N-WASP-dependent branched actin polymerization attenuates B-cell receptor signaling by increasing the molecular density of receptor clusters

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Abstract

Antigen-induced B-cell receptor (BCR) signaling is critical for initiating and regulating B-cell activation. The actin cytoskeleton plays essential roles in BCR signaling. Upon encountering cell-surface antigens, actin-driven B-cell spreading amplifies signaling, while B-cell contraction following spreading leads to signal attenuation. However, the mechanism by which actin dynamics switch BCR signaling from amplification to attenuation is unknown. Here, we show that Arp2/3-mediated branched actin polymerization is required for B-cell contraction. Contracting B-cells generate centripetally moving actin foci from lamellipodial F-actin networks in the B-cell plasma membrane region contacting antigen-presenting surfaces. Actin polymerization driven by N-WASP, but not WASP, generates these actin foci. N-WASP-dependent actin foci facilitate non-muscle myosin II recruitment to the contact zone to create actomyosin ring-like structures. Furthermore, B-cell contraction increases BCR molecular density in individual clusters, leading to decreased BCR phosphorylation. Increased BCR molecular density reduced levels of the stimulatory kinase Syk, the inhibitory phosphatase SHIP-1, and their phosphorylated forms in individual BCR clusters. These results suggest that N-WASP-activated Arp2/3 generates centripetally moving foci and contractile actomyosin ring-like structures from lamellipodial networks, enabling contraction. B-cell contraction attenuates BCR signaling by pushing out both stimulatory kinases and inhibitory phosphatases from BCR clusters, providing novel insights into actin-facilitated signal attenuation.

Introduction

B-cell-mediated antibody responses are essential for eliminating invading pathogens. B-cell receptors (BCRs) expressed on the B-cell surface detect the presence of cognate antigens. The binding of antigen to the BCR leads to the activation of signaling cascades (Reth and Wienands 1997; Dal Porto *et al.* 2004; Kwak *et al.* 2019), which induce transcriptional programs that prepare B-cells for proliferation and differentiation (Kurosaki *et al.* 2010; Shlomchik *et al.* 2019; Wang *et al.* 2020). BCR signaling also induces rapid antigen internalization, processing, and presentation for T-cell recognition, which provides the second signal required for B-cell clonal expansion and differentiation to high-affinity antibody-secreting cells and memory B-cells (Song *et al.* 1995; Gitlin *et al.* 2014). BCR signaling is tightly regulated by various external and internal factors to activate antibody responses that are specific and also qualitatively and quantitatively matched to the encountered antigen.

Antigen-induced receptor reorganization activates BCRs at the B-cell surface. Antigen binding leads to BCR clustering at lipid rafts, which enables raft-resident kinases, such as the Src kinases Lyn and Fyn, to phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains of the BCR signaling subunit CD79a/b heterodimer (Reth 1994; Pierce 2002; Sohn *et al.* 2008). Doubly phosphorylated ITAMs recruit and activate spleen tyrosine kinase (Syk), which in turn activates multiple downstream signaling pathways, including phospholipase Cγ2 (PLCγ2), Bruton's tyrosine kinase (Btk), Ras-GTPase, and phosphatidylinositol-3 kinase (PI3K), initiating signaling cascades (Kurosaki 2000; Dal Porto *et al.* 2004; Tanaka and Baba 2020). Signaling activation also induces the recruitment and activation of inhibitory phosphatases, such as SH2-containing tyrosine phosphatase-1 (SHP-1) and phosphatidylinositol-5 phosphatase-1 (SHIP-1), to BCR signaling complexes (Brauweiler *et al.* 2000; Gross *et al.* 2009; Franks and Cambier 2018). SHIP-1 and SHP-1 negatively regulate

64 BCR signaling by inactivating the plasma membrane docking lipids for stimulatory kinases, such
65 as Btk and Akt (Aman *et al.* 1998; Bolland *et al.* 1998) and dephosphorylating BCR and its
66 downstream signaling molecules (Mizuno *et al.* 2000; Adachi *et al.* 2001), respectively. The
67 interplay between the stimulatory kinases and the inhibitory phosphatases controls the balance
68 of antibody responses against pathogens and self. Deficiencies in stimulatory kinases by
69 mutations, such as Btk, cause X-linked agammaglobulinemia (XLA) (Kinnon *et al.* 1993), while
70 deficiencies in the inhibitory phosphatases SHP-1 or SHIP-1 result in autoimmune diseases
71 (Pao *et al.* 2007; Leung *et al.* 2013). However, the mechanisms by which BCR signaling
72 attenuation is initiated and regulated remain elusive.
73
74 B-cells encounter both soluble and membrane-associated antigens *in vivo*. Membrane-
75 associated antigens include antigens on antigen-presenting cells, like follicular dendritic cells in
76 B-cell follicles or germinal centers, and pathogenic cells, like bacteria, parasites, and cancer
77 cells (Batista and Harwood 2009; Depoil *et al.* 2009; Gonzalez *et al.* 2009; Cyster 2010). The
78 binding of multi-valent soluble antigen and membrane-associated antigen with any valency
79 induces dynamic reorganization of surface BCRs into microclusters, triggering BCR signaling.
80 Subsequently, surface BCRs continue moving to the plasma membrane region contacting
81 antigen-presenting surfaces or to one B-cell pole in the case of soluble antigen, which leads to
82 the growth and merger of BCR microclusters and the formation of immunological synapses
83 (Carrasco *et al.* 2004; Harwood and Batista 2010) or supra-molecular activation complexes
84 (Unanue and Karnovsky 1973; Schreiner and Unanue 1977; Tolar *et al.* 2009b; Harwood and
85 Batista 2010).
86
87 Multiple mechanisms have been proposed for signaling initiation by antigen-induced surface
88 BCR reorganization. The binding of surface BCRs to membrane-associated antigen has been
89 shown to induce a conformational change in the BCR extracellular domain, exposing a proximal

membrane region of membrane IgM that promotes receptor clustering (Tolar *et al.* 2009a; Shen *et al.* 2019). Antigen-binding also changes the conformation of the BCR cytoplasmic domains from a closed to an open form, facilitating the recruitment of signaling molecules (Tolar *et al.* 2005). Alternatively, antigen-BCR interaction can increase the molecular spacing of BCRs in clusters, facilitating BCR interaction with signaling molecules (Yang and Reth 2010; Kläsener *et al.* 2014). The inhibitory phosphatases SHP-1 and SHIP-1 are also recruited to BCR clusters (Adachi *et al.* 2001; Seeley-Fallen *et al.* 2014). However, the mechanisms underlying phosphatase recruitment and its relationship with BCR signaling activation complexes are unknown.

The actin cytoskeleton is essential for antigen-induced BCR reorganization on the B-cell surface in response to both soluble and membrane-associated antigen (Harwood and Batista 2011; Liu *et al.* 2013b; Song *et al.* 2013; Hoogeboom and Tolar 2016). Early signaling of the BCR triggers transient actin depolymerization via Rap GTPase and its downstream target cofilin (Freeman *et al.* 2011), which disassembles the existing cortical actin network that confines the lateral movement of surface BCRs (Treanor *et al.* 2010). Following this transient depolymerization, BCR signaling activates rapid actin polymerization through the actin nucleation promoting factors Wiskott-Aldrich Syndrome Protein (WASP) and neuronal-WASP (N-WASP), modulating BCR mobility and leading to F-actin accumulation at BCR-antigen interaction sites (Liu *et al.* 2013a; Rey-Suarez *et al.* 2020). Actin polymerization and treadmilling in B-cells stimulated by soluble antigen drive surface BCRs to cluster and move to one pole of the B-cell, forming a BCR cap (Schreiner and Unanue 1977; Liu *et al.* 2012a). Upon interacting with membrane-associated antigen, B-cells organize actin into two dynamic structures. One treadmills outwards, driving B-cell membrane spreading and expanding the B-cell membrane region contacting the antigen-presenting surface (contact zone), which enables more BCRs to engage antigen (Bolger-Munro *et al.* 2019). The other creates retrograde flow towards the center of the B-cell contact zone,

116 driving the centripetal movement and growth of BCR microclusters (Liu *et al.* 2012b). Together,
117 these processes amplify BCR signaling (Batista *et al.* 2010; Harwood and Batista 2011). The
118 extent of B-cell spreading and the amount of BCR-antigen complexes gathered in the contact
119 zone depend on BCR binding affinity and the density of antigen on the membrane (Fleire *et al.*
120 2006). Subsequent to spreading, B-cells contract, reducing the contact area, which drives BCR
121 microclusters to merge into central clusters to form immunological synapses (Fleire *et al.* 2006;
122 Liu *et al.* 2013a).
123
124 The actin cytoskeleton closely interplays with BCR signaling. We have previously shown that
125 mouse primary B-cells with Btk deficiency (Liu *et al.* 2011) or double knockouts of the actin
126 nucleation promoting factors WASP and N-WASP (Liu *et al.* 2013a) fail to spread on antigen-
127 presenting surfaces and establish stable interactions with membrane-associated antigen,
128 drastically reducing BCR signaling. Btk can activate WASP and N-WASP through Vav, the
129 guanine nucleotide exchange factor of Cdc42 and Rac and phosphatidylinositol-5 kinase that
130 generates phosphatidylinositol-4,5-biphosphate (PI4,5P_2) (Sharma *et al.* 2009; Padrick and
131 Rosen 2010). Surprisingly, B-cell-specific knockout of N-WASP (cNKO) but not WASP germline
132 knockout (WKO) enhances B-cell spreading, delays B-cell contraction, and inhibits the
133 centralization of BCR clusters at the contact zone (Liu *et al.* 2013a). Consistent with these
134 findings, Bolger-Munro et al. have shown that knockdown of the actin nucleation factor Arp2/3
135 downstream of WASP and N-WASP disrupts the dynamic actin reorganization induced by
136 membrane-associated antigen required for BCR microcluster growth and merger (Bolger-Munro
137 *et al.* 2019). Using B-cell-specific and germline knockouts, we showed that in addition to N-
138 WASP, the actin motor non-muscle myosin IIA (NMIIA) (Seeley-Fallen *et al.* 2022) and the
139 actin-binding adaptor protein Abp1 (Seeley-Fallen *et al.* 2014) are required for B-cell contraction
140 and BCR central cluster formation. Finally, Wang et al. recently showed that in the presence of
141 the adhesion molecule ICAM on the antigen-presenting surface, B-cells form contractile

142 actomyosin arcs, driving centripetal movement of BCR clusters in the B-cell contact zone (Wang
143 *et al.* 2022). Together, these findings indicate a critical role of different actin networks in B-cell
144 contraction and BCR signaling.
145
146 Our previously studies demonstrate that B-cell contraction is critical for BCR signaling
147 attenuation. Delay and inhibition of B-cell contraction through deficiencies of the actin regulators
148 N-WASP, Abp1, or NMIIA, prolongs and/or increases BCR signaling by enhancing the activation
149 of stimulatory kinases and suppressing the activation of inhibitory phosphatases, which elevates
150 the production of autoantibody levels in mice (Liu *et al.* 2013a; Seeley-Fallen *et al.* 2014;
151 Seeley-Fallen *et al.* 2022). N-WASP and NMIIA are not known to interact with kinases and
152 phosphatases directly. The growth, merger, and centralization of antigen-BCR complexes at the
153 B-cell contact zone during the B-cell contraction phase are associated with the reduced BCR
154 signaling capability (Liu *et al.* 2013a). These findings suggest that B-cell contraction is one of
155 the mechanisms for inducing BCR signaling attenuation. However, how the actin cytoskeleton
156 facilitates the transition of the spreading to contraction phase and how B-cell contraction
157 switches BCR signaling from amplification to attenuation is unknown.
158
159 This study explores the mechanisms by which the actin cytoskeleton remodels from spreading
160 lamellipodia to contractile structures and by which B-cell contraction suppresses BCR signaling.
161 Our results show that the contractile actomyosin structures responsible for B-cell contraction
162 originate from branched actin at spreading lamellipodia. N-WASP/Arp2/3- mediated actin
163 polymerization prolongs the lifetime of the lamellipodial actin structures, enables NMIIA
164 recruitment, and drives their movement to the center of the B-cell contact zone. B-cell
165 contraction increases the molecular density within individual BCR-antigen clusters, which
166 promotes the disassociation of both the stimulatory kinase Syk and the inhibitory phosphatases

167 SHIP-1 from BCR clusters, leading to signal attenuation. Our study reveals a new mechanism
168 underlying BCR signal downregulation.
169
170 **Results**
171 **Arp2/3, activated by N-WASP but not WASP, is required for B-cell contraction**
172 Arp2/3-generated branched F-actin is known to drive lamellipodial expansion for B-cell
173 spreading (Bolger-Munro *et al.* 2019). However, whether branched F-actin is involved in the
174 subsequent contraction phase is unknown. To address this, we perturbed the polymerization of
175 branched F-actin using the Arp2/3 inhibitor CK-666 (50 μM) while using its inactive derivative
176 CK-689 as a control. Pre-warmed splenic B-cells from WT C57BL/6 mice were incubated with
177 monobiotinylated Fab' fragment of anti-mouse IgM+G attached to planar lipid bilayers (Fab'-
178 PLB) by biotin-streptavidin interaction and imaged live at 37°C using interference reflection
179 microscopy (IRM). The contact area of B-cells treated with CK-689 rapidly increased upon
180 contacting Fab'-PLB and reached a maximum at ≥0.5 min after the initial contact (*Figure 1A,*
181 *Figure 1-figure supplement 1, and Figure 1-Video 1A*). Following maximal spreading, most B-
182 cells reduced the area of their contact zone, indicating contraction (*Figure 1A, Figure 1-figure*
183 *supplement 1, and Figure 1-Video 1A*). We classified a B-cell as contracting if its contact zone
184 area reduced by ≥5% for at least 10 sec after reaching a maximum value. Based on the average
185 timing for B-cell maximal spreading, we treated B-cells with CK-666 at the beginning of the
186 incubation (Time 0), before B-cell spreading initiation, or at 2 min, when all B-cells had already
187 spread. The effectiveness of CK-666 was detected by reduced Arp2/3 staining in the contact
188 zone (*Figure 1-figure supplement 2*). As expected, CK-666 treatment at 0 but not 2 min reduced
189 the kinetics of B-cell spreading (*Figure 1-figure supplement* 3). Importantly, CK-666 treatment at
190 0 and 2 min both significantly reduced the percentage of B-cells undergoing contraction (*Figure*
191 *1A-C and Figure 1-Video 1A*). The inhibitory effect of CK-666 on B-cell contraction, particularly

192 CK-666 treatment after B-cell spreading, suggests that Arp2/3-mediated branched actin
193 polymerization is required for B-cell contraction.
194
195 WASP and N-WASP are actin nucleation-promoting factors upstream of Arp2/3 that are
196 expressed in B-cells (Padrick and Rosen 2010). To determine if either or both were responsible
197 for activating Arp2/3 for B-cell contraction, we utilized the N-WASP inhibitor wiskostatin (Wisko),
198 and splenic B-cells from B-cell-specific N-WASP knockout mice (cNKO) and germline WASP
199 knockout (WKO) mice (Westerberg *et al.* 2012; Liu *et al.* 2013a). Wisko has been shown to
200 inhibit N-WASP activation while enhancing WASP activation in B-cells (*Figure 1-figure*
201 *supplement 4*) (Liu *et al.* 2013a). We found that both Wisko (10 μM) (*Figure 1D and E and*
202 *Figure 1-Video 1B*) and cNKO (*Figure 1F and G and Figure 1-Video 1C*), but not WKO (*Figure*
203 *1H and I and Figure 1-Video 1D*), significantly reduced the percentage of B-cells undergoing
204 contraction, compared to the vehicle, flox, or WT controls. These results suggest that N-WASP-
205 but not WASP-activated Arp2/3 mediates branched actin polymerization for B-cell contraction.
206
207 **Arp2/3, downstream of N-WASP, generates inner F-actin foci, driving B-cell contraction**
208 To understand how Arp2/3 drives B-cells to transition from spreading to contraction, we
209 identified F-actin structures associated with contracting B-cells that were sensitive to CK-666
210 treatment. We visualized F-actin by phalloidin staining and compared F-actin organization in the
211 contact zone of B-cells at the spreading (2 min) and contraction (4 min) phases using TIRF.
212 While B-cells in both spreading and contraction phases exhibited phalloidin staining outlining the
213 contact zone (*Fig. 2A, green arrows*), only B-cells in the contracting phase showed interior
214 phalloidin patches brighter than the phalloidin staining at the periphery of the contact zone
215 (*Figure 2A, purple arrows*). These F-actin patches were organized into a ring-like structure and
216 resided ~1 μm behind the spreading front, surrounding an F-actin-poor center (*Figure 2A, purple*
217 *arrows*). Here, we refer these F-actin patches as inner F-actin foci. We identified inner F-actin

218 foci based on whether their peak fluorescence intensity (FI) was ≥2 fold of the mean
219 fluorescence intensity (MFI) of phalloidin in the no-foci area, had diameters of ≥250 nm, and
220 were located 1 μm away from the edge of the contact zone. We found that such inner F-actin
221 foci were detected in >60% of B-cells in the contracting phase (4 min) but only in <20% of B-
222 cells in the spreading phase (2 min) (*Figure 2B*). CK-666 treatment at 0 min, which inhibited B-
223 cell contraction, significantly reduced the percentage of B-cells showing inner F-actin foci at 4
224 min but not at 2 min (*Figure 2B*). CK-666 treatment did not affect the phalloidin staining outlining
225 the contact zone (*Figure 2A*). Similarly, the percentage of cNKO B-cells showing inner F-actin
226 foci was drastically reduced at 4 min but not at 2 min (*Figure 2C*). These results suggest that the
227 formation of these inner F-actin foci is associated with B-cell contraction.
228
229 We further quantified the number of inner F-actin foci in individual B-cells from mice expressing
230 the LifeAct-GFP transgene (Riedl *et al.* 2010) (*Figure 2D, E, and G*), which allowed us to
231 monitor F-actin reorganization using live-cell imaging (*Figure 2-Video 1*), or using phalloidin
232 staining in flox control and cNKO B-cells (*Figure 2F*). Consistent with the results with phalloidin
233 staining, CK-666 treatment at time 0 significantly reduced the number of inner F-actin foci in the
234 contact zone (*Figure 2D and Figure 1-Video 1A and B*). When we followed the same B-cells
235 before and after CK-666 treatment at 2 min, many of the inner F-actin foci formed before the
236 treatment (1 min 50 sec) disappeared after the treatment (2 min 20 sec), significantly reducing
237 the number of inner F-actin foci (*Figure 2E*). Similar to the CK-666 treatment, cNKO cells
238 significantly reduced the number of inner F-actin foci in the contact zone, compared to flox
239 controls (*Figure 2F*). In contrast, WKO, which does not affect B-cell contraction, did not
240 significantly change the number of inner F-actin foci, compared to WT control B-cells (*Figure 2G*
241 *and Figure 2-Video 1C and D*). Thus, N-WASP- but not WASP-activated Arp2/3 drives the
242 formation and the maintenance of contraction-associated inner F-actin foci.
243

244 **Inner F-actin foci are derived from lamellipodial actin networks supporting the spreading**
245 **membrane**
246 We next examined the formation of contraction-associated inner F-actin foci utilizing live-cell
247 TIRF imaging of B-cells from mice expressing LifeAct-GFP. We generated kymographs along
248 eight lines from the center of each contact zone using time-lapse images of LifeAct-GFP (*Figure*
249 *3A and B and Figure 3-figure supplement 1*). Analysis of these kymographs showed that most
250 F-actin foci were first detected closely behind lamellipodial F-actin networks. Following maximal
251 spreading, these F-actin foci moved centripetally while increasing in intensity, becoming inner F-
252 actin foci, in cells transitioning from spreading to contraction (*Figure 3B, top panel, and Figure*
253 *3-figure supplement 1*). However, such centripetally moving F-actin foci were not detected in B-
254 cells that did not undergo contraction (*Figure 3B, bottom panel*). We quantified the percentage
255 of the eight kymographs from each cell that exhibited lamellipodia-derived inner F-actin foci and
256 found, on average, that six out of eight kymographs from contracting cells showed lamellipodia-
257 derived inner F-actin foci, compared to only one or two kymographs from non-contracting cells
258 (*Figure 3C*). To examine the temporal relationship between the generation of lamellipodia-
259 derived inner F-actin foci and contraction, we plotted the percentage of kymographs with
260 lamellipodia-derived inner F-actin foci over time with the spreading to contracting transition time
261 set as 0 (*Figure 3D*). We found that the percentage of kymographs showing lamellipodia-derived
262 inner F-actin foci peaked at almost the same time when the spreading transitioned to
263 contraction (*Figure 3D*), suggesting a close temporal relationship between the two events.
264 Furthermore, the N-WASP inhibitor Wisko, but not WKO, significantly inhibited the formation of
265 lamellipodia-derived inner F-actin foci (*Figure 3E*). Thus, inner F-actin foci originate from
266 branched actin driven lamellipodia and form simultaneously with the transition of B-cell
267 spreading to contraction.
268

269 **N-WASP-activated Arp2/3 generates inner F-actin foci by sustaining the lifetime and the**
270 **centripetal movement of lamellipodial F-actin**

271 We examined the mechanism by which N-WASP and Arp2/3 generate inner F-actin foci by
272 measuring their relative lifetime and mobility using kymographs generated from TIRF time-lapse
273 images of B-cells expressing LifeAct-GFP. Inner F-actin foci were identified as described above,
274 and their tracks were manually determined (*Figure 4A, black dashed lines*). The time window in
275 which an inner F-actin patch could be detected in a kymograph was measured as the relative
276 lifetime, as actin foci could move away from the kymograph line or the TIRF evanescent field
277 (*Figure 4A, right panels*). The distance each F-actin focus moved during its lifetime was used to
278 calculate its speed (*Figure 4A, right panels*). Compared to CK-689-treated B-cells, CK-666
279 treatment at time 0 significantly reduced the relative lifetime and the centripetal speed of inner
280 F-actin foci (*Figure 4B-D*). After B-cells were treated with CK-666 at 2 min (the average time for
281 B-cells to reach the maximal spreading), the relative lifetime and the centripetal speed of inner
282 F-actin foci were significantly lower than before the treatment in the same cell (*Figure 4E-G,*
283 *line-linked dots*). Similarly, the N-WASP inhibitor Wisko significantly reduced the relative lifetime
284 and the centripetal speed of inner F-actin foci (*Figure 4H-J*); however, WKO had no significant
285 effect (*Figure 4K-M*). These results show that N-WASP and Arp2/3 mediated branched actin
286 polymerization prolongs the lifetime of lamellipodia-derived F-actin foci and drives them to move
287 inward in the B-cell contact zone.

288

289 **Inner F-actin foci, generated by N-WASP-activated Arp2/3, facilitate NMII recruitment and**
290 **reorganization into ring-like structures**

291 As non-muscle myosin II (NMII) is required for B-cell contraction (Seeley-Fallen *et al.* 2022), we
292 examined the relationship between the formation of inner F-actin foci and the recruitment and
293 reorganization of NMII in the contact zone using TIRF imaging of B-cells from mice expressing a
294 GFP-NMIIA transgene. Upon interacting with Fab'-PLB, the GFP-NMIIA MFI in the contact zone

295 of untreated WT B-cells increased rapidly in the first minute and slowly afterward (*Figure 5A and*
296 *B and Figure 5-Video 1*). Wiskostatin treatment significantly reduced the GFP-NMIIA MFI in the
297 contact zone (*Figure 5B*) and its initial rate of increase (as determined by the slope of the GFP-
298 NMIIA MFI increase at 0~30 sec time window) (*Figure 5C*). Kymographs generated from time-
299 lapse TIRF images of B-cells from mice expressing GFP-NMIIA and LifeAct-RFP showed that
300 recruited NMIIA accumulated between lamellipodia and inner F-actin foci when the foci moved
301 centripetally away from lamellipodia (*Figure 5D, white arrow, and Figure 5-Video 1*). Recruited
302 NMIIA reorganized with inner F-actin foci to form a ring-like structure in the contact zone (*Figure*
303 *5A, D, and E, and Figure 5-Video 1*). The percentage of B-cells with NMIIA ring-like structures,
304 visualized by immunostaining, increased over time as more B-cells underwent contraction
305 (*Figure 5E and F*). Compared to flox controls, the percentage of cNKO B-cells with NMIIA ring-
306 like structures was significantly decreased (*Figure 5E and F*). Wiskostatin treatment also
307 reduced NMIIA recruitment and ring-like structure formation (*Figure 5-Video 1*). Surprisingly, the
308 percentage of WKO B-cells with NMIIA ring-like structure was higher than that of flox control B-
309 cells (*Figure 5E and F*). Thus, N-WASP and Arp2/3 mediated branched actin polymerization
310 promotes the recruitment and the reorganization of NMII ring-like structures by generating inner
311 F-actin foci in the contact zone.
312
313 **B-cell contraction increases the BCR molecular density in individual clusters**
314 To understand how B-cell contraction promotes BCR signaling attenuation, we examined the
315 impact of B-cell contraction on the properties of BCR clusters. We first measured the MFI of
316 AF546-Fab' attached to PLB gathered by B-cells into the contact zone as an indication of the
317 overall BCR molecular density. The clustering of AF546-Fab' on PLB by B-cell binding reflects
318 surface BCR clustering, as B-cell binding to transferrin (Tf)-tethered PLB does not cause
319 surface BCRs to cluster and be phosphorylated (*Figure 6-figure supplement 1*) (Liu *et al.* 2011;
320 Liu *et al.* 2013a). The MFI of AF546-Fab' in the B-cell contact zone increased over time.

321 Treatment with CK-666, Wisko, or cNKO all reduced the AF546-Fab' MFI, particularly during the
322 time window of B-cell contraction in controls (*Figure 6A-G, purple rectangles, and Figure 6-*
323 *Video 1*). Notably, the rates of increase in AF546-Fab' MFI, calculated from the slopes of
324 AF546-Fab' MFI versus time plots in individual cells, were significantly higher during B-cell
325 contraction than before B-cell contraction in control cells and conditions (*Figure 6D-K*).
326 Significantly, inhibiting B-cell contraction by CK-666 treatment at 0 (*Figure 6A, D, and H, and*
327 *Figure 6-Video 1A and D*) or 2 min (*Figure 6A, E, and I*), Wisko (*Figure 6B, F, and J, and Figure*
328 *6-Video 1B and E*), and cNKO (*Figure 6C, G, and K, and Figure 6-Video 1C and F*) abolished
329 the increases in AF546-Fab' accumulation rates. We further examined the peak FI of AF546-
330 Fab' in individual microclusters as a measure of the BCR molecular density in individual
331 clusters. AF546-Fab' clusters were identified based on their diameters ≥250 nm, peak FI ≥1.1
332 fold outside the B-cell contact zone, and trackable for ≥20 sec (*Figure 6L and Figure 6-figure*
333 *supplement 2*). AF546-Fab' microclusters could not be identified during the early stage of B-cell
334 spreading. Time-lapse imaging by TIRF enabled us to measure the rate of increase in AF546-
335 Fab' peak FI in individual clusters (*Figure 6M and Figure 6-figure supplement 2*). Consistent
336 with our observation of AF546-Fab' MFI increase in the contact zone, the peak FI of individual
337 clusters increased at a faster rate during contraction than after contraction (when the contact
338 area no longer decreased) (*Figure 6N*). Furthermore, CK-666 (*Figure 6O*), Wisko (*Figure 6P*),
339 and cNKO (*Figure 6Q*) all significantly reduced the rate of increase in AF546-Fab' peak FI in
340 individual clusters. These results show that B-cell contraction significantly increases the
341 molecular density of BCRs in BCR clusters.
342

343 **Increased BCR molecular density by B-cell contraction reduces BCR phosphorylation**
344 **levels in individual microclusters**

345 To examine how BCR molecular density influenced BCR signaling capability, we
346 immunostained B-cells interacting with AF546-Fab'-PLB for 1, 3, 5, and 7 min, for

347 phosphorylated CD79a (pCD79a, Y182) and performed IRM and TIRF imaging (*Figure 7A and*
348 *H*). We analyzed equal numbers of AF546-Fab' clusters selected randomly, in the contact zone
349 of B-cells interacting with Fab'-PLB for 1, 3, 5, and 7 min, using a gradient threshold of their
350 AF546-Fab' MFI, 1.1 to 4.1 fold of the background, and with diameter ≥250 nm (*Figure 6-figure*
351 *supplement 2*). We determined the MFI of pCD79a and AF546-Fab' in individual clusters and
352 plotted the MFI ratio of pCD79a relative to AF546-Fab' (reflecting the relative level of BCR
353 phosphorylation) versus the AF546-Fab' peak FI (reflecting BCR molecular density) in individual
354 clusters (*Figure 7B and C*). The dot plots show that when the AF546-Fab' peak FI of individual
355 clusters was relatively low, the MFI ratios of pCD79a to AF546-Fab' increased with the AF546-
356 Fab' peak FI in individual clusters of flox control B-cells (*Figure 7B*). When the AF546-Fab' peak
357 FI reached a certain level, the MFI ratios of pCD79a to AF546-Fab' decreased as the AF546-
358 Fab' peak FI in individual clusters further increased (*Figure 7A and B*). Inhibition of B-cell
359 contraction by cNKO reduced the AF546-Fab' peak FI in individual clusters, maintaining it within
360 a relatively low range, where the MFI ratios of pCD79a to AF546-Fab' increased with the
361 AF546-Fab' peak FI (*Figure 7C and D*). Additionally, the average pCD79a to Fab' MFI ratios
362 were much higher in cNKO B-cells than flox control B-cells, when comparing AF546-Fab'
363 clusters with the same range of peak FI (*Figure 7B-D*). During the contraction stage (5 and 7
364 min), flox control B-cells exhibited increased AF546-Fab' MFI (*Figure 7E*) but decreased
365 pCD79a MFI (*Figure 7F*) and the MFI ratios of pCD79a relative to AF54-Fab' (*Figure 7G*) in
366 individual clusters, compared to the spreading stage (1 and 3 min). In contrast, non-contracting
367 cNKO B-cells only slightly increased AF546-Fab' MFI (*Figure 7E*) but significantly increased
368 pCD79a MFI (*Figure 7F*) and the pCD79a to AF546-Fab' MFI ratios (*Figure 7G*) in individual
369 clusters at the contraction stage compared to the spreading stage. Consequently, individual
370 clusters in non-contracting cNKO B-cells had significantly lower AF546-Fab' MFI but
371 significantly higher pCD79a MFI and pCD79a to AF546-Fab' FIRs than contracting flox control
372 B-cells at 5 and 7 min but not at 1 and 3 min (*Figure 7E-G*). Similarly, inhibiting B-cell

373 contraction by treatment with the Arp2/3 inhibitor CK-666 at 2 min post-stimulation reduced
374 AF546-Fab' MFI (*Figure 7I*) but increased pCD79a MFI (*Figure 7J*) and the MFI ratios of
375 pCD79a relative to AF546-Fab' (*Figure 7K*) in individual clusters. These data suggest that
376 increases in BCR molecular density in BCR clusters during B-cell contraction inhibit BCR
377 phosphorylation.
378
379 **Increased BCR molecular density by B-cell contraction promotes the disassociation of**
380 **the stimulatory kinase Syk from BCR microclusters**
381 Increased BCR molecular density may promote signaling attenuation by inducing the
382 disassociation and/or dephosphorylation of stimulatory kinases from and at BCR clusters. To
383 test this hypothesis, we analyzed the relative amounts of Syk, a major stimulatory kinase in the
384 BCR signaling pathway, and its phosphorylated form pSyk (Y519/520) in individual BCR
385 clusters in relation to the molecular density of BCRs. Splenic B-cells were incubated with Fab'-
386 PLB for 3 and 7 min (when most cells were at the spreading and contraction phase,
387 respectively), fixed, permeabilized, stained for Syk or pSyk, and imaged using TIRF. We
388 measured MFI ratios of Syk relative to AF546-Fab' in individual BCR clusters to reflect the
389 relative amount of Syk associated with individual BCR clusters and analyzed their relationship
390 with AF546-Fab' peak FI (reflecting the molecular density within BCR clusters). AF546-Fab'
391 clusters were detected and analyzed as described above. In flox control B-cells, the highest
392 fractions of BCR clusters had an AF546-Fab' peak FI at the150~200 range (*Figure 8A, brown*
393 *line and symbol*s). The average MFI ratios of Syk to Fab' in individual clusters increased at a
394 low peak FI range (<140) and did not significantly decrease until Fab' peak FI reached a
395 relatively high range (>280) (*Figure 8A, black line and symbol*). B-cells from cNKO mice
396 exhibited a reduction in the Fab' peak FI of clusters (*Figure 8B and C, brown line and symbol*)
397 but an increase in the average Syk to Fab' MFI ratios in clusters in a wide range of Fab' peak FI,
398 when compared to flox control B-cells (*Figure 8B and C, black lines and symbols*). However, the

399 average Syk to Fab' MFI ratios of cNKO B-cells decreased in clusters with Fab' peak FI ≥220
400 and reduced to levels similar to those in flox control B-cells in clusters with Fab' peak FI ≥240
401 (*Figure 8B and C, black lines and symbols*). These data suggest that increases in the molecular
402 density of BCR clusters, reflected by Fab' peak FI, induce disassociation of Syk from BCR
403 clusters in both flox control and cNKO B-cells. Our results also show that BCR clusters in cNKO
404 B-cells have significantly higher levels of Syk association than in flox control B-cells, even
405 though they have similar Fab' peak intensities.
406
407 We next analyzed the relationship of the pSyk level in individual clusters with BCR molecular
408 density using the method described above. We found that the MFI ratios of pSyk relative to
409 AF546-Fab' gradually decreased with increases in Fab' peak FI in both flox control and cNKO
410 B-cells (*Figure 8D and E, black lines and symbols*), even though we did not observe an increase
411 in pSyk to Fab' MFI ratio at the low Fab' peak FI range. Similar to the Syk to Fab' MFI ratio, the
412 average pSyk to Fab' MFI ratios of individual clusters in cNKO B-cells were much higher than
413 those in flox control B-cells, except for those at the high Fab' peak FI range (*Figure 8D-F*). To
414 confirm this result, we analyzed equal numbers of pSyk clusters in the same cells, based on the
415 criteria of ≥1.3 fold increase in the peak FI compared to the background outside the contact
416 zone with a diameter of ≥250 nm. Similar to clusters identified by AF546-Fab', the average MFI
417 ratios of pSyk to Fab' in these pSyk clusters decreased with increase in their Fab' peak FI in
418 both flox control and cNKO B-cells (*Figure 8G-I, black lines and symbols*). Again, the average
419 pSyk to Fab' MFI ratios of pSyk clusters were much higher in cNKO than flox control B-cells, but
420 were reduced to similar levels in clusters with relatively high Fab' peak FI (*Figure 8I, black line*
421 *and symbols*). Similar reductions of total Syk and pSyk with increasing molecular density of
422 BCR clusters suggest that the disassociation of Syk from BCR clusters, caused by cell
423 contraction-induced increases in molecular density, contributes to BCR signaling attenuation.

424

425 **Increased BCR molecular density by B-cell contraction promotes disassociation of the**
426 **inhibitory phosphatase SHIP-1 from BCR microclusters**

427 The inhibitory phosphatase SHIP-1 is essential for B-cell signaling attenuation (Brauweiler *et al.*
428 2000; Liu *et al.* 2011), suggesting that increases in the molecular density of BCR clusters by B-
429 cell contraction may promote SHIP-1 recruitment. We used the methods described above to
430 address this hypothesis, staining cells for total SHIP-1 and phosphorylated SHIP-1 (pSHIP-1
431 Y1020). We found that the average SHIP-1 to Fab' MFI ratios in both flox control and cNKO B-
432 cells decreased with Fab' peak FI at similar rates (*Figure 9A-C, black lines and symbols*), even
433 though inhibition of contraction by cNKO reduced the Fab' peak FI of BCR clusters (*Figure 9A-*
434 *C, brown lines and symbols*). Notably, the reduction in the SHIP-1 to Fab' MFI ratios with
435 increasing Fab' peak FI occurred at the lowest detectable Fab' peak PI (*Figure 9A-C, brown*
436 *lines and symbols*), when the Syk to Fab' MFI ratios increased and were sustained (*Figure 8A-*
437 *C*). Furthermore, this reciprocal relationship between the SHIP-1 to Fab' MFI ratio and Fab'
438 peak FI continued over the entire Fab' peak FI range. It also remained the same in both flox
439 control and cNKO B-cells (*Figure 9A-C*). These results suggest that SHIP-1 disassociates from
440 BCR clusters as their molecular density increases, and that the SHIP-1 disassociation is more
441 sensitive to the molecular density of BCR clusters than Syk disassociation.

442

443 Similar to the relationship of total SHIP-1 with BCR molecular density, the average MFI ratios of
444 pSHIP-1 relative to AF546-Fab' in individual Fab' clusters decreased with increases in Fab'
445 peak FI at similar rates in flox control and cNKO B-cells (*Figure 9D-F, black lines and symbols*),
446 even though inhibition of contraction by cNKO reduced Fab' peak FI of BCR clusters (*Figure*
447 *9D-F, brown lines and symbols*). The average pSHIP-1 to Fab' MFI ratios in individual pSHIP-1
448 clusters, detected and analyzed in the same way as pSyk clusters, showed the same decrease
449 with increases in their Fab' peak FI in both flox control and cNKO B-cells (*Figure 9G-I, black*

450 *lines and symbols*). Notably, the average pSHIP-1 to Fab' MFI ratios in individual Fab' or
451 pSHIP-1 clusters in flox control and cNKO B-cells were at similar levels at the same Fab' peak
452 FI ranges (*Figure 9F and I, black lines and symbols*). These results indicate that contraction-
453 induced molecular density increases within individual BCR clusters do not induce preferential
454 recruitment of SHIP-1; instead, it promotes the disassociation of SHIP-1 from BCR clusters.
455

456 **Discussion**

457 When binding membrane-associated antigen, B-cells undergo actin-mediated spreading
458 followed by a contraction, which amplifies BCR signaling and promotes immunological synapse
459 formation (Fleire *et al.* 2006; Harwood and Batista 2011). We previously showed that B-cell
460 contraction after spreading on antigen-presenting surfaces promotes BCR signaling attenuation
461 (Liu *et al.* 2013a; Seeley-Fallen *et al.* 2022). However, how the actin cytoskeleton reorganizes
462 as B-cells transition from spreading to contraction and how B-cell contraction downregulates
463 BCR signaling have been elusive. Here we demonstrate that inner F-actin foci formed at the
464 contact zone distal to the lamellipodial F-actin network promote B-cells to switch from spreading
465 to contraction. These inner foci are derived from the lamellipodial F-actin network that mediates
466 spreading, are generated by N-WASP- but not WASP-activated Arp2/3-mediated branched actin
467 polymerization, and facilitate NMII recruitment, enabling B-cell contraction. B-cell contraction
468 increases BCR molecular density in existing clusters, which promotes the disassociation of both
469 the stimulatory kinase Syk and the inhibitory phosphatase SHIP-1, leading to signaling
470 attenuation.
471

472 One significant finding of this study is that Arp2/3-mediated polymerization of branched actin is
473 required for B-cell contraction. Arp2/3-mediated branched actin polymerization is known to drive
474 B-cell spreading and to create actin centripetal flow at the contact zone between B-cells and
475 antigen-presenting surface (Bolger-Munro *et al.* 2019). The actin structure that supports B- and

476 T-cell spreading to form the immunological synapse with antigen-presenting cells is similar to
477 lamellipodial F-actin networks found in adherent cells (Bunnell *et al.* 2001; Koestler *et al.* 2008;
478 Bolger-Munro *et al.* 2019). Lamellipodial F-actin networks consist primarily of branched actin
479 filaments polymerizing against the plasma membrane interspersed with bundled actin filaments
480 (Krause and Gautreau 2014; Skau and Waterman 2015). Contractile actin structures, such as
481 stress fibers, are typically generated from bundled actin filaments, as observed in adherent and
482 migrating cells (Levayer and Lecuit 2012; Tojkander *et al.* 2015; Hammer *et al.* 2019). Here, we
483 show that inner F-actin foci, generated by Arp2/3-mediated actin polymerization, transition B-
484 cells from spreading to contraction. Furthermore, these inner F-actin foci are directly derived
485 from lamellipodial F-actin networks. Based on the observed dynamics, we infer that instead of
486 polymerizing against the plasma membrane, Arp2/3 appears to nucleate actin polymerization in
487 the opposite direction, sustaining actin foci and their movement away from the lamellipodia.
488 However, this study does not exclude the involvement of bundled actin filaments. Based on the
489 requirement of formin-activated bundled actin filaments for lamellipodial F-actin networks and B-
490 cell spreading (Wang *et al.* 2022), we can speculate that bundled actin filaments may play a role
491 in the formation and movement of these inner F-actin foci, as well as in NMII recruitment.
492
493 Our work provides new insights into distinct functions of the actin nucleation-promoting factors
494 WASP and N-WASP in controlling cell morphology and signaling. Immune cells, including B-
495 cells, express both hematopoietic-specific WASP and the ubiquitous homolog of WASP, N-
496 WASP. These two share high sequence homology, activation mechanism, and Arp2/3 activation
497 function (Padrick and Rosen 2010). We previously identified distinct functions of these two
498 factors that are unique to B-cells. While both are required for B-cell spreading, N-WASP plays a
499 unique role in B-cell contraction. WASP and N-WASP double knockout B-cells fail to spread on
500 antigen-presenting surfaces, but B-cell-specific N-WASP knockout enhances B-cell spreading
501 and delays B-cell contraction (Liu *et al.* 2013a). Furthermore, these two factors reach peak

502 activation at different times (phosphorylated WASP peaks during B-cell spreading, while
503 phosphorylated N-WASP peaks during contraction) and are detected at distinct locations (the
504 majority of WASP is at the periphery, and N-WASP is scattered across the B-cell contact zone)
505 (Liu *et al.* 2013a). Here, we reveal the exact role of N-WASP in B-cell contraction – to generate
506 inner F-actin foci from lamellipodial F-actin networks by activating Arp2/3-mediated actin
507 polymerization. N-WASP- but not WASP-activated actin polymerization prolongs the relative
508 lifetime of actin foci and mediates their inward motion toward the center of the contact zone. The
509 delayed activation time and the location in the interior of the contact zone likely give N-WASP a
510 unique opportunity to generate inner F-actin foci. As WASP and N-WASP suppress each other
511 for activation in B-cells (Liu *et al.* 2013a), the timing and the location of their activation may
512 depend on the relative levels of their expression and their abilities to compete for upstream and
513 downstream molecules. As cNKO only delays and reduces but does not block B-cell contraction
514 and inner F-actin foci formation, other actin factors may be involved, such as the WASP-family
515 verprolin-homologous protein (WAVE) (Rotty *et al.* 2013).
516
517 Our recently published data show that NMII is required for B-cell contraction. Activated NMII is
518 recruited to the B-cell contact zone in a SHIP-1-dependent manner and forms a peripheral ring
519 surrounding the contact zone during B-cell contraction (Seeley-Fallen *et al.* 2022). Here, we
520 further show that in addition to SHIP-1, N-WASP but not WASP is also involved in NMII
521 recruitment and NMII ring-like structure formation, probably by generating inner F-actin foci.
522 Collectively, these findings suggest that SHIP-1 coordinates with N-WASP to recruit NMII. Our
523 previous finding that SHIP-1 promotes B-cell contraction by facilitating N-WASP activation (Liu
524 *et al.* 2011; Liu *et al.* 2013a) supports this notion. However, it is surprising that the recruitment of
525 NMII activated by BCR signaling to the contact zone is associated with inhibitory signaling
526 molecules. BCR downstream signaling, including Ca^{2+} fluxes and Rho-family GTPase activation,
527 likely activates NMII motor activity (Vicente-Manzanares *et al.* 2009). The activation switches

528 NMII from the incompetent folded conformation to the competent extended conformation,
529 enabling NMII molecules to bind to F-actin and assemble into contractible bipolar filaments and
530 stacks (Matsumura 2005). A denser F-actin organization has been shown to increase NMII
531 filament stacking (Fenix and Burnette 2018). Here, we showed that the inner F-actin foci
532 generated by N-WASP-activated Arp2/3 are more stable and denser and thus likely promote
533 NMII binding and stacking more efficiently than F-actin generated by WASP-activated Arp2/3.
534 Indeed, our kymograph analysis revealed similar time windows and spatial locations for NMII
535 accumulation and for generating inner F-actin foci from lamellipodial F-actin, supporting our
536 hypothesis.
537
538 Wang et al. (Wang *et al.* 2022) recently showed that B-cells generate actomyosin arcs when
539 interacting with membrane-associated antigen in the presence of adhesion molecules, as when
540 interacting with professional antigen-presenting cells. In contrast to T-cells, B-cells can also
541 respond to membrane-associated antigen without the help of adhesion molecules, when
542 engaging antigen on pathogenic cells, like bacteria, parasites, and cancer cells. While the
543 relationship between actomyosin arcs and inner F-actin foci remains to be explored, the role of
544 inner F-actin foci in the formation of NMII ring-like structures revealed in our study suggests that
545 the inner F-actin foci in the absence of adhesion molecules may be the facilitator or precursors
546 of the actomyosin arcs. These structures can potentially be enhanced and matured by
547 interactions between B-cells and antigen-presenting membranes mediated by adhesion
548 molecules.
549
550 Our findings suggest an increase in the molecular density within BCR clusters or B-cell
551 synapses as one of the mechanisms by which B-cell contraction promotes BCR signaling
552 attenuation. During contraction, the molecular density of individual BCR clusters, measured by
553 their MFI and peak FI of BCR clustered Fab', increased faster than during the spreading and

554 post-contraction phases. Surprisingly, increases in molecular density induced disassociation of
555 both the stimulatory kinase Syk and the inhibitory phosphatase SHIP-1, contradicting the
556 existing dogma of sequential association of stimulatory and inhibitory signaling molecules with
557 the BCR (Franks and Cambier 2018). Interestingly, the disassociation of SHIP-1 is much more
558 sensitive to BCR molecular density than Syk disassociation. Syk does not disassociate until the
559 molecular density of BCR clusters reaches the top 10% range, while SHIP-1 disassociates from
560 BCR clusters with increases over the entire detectable range of molecular densities. When B-
561 cell contraction is inhibited, the molecular density of BCR clusters is reduced to a range with
562 limited Syk disassociation and normal SHIP-1 disassociation, resulting in high Syk to SHIP-1
563 molecular ratios in BCR clusters than contracting B-cells, increasing BCR signaling levels. The
564 resolution of our widefield TIRF imaging limits our ability to examine nascent BCR clusters that
565 actively recruit signaling molecules. However, the consistency of the data from AF546-Fab' and
566 pSyk or pSHIP-1 clusters confirms our results. How the molecular density within BCR clusters
567 promotes disassociation of signaling molecules is unknown. We speculate that molecular
568 crowding may displace signaling molecules out of BCR clusters. Supporting this possibility,
569 disassociation of the 145 kDa SHIP-1 is likely to be more sensitive to changes in the molecular
570 density of BCR clusters, compared to disassociation of the 72 kDa Syk. However, these
571 hypotheses need to be further examined.
572
573 An interesting finding of this study is that the pCD79a and pSyk levels were higher in individual
574 BCR clusters of cNKO B-cells than those of flox control B-cells, even when BCR clusters had
575 similar molecular densities. These data suggest additional mechanisms for B-cell contraction to
576 promote BCR signaling attenuation. In addition to increasing molecular density within BCR
577 clusters, the contractile forces generated by actomyosin rings on the B-cell membrane and BCR
578 clusters may cause changes to the conformations of both BCRs and associated signaling
579 molecules and their lateral interactions in the membrane. These changes may favor the

580 disassociation rather than the association of signaling molecules with BCR clusters. Recently
581 solved molecular assembly structures of both human and mouse BCRs (Dong *et al.* 2022; Su *et*
582 *al.* 2022) support conformational and lateral molecular interaction changes as possible
583 mechanisms for BCR signaling regulation. Actomyosin-mediated contractile forces were also
584 shown to be required for BCR-mediated endocytosis of antigen (Natkanski *et al.* 2013;
585 Hoogeboom *et al.* 2018; Maeda *et al.* 2021), which removes the BCR from the cell surface,
586 downregulating the BCR surface signaling. Whether the inner F-actin foci identified here play
587 any role in BCR endocytosis remains an interesting question. Our early finding that N-WASP is
588 required for BCR endocytosis (Liu *et al.* 2013a) supports this notion. Similar to the differential
589 impact of the molecular density on the disassociation of Syk and SHIP-1 from BCR clusters, B-
590 cell contraction may differentially affect the interaction of surface BCRs with cytoplasmic and
591 membrane-anchored signaling molecules, such as the lipidated and lipid raft-resident Src kinase
592 Lyn that is responsible for phosphorylating both the immunoreceptor tyrosine-based activation
593 motif (ITAM) of CD79a and the immunoreceptor tyrosine-based inhibitory motif (ITIM) that SHIP-
594 1 binds to (Franks and Cambier 2018).
595
596 The results presented here have revealed novel insights into the mechanisms underlying actin-
597 facilitated signaling attenuation of the BCR. Taking our previous and current data together, we
598 propose a new working model for such actin-mediated signaling downregulation. Upon maximal
599 spreading, N-WASP distal to lamellipodial networks activates Arp2/3-mediated branched actin
600 polymerization, which sustains actin foci originating from lamellipodia and their movement away
601 from the lamellipodia towards the center of the B-cell contact zone, generating inner F-actin foci.
602 NMII is then preferentially recruited to these relatively stable inner foci, forming ring-like
603 actomyosin structures, which enable B-cell contraction. B-cell contraction pushes the BCR
604 microclusters formed during B-cell spreading to the center of the contact zone, increasing their
605 molecular density. Increased molecular density promotes disassociation of signaling molecules

606 from BCR clusters, probably due to crowding and conformational changes, leading to signal
607 downregulation. Inhibitory signaling molecules likely activate the actin reorganization that drives
608 B-cell contraction, as both the activation of N-WASP and the recruitment of NMII to the B-cell
609 contact zone depend on SHIP-1 (Liu *et al.* 2013a; Seeley-Fallen *et al.* 2022). Thus, actin
610 reorganization downstream of inhibitory phosphatases reinforces signaling attenuation by
611 driving B-cell contraction.
612

613 **Materials and Methods**

614 Key Reagents Table

Reagent type	Designation	Source	Identifiers	Additional Information
B-cells from C57BL/6 mice	WT	Jackson Laboratories	000664	
B-cells from WASP$^{-/-}$ mice	WKO	Jackson Laboratories	019458	
B-cells from CD19$^{Cre/+}$N-WASP$^{flox/flox}$ mice	cNKO	Lisa Westerberg laboratory		
B-cells from N-WASP$^{flox/flox}$ mice	Flox control	Lisa Westerberg laboratory		
B-cells from LifeAct-GFP mice	LifeAct-GFP	Roberto Weigert laboratory		
B-cells from LifeAct-RFP mice	LifeAct-RFP	Klaus Ley laboratory		
B-cells from GFP-NMIIA mice	GFP-NMIIA	Robert Adelstein laboratory		
Reagent	1,2-dioleoyl-sn-glycero-3-phosphocholine	Avanti Polar Lipids	850375 P	5 mM
Reagent	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-cap-biotin	Avanti Polar Lipids	870273 C	50 µM
Reagent	Streptavidin	Jackson Immuno Research	016-000-084	1 µg/ml
Antibody	F(ab')$_2$ fragment of goat IgG anti-mouse Ig(G+M) (polyclonal)	Jackson Immuno Research	115-006-068	-
Antibody	Cy3-Fab fragment of goat anti-mouse IgG+M (polyclonal)	Jackson Immuno Research	115-167-020	2.5 µg per 1 x 10^6 cells

Chemical	2-Mercaptoethylamine HCL	Thermo Fisher Scientific	20408	50 mM
Reagent	EZ-Link™ Maleimide-PEG$_2$-biotin	Thermo Fisher Scientific	A39261	20 mM per mM of protein
Commercial Kit	Alexa Fluor 546 antibody labeling kit	Thermo Fisher Scientific	A20183	-
Ligand	Biotinylated holo-transferrin	Jackson Immuno Research	015-060-050	-
Antibody	Rat IgG$_{2b}$ anti-mouse CD90.2 (Thy1.2) (monoclonal)	Biolegend	105351	1 µl per 2x10^6 cells
Inhibitor	CK-689	Millipore Sigma	182517-25MG	50 µM
Inhibitor	CK-666	Millipore Sigma	SML0006-5MG	50 µM
Inhibitor	Wiskostatin	Millipore Sigma	W2270-5MG	10 µM
Antibody	Rabbit IgG anti-mouse Arp2 antibody (polyclonal)	Abcam	ab47654	1:100
Antibody	Rabbit IgG anti-mouse pWASP antibody (polyclonal)	Thermo Fisher Scientific	PA5-105572	1:100
Antibody	Rabbit IgG anti-mouse pN-WASP antibody (polyclonal)	Thermo Fisher Scientific	PA5-105307	1:100
Antibody	AF488-goat IgG anti-rabbit IgG antibody (polyclonal)	Thermo Fisher Scientific	A-11034	1:200
Antibody	AF546-goat IgG anti-rabbit IgG antibody (polyclonal)	Thermo Fisher Scientific	A-11035	1:200
Reagent	Acti-stain-488 Phalloidin	Cytoskeleton	PHDG1-A	200 nM
Reagent	Acti-stain-555 Phalloidin	Cytoskeleton	PHDH1-A	200 nM
Antibody	Rabbit IgG anti-mouse NMIIA antibody (polyclonal)	Abcam	ab75590	1:100
Antibody	Rabbit IgG anti-mouse pCD79a (Y182) (monoclonal)	Cell Signaling Technology	14732S	1:100
Antibody	Rabbit IgG anti-mouse pSyk (Y519/520) (monoclonal)	Cell Signaling Technology	2710S	1:100
Antibody	Rabbit IgG anti-mouse pSHIP-1 (Y1020) (polyclonal)	Cell Signaling Technology	3941S	1:100
Antibody	Rabbit IgG anti-mouse Syk (polyclonal)	Thermo Fisher Scientific	PA5-17812	1:100
Antibody	Rabbit IgG anti-mouse SHIP-1 (polyclonal)	Thermo Fisher Scientific	PA5-115894	1:100

615

616 **Mice and B-cell isolation**

617 Wild-type (WT) C57BL/6 mice and WASP knockout (WKO) mice on the C57BL/6 background
618 were purchased from Jackson Laboratories. C56BL/6 mice expressing LifeAct-GFP transgene
619 (Riedl *et al.* 2010) were kindly provided by Dr. Roberto Weigert at National Cancer Institute,
620 USA. C56BL/6 mice expressing the LifeAct-RFP transgene were kindly provided by Dr. Klaus

621 Ley at La Jolla Institute for Allergy & Immunology. C57BL/6 mice expressing the GFP-non-
622 muscle myosin IIA (NMIIA) transgene were kindly provided by Dr. Robert Adelstein at the
623 National Heart, Lung, and Blood Institute, USA. WKO mice were bred with LifeAct-GFP mice to
624 obtain LifeAct-GFP-expressing WKO mice. GFP-NMIIA mice were crossed with LifeAct-RFP
625 mice to generate mice expressing both transgenes. N-WASP$^{flox/flox}$ on a 129Sv background
626 (Cotta-de-Almeida *et al.* 2007) were kindly provided by Dr. Lisa Westerberg at Karolinska
627 Institute, Sweden. B-cell-specific N-WASP knockout mice (cNKO, CD19$^{Cre/+}$N-WASP$^{flox/flox}$) and
628 floxed littermate controls (N-WASP$^{flox/flox}$) were obtained by breeding N-WASP$^{flox/flox}$ mice with
629 CD19$^{Cre/+}$ mice on a C57BL/6 background.
630
631 Primary B-cells were isolated from the spleens of 6 to 18 week-old male or female mice, using a
632 previously published protocol (Sharma *et al.* 2009). Briefly, mononuclear cells were isolated by
633 Ficoll density-gradient centrifugation (Millipore Sigma), and T-cells were eliminated by
634 complement-mediated cytolysis with anti-mouse CD90.2 mAb (BD Biosciences) and guinea pig
635 complement (Innovative Research Inc). Monocytes and dendritic cells were eliminated by
636 panning at 37°C and 5% CO_2. Isolated B-cells were kept on ice in DMEM (Lonza) supplemented
637 with 0.6% BSA (Thermo Fisher Scientific). All work involving mice was approved by the
638 Institutional Animal Care and Usage Committee of the University of Maryland.
639
640 **Pseudo-antigen-coated planar lipid bilayers**
641 Planar lipid bilayers (PLB) were prepared using a previously described method (Dustin *et al.*
642 2007; Liu *et al.* 2012a). Briefly, liposomes were generated from a mixture of 5 mM (total
643 concentration) 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-
644 phosphoethanolamine-cap-biotin (Avanti Polar Lipids) at a 100:1 molar ratio by sonication.
645 Glass coverslips, cleaned overnight with Piranha solution (KMG chemicals), were attached to 8-
646 well chambers (Lab-Tek) and incubated with liposomes (0.05 mM) in PBS for 20 min at room

647 temperature and washed with PBS. The chambers were then incubated with 1 μg/ml
648 streptavidin (Jackson ImmunoResearch Laboratories) for 10 min, washed with PBS, and then
649 incubated with 10 μg/ml mono-biotinylated Fab' fragment of goat anti-mouse Ig(G+M) (pseudo-
650 antigen) (Fab'-PLB) for 10 min, followed by PBS wash. For transferrin-coated PLB (Tf-PLB), 16
651 16 μg/ml biotinylated holoTF was used. To visualize Fab' clustering, a mixture of 0.5 μg/ml
652 Alexa Fluor (AF) 546-labeled and 9.5 μg/ml unlabeled mono-biotinylated Fab' fragment of goat
653 anti-mouse Ig(G+M) was used. The lateral mobility of AF546-Fab' on the PLB was tested using
654 fluorescence recovery after photobleaching (FRAP) to ensure ≥85% AF546 fluorescence
655 recovery within 1 min after photobleaching using a Zeiss LSM 710 equipped with a 60X oil-
656 immersion objective.
657
658 Mono-biotinylated Fab' fragments were generated as previously described (Liu *et al.* 2011).
659 Briefly, the disulfide bond linking the two Fab fragments of $F(ab')_2$ goat anti-mouse Ig(G+M) was
660 reduced using 2-mercaptoethylamine HCL (Thermo Fisher Scientific) and biotinylated by using
661 maleimide-PEG_2-biotin (Thermo Fisher Scientific). Mono-biotinylated Fab' fragments of goat
662 anti-mouse Ig(G+M) were labeled with AF546 using an Alexa Fluor 546 antibody labeling kit
663 (Thermo Fisher Scientific). The molar ratio of AF546 to Fab' in the AF546-labeled Fab' was ~2
664 determined by a Nanodrop spectrophotometer (Nanodrop Technologies).
665
666 **Total internal fluorescence microscopy**
667 To visualize molecules proximal to interacting sites between B-cells and Fab'- or Tf-PLB, we
668 utilized total internal reflection fluorescence microscopy (TIRF) and interference reflection
669 microscopy (IRM). Images were acquired using a Nikon TIRF system on an inverted microscope
670 (Nikon TE2000-PFS, Nikon Instruments Inc.) equipped with a 60X, NA 1.49 Apochromat TIRF
671 objective (Nikon), a Coolsnap HQ2 CCD camera (Roper Scientific), and two solid-state lasers of
672 wavelength 491 and 561 nm. IRM, AF488, and AF546 images were acquired sequentially.

673

674 The plasma membrane area of B-cells contacting PLB was determined using IRM images and
675 custom MATLAB codes. Whether a B-cell contracted or not was determined using the area
676 versus time plots, wherein if a B-cell's contact zone area reduced by ≥5% for at least 10 sec
677 after reaching a maximum value, it was classified as contracting. To image intracellular
678 molecules, B-cells were incubated with Fab'-PLBs for varying lengths of time in PBS at 37°C,
679 fixed with 4% paraformaldehyde, permeabilized with 0.05% saponin, and stained for various
680 molecules. For live-cell imaging, B-cells were pre-warmed to 37°C and imaged as soon as B-
681 cells were dropped into coverslip chambers coated with Fab'-PLB containing PBS in a humidity
682 chamber at 37°C, at 2 sec per frame, up to 7 min. All images from multiple independent
683 experiments were analyzed using NIH ImageJ and custom MatLab codes. Acquired
684 fluorescence intensity (FI) data were normalized to the one with the lowest FI.

685

686 **Inhibitors**

687 CK-666 (50 μM, Millipore Sigma) was used to perturb Arp2/3 activity (Nolen *et al.* 2009), and its
688 non-functional derivative CK-689 (50 μM, Millipore Sigma) as a control. CK-666 or CK-689 was
689 added at either 0 min, the start of incubation with Fab'-PLB, or at 2 min when most B-cells
690 reached maximum spreading. Notably, B-cells take approximately 1 to 1.5 min to land on PLB.
691 The time when B-cells were added to Fab'-PLB is referred to as 0 min, and the time when B-
692 cells landed on PLB as the start of spreading. The effectiveness of CK-666 was determined by
693 its inhibitory effects on the recruitment of Arp2/3, stained by an anti-Arp2 antibody (Abcam), to
694 the B-cell contact zone using TIRF (*Figure 1-figure supplement 2*). Wiskostatin (Wisko, 10 μM,
695 Millipore Sigma) was used to perturb N-WASP activity in B-cells (Peterson *et al.* 2004), with
696 DMSO used as a vehicle control. Splenic B-cells were pretreated with Wisko for 10 min at 37°C
697 before and during incubation with Fab'-PLB. The effectiveness of Wisko was determined by its

698 inhibitory effects on the level of phosphorylated N-WASP in the B-cell contact zone using
699 immunostaining and TIRF (*Figure 1-figure supplement 4A and C)*. Possible effects of Wisko on
700 WASP activation were evaluated by measuring the mean fluorescence intensity (MFI) of
701 phosphorylated WASP in the contact zone of B-cells treated with or without Wisko for 10 min
702 (*Figure 1-figure supplement 4B and D*).
703
704 **Analysis of the actin cytoskeleton**
705 F-actin was visualized by phalloidin staining in fixed cells and by LifeAct-GFP or LifeAct-RFP
706 expressed by primary B-cells from transgenic mice in live cells. Inner F-actin foci, visualized by
707 both phalloidin staining and LifeAct-GFP, were identified using NIH ImageJ based on the
708 following three criteria: 1) ≥250 nm in diameter, 2) peak fluorescence intensity (FI) ≥2 fold higher
709 than the FI of a nearby area containing no foci, and 3) ≥1 μm away from the outer edge of the B-
710 cell contact zone. The horizontal or vertical length of a focus was measured, and the lesser of
711 the two values was used as its diameter. To analyze the spatiotemporal relationship between
712 inner F-actin foci and the lamellipodial F-actin network, we generated 8 radially and equally
713 spaced kymographs from each cell using time-lapse images and MATLAB. Each kymograph
714 was either classified as contracting or not contracting based on the movement of the leading
715 edge of the B-cell contact zone. The percentage of 8 kymographs from each cell exhibiting inner
716 F-actin foci that could be traced back to lamellipodial F-actin when the B-cell switched from
717 spreading to contraction was determined.
718 The relative speeds and lifetimes of F-actin foci were determined using three randomly
719 positioned kymographs from each B-cell and F-actin focuses emerging during a 60-sec window
720 right after B-cell maximal spreading. Only those remaining visible in individual kymographs for at
721 least 4 sec were analyzed. The time duration of each F-actin foci detected in a kymograph was
722 used to determine the relative lifetime. The distances individual F-actin foci moved during their
723 lifetimes were used to determine the relative speed. The term ‘relative’ has been used because

724 the disappearance of an F-actin focus could be due to its movement away from the region used
725 for the kymograph or vertically from the TIRF evanescent field of excitation.
726 To determine the rate of myosin recruitment, GFP-NMIIA MFI in the B-cell contact zone was
727 plotted over time, and the slope for the initial segment of the MFI versus the time plot was
728 determined using linear regression. The percentage of B-cells with the NMIIA ring was
729 determined by visual inspection.
730
731 **Analysis of BCR-Fab' clusters**
732 BCR clusters were identified by clustering of AF546-Fab' on PLBs using custom code by
733 MATLAB. We utilized the median FI of the Fab'-PLB within the same area outside but near the
734 B-cell contact zone as the background. The median, but not mean FI value was used for
735 background FI calculations to minimize fluctuations due to debris. To ensure that individual
736 clusters with varying FI were detected as distinct objects, we used 16 graded thresholds from
737 1.1 to 4.1 fold of the background (0.2 fold apart) to acquire 16 sets of binary masks for each
738 frame of time-lapse images from each cell. When clusters were detected by multiple thresholds
739 at the same location, only the one identified by the highest threshold was retained. Objects that
740 were smaller than 250 nm in diameter or could not be tracked for at least 20 sec were
741 eliminated. This allowed us to detect the position of BCR-Fab' clusters and track them over time
742 until they merged with other clusters. The horizontal or vertical length of a focus was measured,
743 and the lesser of the two values was used as its diameter. We chose the peak FI as a metric for
744 the extent of Fab' clustering by the BCR, because it does not rely on the area occupied by each
745 cluster. The rate of peak FI increase was determined by linear regression of peak FI versus time
746 curves for each cluster for a given window of time.
747
748 **BCR signaling**

749 Splenic B-cells were incubated with AF546-Fab'-PLB at 37°C and fixed at 1, 3, 5, 7, and 9 min.
750 After fixation, cells were stained for phosphorylated CD79a (pCD79a, Y182) (Cell Signaling
751 Technology), Syk (pSyk, Y519/520) (Cell Signaling Technology), SHIP-1 (pSHIP-1, Y1020) (Cell
752 Signaling Technology), or total Syk or SHIP-1 proteins (Thermo Fisher Scientific). We identified
753 BCR-Fab' clusters as described. We determined the peak FI of AF546-Fab' and the MFI of
754 AF546-Fab', pCD79a, pSyk, pSHIP-1, Syk, or SHIP-1 within each cluster. The ratio of pCD79a
755 MFI to AF546-Fab' MFI in the same cluster was used to estimate the relative phosphorylation
756 level of BCRs in that cluster. This MFI ratio was plotted against AF546-Fab' peak FI to analyze
757 the relationship between BCR phosphorylation and BCR density of individual clusters. We
758 calculated the MFI ratio of Syk to Fab' or SHIP-1 to Fab' in individual clusters to estimate the
759 relative recruitment level of Syk or SHIP-1 to BCR clusters. We plotted these MFI ratios against
760 Fab' peak FI to determine the relationship between Syk and SHIP-1 recruitment levels and BCR
761 density in individual clusters. We calculated the MFI ratio of pSyk to Fab' or pSHIP-1 to Fab' to
762 estimate the amount of pSyk or pSHIP-1 relative to BCR in individual clusters. We plotted these
763 MFI ratios against Fab' peak FI to determine the relationship between Syk or SHIP-1
764 phosphorylation and BCR density in individual clusters. We identified pSyk or pSHIP-1 puncta
765 using the criteria: ≥1.3 fold of the background staining outside the B-cell contact zone and ≥250
766 nm in diameter. We calculated the MFI ratio of pSyk to Fab' or pSHIP-1 to Fab' in individual
767 puncta to estimate the amount of pSyk or pSHIP-1 relative to BCR in individual puncta. We
768 plotted these MFI ratios against Fab' peak FI to determine the relationship between Syk or
769 SHIP-1 phosphorylation and BCR density in individual puncta.
770
771 **Statistical analysis**
772 Statistical analysis was performed using the Mann-Whitney U non-parametric test for unpaired
773 groups having different sample sizes or the Student's *t*-test for paired groups with the same
774 sample size. To compare curves, Kolmogorov-Smirnov test was used. Statistical analyses were

775 performed in Microsoft Excel, GraphPad Prism, and MATLAB. All data are presented as mean ±
776 SEM (standard error of the mean). When testing multiple hypotheses, *p*--values acquired using
777 t-tests were corrected using the Benjamini-Hochberg/Yekutieli method for false discovery rate
778 control.
779

780 **MATLAB scripts**

781 All MATLAB scripts used for this study are available as supplemental materials.
782

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789

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1117

1118 **Figure legend**

1119 **Figure 1. Arp2/3, activated by N-WASP but not WASP, is required for B-cell contraction.**
1120 Splenic B-cells were incubated with planar lipid bilayers coated with monobiotinylated Fab'
1121 fragment of goat anti-mouse IgG+M (Fab'-PLB) in the absence and presence of various
1122 inhibitors and imaged live at 37°C by interference reflection microscopy (IRM). The B-cell
1123 plasma membrane area contacting Fab'-PLB (B-cell contact zone) was measured using IRM
1124 images and custom MATLAB scripts. (**A**) Representative IRM images of splenic B-cells from
1125 C57BL/6 mice treated with CK-689 or CK-666 (50 μM) before (0 min) and after maximal
1126 spreading (2 min). (**B**) Representative plots of the B-cell contact area versus time from one
1127 contracting cell and one non-contracting cell. (**C**) Percentages (±SEM) of B-cells that underwent
1128 contraction after treatment with CK-666 or CK-689. (**D**) Representative IRM images of splenic
1129 B-cells from C57BL/6 mice treated with DMSO or Wiskostatin (Wisko, 10 μM) 10 min before and
1130 during incubation with Fab'-PLB. (**E**) Percentages (±SEM) of B-cells that underwent contraction
1131 after treatment with Wisko or DMSO. (**F**) Representative IRM images of splenic B-cells from flox
1132 control and B-cell-specific N-WASP knockout (cNKO) mice. (**G**) Percentages (±SEM) of cNKO
1133 or flox control B-cells that underwent contraction. (**H**) Representative IRM images of splenic B-
1134 cells from WT or WASP knockout mice (WKO). (**I**) Percentages (±SEM) of WKO or WT B-cells
1135 that underwent contraction. Data were generated from three independent experiments, with ~25
1136 cells per condition, per experiment. Scale bar, 2 μm. *p <0.05, ***p<0.001, by paired student's t-
1137 test.

1138 **Figure supplement 1.** B-cells spread and contract on Fab'-coated-planar lipid bilayers.

1139 **Figure supplement 2.** CK-666 significantly decreases Arp2/3 recruitment to the B-cell contact
1140 zone.

1141 **Figure supplement 3.** CK-666 treatment before but not after maximal B-cell spreading
1142 decreases the spreading kinetics.

1143 **Figure supplement 4.** Wiskostatin treatment inhibits N-WASP activation while enhancing
1144 WASP activation in B-cells.
1145 **Figure 1–Video 1.** Effects of CK-666, Wiskostatin, conditional N-WASP knockout, and WASP
1146 knockout on B-cell contraction.
1147
1148 **Figure 2. Arp2/3, downstream of N-WASP, generates inner F-actin foci, driving B-cell**
1149 **contraction.** (**A-C**) WT splenic B-cells were treated with CK-689 or CK-666 (50 μM) during
1150 incubation with Fab'-PLB (**A and B**), and flox control and cNKO B-cells were incubated with
1151 Fab'-PLB at 37°C **(C)**. Cells were fixed at 2 and 4 min, permeabilized, stained for F-actin with
1152 phalloidin, and analyzed using TIRF. Shown are representative TIRF images of phalloidin
1153 staining in the contact zone of CK-689 and CK-666-treated B-cells (**A,** left panels) and
1154 fluorescence intensity (FI) profiles of phalloidin staining along a line crossing cells (**A,** right
1155 panels). Green arrows indicate lamellipodial F-actin, and purple arrows indicate inner F-actin
1156 foci forming a ring-like structure. Percentages of cells (per image) (±SEM) with inner F-actin foci
1157 forming ring-like distribution among CK-689- versus CK-666-treated cells (**B**) and flox control
1158 versus cNKO B-cells (**C**) before (2 min) and after (4 min) contraction were determined by visual
1159 inspection of phalloidin FI line-profiles across the B-cell contact zones. Data were generated
1160 from 3 independent experiments with 5 images per condition per experiment and ~15 cells per
1161 image. (**D-G**) Inner F-actin foci were identified by their diameter (≥250 nm), peak FI (≥2 fold of
1162 no foci area, and location (1 μm away from the outer edge) and quantified as the number per
1163 cell using TIRF images. (**D**) Shown are representative images of splenic B-cells from LifeAct-
1164 GFP-expressing mice treated with CK-689 or CK-666 from 0 min during incubation with Fab'-
1165 PLB at 37°C (top) and the average number (±SEM) of inner LifeAct-GFP foci per cell (bottom) at
1166 2 min. (**E**) LifeAct-GFP B-cells were treated with CK-666 at 2 min. Shown are representative
1167 TIRF images of LifeAct-GFP in the contact zone of B-cells (top) and the average number
1168 (±SEM) of inner LifeAct-GFP foci (bottom) in the same cell 10s before and 20s after CK-666

1169 treatment. (**F**) Shown are representative TIRF images of phalloidin-staining in the contact zone
1170 of flox control and cNKO B-cells after incubating with Fab'-PLB for 2 min (top) and the average
1171 number (±SEM) of inner F-actin foci per cell (bottom). (**H**) Shown are representative TIRF
1172 images of WT and WKO B-cells expressing LifeAct-GFP incubated with Fab'-PLB for 2 min and
1173 the average number (±SEM) of inner F-actin foci per cell (bottom). Data points represent
1174 individual cells from three independent experiments with 10 (**D, F, and G**) or 6 (**E**) cells per
1175 condition per experiment. Scale bar, 2 μm. ** p <0.01, *** p<0.001, by non-parametric
1176 student's *t*-test.
1177 **Figure 2-Video 1.** Effects of CK-666 and WKO on F-actin foci formation.
1178
1179 **Figure 3. Inner F-actin foci are originated from lamellipodia behind the spreading**
1180 **membrane.** Mouse splenic B-cells from LifeAct-GFP transgenic mice were treated with DMSO
1181 or Wisko (10 μM), imaged live using TIRF during incubation with Fab'-PLB at 37°C, and
1182 analyzed using kymographs generated by NIH ImageJ. (**A**) One frame of TIRF time-lapse
1183 images of LifeAct-GFP in the contact zone of B-cells treated with DMSO or Wisko. Lines
1184 indicate eight kymographs that were randomly generated from each cell. (**B**) Representative
1185 kymographs were generated from TIRF time-lapse images of LifeAct-GFP at the red line in (A).
1186 Top panel, a contracting cell. Arrows indicate the start of contraction with inner F-actin foci
1187 originating from lamellipodia. Bottom panel, a non-contracting cell. Lamellipodia-derived inner F-
1188 actin foci were identified by their LifeAct-GFP FI ≥2 fold of their nearby region, migrating out of
1189 the lamellipodial F-actin toward the center of the contact zone, and trackable for >8 sec. (**C**)
1190 Percentages (±SEM) of kymographs showing inner F-actin foci originating from lamellipodia per
1191 cell that did and did not undergo contraction. Data were generated from 3 independent
1192 experiments with ~10 cells per condition per experiment**.** (**D**) A histogram of inner F-actin foci
1193 emerging (expressed as percentages of the total events, blue line) over time relative to the time
1194 of B-cell contraction (defined as 0 sec, indicated by a purple dash line and arrow). Data were

1195 generated from 5 independent experiments with ~9 cells per condition per experiment**.** (**E**)
1196 Percentage (±SEM) of inner F-actin foci originated from lamellipodia observed in 8 randomly
1197 positioned kymographs of each DMSO- or Wisko-treated WT or untreated WKO B-cell. Data
1198 were generated from 3 independent experiments with ~10 cells per condition per experiment**.**
1199 Scale bars, 2 μm. *** $p<0.001$, by non-parametric student's *t*-test.
1200 **Figure supplement 1.** Emerging of Inner F-actin foci from lamellipodia.
1201
1202 **Figure 4. N-WASP-activated Arp2/3 sustains the lifetime and the centripetal movement of**
1203 **inner F-actin foci.** LifeAct-GFP-expressing B-cells were incubated with or without various
1204 inhibitors and imaged live by TIRF during interaction with Fab'-PLB at 37°C. Three randomly
1205 positioned kymographs were generated for each cell using time-lapse images. (**A**) A
1206 representative kymograph from TIRF time-lapse images of a DMSO-treated B-cell (Left panels).
1207 Inner F-actin foci were identified as described in Figure 3, and those that emerged during the 60
1208 sec window after maximal spreading (white rectangles in B) and can be tracked for ≥ 4 sec
1209 (dashed lines) in individual kymographs were analyzed. Relative lifetimes of inner F-actin foci
1210 were measured using the duration each focus could be detected in a kymograph. The relative
1211 distances traveled by the foci were measured using the displacement of each focus in a
1212 kymograph. Relative speed was calculated for each inner F-actin focus by dividing its relative
1213 distance by its relative lifetime (right panels). (**B-D**) B-cells were treated with CK-689 or CK-666
1214 (50 μM) from the beginning of the incubation with Fab'-PLB (0 min). Shown are representative
1215 kymographs (**B**), relative lifetimes (**C**), and relative speed (**D**) of inner F-actin foci in CK-689-
1216 versus CK-666-treated B-cells. (**E-G**) B-cells were treated with CK-666 at maximal spreading (2
1217 min). Shown is a representative kymograph of a CK-666-treated cell (**E**), relative lifetimes (**F**),
1218 and relative speeds (**G**) of inner F-actin foci in 30-sec windows before the inhibition and 10 sec
1219 after the inhibition in the same cells (linked by blue lines). (**H-J**) B-cells were treated with DMSO
1220 or Wisko (10 μM) 10 min before and during interaction with Fab'-PLB. Shown is a representative

1221 kymograph of a Wisko-treated B-cell **(H)**, relative lifetime **(I)**, and relative speed **(J)** of inner F-
1222 actin foci in DMSO versus Wisko-treated B-cells. (**K-M**) LifeAct-GFP-expressing WT and WKO
1223 B-cells were incubated with Fab'-PLB. Shown are a representative kymograph of a WKO B-cell
1224 (**K**), relative lifetime (**L**), and relative speed (**M**) of WT versus WKO B-cells. Data points
1225 represent the averaged values from inner F-actin foci in individual cells, with 3 kymographs per
1226 cell and ~12 cells per condition per experiment from 3 independent experiments. Scale bar, 2
1227 μm. * p <0.05, ** p <0.01, *** p<0.001, by non-parametric and paired student's t-test.
1228

1229 **Figure 5. Inner F-actin foci, generated by N-WASP-activated Arp2/3, facilitate NMII**
1230 **recruitment and ring-like structure formation.** (**A-C**) B-cells from mice expressing the GFP
1231 fusion of non-muscle myosin IIA (GFP-NMIIA) transgene were treated with DMSO or Wisko (10
1232 μM) 10 min before and during incubation with Fab'-PLB. The B-cell contact zones were imaged
1233 live using TIRF. Shown are representative TIRF images of DMSO- and Wisko-treated B-cells at
1234 30 sec (during spreading) and 2 min 30 sec (after maximal spreading) post landing (**A,** Scale
1235 bars, 2 μm), the averaged GFP-NMIIA MFI (±SEM) (**B**), and the initial rates of increasing
1236 (±SEM) of GFP-NMIIA in the contact zone (the slope of the initial GFP-NMIIA MFI versus time
1237 curves of individual cells) (**C**). Data points represent individual cells from 3 independent
1238 experiments with ~6 cells per condition per experiment. * p <0.05, ***p<0.001, by Kolmogorov-
1239 Smirnov test (**B**) or non-parametric student's t-test (**C**). (**D**) Primary B-cells from mice
1240 expressing both GFP-NMIIA and LifeAct-RFP transgenes were incubated with Fab'-PLB at 37°C
1241 and imaged live by TIRF. Shown are a representative TIRF image of a cell and a kymograph
1242 generated from time-lapse TIRF images at the yellow line. The purple arrow indicates the
1243 starting point of contraction, the white arrow GFP-NMIIA recruitment proximal to the spreading
1244 membrane, and the yellow arrow an F-actin (LifeAct-RFP) focus originating at the lamellipodia
1245 and moving away from the spreading membrane. (**E, F**) Primary B-cells from flox control, WKO,
1246 and cNKO mice were incubated with Fab'-PLB for indicated times. Cells were fixed,

1247 permeabilized, stained for NMII light chain, and imaged by IRM and TIRF. Shown are
1248 representative IRM and TIRF images (**E**) and percentages (±SD) of B-cells with the NMII ring-
1249 like structure in individual images (**F**), identified by visual inspection. The data were generated
1250 from 3 independent experiments with 5 images per condition per experiment. Scale bars, 2 μm.
1251 * p <0.05, ** p <0.01, *** p<0.001, by non-parametric student's t-test.
1252 **Figure 5-Video 1.** Wiskostatin treatment inhibits NMII ring-like structure formation.
1253
1254 **Figure 6. B-cell contraction increases the molecular density within BCR clusters.** Primary
1255 B-cells from WT mice were treated with CK-689 or CK-666 from the beginning of the incubation
1256 with AF546-Fab'-PLB (0 min) or at the maximal B-cell spreading (2 min) (**A, D, E, H, I, N, O**).
1257 WT B-cells were treated with DMSO or Wisko (10 μM) 10 min before and during the incubation
1258 with AF546-Fab'-PLB (**B, F, J, P**). B-cells from flox control and cNKO mice were incubated with
1259 AF546-Fab'-PLB (**C, G, K, Q**). The B-cell contact zones were imaged live by TIRF. (**A-C**)
1260 Representative time-lapse images at 30 sec (during B-cell spreading) and 2 min 30 sec (after
1261 maximal spreading) after cell landed. Scale bars, 2 μm. (**D-G**) The MFI of AF546-Fab' in the
1262 contact zone was plotted over time. Purple rectangles indicate the contraction phase. (**H-K**)
1263 Rates of AF546-Fab' MFI increases in the B-cell contact zone before and during contraction
1264 were determined by the slope of AF546-Fab' MFI versus time plots. Data points represent
1265 individual cells and were generated from 3 independent experiments with 6-12 cells per
1266 experiment. * p <0.05, ** p <0.01, *** p<0.001, by Kolmogorov-Smirnov test (D-G) or paired
1267 student's t-test (H-K). (**L**) A representative frame from a time-lapse of a CK-689-treated B-cell
1268 (left) shows AF546-Fab' clusters 30 sec after contraction began, and enlarged time-lapse
1269 images (right) show a single AF546-Fab' cluster over a 40-sec time window after contraction
1270 began. AF546 clusters were identified using the criteria: ≥250 nm in diameter, ≥1.1 fold of FI
1271 outside the B-cell contact zone, and trackable for ≥ 20 sec. Scale bars, 2 μm. (**M**) The peak FI of

1272 an AF546-Fab' cluster was measured over time, and the increasing rate of AF546-Fab' peak FI
1273 of this cluster was determined by the slope of the plot. (**N-Q**) The rates (±SEM) of increase in
1274 AF546-Fab' peak FI in individual clusters were compared between during and after contraction
1275 (**N**), between B-cells treated with CK-689 and CK-666 from 0 min (**O**), between DMSO- and
1276 Wisko-treated B-cells (**P**), and between flox control and cNKO B-cells (**Q**) after B-cells reached
1277 maximal spreading. Data points represent individual cells, the averaged slopes of clusters
1278 detected in one B-cell, from 3 independent experiments with 6~12 cells per condition per
1279 experiment. *p <0.05, ** p <0.01, ***p<0.001, by non-parametric student's t-test.
1280 **Figure supplement 1.** Fab'-PLB, but not Tf-PLB, induces BCR clustering and phosphorylation.
1281 **Figure supplement 2.** Tracking and analyzing AF546-Fab' clusters in the B-cell contact zone.
1282 **Figure 6-Video 1.** Inhibition of B-cell contraction reduces the molecular density within BCR
1283 clusters.
1284
1285 **Figure 7. Increased molecular density in BCR clusters leads to reductions in BCR**
1286 **phosphorylation.** (**A-G**) Flox control and cNKO B-cells incubated with AF546-Fab'-PLB were
1287 fixed at 1, 3, 5, and 7 min, permeabilized, stained for phosphorylated CD79a (pCD79a,
1288 Tyr182), and imaged using TIRF and IRM. (**A**) Representative IRM and TIRF images of a flox
1289 control versus a cNKO B-cell at 7 min. Scale bars, 2 μm. (**B-D**) Ratios of pCD79a MFI relative to
1290 AF546-Fab' MFI were plotted against AF546-Fab' peak FI in individual AF546-Fab' clusters in
1291 the contact zone of flox control (**B**), cNKO B-cells (**C**), or flox control and cNKO B-cells overlay
1292 (**D**). AF546-Fab' clusters were identified as described in Figure 6 and Figure 6-figure
1293 supplement 2, from an equal number of cells at the 4 time points. Data points represent
1294 individual AF546-Fab' clusters with an equal number of clusters from each time point. The black
1295 diamond symbols represent the average ratios of pCD79a MFI to Fab' MFI in individual BCR-
1296 Fab' clusters within the indicated Fab' peak FI range. The brown square symbols represent the
1297 fraction of the AF546-Fab' clusters out of the total, within the indicated Fab' peak FI range. Data

1298 were generated from 3 independent experiments with ~20 cells and ≥125 clusters per condition
1299 per experiment. * p <0.05, *** p<0.001, by non-parametric student's t-test. (**E-G**) The MFI
1300 (±SEM) of AF546-Fab' (**E**) and pCD79a (**F**) and the MFI ratio (±SEM) of pCD79a relative to
1301 AF546-Fab' (**G**) in individual AF546-Fab' clusters at indicated times were compared between
1302 flox control and cNKO B-cells and between different times. (**H-K**) WT B-cells treated with CK-
1303 689 or CK-666 after 2 min-incubation with AF546-Fab'-PLB. (**H**) Representative IRM and TIRF
1304 images of a CK-689- versus a CK-666-treated B-cell at 7 min. Scale bars, 2 μm. (**I-K**) The MFI
1305 (±SEM) of AF546-Fab' (**I**) and pCD79a (**J**) and the MFI ratio (±SEM) of pCD79a relative to
1306 AF546-Fab' (**K**) in individual AF546-Fab' clusters were compared between CK-689- and CK-
1307 666-treated B-cells after 7 min stimulation. Data points represent individual clusters. Horizontal
1308 solid lines in the violin plots represent the mean, while the dotted lines represent the quartiles of
1309 the distribution. Data were generated from 3 independent experiments with ~20 cells per
1310 condition per experiment. * p <0.05, *** p<0.001, by non-parametric student's t-test. The p-
1311 values in **D** were corrected using the Benjamini-Hochberg/Yekutieli method for false discovery
1312 rate control.
1313
1314 **Figure 8. Effects of BCR-Fab' density on the association of Syk with BCR-Fab' clusters**
1315 **and its phosphorylation.** Primary B-cells from flox control and cNKO mice were incubated with
1316 AF546-Fab'-PLB at 37°C, fixed at 3 or 7 min, stained for total Syk and phosphorylated Syk
1317 (pSyk Y519/520), and imaged by TIRF. (**A-C**) Ratios of Syk MFI relative to AF546-Fab' MFI
1318 were plotted against AF546-Fab' peak IF in individual AF546-Fab' clusters in the contact zone
1319 of Flox control (**A**) and cNKO B-cells (**B**) and their overlay (**C**). AF546-Fab' clusters were
1320 identified as described in Figure 6 and Figure 6-figure supplement 2 from an equal number of
1321 cells after 3- and 7-min stimulation. Data points represent individual AF546-Fab' clusters with an
1322 equal number of clusters from each time point. The black diamond symbols represent the
1323 average ratios of Syk MFI to Fab' MFI in individual AF546-Fab' clusters with indicated Fab' peak

1324 FI ranges. The brown square symbols represent the fraction of the AF546-Fab' clusters out of
1325 the total at indicated Fab' peak PI ranges. (**D-F**) Ratios of pSyk MFI relative to AF546-Fab' MFI
1326 were plotted against AF546-Fab' peak IF in individual AF546-Fab' clusters in the contact zone
1327 of flox control (**D**) and cNKO B-cells (**E**) and their overlay (**F**). (**G-I**) MFI ratios of pSyk relative to
1328 AF546-Fab' were plotted against AF546-Fab' peak IF in individual pSyk puncta in the contact
1329 zone of Flox control (**G**) and cNKO B-cells (**H**) and their overlay (**I**)**.** pSyk puncta were identified
1330 using the criteria: FI ≥1.3 fold of the background outside the B-cell contact zone and diameter
1331 ≥250 nm. The black diamond symbols represent the average ratios of pSyk MFI to Fab' MFI in
1332 individual AF546-Fab' clusters (**D-F**) or individual pSyk puncta (**G-I**) at indicated Fab' peak FI
1333 ranges. The brown square symbols represent the fraction of the AF546-Fab' clusters out of the
1334 total at indicated Fab' peak PI ranges. Data points represent individual AF546-Fab' clusters (**D-**
1335 **F**) or pSyk puncta (**G and H**), collected from an equal number of cells at 3 and 7 min. Data were
1336 generated from 3 independent experiments with ~23 cells per condition per experiment. * p
1337 <0.05, ** p <0.01, *** p<0.001, by non-parametric student's t-test, between AF546-Fab' cluster
1338 group with different Fab' peak FI ranges. The p-values in **C, F** and **I** were corrected using the
1339 Benjamini-Hochberg/Yekutieli method for false discovery rate control.
1340
1341 **Figure 9. The effects of BCR-Fab' density on the association of SHIP-1 with BCR-Fab'**
1342 **clusters and its phosphorylation.** Primary B-cells from flox control and cNKO mice were
1343 incubated with AF546-Fab'-PLB at 37°C, fixed at 3 or 7 min, stained for total SHIP-1 and
1344 phosphorylated SHIP-1 (pSHIP-1 Tyr1020), and imaged by TIRF. (**A-C**) Ratios of SHIP-1 MFI
1345 relative to AF546-Fab' MFI were plotted against AF546-Fab' peak IF in individual AF546-Fab'
1346 clusters in the contact zone of Flox control (**A**) and cNKO B-cells (**B**). An overlay of flox control
1347 and cNKO plots is also shown (**C**). AF546-Fab' clusters were identified as described in Figure 6
1348 and Figure 6-figure supplement 2 from an equal number of cells after 3 and 7 min stimulation.

1349 Data points represent individual AF546-Fab' clusters with an equal number of clusters from
1350 each time point. The black diamond symbols represent the average ratios of SHIP-1 MFI to Fab'
1351 MFI in individual AF546-Fab' clusters within indicated Fab' peak FI ranges. The brown square
1352 symbols represent the fraction of the AF546-Fab' clusters out of the total at indicated Fab' peak
1353 FI ranges. (**D-F**) Ratios of pSHIP-1 MFI relative to AF546-Fab' MFI were plotted against AF546-
1354 Fab' peak IF in individual AF546-Fab' clusters in the contact zone of flox control **(D)** and cNKO
1355 B-cells (**E**) and their overlay (**F**). (**G-I**) MFI ratios of pSHIP-1 relative to AF546-Fab' were plotted
1356 against AF546-Fab' peak IF in individual pSHIP-1 puncta in the contact zone of Flox control **(G)**
1357 and cNKO B-cells (**H**) and their overlay (**I**). The black diamond symbols represent the average
1358 ratios of pSHIP-1 MFI to Fab' MFI in individual AF546-Fab' clusters (**D-F**) or individual pSHIP-1
1359 puncta (**G-I**) at indicated Fab' peak FI ranges. The brown square symbols represent the fraction
1360 of the total clusters at indicated Fab' peak FI ranges. pSHIP-1 puncta were identified using the
1361 criteria: FI ≥1.5 fold of the background outside the B-cell contact zone and diameter ≥250 nm.
1362 Data points represent individual AF546-Fab' clusters (**D-F**) and pSHIP-1 puncta (**G and H**),
1363 collected from an equal number of cells at 3- and 7-min. Data were generated from 3
1364 independent experiments with ~23 cells per condition per experiment. * p <0.05, ** p <0.01, ***
1365 p<0.001, by non-parametric student's *t*-test, between AF546-Fab' cluster group with different
1366 Fab' peak FI ranges. The p-values in **C, F,** and **I** were corrected using the Benjamini-
1367 Hochberg/Yekutieli method for false discovery rate control.
1368

Figure 1

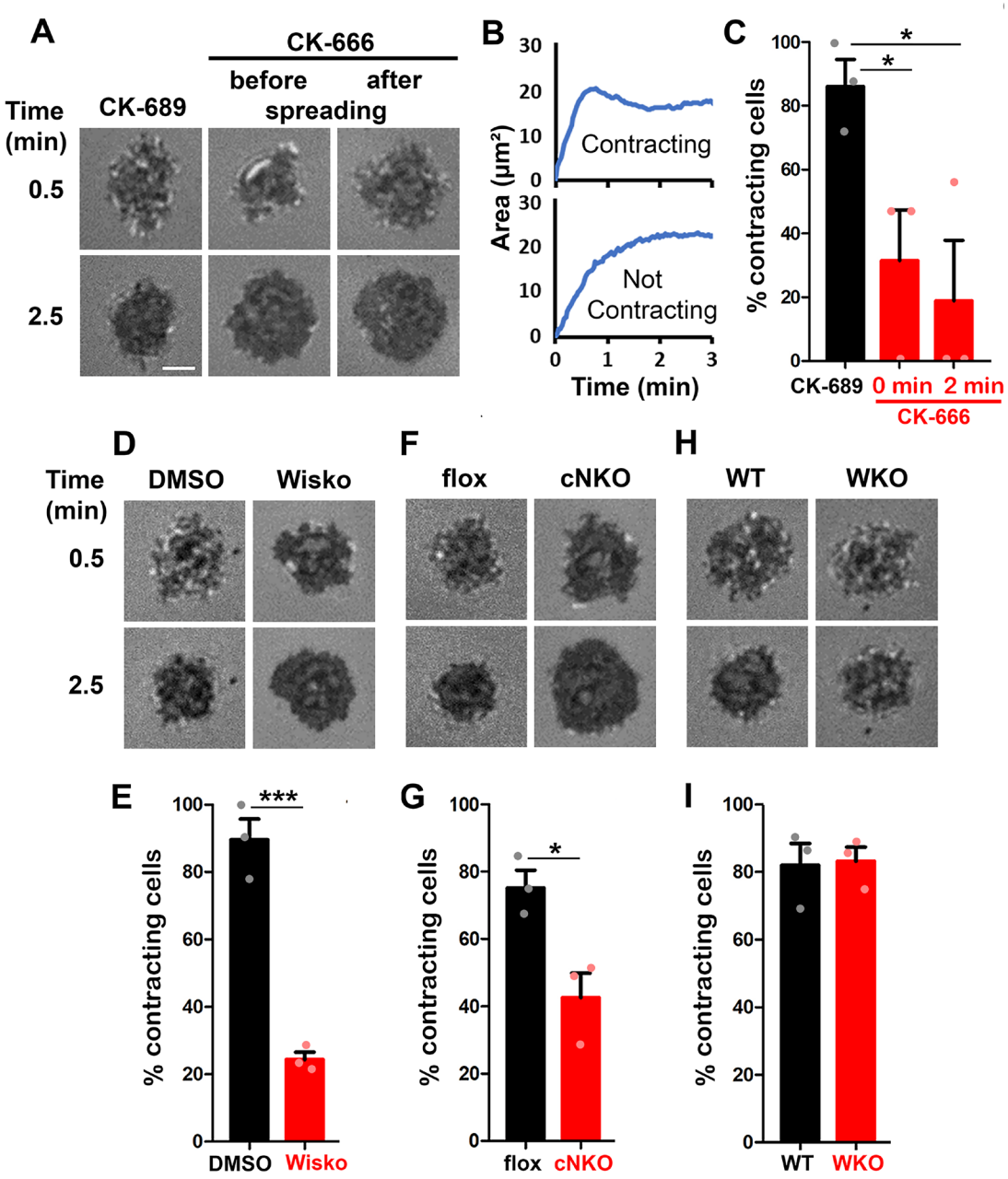

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Figure 2

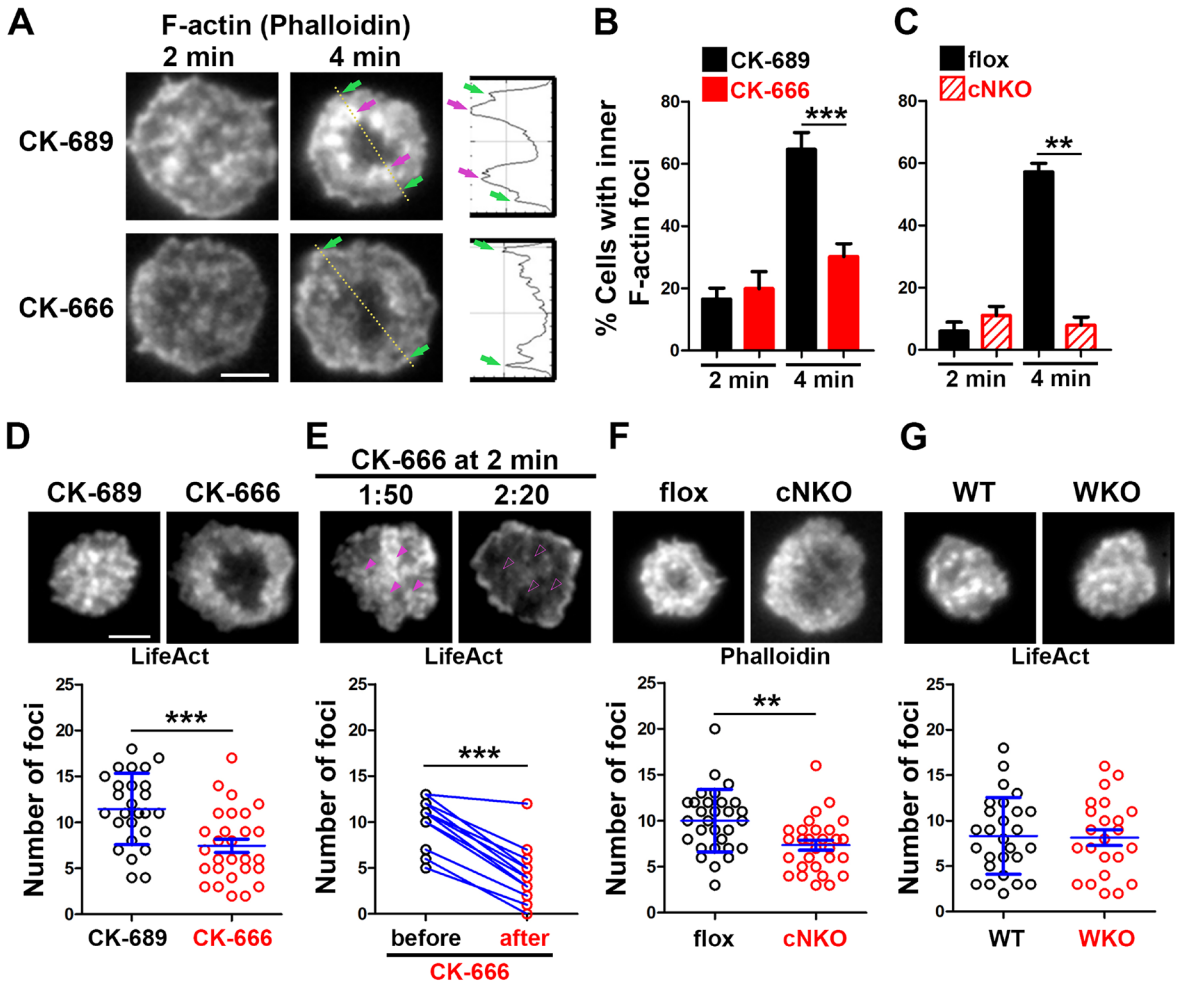

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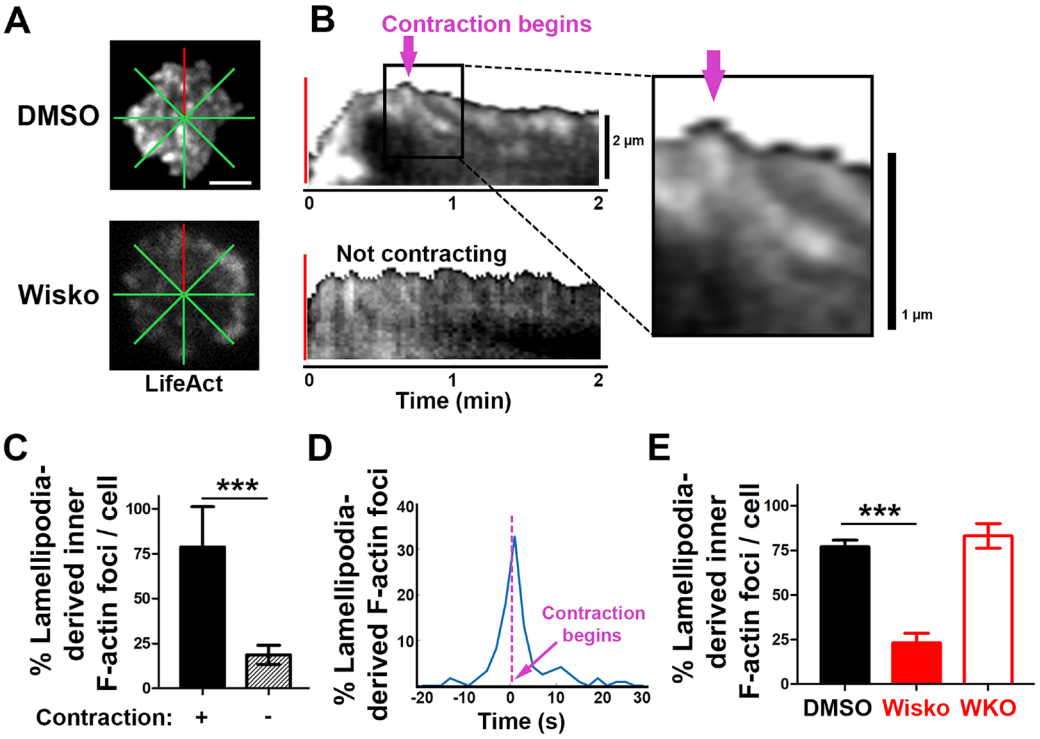
Figure 3



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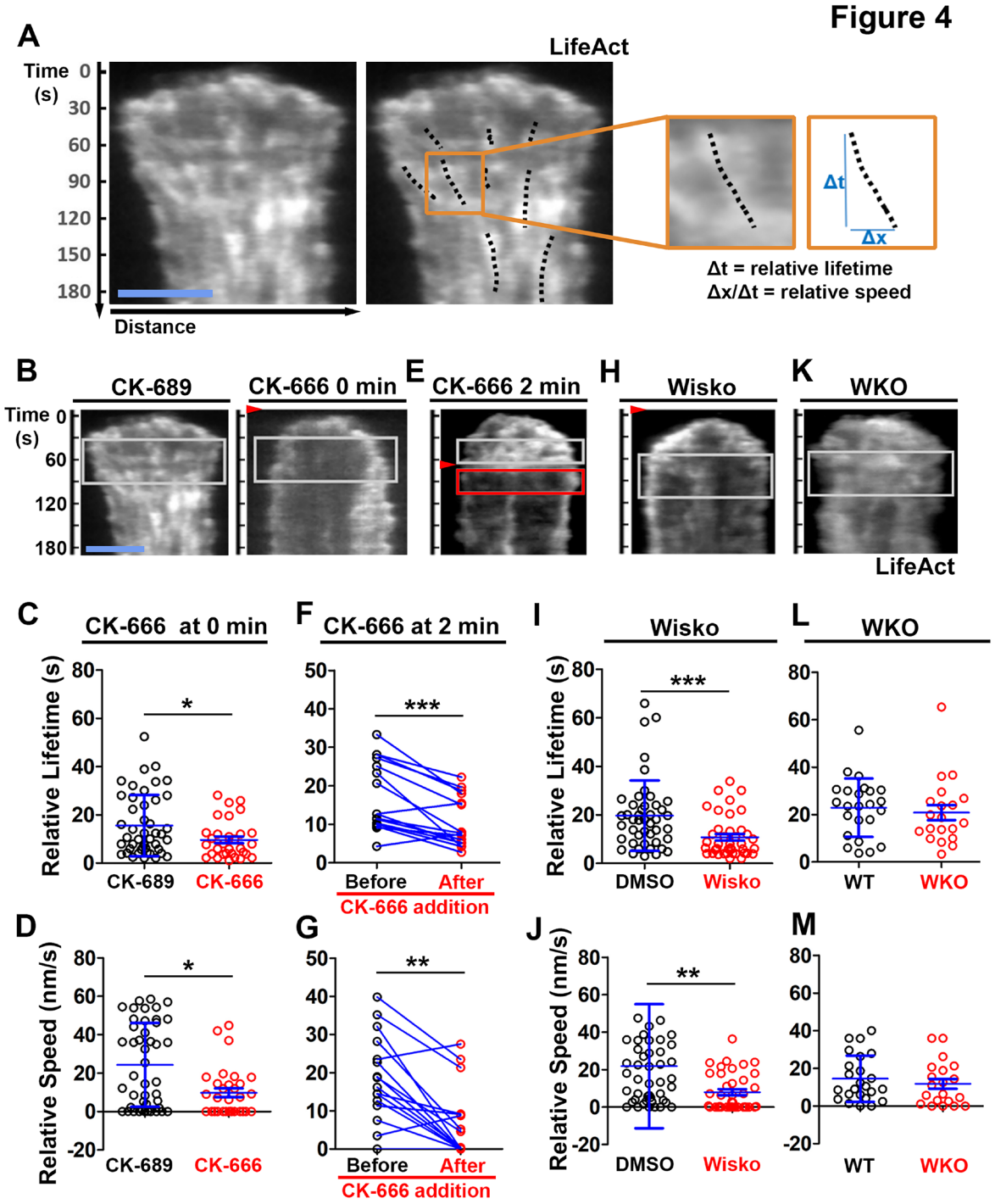
Figure 4
A
LifeAct
Time (s)
0
30
60
90
120
150
180
Distance
Δt
Δx
Δt = relative lifetime
Δx/Δt = relative speed
B
CK-689
CK-666 0 min
E
CK-666 2 min
H
Wisko
K
WKO
LifeAct
C
CK-666 at 0 min
Relative Lifetime (s)
CK-689
CK-666
F
CK-666 at 2 min
Before
After
CK-666 addition
I
Wisko
DMSO
Wisko
L
WKO
WT
WKO
D
Relative Speed (nm/s)
G
J
M

1375

1376

Figure 5

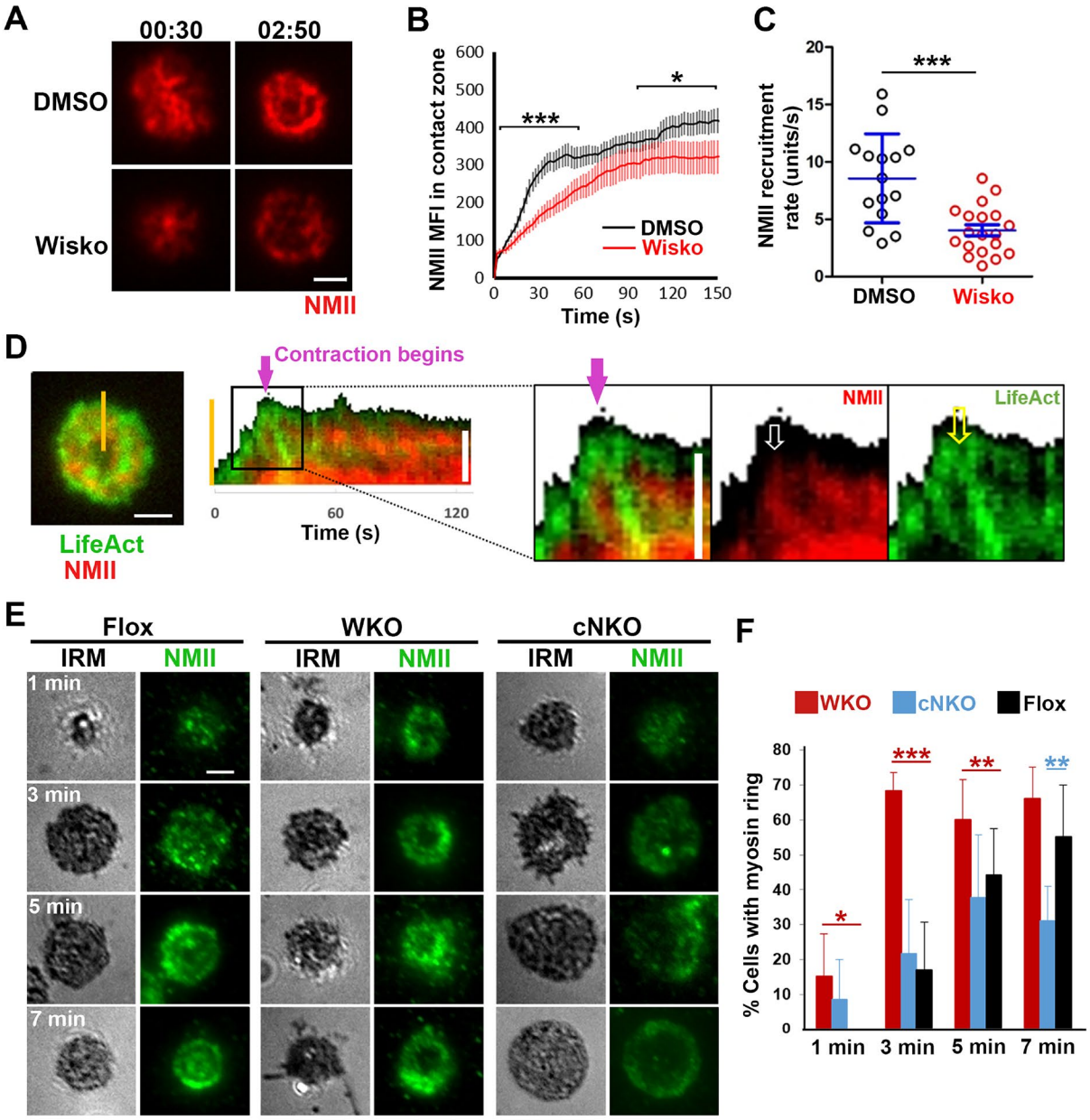

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Figure 6

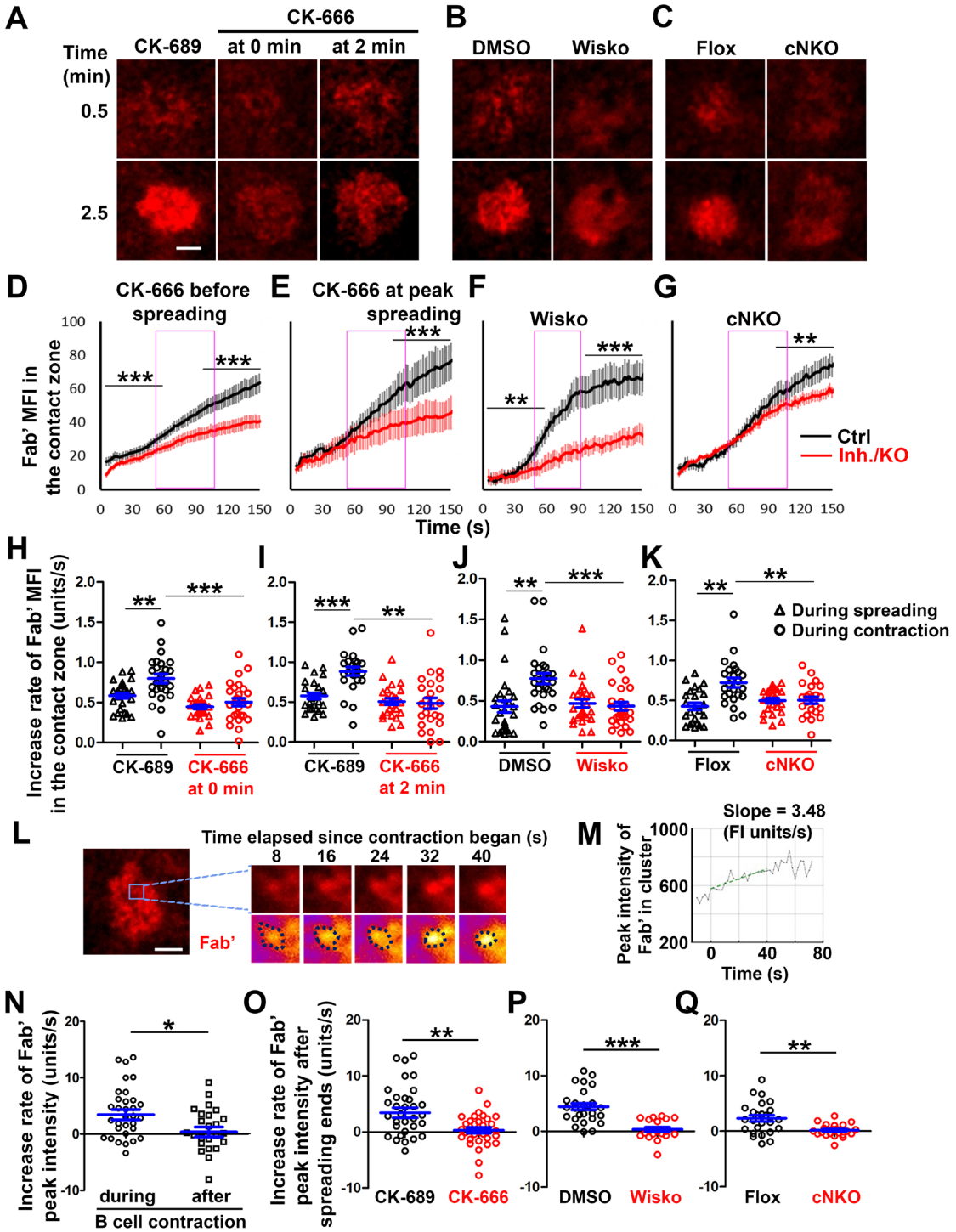
A
CK-666
Time (min)
CK-689
at 0 min
at 2 min
0.5
2.5
B
DMSO
Wisko
C
Flox
cNKO
D
CK-666 before spreading
E
CK-666 at peak spreading
F
Wisko
G
cNKO
Fab' MFI in the contact zone
Time (s)
Ctrl
Inh./KO
H
I
J
K
Increase rate of Fab' MFI in the contact zone (units/s)
CK-689
CK-666 at 0 min
CK-666 at 2 min
DMSO
Wisko
Flox
cNKO
During spreading
During contraction
L
Time elapsed since contraction began (s)
8
16
24
32
40
Fab'
M
Slope = 3.48 (FI units/s)
Peak intensity of Fab' in cluster
Time (s)
N
Increase rate of Fab' peak intensity (units/s)
during
after
B cell contraction
O
Increase rate of Fab' peak intensity after spreading ends (units/s)
CK-689
CK-666
P
DMSO
Wisko
Q
Flox
cNKO

Figure 7

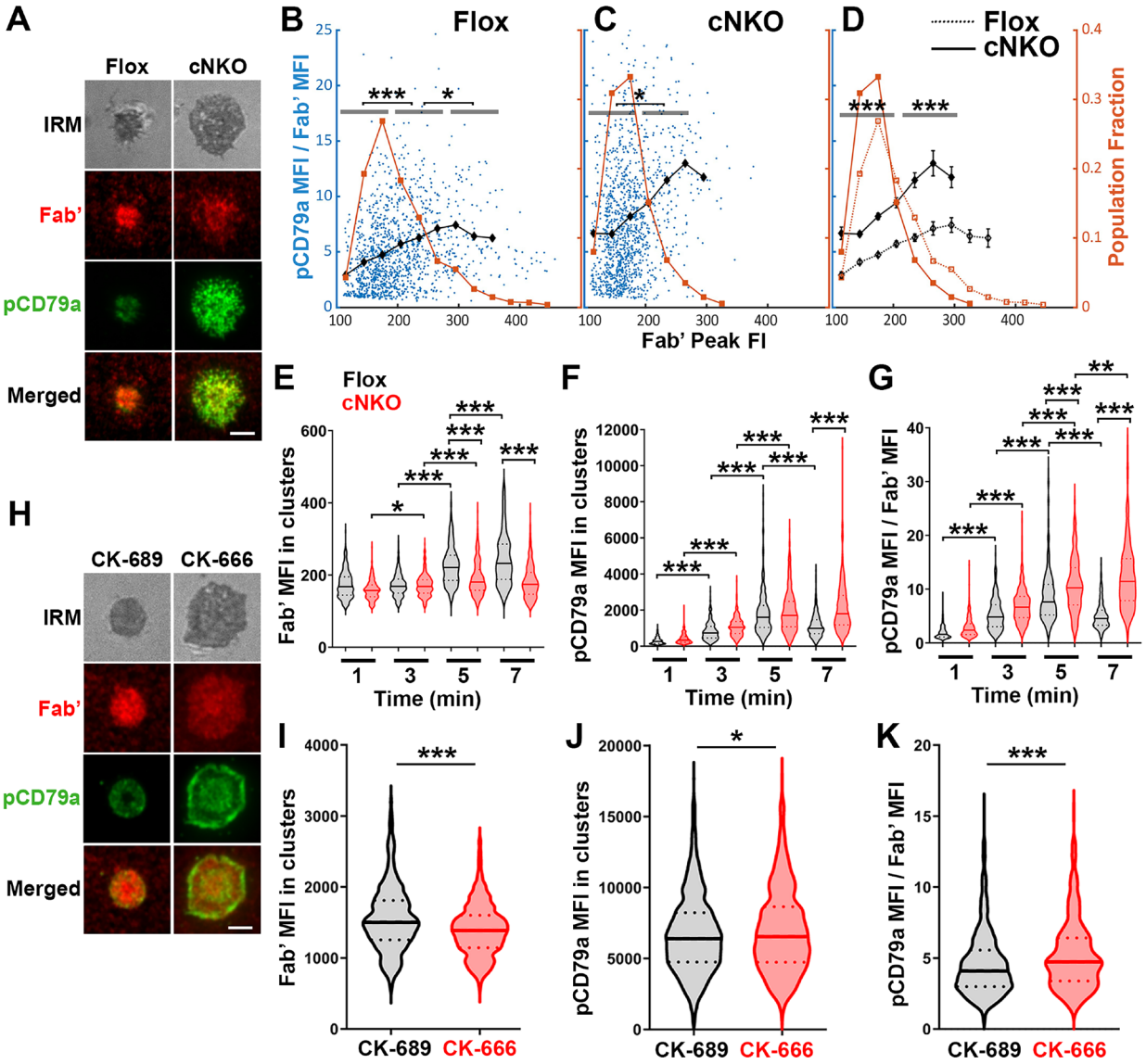

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Figure 8

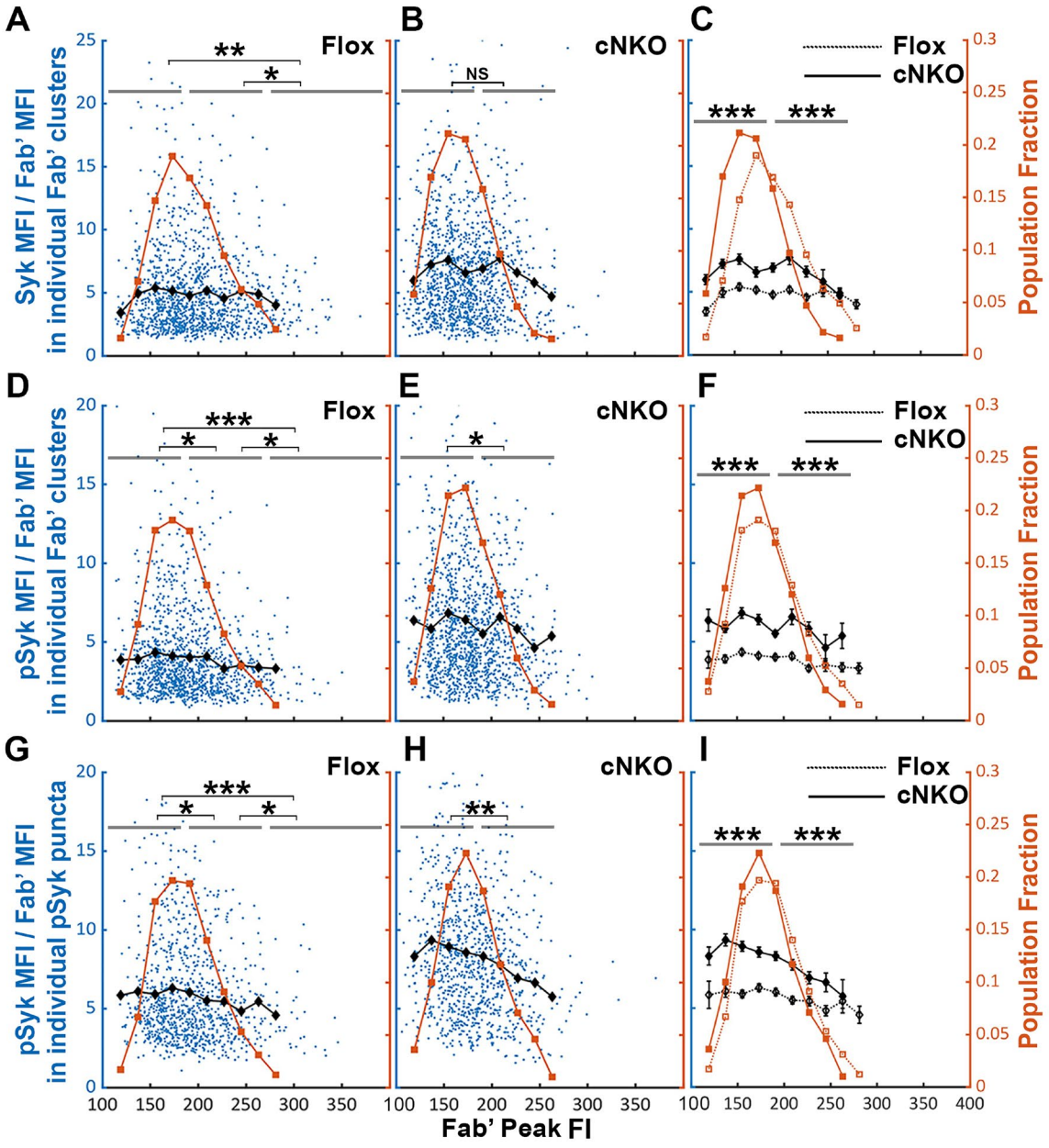

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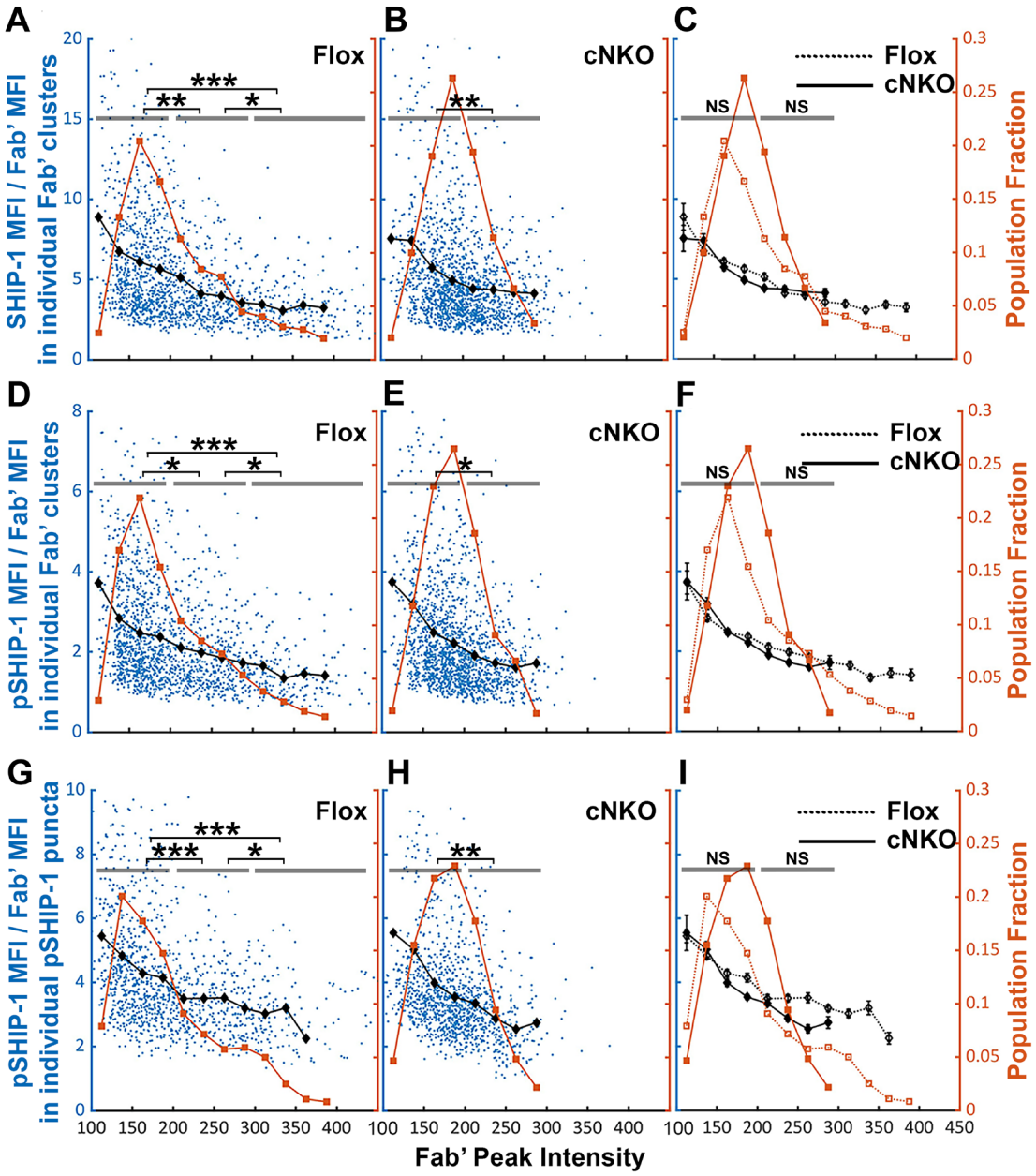
Figure 9



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