naïve T cells, Th17 cells, and  
408 iTregs. These experiments demonstrate the sensitivity, brightness, uniformity of  
409 labeling, and ease of detecting dynamic  $\text{Ca}^{2+}$  signals using Salsa6f.

410 A primary advance of this work is to take the in vitro capabilities of an excellent  
411  $\text{Ca}^{2+}$  indicator and bring these into the realm of in vivo imaging. Within tissues, cells at a  
412 given position exhibit differences, ranging from subtle to dramatic, in morphology,  
413 connectivity, and molecular profile. The red fluorescence of Salsa6f, combined with  
414 genetic Salsa6f labeling, associates these characteristics with readout of cellular  $\text{Ca}^{2+}$   
415 signaling. Red fluorescence of Salsa6f is well excited by the same wavelength used for  
416 2-photon imaging of GCaMP6f, 920 nm, enabling imaging hundreds of micrometers

417 deep into lymph nodes. The immune system poses additional challenges for imaging  
418 because the constituent cells are highly motile in lymphoid organs. Indeed, direct cell  
419 interactions of motile immune cells form the basis of immune surveillance. We were  
420 able to identify red fluorescent Salsa6f T cells easily in intact lymph nodes upon  
421 adoptive transfer. Our images reveal uniform red fluorescence labeling by Salsa6f with  
422 clear subcellular morphology in imaging sessions encompassing hundreds of time lapse  
423 images. Images of lymph nodes from CD4-Salsa6f<sup>+/+</sup> mice display dozens to hundreds  
424 of cell-wide  $\text{Ca}^{2+}$  responses even in the absence of antigen. These capabilities indicate  
425 that Salsa6f transgenic mice could be used to associate  $\text{Ca}^{2+}$  signaling with T cell  
426 behavior *in vivo*.

427 Salsa6f offers the opportunity not only to record fluctuations in relative  $\text{Ca}^{2+}$   
428 levels over time, but also to read out  $\text{Ca}^{2+}$  concentrations within cells. We have  
429 measured the affinity of Salsa6f in intact cells; use of this approach will allow other  
430 microscope systems to be calibrated for measuring absolute  $\text{Ca}^{2+}$  concentrations with  
431 Salsa6f. Knowledge of absolute  $\text{Ca}^{2+}$  concentrations is necessary to develop  
432 quantitative models of  $\text{Ca}^{2+}$  signaling and cell behavior. Indeed, we demonstrate that  
433 clear Salsa6f ratio images can be generated from motile T cells in intact lymph nodes.

434 Our Salsa6f transgenic mouse line enables more sophisticated experimental  
435 approaches. One is the ability to detect rare  $\text{Ca}^{2+}$  signaling events. The high brightness  
436 and dynamic range of modern GECIs like Salsa6f contribute to detection of rare  $\text{Ca}^{2+}$   
437 signaling events inside intact tissues or even whole transgenic animals (Kubo, Hablitzel  
438 et al. 2014, Portugues, Feierstein et al. 2014). Detecting rare events is made harder by  
439 inhomogeneities in cell populations of the lymph node as well as the movement of

440 immune cells therein. Because of the one-to-one correspondence of tdTomato and  
441 GCaMP6f in Salsa6f, we were able to estimate and subtract resting GCaMP6f  
442 fluorescence even in motile cells. This approach substantially improves the uniformity of  
443 the fluorescence background upon which rare  $\text{Ca}^{2+}$  signaling events are detected.  
444 Reliable and uniform cytosolic labeling contributes as well. Combined, these factors  
445 enabled us to detect not only sporadic cell-wide  $\text{Ca}^{2+}$  elevations, but also unexpectedly  
446 sparkles, much smaller sporadic local  $\text{Ca}^{2+}$  signals. The sensitivity and resolution of  
447 these images are sufficient to map local signals from intact lymph nodes to sub-regions  
448 of T cells, which are some of the smallest cells of the body. Moreover, while we focused  
449 upon the brightest local  $\text{Ca}^{2+}$  signals to demonstrate their existence, we expect that  
450 Salsa6f will enable lower intensity  $\text{Ca}^{2+}$  signals to be linked to subcellular mechanisms  
451 and, ultimately, resulting cell behaviors. In a companion paper we relate  $\text{Ca}^{2+}$  signals  
452 detected by Salsa6f, both global and local, to T cell motility in the lymph node.

453 In conclusion, here we demonstrate the utility our  $\text{Ca}^{2+}$  indicator Salsa6f, and the  
454 transgenic mouse line which expresses Salsa6f, for studies of immune cell function.  
455 Use of Salsa6f improves assays of  $\text{Ca}^{2+}$  signaling in immune cell function, both in  
456 purified cell populations as well as in vivo, and for the first time to our knowledge, we  
457 detected  $\text{Ca}^{2+}$  influx associated with Piezo1 channel opening in T cells.  $\text{Ca}^{2+}$  signals  
458 were detected in T cells in lymph nodes under basal steady state conditions in the  
459 absence of antigen. Many of these  $\text{Ca}^{2+}$  signals were localized to sub-regions of T cells.  
460 Finally, we anticipate that this new probe of  $\text{Ca}^{2+}$  signaling will be widely applicable for  
461 studies of other cell types in other tissues.

462

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472

473 **Methods**

474 **GECI screening and Salsa6f plasmid generation**

475 Plasmids encoding GECIs (GECO and GCaMP6) were obtained from Addgene for  
476 screening in live cells. Each probe was cotransfected with Orai1 and STIM1 into HEK  
477 293A cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 48 hr before  
478 screening on an epifluorescence microscope. For construction of Salsa6f, a plasmid for  
479 tdTomato (Addgene, Cambridge, MA) and the pEGP-N1 vector (Clontech, Mountain  
480 View, CA) was used as a backbone. GCaMP6f was amplified via PCR with N- and C-  
481 terminal primers (5' CACAAACCGGTCGCCACCATGGTCGACTCATCACGTC 3' and 5'  
482 AGTCGCGGCCGCTTAAAGCTTCGCTGTCATCATTGTAC 3') and ligated into  
483 pEGFP-N1 at the AgeI/NotI sites to replace the eGFP gene, while tdTomato was  
484 amplified via PCR with N- and C-terminal primers (5'  
485 ATCCGCTAGCGCTACCGGGTCGCC 3' and 5'  
486 TAACGAGATCTGCTTGTACAGCTCGTCCATGCC 3') and ligated into the backbone at  
487 the NheI/BglII sites. An oligo containing the V5 epitope tag was synthesized with sense  
488 and antisense strands (5'  
489 GATCTCGGGTAAGCCTATCCCTAACCCCTCTCCTCGGTCTCGATTCTACG 3' and 5'  
490 GATCCGTAGAATCGAGACCGAGGGAGAGGGTTAGGGATAGGCTTACCGA 3') and  
491 ligated into the backbone at the BglII/BamHI sites, linking tdTomato to GCaMP6f and  
492 creating Salsa6f. The amplified regions of the construct were verified by sequencing  
493 (Eton Bioscience Inc., San Diego, CA). This plasmid, driven by the CMV promoter, was  
494 used for transient transfections in HEK 293A cells with Lipofectamine 2000 and in  
495 primary human T cells with Amaxa Nucleofection.

496 **Transgenic mouse generation and breeding**

497 The transgenic cassette in **Figure 2B** was generated by inserting Salsa6f, from the  
498 plasmid described above, into the Ai38 vector (Addgene Plasmid #34883) and replacing  
499 GCaMP3. The final targeting vector included the CAG (cytomegalovirus early  
500 enhancer/chicken  $\beta$ -actin) promoter, an LSL sequence with LoxP-STOP-LoxP, the  
501 Salsa6f probe (tdTomato-V5-GCaMP6f), the woodchuck hepatitis virus  
502 posttranscriptional regulatory element (WPRE), and a neomycin resistance gene  
503 (NeoR), all flanked by 5' and 3' Rosa26 homology arms of 1.1 and 4.3 kb. The targeting  
504 vector was linearized with Pvul and electroporated into JM8.N4 mouse embryonic stem  
505 (ES) cells of C57BL/6N background. Following selection with G418, clones were  
506 screened by Southern blotting after digestion with HindIII for the 5' end or BgII for the 3'  
507 end. Four correctly targeted clones were expanded and checked by chromosome  
508 counting, then two clones with >90% euploidy were further expanded and injected into  
509 C57BL/6J blastocysts for implantation into pseudopregnant foster mothers. Presence of  
510 the Salsa6f transgenic cassette was detected in the resulting chimeric pups by PCR  
511 screening for the *Nnt* gene, as the initial JM8.N4 ES cells are *Nnt*<sup>+/+</sup> while the C57BL/6J  
512 blastocysts are *Nnt*<sup>-/-</sup>. Finally, positive chimeras were bred to R26 $\Phi$ C31o mice (JAX  
513 #007743) to remove the neomycin resistance gene flanked by AttB and AttP sites in the  
514 original transgenic cassette, and to produce Salsa6f<sup>LSL/-</sup> F1 founders carrying the allele  
515 for LSL-Salsa6f at the Rosa26 locus. These F1 founders were then bred to  
516 homozygosity to generate Salsa6f<sup>LSL/LSL</sup> mice, and subsequently crossed to  
517 homozygotic CD4-Cre mice (JAX #017336) to generate CD4-Salsa6f<sup>+//-</sup> mice expressing

518 Salsa6f only in T cells. CD4-Salsa6f<sup>+/−</sup> mice were further bred to generate homozygotic  
519 CD4-Salsa6f<sup>+/+</sup> mice for increased Salsa6f expression and fluorescence.

520 **T cell proliferation and differentiation**

521 *For T cell proliferation:* CD4 T cells were isolated from spleen and lymph nodes of 6-10  
522 week old mice using negative selection (StemCell Technologies, Cambridge, MA).  
523 CellTrace Violet (CTV)-labeled T cells were co-cultured with αCD3/CD28 coated  
524 dynabeads (Life Technologies Corp., Grand Island, NY) at 1:1 ratio according to the  
525 manufacturer's protocol in a U bottom 96 well plate. *For T cell differentiation:* Naïve CD4  
526 T cells were differentiated on activating polystyrene surface (Corning Inc., Corning, NY)  
527 with plate-bound αCD3 (2.5 µg/ml) and αCD28 (2.5 µg/ml) in the presence of cytokines  
528 for 6 days (Yosef, Shalek et al. 2013). For Th1 differentiation: 25 ng/mL rmIL-12  
529 (BioLegend, San Diego, CA), 10 µg/mL rmouse IL4 (Biolegend). For Th17  
530 differentiation: 2.5 ng/mL rhTGF-β1 (Tonbo Biosciences, San Diego, CA), 50 ng/mL  
531 rmIL-6 (Tonbo Biosciences), 25 ng/ml rmIL-23 (Biolegend), and 25 ng/ml rmIL-β1  
532 (Biolegend). For iTreg differentiation: 10 ng/mL rhTGF-β1, 100 units/mL of rmIL-2  
533 (Biolegend), 5 µM Retinoic Acid (Sigma, St. Louis, MO).

534 **Flow cytometry.**

535 CTV dilution assay was performed in live cells (Fixable Viability Dye eFluor® 780  
536 negative gating; ThermoFisher Scientific Inc., Grand Island, NY). To detect intracellular  
537 cytokines, 6 day differentiated cells were stimulated in with 25 ng of phorbol 12-  
538 myristate 13-acetate (PMA), 1 µg ionomycin (Sigma), and monensin (Golgistop® BD  
539 biosciences) for 4 hr at 37 °C. Dead cells were labeled with Ghost dye 780 (BioLegend),  
540 then washed, fixed, permeabilized using FoxP3 staining buffer set (ThermoFisher Inc).

541 The following antibodies were used to detect intracellular cytokines: IL-17A-APC (clone  
542 TC11-18H10.1, BioLegend); IFN $\gamma$ -Pacific Blue (clone XMG1.2, BioLegend); Foxp3-PE  
543 (clone FJK16s, ThermoFisher Scientific Inc.); in permeabilization buffer (eBioscience).  
544 Data were acquired using NovoCyte flow cytometer (ACEA Biosciences) and analyzed  
545 using FlowJo.

546 **T-cell preparation for live cell imaging**

547 CD4 T cells were activated by plating on 6 well plates coated overnight with 2.5  $\mu$ g/mL  
548  $\alpha$ CD3/ $\alpha$ CD28 (Invivogen, San Diego, CA) at 4° C. Cells were cultured in RPMI medium  
549 (Lonza) containing 10% FCS, L-glutamine, Non-essential amino acids, Sodium  
550 pyruvate,  $\beta$ -mercaptoethanol and 50 U/mL of IL-2 at 37° C in 5% CO<sub>2</sub> incubator.  
551 Following 2 days of culture, cells were plated on either poly-L-lysine or 1  $\mu$ g/mL  $\alpha$ -  
552 CD3/ $\alpha$ 28 coated 35mm glass chambers (Lab-Tek, ThermoFisher Inc.) for imaging. RPMI  
553 medium with 2% FCS and L-glutamine containing 2 mM Ca<sup>2+</sup> was used for imaging  
554 experiments. For experiments involving calibration and characterization of the Salsa6f  
555 probe in CD4-Salsa6f<sup>+/−</sup> cells, Ringer solution containing various concentrations of Ca<sup>2+</sup>  
556 was used. For Ca<sup>2+</sup> imaging of different T cell subsets, Th17 cells and iTregs were  
557 differentiated as described above.

558 **Confocal imaging and analysis**

559 For Ca<sup>2+</sup> imaging of CD4<sup>+</sup> T cells from CD4-Salsa6f mice, we used an Olympus  
560 Fluoview FV3000RS confocal laser scanning microscope, equipped with high speed  
561 resonance scanner and the IX3-ZDC2 Z-drift compensator (Olympus Corp., Waltham,  
562 MA). Diode lasers (488 and 561 nm) were used for excitation, and two high-sensitivity  
563 cooled GaAsP PMTs were used for detection. Cells were imaged using the Olympus

564 40x silicone oil objective (NA 1.25), by taking 5 slice z-stacks at 2  $\mu\text{m}/\text{step}$ , at 5 sec  
565 intervals, for up to 20 min. Temperature, humidity, and  $\text{CO}_2$  were maintained using a  
566 Tokai-Hit WSKM-F1 stagetop incubator. Data were processed and analyzed using  
567 Imaris and ImageJ software. Calcium imaging experiments were done at 37° C on 2  
568 day-activated CD4 $^+$  T cells from CD4-Salsa6f $^{+/-}$  mice, unless otherwise indicated.  
569 Salsa6f calibration experiments were done at room temperature.

570 **Two-photon microscopy**

571 Lymph nodes images were acquired using a custom-built two photon microscope based  
572 on Olympus BX51 upright frame, Motorized ZDeck stage (Prior, Rockland, MA), with  
573 excitation generated by a tunable Chameleon femtosecond laser (Coherent, Santa  
574 Clara, CA) (Miller, Wei et al. 2002). The following wavelengths were used to excite  
575 single or combination of fluorophores: 920 nm to excite tdTomato and GCaMP6f; 1040  
576 nm to excite tdTomato alone. 495 nm and 538 nm dichroic filters were arranged in  
577 series to separate blue, green and red signals. Two-photon excitation maxima of  
578 tdTomato and GCaMP6f are 1040 and 920 nm, respectively (Drobizhev, Makarov et al.  
579 2011, Chen, Wardill et al. 2013). Using 1040 nm excitation, tdTomato signals were  
580 readily detected up to 300  $\mu\text{m}$  depth; however, 1040 is not ideal to image Salsa6f  
581 because: 1) Collagen fibers generate second harmonic at 520 nm when excited with  
582 1040 nm, which interferes with simultaneous detection of GCaMP6f (emission maxima,  
583 509 nm); and 2) 1040 nm does not excite GCaMP6f (**Figure 9-figure supplement 1A,**  
584 **top row**). Alternatively, 920 nm optimally excites GCaMP6f, and excites tdTomato  
585 sufficiently, and Salsa6f signals were detected up to 300  $\mu\text{m}$  depth, while second  
586 harmonic collagen signals (460 nm) can be easily separated into blue channel (**Figure**

587 **9-figure supplement 1A, bottom row).** Additionally, autofluorescent structures (LN  
588 resident DCs and fibroblastic reticular cells) show up as yellow bodies when excited  
589 with 920 nm, which serve as a guide to locate the T cell zone (**Figure 9-figure**  
590 **supplement 1B**). Therefore, 920 nm is the ideal two-photon excitation wavelength for  
591 simultaneous imaging of tdTomato and GCaMP6f as component parts of Salsa6f.

592 Lymph nodes were oriented with the hilum away from the water dipping  
593 microscope objective (Nikon 25x, NA 1.05). The node was maintained at 36-37°C by  
594 perfusion with medium (RPMI) bubbled with medical grade carbogen (95% O<sub>2</sub> and 5%  
595 CO<sub>2</sub>) using a peristaltic heated perfusion system (Warner Instruments), with  
596 thermocouple-based temperature sensors placed next to the tissue in a custom built  
597 chamber. 3D image stacks of x=250 μm, y=250 μm, and z=20 or 52 μm (4 μm step  
598 size) were sequentially acquired at 5 or 11 second intervals respectively, using image  
599 acquisition software Slidebook (Intelligent Imaging Innovations) as described previously  
600 (Matheu, Othy et al. 2015). This volume collection was repeated for up to 40 min to  
601 create a 4D data set.

602 **Data analysis and statistical testing**

603 Graphpad Prism was used for statistical analysis and generating figures. p values are  
604 indicated in figures: ns p> 0.05, \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001; and \*\*\*\* p <  
605 0.0001.

606 **Detection of Ca<sup>2+</sup> signals in lymph nodes**

607 Stacks of 6 optical sections 4 μm apart from the T-zone of CD4-Salsa6f<sup>+/−</sup> lymph nodes  
608 were acquired once every 5 sec at a resolution of 0.488 or 0.684 μm per pixel.  
609 Maximum intensity projections of 1 pixel radius median-filtered images were used for

610 subsequent processing and analysis. Autofluorescent cells were identified by averaging  
611 the red or green time lapse image stacks and automated local thresholding (Bernsen 5  
612 pixel radius) using the public domain image processing program ImageJ.  
613 Autofluorescent cell masks were dilated by 4 pixels, regions exhibiting less contrast and  
614 detail due to light scattering manually masked to produce the final time lapse image  
615 mask. Red (tdTomato) channel fluorescence from Salsa6f corresponding to green  
616 (GCaMP6f) channel resting state fluorescence was determined to be 5-fold higher using  
617 our standard 2-photon microscope acquisition settings. Final green images were  
618 produced by subtracting a 0.2x scaled red channel image, and subsequently subtracting  
619 out the average of all green channel time lapse images. The standard deviation (SD) of  
620 each masked green channel time lapse image stack was used to determine thresholds  
621 for local (sparkle) and cell-wide  $\text{Ca}^{2+}$  events. Thresholds for detection of local and cell-  
622 wide  $\text{Ca}^{2+}$  events were 5.4 and 2.1 SD and  $1.4 \mu\text{m}^2$  and  $25 \mu\text{m}^2$ , respectively.  
623 Frequency of background events was calculated using a standard normal distribution  
624 with a Z-score corresponding to the average intensity of local events (6.5 SD), which  
625 was 1 in  $2 \times 10^{10}$  pixels (WolframAlpha).

626

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775 **Figure Legends**

776 **Figure 1. Design of novel tdTomato-V5-GCaMP6f fusion probe “Salsa6f” and**  
777 **characterization in living cells. (A)** Several genetically encoded  $\text{Ca}^{2+}$  indicators were  
778 screened in vitro in HEK 293A cells, by co-transfected with Orai1/STIM1 and measuring  
779  $\text{Ca}^{2+}$  influx after thapsigargin-induced store depletion, showing maximum change in  
780 fluorescence intensity in dark green bars and dynamic range (DR) in light green bars,  
781 with Salsa6f shown in orange bars on right;  $n > 30$  cells per probe, from two different  
782 transfections, error bars indicate SEM. **(B)** Averaged thapsigargin-induced  $\text{Ca}^{2+}$  entry,  
783 measured by change in GFP fluorescence, in GCaMP6f (green,  $11.5 \pm 0.3$ ,  $n = 63$ ) or  
784 Salsa6f (orange,  $10.2 \pm 0.3$ ,  $n = 78$ ) transfected HEK cells; data from two different  
785 transfections, error bars indicate SEM. **(C)** Diagram of Salsa6f construct used in  
786 transfection. **(D)** Two-photon images of Salsa6f co-transfected in HEK cells with  
787 Orai1/STIM1, showing red (tdTomato), green (GCaMP6f), and merged channels, at  
788 baseline in 0 mM extracellular  $\text{Ca}^{2+}$  and after maximum stimulation with 2  $\mu\text{M}$  ionomycin  
789 in 2 mM extracellular  $\text{Ca}^{2+}$ ; scale bar = 20  $\mu\text{m}$ ; see **Video 1**; data representative of at  
790 least three different experiments. **(E)** Confocal time lapse microscopy of human CD4 $^{+}$  T  
791 cells transfected with Salsa6f, then activated for two days on plate-bound  $\alpha$ CD3/28  
792 antibodies; time = min:sec, scale bar = 10  $\mu\text{m}$ . **(F)** Representative cell traces of  
793 activated human T cells transfected with Salsa6f, tracking green fluorescence intensity  
794 only; data representative of at least three different experiments.

795 **Figure 2. Generation of Salsa6f transgenic mouse line targeted to Rosa26 locus.**

796 **(A)** Transgenic targeting vector for Salsa6f, inserted between Rosa26 homology arms  
797 and electroporated into embryonic stem cells. CAG Pr: cytomegalovirus early  
798 enhancer/chicken  $\beta$ -actin promoter; Salsa6f: tdTomato-V5-GCaMP6f; FRT, LoxP, AttB,  
799 AttP: recombinase sites; WPRE: woodchuck hepatitis virus posttranscriptional  
800 regulatory element; pA: bovine growth hormone polyadenylation sequence; NeoR:  
801 neomycin resistance gene. **(B)** Correctly targeted ES cells were screened by Southern  
802 blot after HindIII digest for the 5' end (top) or BglII digest for the 3' end (bottom). The two  
803 clones marked in red failed to integrate at the 5' end. **(C)** PCR screening for chimeras  
804 based on presence of the Nnt mutation, present only in JM8.N4 ES cells but not in the

805 C57BL/6J blastocyst donors. 2540 and 2543 are chimeras. Control lanes on the right  
806 are wildtype ( $Nnt^{+/+}$ ), heterozygous ( $Nnt^{+/+}$ ), or homozygous mutant ( $Nnt^{-/-}$ ).

807 **Figure 3. CD4-Salsa6f mice show normal immune cell development and**  
808 **expression.** (A) Experimental design to target expression of Salsa6f in CD4 cells. (B)  
809 CD4, CD8 and double-positive cells gated on tdTomato (Salsa6f<sup>+</sup> cells) from thymus.  
810 (C) Histograms showing percent of Salsa6f<sup>+</sup> cells in spleen, LN, and thymus. (D) CD4,  
811 CD8, and double positive cells from spleen, gated on tdTomato (Salsa6f<sup>+</sup> cells). (E)  
812 Histograms showing percent of Salsa6f<sup>+</sup> cells within CD4, CD8, CD19, CD11b  
813 populations from spleen. (F) Total number of CD4, CD8, CD19, CD11b cells in the  
814 spleen of CD4-Salsa6f<sup>+/−</sup> mice and CD4-Cre mice (n=6 mice). (G) Relative percentages  
815 of CD4, CD8, CD19, CD11b cells in thymus, lymph nodes, and spleen of CD4-Salsa6f  
816 mice and CD4-Cre mice (n=6).

817 **Figure 4. Functional responses of CD4-Salsa6f T cells in vitro.** (A) Representative  
818 histogram showing cell trace violet (CTV) dilution in CD4-Cre (teal) and CD4-Salsa6f<sup>+/−</sup>  
819 T cells (red) at 92 hours following stimulation with  $\alpha$ CD3/28 Dynabeads (1:1 ratio). (B)  
820 Proliferation index measured on CTV dilution curves (n=10). (C-E) Dot plots showing  
821 differentiation of naive T cells from CD4-Cre and CD4-Salsa6f<sup>+/−</sup> mice into Th1 cells (C),  
822 Th17 cells (D) and iTregs (E) after 6 days (n = 4 mice). Right panels show average  
823 percent of IFN $\gamma$ <sup>+</sup> cells (C), IL-17<sup>+</sup> cells (D) and Foxp3<sup>+</sup> cells (E).

824 **Figure 5. Single-cell readout of Salsa6f calcium signals in T cells.** (A) Confocal  
825 imaging of Ca<sup>2+</sup> signals in activating CD4<sup>+</sup> T cells from CD4-Salsa6f<sup>+/−</sup> mice, after two  
826 day stimulation on plate bound  $\alpha$ CD3/28 antibody, showing merged green (GCaMP6f)  
827 and red (tdTomato) channels; time = min:sec; scale bar = 10  $\mu$ m. (B) Representative  
828 traces from cell #3 in (A), showing total fluorescence intensity changes in GCaMP6f  
829 (green), tdTomato (red), and green/red ratio (G/R, blue). (C) G/R ratios for cells 1, 2,  
830 and 4 from (A). (D) Dynamic range of Salsa6f in resting CD4 T cells, measured as  
831 green/red fluorescence by flow cytometry. Cells were pre-treated with 10  $\mu$ M ionomycin  
832 in Ca<sup>2+</sup>-free solution (white bar), followed by re-addition of 10 mM Ca<sup>2+</sup> (blue bar). (E)  
833 Averaged tdTomato fluorescence in resting T cells from heterozygous CD4-Salsa6f<sup>+/−</sup>  
834 compared to homozygotic CD4-Salsa6f<sup>+/+</sup> mice.

835 **Figure 6. Probe characterization and calibration of  $[\text{Ca}^{2+}]$  in Salsa6f T cells. (A)**  
836 Confocal image of a naïve T cell from a CD4-Salsa6f<sup>+/−</sup> mouse. Upper panel: tdTomato  
837 (left) and GCaMP6f (right) fluorescence intensity in  $\text{Ca}^{2+}$ -free Ringer solution. Lower  
838 panel: same cell treated with 2  $\mu\text{M}$  thapsigargin (TG) in Ringer solution containing 2 mM  
839  $\text{Ca}^{2+}$ . Line scan for each condition is shown adjacent to the images. Scale bar = 2  $\mu\text{m}$   
840 for **A-C**. **(B)** Confocal images of Salsa6f localization in a 2-day activated CD4<sup>+</sup> T-cell  
841 from CD4-Salsa6f<sup>+/−</sup> mouse. **(C)** Confocal image of a Fluo-4 (5  $\mu\text{M}$ ) loaded CD4<sup>+</sup> T cell  
842 from CD4-Cre mouse. **(D)** Average GCaMP6f and tdTomato intensities and G/R ratios  
843 in 2-day activated CD4<sup>+</sup> T cells treated with 2  $\mu\text{M}$  ionomycin in  $\text{Ca}^{2+}$  free buffer ( $F_{\min}$ )  
844 and 20 mM  $\text{Ca}^{2+}$  buffer ( $F_{\max}$ );  $n = 76$  cells, representative of 3 experiments. **(E)**  
845 Average 340 / 380 nm ratios in fura-2 loaded CD4<sup>+</sup> T cells ( $n=59$  cells) and G/R ratios in  
846 Salsa6f CD4<sup>+</sup> T cells ( $n=47$  cells) treated identically with 2  $\mu\text{M}$  ionomycin followed by  
847 graded increases of external  $\text{Ca}^{2+}$  concentration as indicated. **(F)** Steady-state fura-2  
848 and Salsa6f ratios recorded 300 s after solution application and peak Salsa6f ratio from  
849 **6E** plotted as a function of external  $\text{Ca}^{2+}$  concentration. **(G)** Steady-state and peak  
850 Salsa6f ratios plotted as a function of cytosolic  $\text{Ca}^{2+}$  concentrations calculated from the  
851 fura-2 experiment, assuming a fura-2  $K_d$  of 225 nM. The points were fit with a 4  
852 parameter Hill equation to obtain the  $K_d$  for Salsa6f, with the following parameters:  
853 Salsa6f steady-state: Hill coefficient =  $1.49 \pm 0.16$ ;  $K_d = 301 \pm 24$ ; Salsa6f peak: Hill  
854 coefficient =  $0.93 \pm 0.4$ ;  $K_d = 162 \pm 48$ . Data are representative of three experiments.

855 **Figure 6-supplement 1. Comparison of GECI localization in CD4 T cells from**  
856 **Salsa6f mouse and PC::G5-tdT mouse. (A,B)** Confocal images of CD4<sup>+</sup> T cells  
857 purified from a CD4-Salsa6f<sup>+/−</sup> mouse **(A)** or a CD4-Cre 5GtdT<sup>+/−</sup> mouse **(B)**, showing  
858 merged red and green; cells imaged at the same laser and PMT settings; scale bar = 10  
859  $\mu\text{m}$ . **(C,D)** CD4<sup>+</sup> T cells purified from a CD4-Salsa6f<sup>+/−</sup> mouse **(C)** or a 5G-tdT<sup>+/−</sup>-CD4-  
860 Cre<sup>+/−</sup> mouse **(D)**, then activated for 24 hr on plate-bound  $\alpha\text{CD3}/28$  antibodies, and  
861 imaged with confocal microscopy, showing red (tdTomato), green (GCaMP6f or  
862 GCaMP5G), and merged channels; scale bar = 10  $\mu\text{m}$ .

863 **Figure 7.  $\text{Ca}^{2+}$  signals in activated CD4<sup>+</sup> T cells from CD4-Salsa6f<sup>+/−</sup> mice in**  
864 **response to store-depletion, TCR stimulation and Piezo1 channel activation.**

865 Average Salsa6f G/R ratios on left, representative single-cell traces superimposed on  
866 right. Experiments were done in standard Ringer solution (**A**) or in RPMI containing 2%  
867 FCS and 2 mM Ca<sup>2+</sup> (**B-D**). **(A)** Store-operated Ca<sup>2+</sup> entry (SOCE) in CD4<sup>+</sup> T cells (n =  
868 86 cells), induced by depleting ER Ca<sup>2+</sup> stores with TG in Ca<sup>2+</sup>-free buffer followed by  
869 re-addition of Ringer containing 2 mM Ca<sup>2+</sup>. **(B,C)** Ca<sup>2+</sup> responses to TCR stimulation T  
870 cells plated on coverslips coated with 1 µg/ml αCD3/CD28 (**B**) or 1 µg/ml αCD3 alone  
871 (**C**) (n = 90 cells each). **(D)** Ca<sup>2+</sup> elevations during shear stress induced by solution  
872 exchange followed by the Piezo1 agonist Yoda1 (15 µM) in cells plated on αCD3/28 (n  
873 = 79 cells).

874 **Figure 7-figure supplement 1. Store-operated Ca<sup>2+</sup> entry and Piezo1 activation in**  
875 **naïve T cells from CD4-Salsa6f<sup>+/−</sup> mouse. (A)** Average (left panel) and representative  
876 single cell responses (right panel) to TG-induced SOCE (n = 96 cells). **(B)** Average (left  
877 panel) and single cell responses (right panel) to solution exchange of media alone  
878 followed by 15 µM Yoda1 (n = 53 cells).

879 **Figure 8. TCR induced Ca<sup>2+</sup> signals in T cell subsets from CD4-Salsa6f<sup>+/−</sup> mice.**  
880 Average and representative single-cell Ca<sup>2+</sup> traces from confocal time-lapse microscopy  
881 showing changes in Salsa6f green/red (G/R) ratio in naïve T cells (**A**), 5 day  
882 differentiated Th17 cells (**B**), and 5 day differentiated iTregs (**C**) plated on 1 µg/mL  
883 αCD3/28. (n = 90 cells from 2 - 3 experiments each).

884 **Figure 9. Lymph nodes from CD4-Salsa6f<sup>+/+</sup> mice exhibit cell-wide and subcellular**  
885 **Ca<sup>2+</sup> signals. (A)** Median filtered, maximum intensity projection of a red channel image  
886 from a single time point of an explanted lymph node from a CD4-Salsa6f<sup>+/+</sup> mouse. **(B)**  
887 Green channel image corresponding to **A**. Orange arrowhead indicates cell-wide Ca<sup>2+</sup>  
888 signal and gray arrowheads indicate smaller, local transient Ca<sup>2+</sup> signals. **(C, D)**  
889 Enlargements of cell-wide (**C**) and local (**D**; gray arrowheads) Ca<sup>2+</sup> signals. Note the  
890 lower fluorescence intensity in the center of the cell in **C** due to exclusion of Salsa6f  
891 from the nucleus. **(E)** Maximum intensity projection of 214 green channel time points  
892 (every 11.5 seconds over 41 minutes) showing hundreds of small local Ca<sup>2+</sup> signals.  
893 Green channel image series was red channel subtracted and cropped from **B**. Asterisks  
894 indicate regions containing autofluorescent cells that have been cropped out. **(F, G)**

895 Surface plot of maximum green channel intensity over two (**F**) and 50 (**G**) consecutive  
896 time points. Note the presence of four (**F**) and dozens (**G**) of small, discrete, high  
897 intensity peaks of similar intensity. (**H**) Bar graph of relative frequencies of cell-wide and  
898 local  $\text{Ca}^{2+}$  signals. (**I**) Frequency distribution of the area of local  $\text{Ca}^{2+}$  signals. Scale bar  
899 in **A** is 100  $\mu\text{m}$  (applies to **B**); scale bar in **C** is 10  $\mu\text{m}$  (applies to **D**), scale bars in **E** and  
900 in **F** are 50  $\mu\text{m}$  (applies to **G**). (**J**) Trace of fluorescence intensity over 25 minutes at the  
901 location of a transient subcellular  $\text{Ca}^{2+}$  signal (one time point every 5 seconds). (**K**)  
902 Trace of fluorescence intensity of a putative cell process from an autofluorescent cell  
903 drifting in the image field.

904 **Figure 9-figure supplement 1. Imaging lymph nodes of CD4-Salsa6f<sup>+/+</sup>**  
905 **homozygous mice.** Cre-mediated expression of Salsa6f in CD4 T cells reveals  
906 endogenous T cell labeling in lymph node. (**A**) Two-photon images of explanted lymph  
907 node from CD4-Salsa6f<sup>+/+</sup> mouse at various depths (indicated above the image); 1040  
908 nm excitation (top, row) or 920 nm excitation (bottom row). Second harmonic signal  
909 from collagen fibers is collected in green for 1040 nm excitation and in blue for 920 nm  
910 excitation. Salsa6f cells are readily detected up to 275  $\mu\text{m}$  deep. (**B**) Montage image of  
911 a CD4-Salsa6f<sup>+/+</sup> lymph node at 100  $\mu\text{m}$  depth, imaged using 920 nm excitation  
912 showing Salsa6f<sup>+</sup> cells in red, autofluorescent structures in yellow, and the capsular  
913 boundary shown in blue (second-harmonic signal); scale bar = 100  $\mu\text{m}$ .

914 **Figure 9-figure supplement 2. Subtraction of red channel fluorescence improves**  
915 **detection of Salsa6f  $\text{Ca}^{2+}$  signals.** (**A, B**) Median filtered, maximum intensity projection  
916 of a red channel image from a single time point of an explanted lymph node from a  
917 CD4-Salsa6f<sup>+/+</sup> mouse. Panel **A** is enlarged and cropped from panel **B** (gray rectangle).  
918 (**C, E, G**) Green channel images corresponding to **A** with different image processing  
919 protocols. (**C**) Maximum intensity projection without further processing. (**E**) Maximum  
920 intensity projection after subtraction of the average of all green channel frames. (**G**)  
921 Maximum intensity projection after subtraction of the corresponding, scaled red channel  
922 image and subtraction of the subsequent average from all green channel frames. Green  
923 arrows in **C, E, G** indicate a subcellular  $\text{Ca}^{2+}$  signal. (**D, F, H**) Surface plots corresponding  
924 to the images in **C, E, G** respectively showing the subcellular  $\text{Ca}^{2+}$  signal as a green

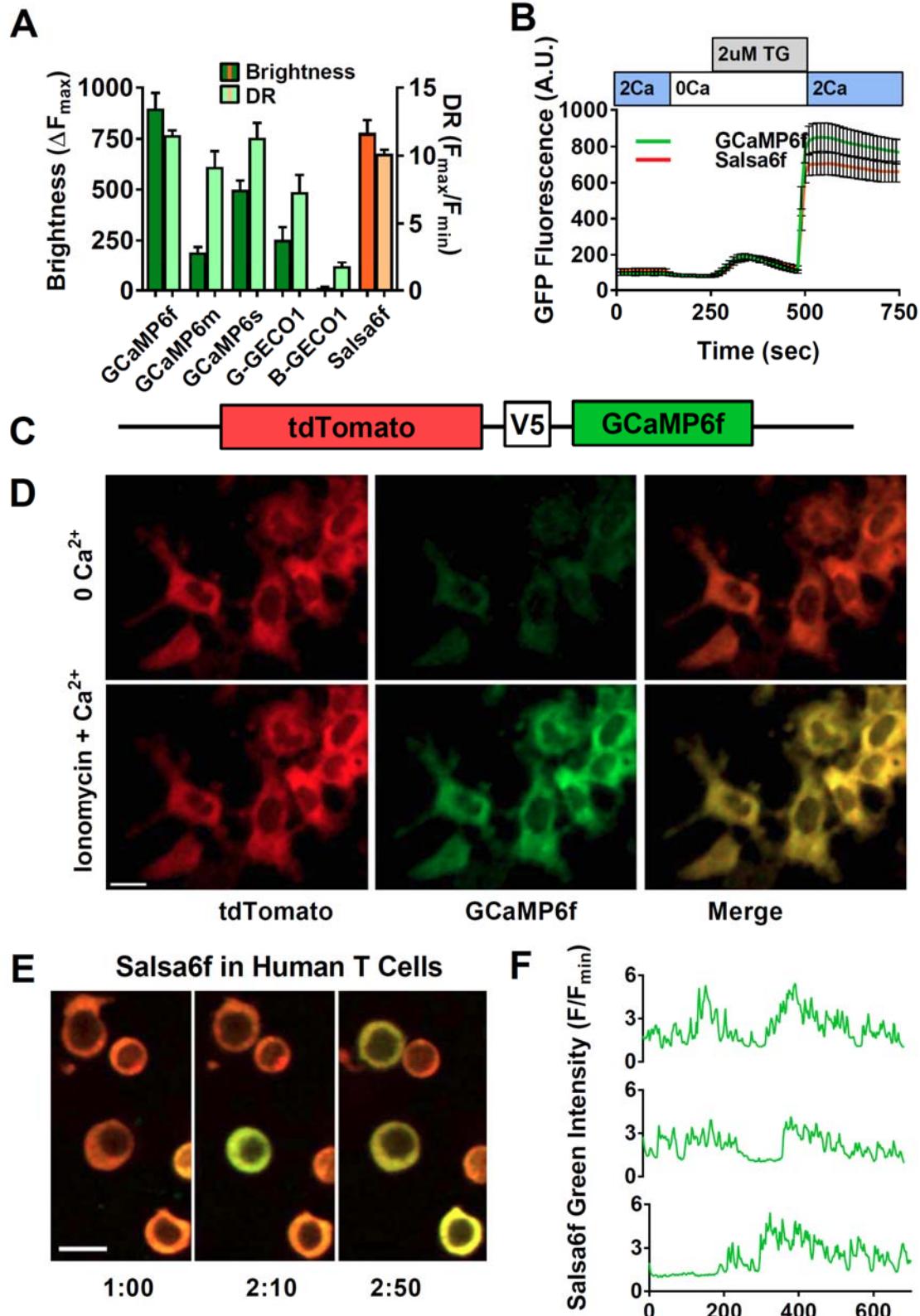
925 peak. Gray arrowheads indicate nearby background cell fluorescence that is  
926 progressively removed by image processing. Asterisk indicates a region containing an  
927 autofluorescent cell that has been cropped out. Scale bar in **A** is 25  $\mu$ m (applies to  
928 **C,E,G**); scale bar in **B** and the horizontal scale bar in **D** are 50  $\mu$ m (applies to **F,H**).

929 **Figure 10. Subcellular  $\text{Ca}^{2+}$  signals map to different regions of motile CD4-  
930 Salsa6f<sup>+/+</sup> T cells.** (A-D) CD4-Salsa6f<sup>+/+</sup> T cell imaged in a wild type lymph node after  
931 adoptive transfer. (A) Red channel fluorescence image. (B) Corresponding  
932 pseudocolored green channel image. (C) Corresponding composite image of gray  
933 pseudocolored red channel image with green channel image. (D) Ratiometric image of  
934 the green divided by the red channel fluorescence image. Gray arrowheads denote a  
935 local  $\text{Ca}^{2+}$  signal at the back of motile T cell and the green arrow denotes a point of  
936 relatively high  $\text{Ca}^{2+}$  concentration at the extreme back of the cell. Look-up table for **B**  
937 corresponds to Arbitrary Units; look-up table for **D** corresponds to green-to-red ratio. (E-  
938 H) Adoptively transferred CD4-Salsa6f<sup>+/+</sup> T cells displaying local  $\text{Ca}^{2+}$  signals at different  
939 subcellular locations. (E) Two local  $\text{Ca}^{2+}$  signals at the back of the cell. (F) Local  $\text{Ca}^{2+}$   
940 signal at the front of a different cell. (G) Same cell as in F five seconds later displaying  
941 local  $\text{Ca}^{2+}$  signals at both the front and back. (H) Different cell displaying local  $\text{Ca}^{2+}$   
942 signal at the side. Location of local  $\text{Ca}^{2+}$  signals indicated by gray arrowheads. For all,  
943 cells are oriented with their front toward the top of the image. Scale bar in A is 5  $\mu$ m  
944 (applies to **B-H**).

945

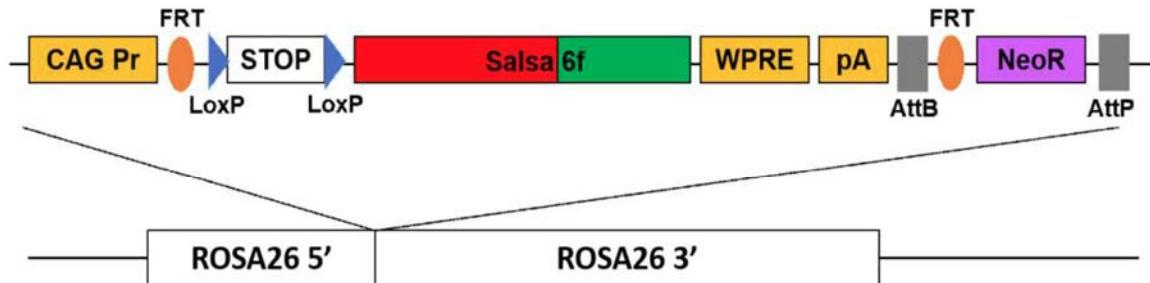
946 **Figure 1**

947

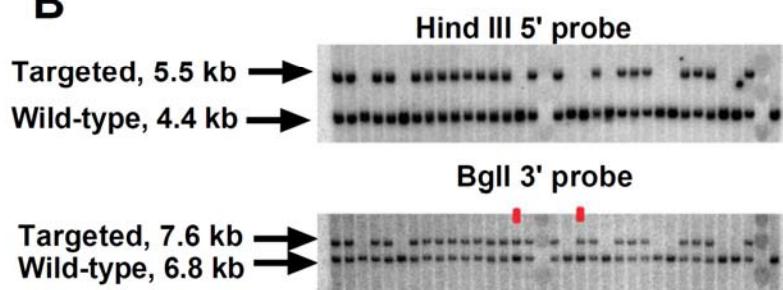


948 **Figure 2**

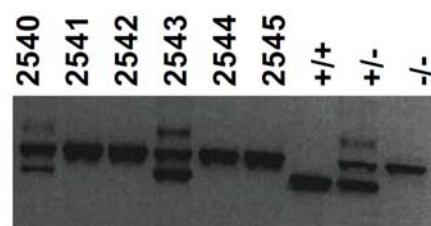
**A**



**B**



**C**

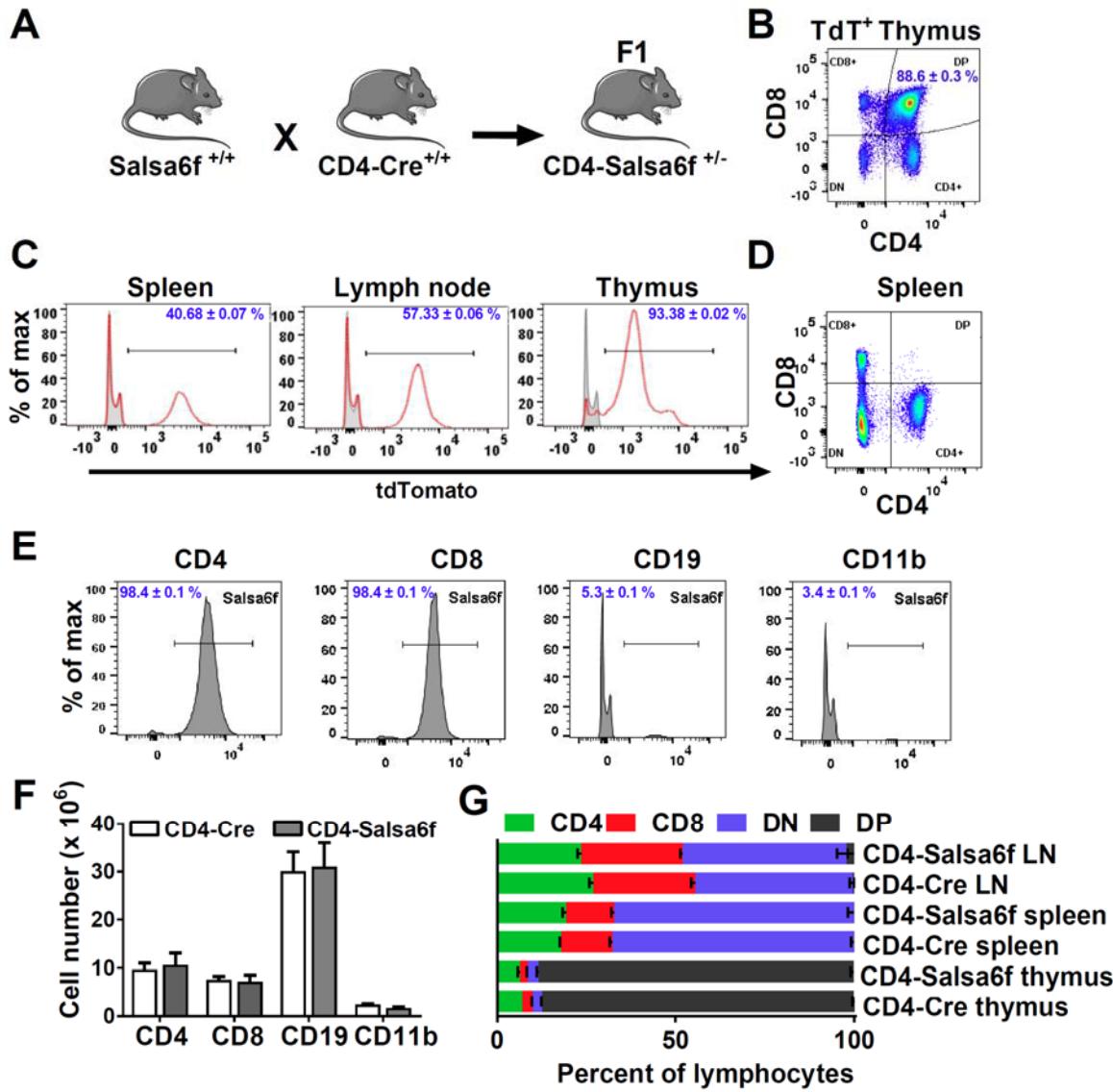


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951 **Figure 3**

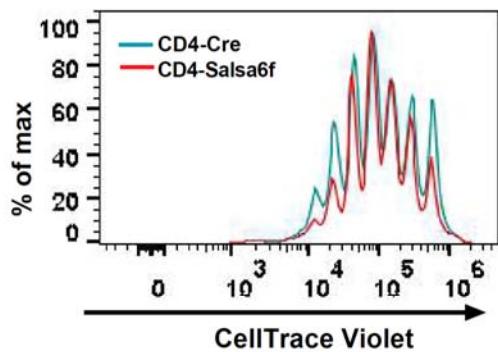
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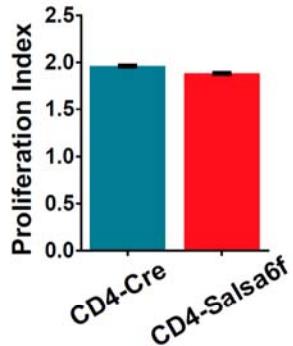
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## Figure 4

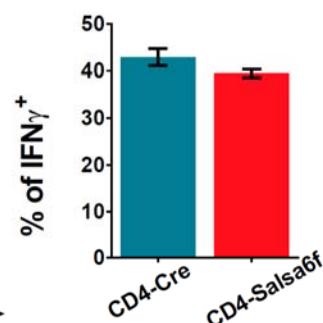
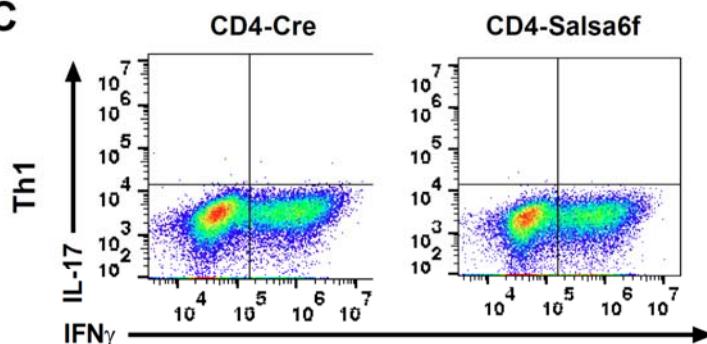
**A**



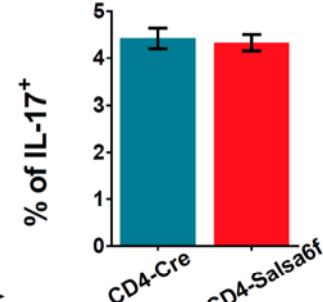
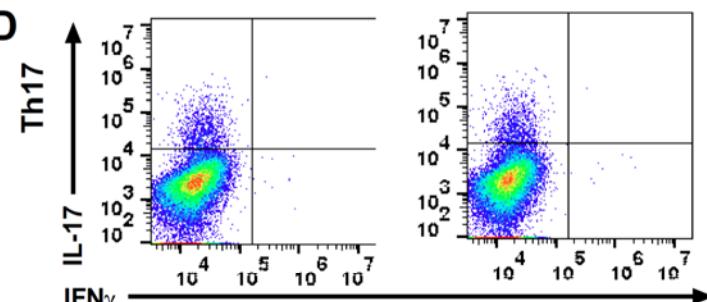
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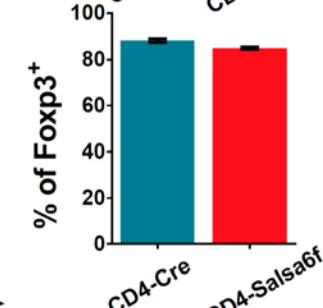
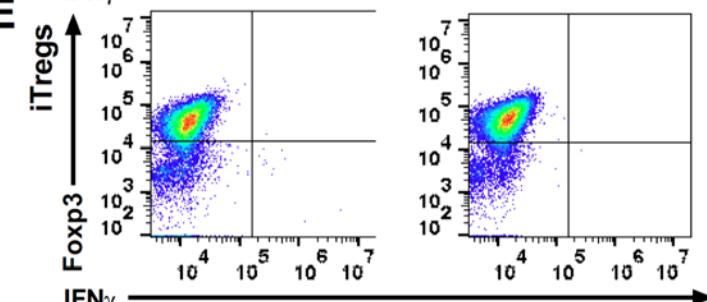
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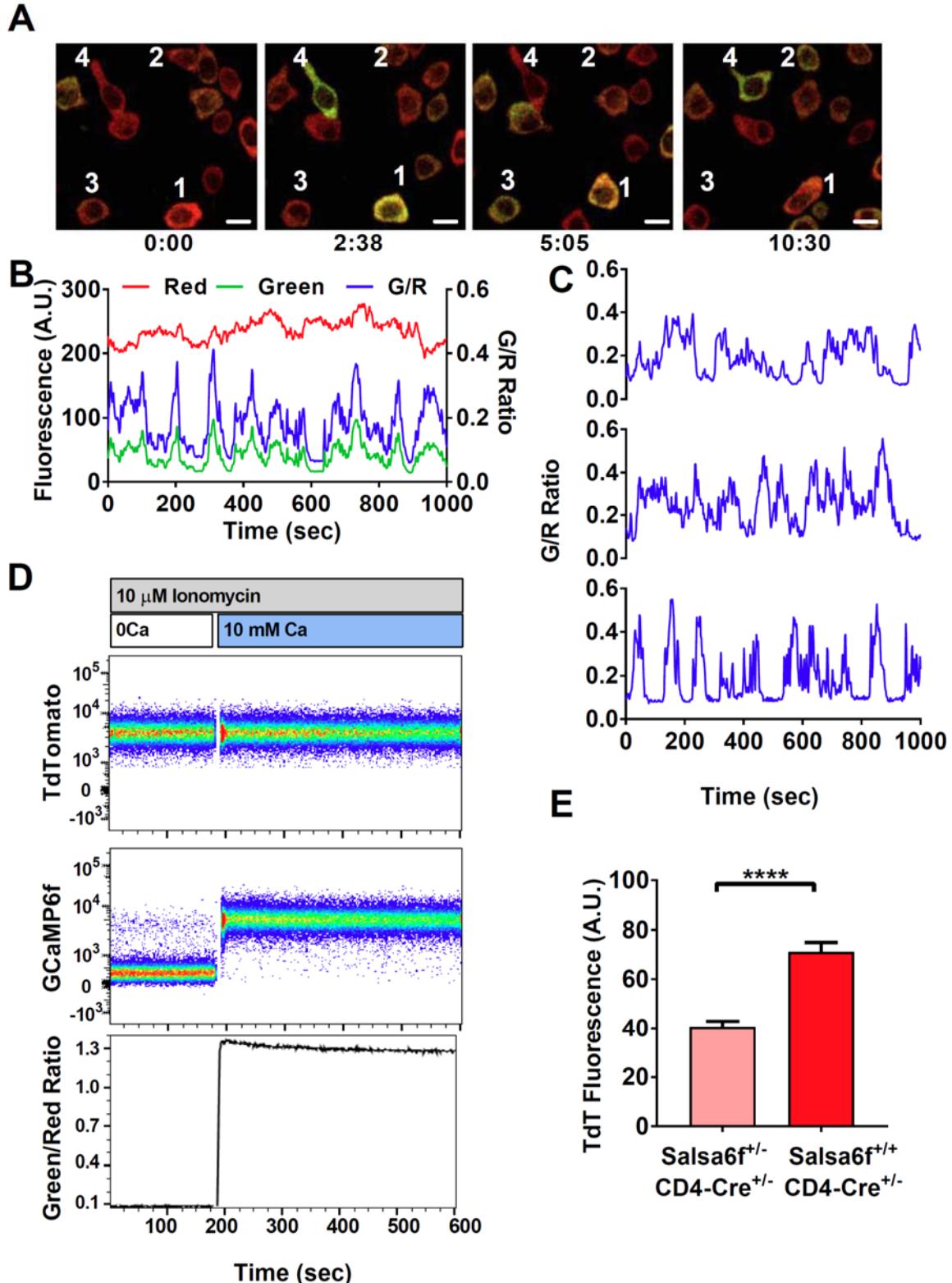
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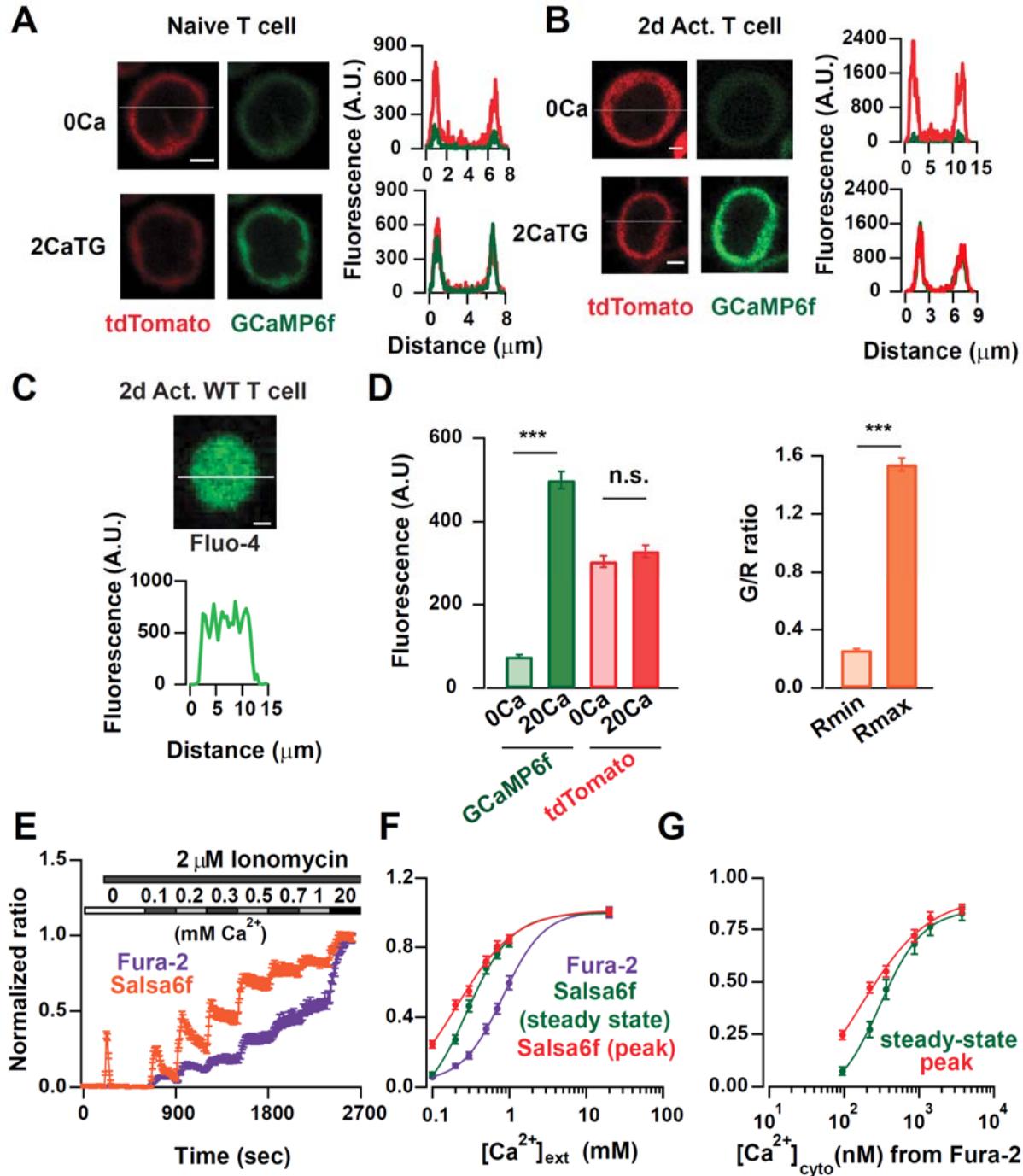
**E**



954 **Figure 5**



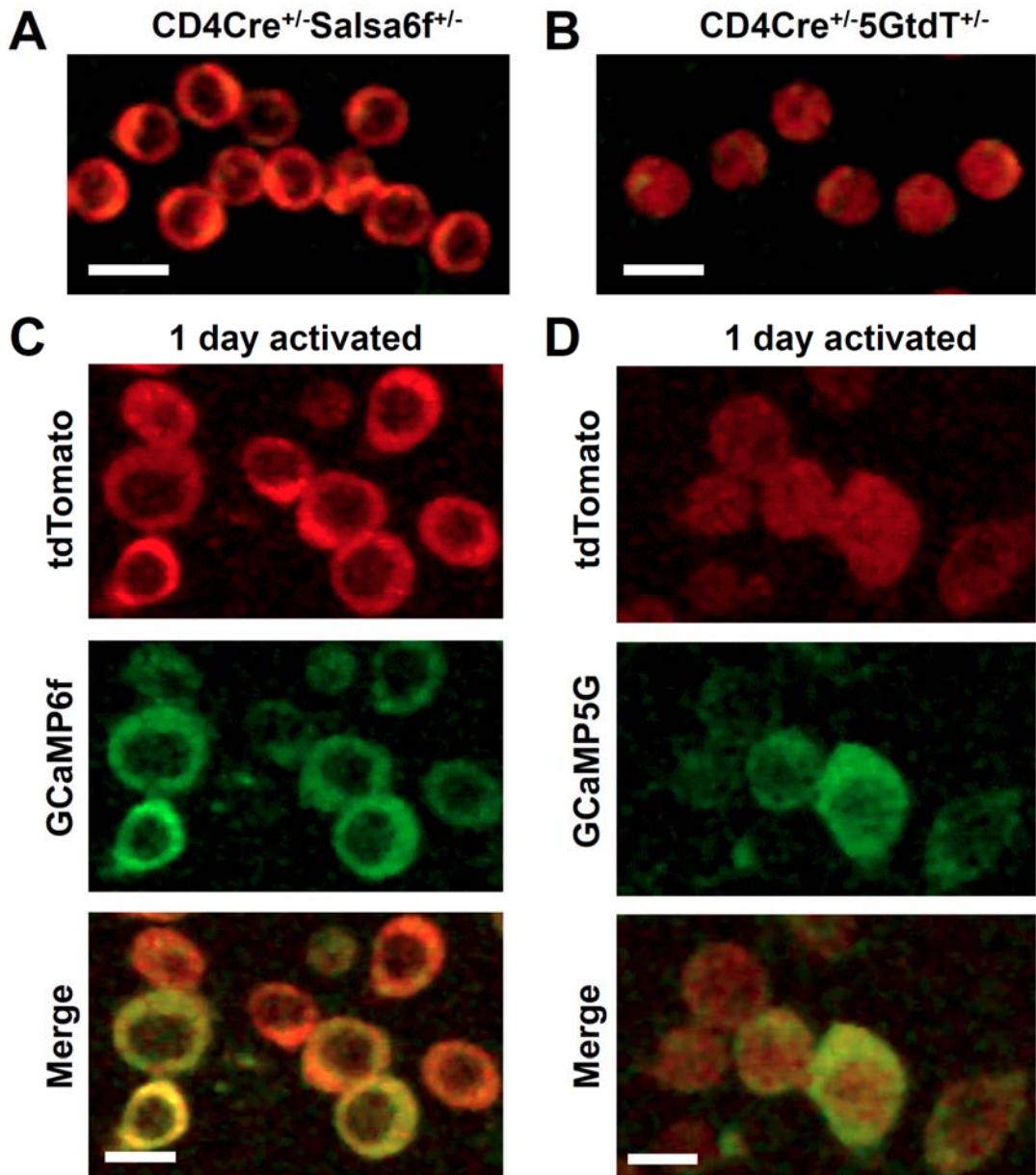
957 **Figure 6**



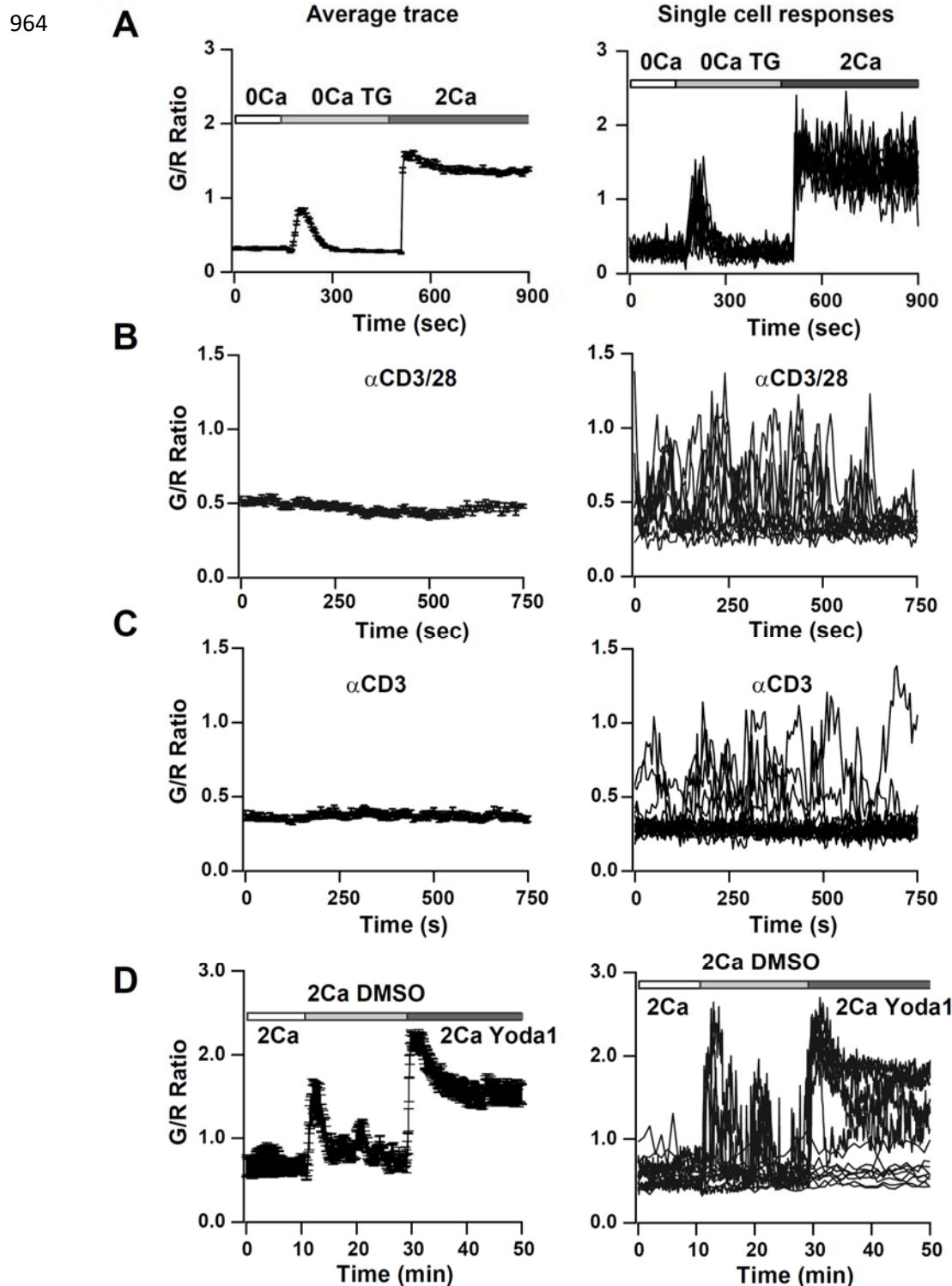
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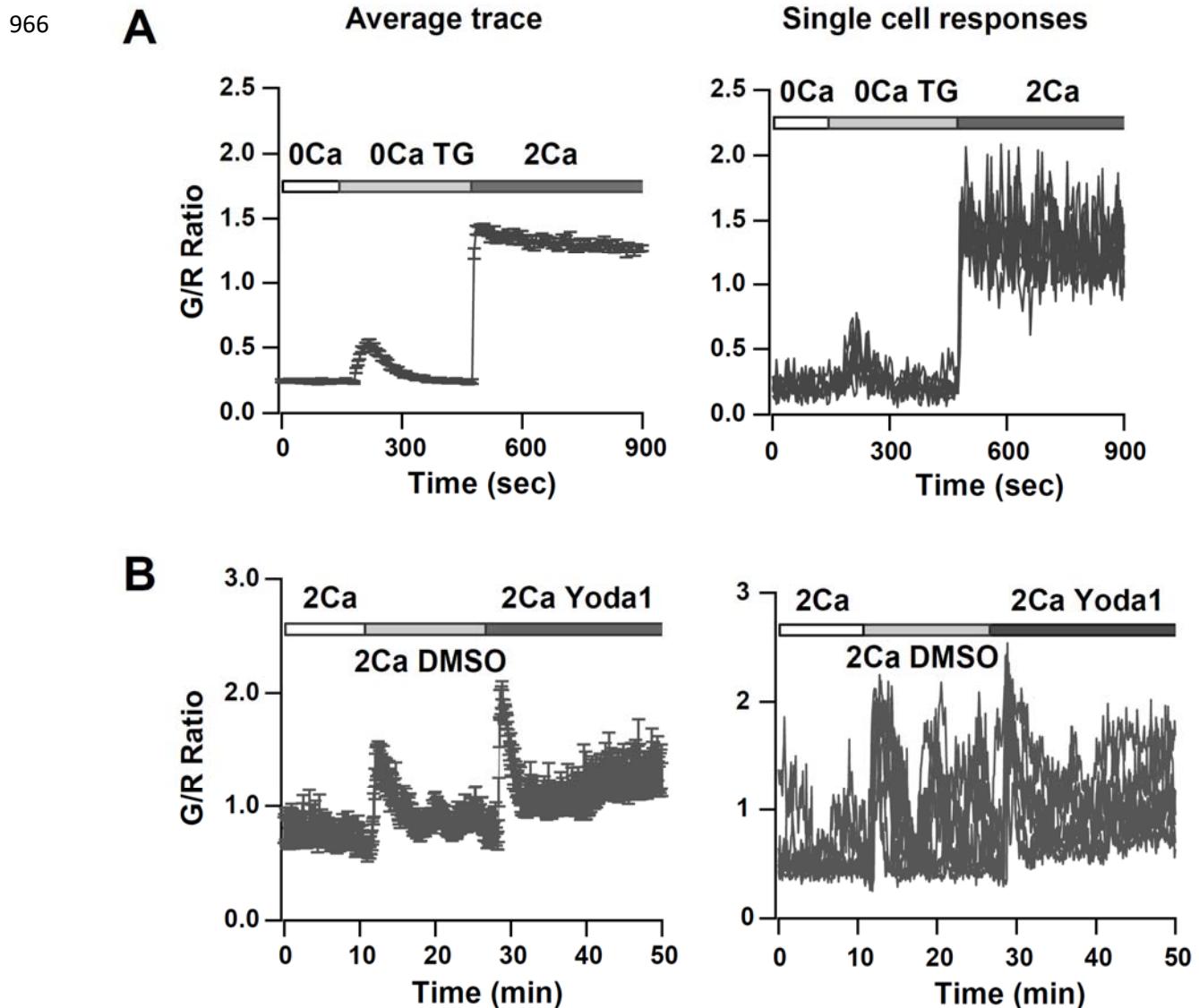
960 **Figure 6 Supplement 1**



963 **Figure 7**

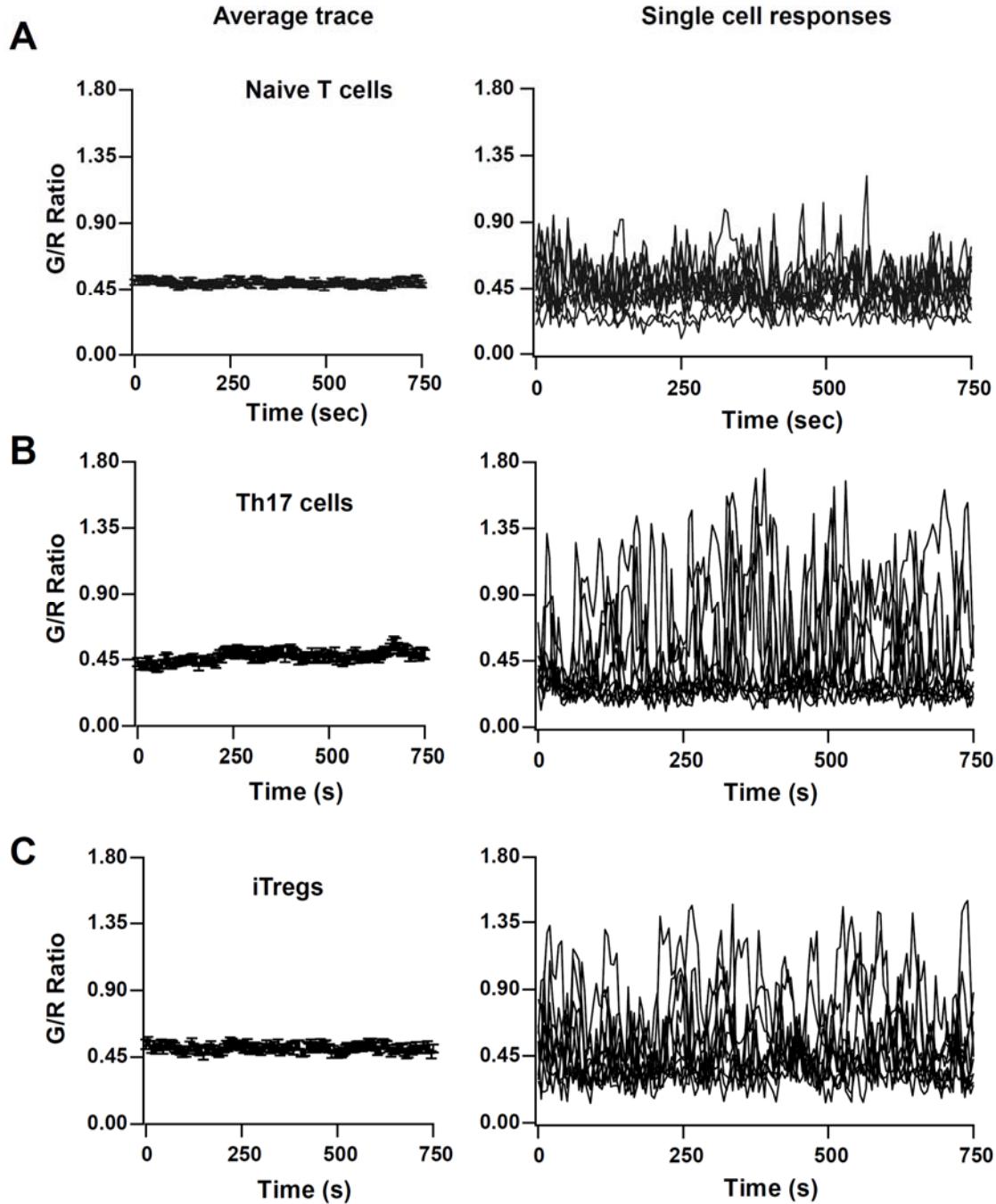


965 **Figure 7 Supplement 1**

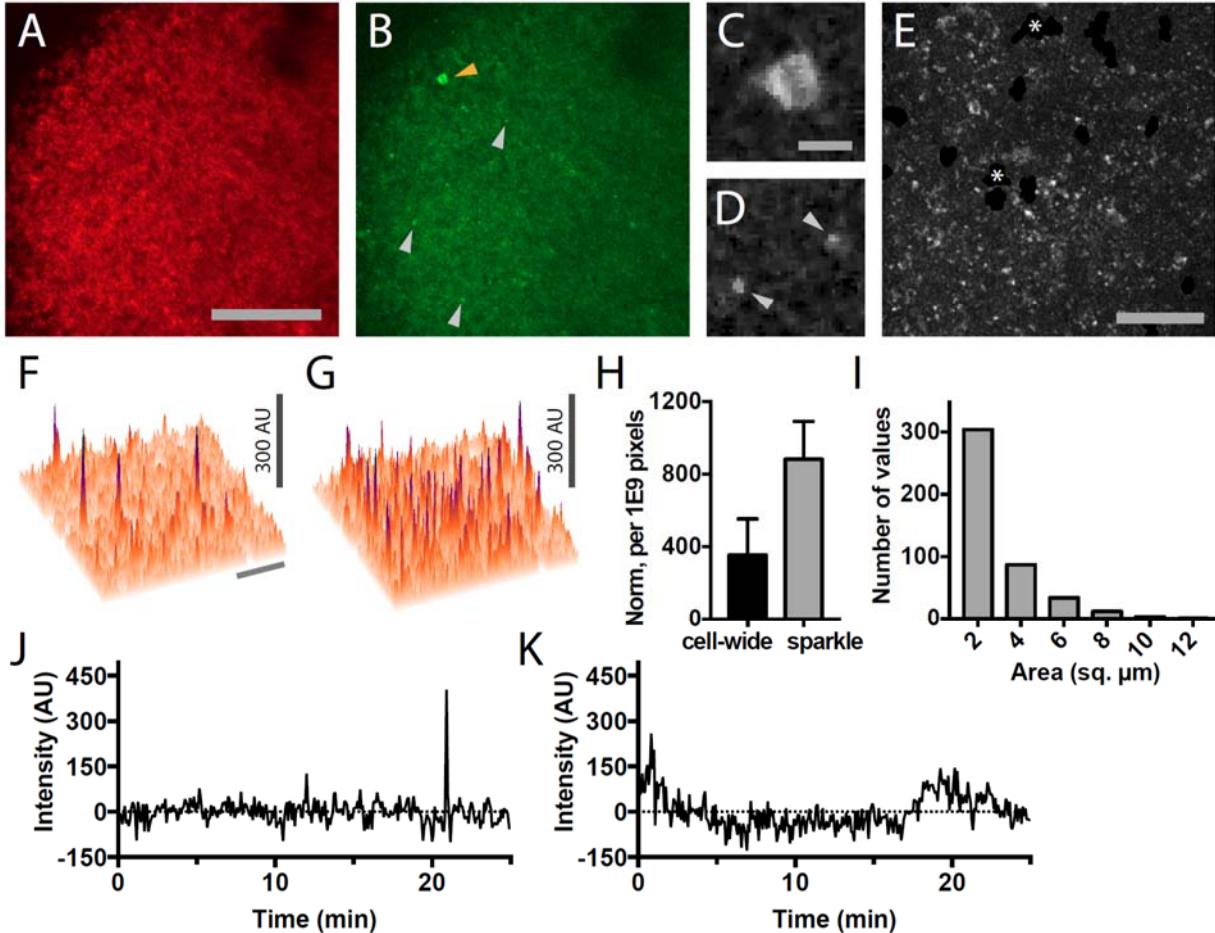


967 **Figure 8**

968



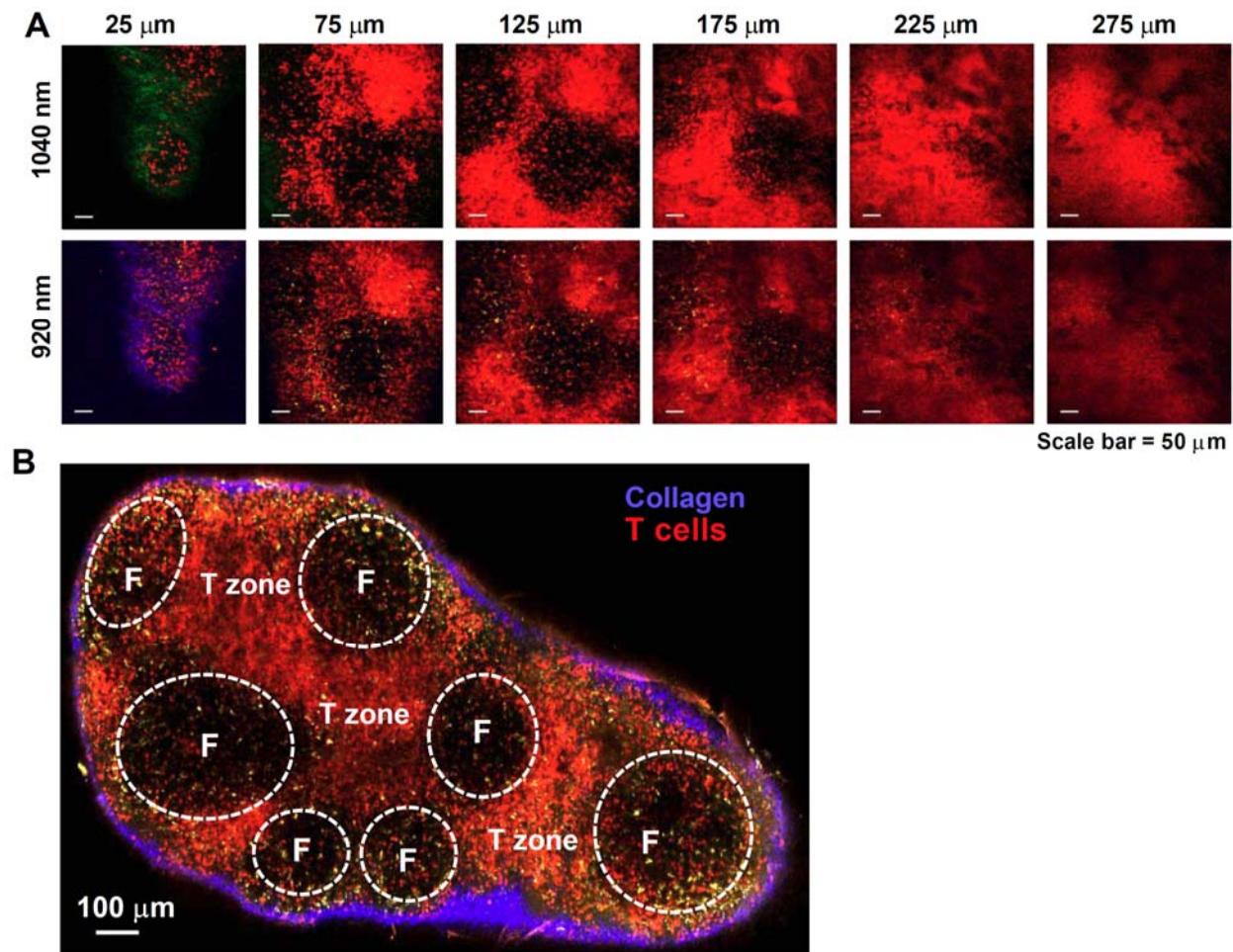
969 **Figure 9**



970

971

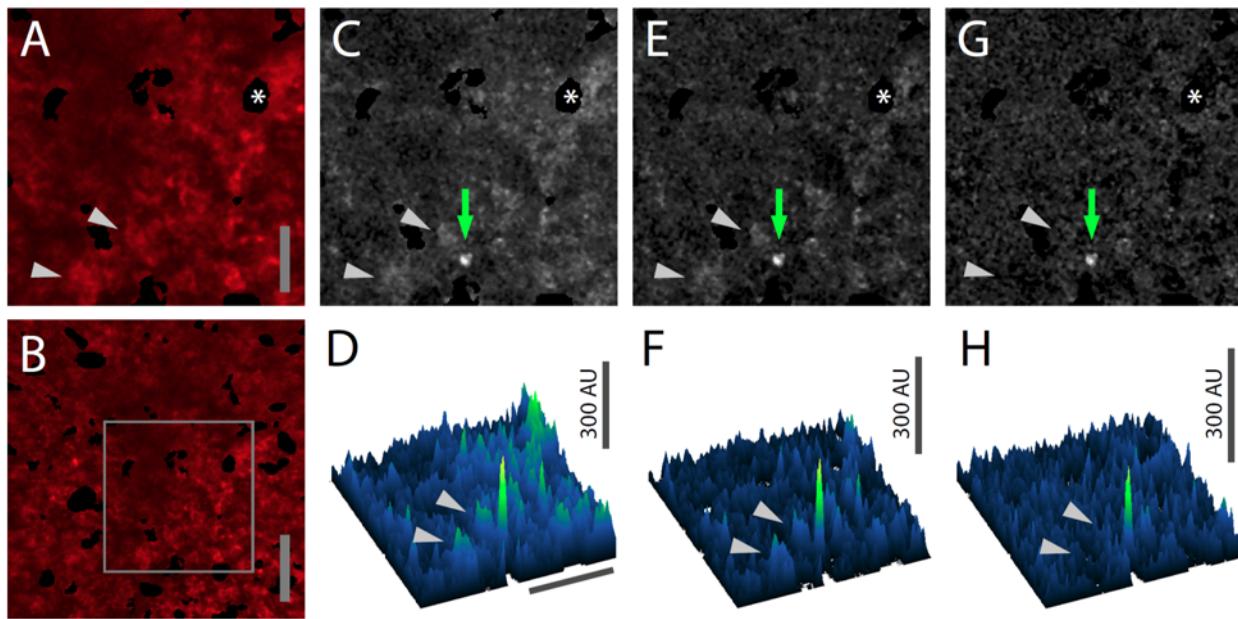
972 **Figure 9 Supplement 1**



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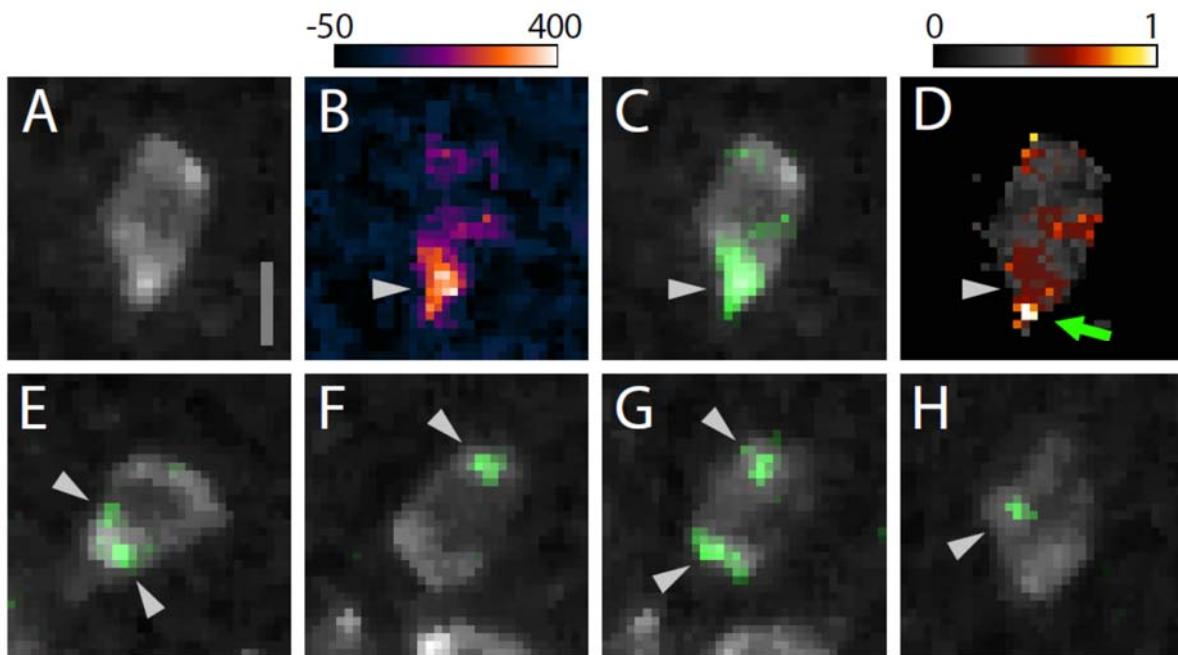
975 **Figure 9 Supplement 2**



976

977

978 **Figure 10**

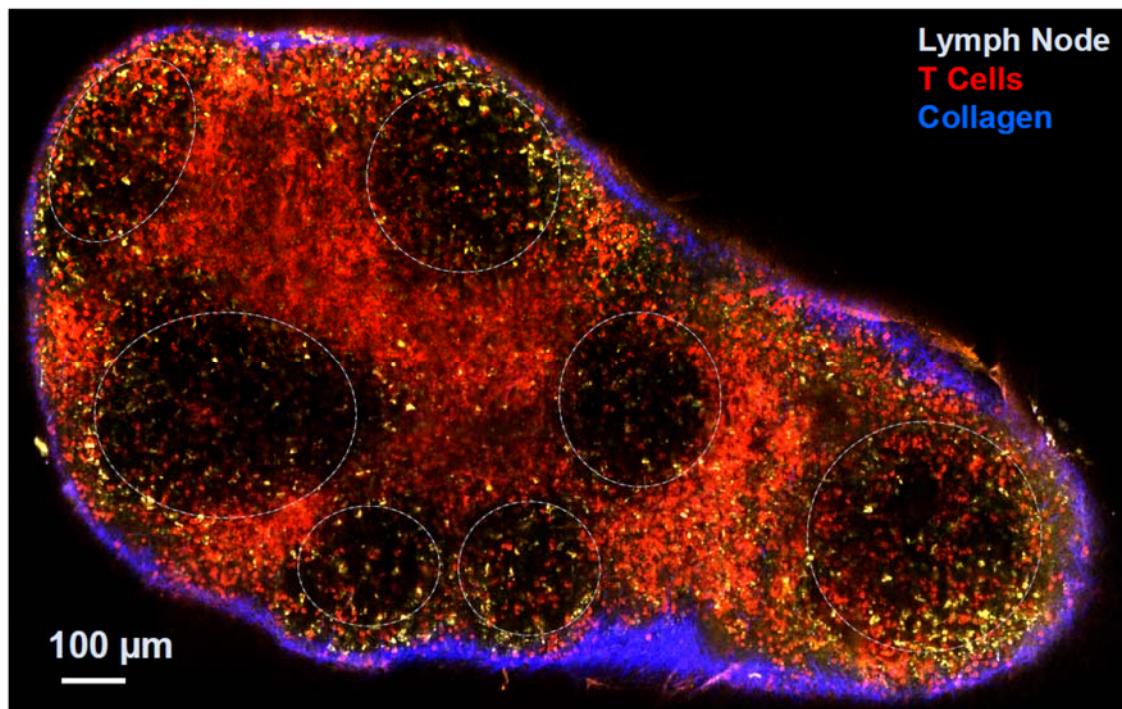


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980

## 981 Proposed Cover Illustration

982 Montage image of a CD4-Salsa6f lymph node at 100  $\mu$ m depth, imaged using 920 nm  
983 excitation showing Salsa6f<sup>+</sup> cells in red, autofluorescent structures in yellow, and the  
984 capsular boundary shown in blue (second-harmonic signal); scale bar = 100  $\mu$ m. CD4-  
985 Salsa6f shows abundant labeling in the T zone and interfollicular region, and scant  
986 labeling in B-cell follicles.



987

988

989 **Video Legends**

990 **Video 1. Calcium readout of Salsa6f probe in HEK cells.** HEK 293A cells transfected  
991 with Salsa6f, first washed with 0 mM Ca<sup>2+</sup> followed by 2  $\mu$ M ionomycin in 2 mM Ca<sup>2+</sup>;  
992 scale bar = 20  $\mu$ m, time shown in hr:min:sec. Images were acquired at 15 second  
993 interval and played back at 15 frames per second. This video corresponds to **Figure**  
994 **1D.**

995 **Video 2. Single-cell readout of activation in transgenic T cells by Salsa6f.** CD4 T  
996 cells from CD4-Salsa6f<sup>+/−</sup> mice were plated on activating surface coated with anti-  
997 CD3/CD28. Images were acquired at 5 second interval and played back at 15 frames  
998 per second. This video corresponds to **Figure 5A.**

999 **Video 3. T cell Ca<sup>2+</sup> response to Ca<sup>2+</sup> store depletion by thapsigargin (TG).** Video of  
1000 maximum intensity projection images of 2 day activated T cells from CD4-Salsa6f<sup>+/−</sup>  
1001 mouse plated on poly-L-lysine. Scale bar = 20  $\mu$ m, time shown in hr:min:sec. 2  $\mu$ M TG  
1002 in Ca<sup>2+</sup> free Ringer's was added at 00:02:30 and 2 mM Ca<sup>2+</sup> was added at 00:08:15.  
1003 Time interval between frames is 5 sec. Play back speed = 50 frames per second. This  
1004 video corresponds to **Figure 7A.**

1005

1006 **Video 4. Activated T cell Ca<sup>2+</sup> responses to TCR stimulation.** Video of maximum  
1007 intensity projection images of 2 day activated T cells from CD4-Salsa6f<sup>+/−</sup> mouse plated  
1008 on anti-CD3/28 coated coverslip. Scale bar = 20  $\mu$ m, time shown in hr:min:sec. Time  
1009 interval between frames is 5sec. Play back speed = 15 frames per second. Video  
1010 corresponds to **Figure 7B.**

1011

1012 **Video 5. T cell Ca<sup>2+</sup> response to shear and Yoda1.** Video of maximum intensity  
1013 projection images of 2 day activated T cells from CD4-Salsa6f<sup>+/−</sup> mouse plated on anti-  
1014 CD3/28 coated coverslip. Scale bar = 20  $\mu$ m, time shown in hr:min:sec. Time interval  
1015 between frames is 5sec. Play back speed = 200 frames per second. Medium was added  
1016 at 00:15:00 and Yoda1 was added at 00:35:00. Video corresponds to **Figure 7C.**

1017

1018 **Video 6. Lymph nodes from CD4-Salsa6f<sup>+/+</sup> mice exhibit cell-wide and subcellular**  
1019 **Ca<sup>2+</sup> signals.** Time shown in hr:min:sec; images were acquired at 5 second intervals. Play  
1020 back speed = 50 frames per second. Red channel is turned off after beginning to  
1021 facilitate visualization of green signals. Video corresponds to **Figure 9B.**