

1 **TITLE:** Coupling of protein condensates to ordered lipid domains determines functional membrane
2 organization

3
4 **SUMMARY:** Membrane-associated protein condensates couple to ordered membrane domains to determine
5 the functional organization of T-cell plasma membranes

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21 **ABSTRACT:**

22 During T-cell activation, the transmembrane adaptor Linker of Activation of T-cells (LAT) forms biomolecular
23 condensates with Grb2 and Sos1, facilitating signaling. LAT has also been associated with cholesterol-rich
24 condensed lipid domains. However, the potential coupling between protein condensation and lipid phase
25 separation and its role in organizing T-cell signaling were unknown. Here, we report that LAT/Grb2/Sos1
26 condensates reconstituted on model membranes can induce and template lipid domains, indicating strong
27 coupling between lipid- and protein-based phase separation. Correspondingly, activation of T-cells induces
28 protein condensates that associate with and stabilize raft-like membrane domains. Inversely, lipid domains
29 nucleate and stabilize LAT protein condensates in both reconstituted and living systems. This coupling of lipid
30 and protein assembly is functionally important, since uncoupling of lipid domains from cytoplasmic protein
31 condensates abrogates T-cell activation. Thus, thermodynamic coupling between protein condensates and
32 ordered lipid domains regulates the functional organization of living membranes.

33

34 **MAIN TEXT:**

35 Spatial compartmentalization is a ubiquitous feature of living systems, with all life on Earth compartmentalized
36 by lipid membranes. Membranes can be further laterally sub-compartmentalized by self-organizing lipid
37 domains. For example, the intrinsic capacity of sterols and tightly packing lipids to preferentially associate into
38 liquid-ordered phases can produce liquid domains in biomimetic systems (1-3), isolated plasma membranes
39 (PMs) (4, 5), and yeast vacuoles (6, 7). Micrometer-scale ordered membrane domains (or “rafts”) have not been
40 directly imaged in living mammalian cells, but accumulating evidence supports the involvement of nanometer
41 scale, dynamic lipid domains in signaling and trafficking (8).

42 A conceptually analogous organizing principle is cytoplasmic compartmentalization via biomolecular
43 condensates, which concentrate molecules in the absence of an encapsulating membrane (9, 10). Similar to lipid
44 self-organization (11), some condensates form through weak, multivalent interactions between
45 biomacromolecules, which drive liquid-liquid phase separation to produce dynamic mesoscale compartments
46 (10). Condensates have become extensively implicated in cellular functions, including embryonic development
47 (12), synaptic organization (13, 14), nuclear organization, gene regulation (15, 16), and signaling at the plasma
48 membrane (PM) (17-19). A prominent example of the latter is the PM module consisting of LAT and two
49 cytoplasmic adaptors Grb2 (growth factor receptor-bound) and Sos1 (son of sevenless), which links T-cell
50 immune receptor engagement with downstream pathways for activation (i.e. proliferation, cytokine secretion,
51 etc.) (20). Interactions between LAT/Grb2/Sos1 produce liquid condensates in reconstituted and living systems
52 (18, 19), which affect signaling by (a) concentrating reactants (18, 21); (b) excluding negative regulators, e.g.
53 the phosphatase CD45 (18); (c) coupling to cytoskeletal dynamics (19); and (d) kinetically proofreading
54 activation (21).

55 A central outstanding question concerns the biophysical and functional coupling between membrane lipid
56 domains and cytoplasmic condensates (22, 23). Lying near phase coexistence boundaries, both the PM (24, 25)
57 and cytoplasm (9) appear poised for large-scale structural rearrangements, such that phase separation of one

58 could produce significant responses in the other. We hypothesized that LAT could produce such coupling,
59 because it participates in both cytoplasmic condensates via its disordered cytoplasmic tail (18, 19) and ordered
60 lipid domains via its transmembrane helix (26-29). Through a combination of *in vitro* reconstitution and cellular
61 experiments, we show that LAT condensates are thermodynamically coupled with ordered membrane domains
62 during T-cell activation, providing direct evidence of convergence between phase separation of membrane lipids
63 and protein condensation. We further show that this coupling is functionally important, as uncoupling abrogates
64 cell activation downstream of T-cell receptor engagement.-These observations indicate that protein condensates
65 can regulate the functional organization of lipid membranes, and conversely, that lipid phase separation can
66 potentiate membrane protein condensation.

67

68 ***Mutual templating between protein condensates and membrane domains in vitro.*** Investigations of
69 membrane-associated protein condensates have often relied on planar lipid bilayers formed by fusion of
70 liposomes onto solid supports (18, 19). This method is not amenable for studying ordered membrane domains
71 because cholesterol-rich mixtures fuse poorly (30) and domain properties are severely affected by the solid
72 substrate (31, 32). To overcome these limitations, we generated phase-separating lipid multi-bilayers by spin-
73 coating mixtures of cholesterol, saturated phosphatidylcholine (DPPC), and unsaturated phosphatidylcholine
74 (DOPC), which form dynamic, temperature-reversible liquid-ordered (Lo) and -disordered (Ld) domains (33)
75 (Fig S1). The phosphorylated intracellular domain of LAT (pLAT) was bound to the topmost leaflet of the multi-
76 bilayer through lipids with a His-chelating headgroup (e.g. DSIDA, which concentrates in Lo domains) (34)
77 (Fig S2).

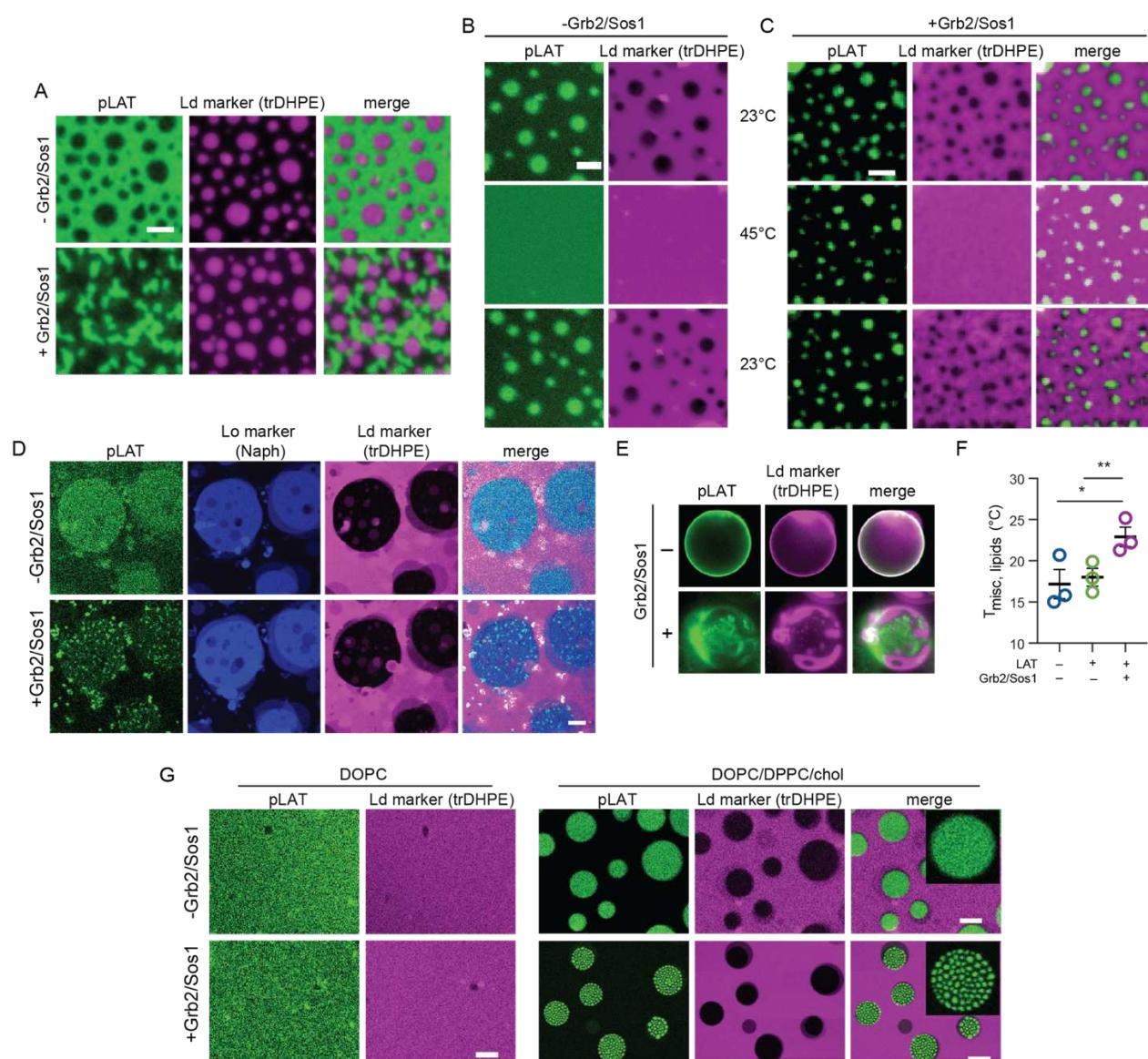
78 As previously shown (18, 19), pLAT coupled to a DOPC membrane phase separates to form micron-sized
79 condensates within minutes of introduction of Grb2+Sos1 (Fig S3) (throughout the text we invoke phase
80 separation for *in vitro* experiments where this mechanism of protein assembly has been clearly established and
81 the more generic ‘condensate’ for cellular results where the mechanism of assembly is less definitive). On more

82 biomimetic, phase-separated membranes, DSIDA-anchored pLAT was uniformly distributed in the Lo phase in
83 the absence of Grb2+Sos1 (Fig 1A, top), evidenced by its segregation from the Ld domain marker (TR-DHPE)
84 (see figure legends and Supplementary methods for lipid and protein concentrations). Addition of Grb2+Sos1
85 induced pLAT coalescence into large condensates that were exclusively overlying the Lo regions (Fig 1A,
86 bottom). These condensate-rich regions existed alongside condensate-poor Lo regions, consistent with three-
87 phase (condensate-rich Lo, condensate-poor Lo, and Ld) coexistence predicted by recent theory (35).

88 At this membrane composition, Lo domains comprise most of the bilayer area; to determine whether
89 condensates could recruit Lo domains, the abundance of unsaturated DOPC was increased to produce Ld-
90 majority bilayers (Fig 1B). In these, pLAT was confined to small Lo domains (Fig 1B, top) and addition of
91 Grb2+Sos1 did not affect its superficial appearance (Fig 1C, top). However, Grb2+Sos1 did induce LAT
92 condensation, as evidenced by the persistence of the pLAT clusters upon melting of the underlying membrane
93 domains at 45°C (Fig 1C, middle). This behavior contrasts with that of pLAT alone, which disperses together
94 with domains at 45°C (Fig 1B, middle). When membranes were again cooled below the miscibility transition
95 temperature (T_{misc}), Lo domains reappeared exclusively beneath the pLAT/Grb2/Sos1 condensates, but
96 randomly on bilayers containing only pLAT (Fig 1B-C, bottom). These observations were mirrored when pLAT
97 was instead attached to the Ld phase (Fig S4). Thus, lipid domains and LAT/Grb2/Sos1 condensates can
98 mutually template each other's localization and morphology.

99 We next tested how condensation would affect pLAT organization when monomers were not confined to a single
100 phase, but rather partitioned more like in isolated plasma membranes, where it is enriched by 20-50% in the raft
101 phase (26, 27, 36). To that end, pLAT was coupled to bilayers via a mixture of saturated (DP-NTA) and
102 unsaturated (DO-NTA) tail lipids. At a ratio of 2:1 DP-NTA:DO-NTA, pLAT partitioned to both phases with a
103 moderate preference for the Lo phase ($K_{p,Lo} = 2.1 \pm 0.4$), Fig 1D, S5A-B), as in natural systems. Strikingly,
104 LAT/Grb2/Sos1 condensates were observable exclusively in the Lo phase (Fig 1D). Even when condensates
105 formed in regions where Ld phases dominated, they appeared to induce the formation of small Lo domains (Fig

106 S5A). We hypothesize that this high affinity of condensates for Lo domains is driven by oligomerization: the
107 partitioning free energy of individual LAT monomers (into Lo) is additive, meaning that partition coefficients
108 multiply such that $K_{p, \text{oligomer}}$ goes as $K_{p, \text{monomer}}^N$, where $N = \text{oligomer number}$. Thus, even for weakly partitioning
109 monomers, this exponential dependence on oligomer number would dramatically enhance partitioning for large
110 oligomers like condensates.



111
112 **Figure 1 - *in vitro* coupling between protein condensates and membrane domains.** (A) pLAT is uniformly
113 distributed in Lo domains in a phase separated membrane when conjugated to DSIDA. Addition of Grb2/Sos1
114 produces protein condensates exclusively on top of Lo domains, i.e. completely excluded from Ld domains
115 (marked by trDHPE). (B) Lo domains are present at 23°C and disperse at 45°C. (C) Condensates recruit nascent
116 Lo domains. LAT/Grb2/Sos1 condensates form in small Lo domains in majority-Ld membranes. Lipid domains
117 are dissolved by increasing temperature above the miscibility transition threshold (~37°C for this composition,

118 see Supp Methods for detailed lipid compositions); pLAT condensates were not notably affected at these
119 temperatures. Cooling membranes below the transition temperature induces Lo domain formation exclusively
120 beneath protein condensates. (D) pLAT was bound to both phases by 2% DP-NTA and 1% DO-NTA in the
121 bilayer, yielding ~2-fold Lo enrichment of LAT. Adding Grb2/Sos1 produces condensates exclusively in the Lo
122 phase. (E) Protein condensates can induce lipid phase separation in GUVs. (F) Protein condensates enhance
123 lipid phase separation, evidenced by significantly increased T_{misc} (temperature at which 50% of GUVs show lipid
124 phase separation). (G) Lipid phase separation facilitates protein condensation. Low concentrations of pLAT (20
125 nM), Grb2 (100 nM), and Sos1 (100 nM) do not produce pLAT condensates on DOPC membrane (left panel),
126 whereas the same protein mixture undergoes Lo-confined condensation on phase separated membranes (right
127 panel). Scale bars are A/C/D/E/H = 5 μm , B = 2 μm .

128

129 ***Reciprocal stabilization between protein condensates and membrane domains.*** To investigate potential
130 thermodynamic coupling between protein phase separation and lipid phase separation, we measured the stability
131 of membrane domains in Giant Unilamellar Vesicles (GUVs). GUVs composed of DOPC, DPPC, and 40% chol
132 were not phase separated (homogeneously distributed fluorescent lipids and DSIDA-coupled pLAT) at 23°C
133 (Fig 1E). As on supported bilayers, addition of Grb2+Sos1 coalesced pLAT into condensates, but also induced
134 phase separation in the lipids, with complete segregation between an Ld phase marker (trDHPE) and protein
135 condensates (Fig 1E, bottom). These observations suggest that protein condensates can stabilize membrane
136 domains, consistent with reports that clustering of membrane components can induce domain formation in
137 cholesterol-containing membranes (25, 37, 38). To define the magnitude by which protein condensates
138 potentiated membrane phase separation, we measured condensate-dependent phase separation in GUVs (Fig 1F
139 and S6). At low temperatures, GUVs separate into coexisting Lo/Ld domains, which ‘melt’ (i.e. become miscible)
140 at higher temperatures. The temperature at which 50% of vesicles are phase separated is defined as the
141 miscibility transition temperature (T_{misc}), which in the absence of LAT was 17°C. pLAT conjugated to the Lo
142 phase via DSIDA did not affect the thermotropic phase transition, whereas condensates induced by Grb2+Sos1
143 increased T_{misc} to 22°C (Fig 1F and S6), indicating robust regulation of lipid phase transition by LAT
144 condensates, as also recently reported (39). Similar effects were observed when pLAT was initially coupled to

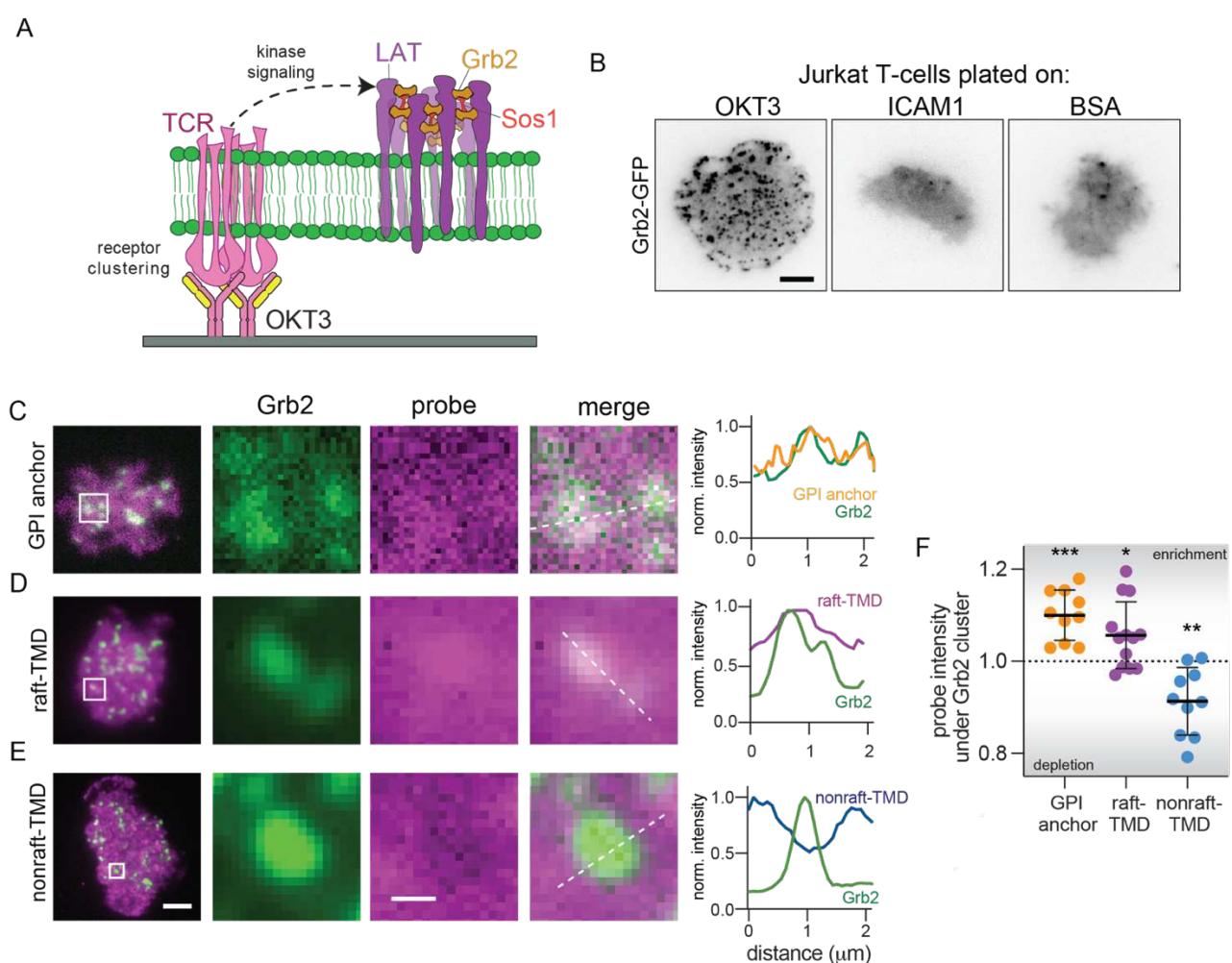
145 both phases via the mixture of saturated/unsaturated-lipid-NTA, as in Fig 1D (Fig S5B-C). Thus, protein
146 condensates enhance membrane phase separation, likely via clustering domain-associated components, as
147 previously observed for membrane-bound actin filaments (40-43), glycolipids (25), and other condensing
148 proteins (39, 44).

149 Inversely, we predicted that enrichment of LAT into ordered domains, as believed to occur in living cells (27,
150 29), would increase its propensity to form condensates. We tested this prediction by lowering the concentration
151 of LAT/Grb2/Sos1 to a regime where no condensates are observable on a uniform Ld membrane (Fig 1G, left).
152 The same protein mix applied to a phase-separated membrane robustly induced condensates confined within Lo
153 domains (Fig 1G, right, Fig S7). Thus, protein condensates and membrane domains can reciprocally stabilize
154 each other, i.e. they are thermodynamically coupled.

155
156 ***Grb2 condensates recruit ordered membrane environments in cells.*** We next investigated coupling of
157 LAT/Grb2/Sos1 condensates with membrane domains in living cells. To induce LAT condensates, Jurkat T-cells
158 were activated by seeding onto coverslips coated with OKT3, an α -CD3 antibody. Crosslinking of CD3 triggers
159 a signaling cascade that converges on LAT phosphorylation (45), which in turn recruits Grb2 and Sos1 to form
160 cytoplasmic condensates analogous to those observed *in vitro* (18) (Fig 2A-B). To evaluate the coupling of these
161 condensates with membrane domains, we employed genetically encoded probes that differentially partition to
162 membrane phases (26, 27, 36). As reporters of ordered membrane regions, we used the transmembrane domain
163 (TMD) α -helix of LAT (raft-TMD) (26, 27, 36) or a saturated lipid-anchor (GPI, glycophosphatidylinositol). To
164 mark disordered regions, we used a TMD consisting of 22 Leu residues (nonraft-TMD) (26, 27). All probes
165 were fused to fluorescent proteins for visualization and validated for expected raft affinity (Table S1). Grb2
166 condensates were coincident with regions of clear enrichment of the two raft probes (raft-TMD and GPI) and
167 were depleted of the nonraft-TMD (Fig 2C-F). The magnitudes of enrichment/depletion were consistent with
168 previous reports for enrichment of raft-associated markers around activated immune receptors by super-

169 resolution localization microscopy (46). Similar sorting of raft markers with Grb2 condensates was observed
170 by inducing condensates on non-activating surfaces (ICAM1) with the phosphatase inhibitor pervanadate (Fig
171 S8). Lipid fluorophores that selectively enrich in Lo or Ld domains were also sorted in accordance with raft-
172 enrichment at condensates. (Fig S9). Thus, activation-induced protein condensates are co-localized with raft-
173 like membrane environments in living Jurkat T-cells, in agreement with reconstitution experiments.

174



175

176 **Figure 2 - *in situ* recruitment of raft marker proteins to Grb2 condensates in cells.** (A) Schematic of LAT
177 condensate formation in activated Jurkats: T-cell receptor (TCR) clustering by OKT3 induces signaling that
178 leads to phosphorylation of LAT and multivalent recruitment of Grb2/Sos1 to produce liquid condensates. (B)
179 TIRF imaging of Grb2-rich condensates formed in OKT3-activated Jurkats, but not in non-activated cells (BSA-
180 or ICAM1-coated coverslip). (C-E) TIRF images of Grb2 condensates relative to GPI-GFP, raft-TMD, and
181 nonraft-TMD. Scale bars are 5 μm . Enlarged images of white squares demonstrate recruitment of raft-TMD /
182 GPI-GFP and exclusion of nonraft-TMD under Grb2 condensates. Scale bar is 0.5 μm . (right) line scans showing

183 probe enrichment under Grb2 condensates. (F) Quantification of relative enrichment in cells imaged at room
184 temperature (imaging at 37°C gave similar results, Fig S10). Each point represents the mean enrichment (>1)
185 or depletion (<1) of probes under Grb2 condensates relative to adjacent region for individual cells across 3
186 independent experiments. Each cell included >10 Grb2 condensates. ***p<0.001, **p<0.01, *p<0.05 for
187 difference from 1 (no enrichment/depletion) of means of individual cells.

188

189 ***Cooperative recruitment between Grb2 condensates and micron-sized membrane domains in live cells.*** Raft
190 probe enrichments were relatively subtle and only observable by using Grb2 condensates as fiducial markers,
191 putatively because the probes used all have relatively low selectivity for raft domains (26, 36). To more clearly
192 detect coupling, we relied on a previously described strategy to enhance probe raft affinity by oligomerization
193 (1, 36). To this end, we used antibodies to crosslink an endogenous T-cell raft component, the GPI-anchored
194 protein (GPI-AP) Thy1 (47). The efficacy of this approach was confirmed in cell-derived Giant Plasma
195 Membrane Vesicles (GPMVs), where primary antibody-crosslinked Thy1 had significantly higher raft phase
196 affinity than monomeric GPI-AP (Fig 3A-B). Further clustering of Thy1 by secondary antibodies produced large
197 clusters that precluded accurate raft affinity measurements; however, essentially all clusters were found in the
198 GPMV raft phase (Fig 3A).

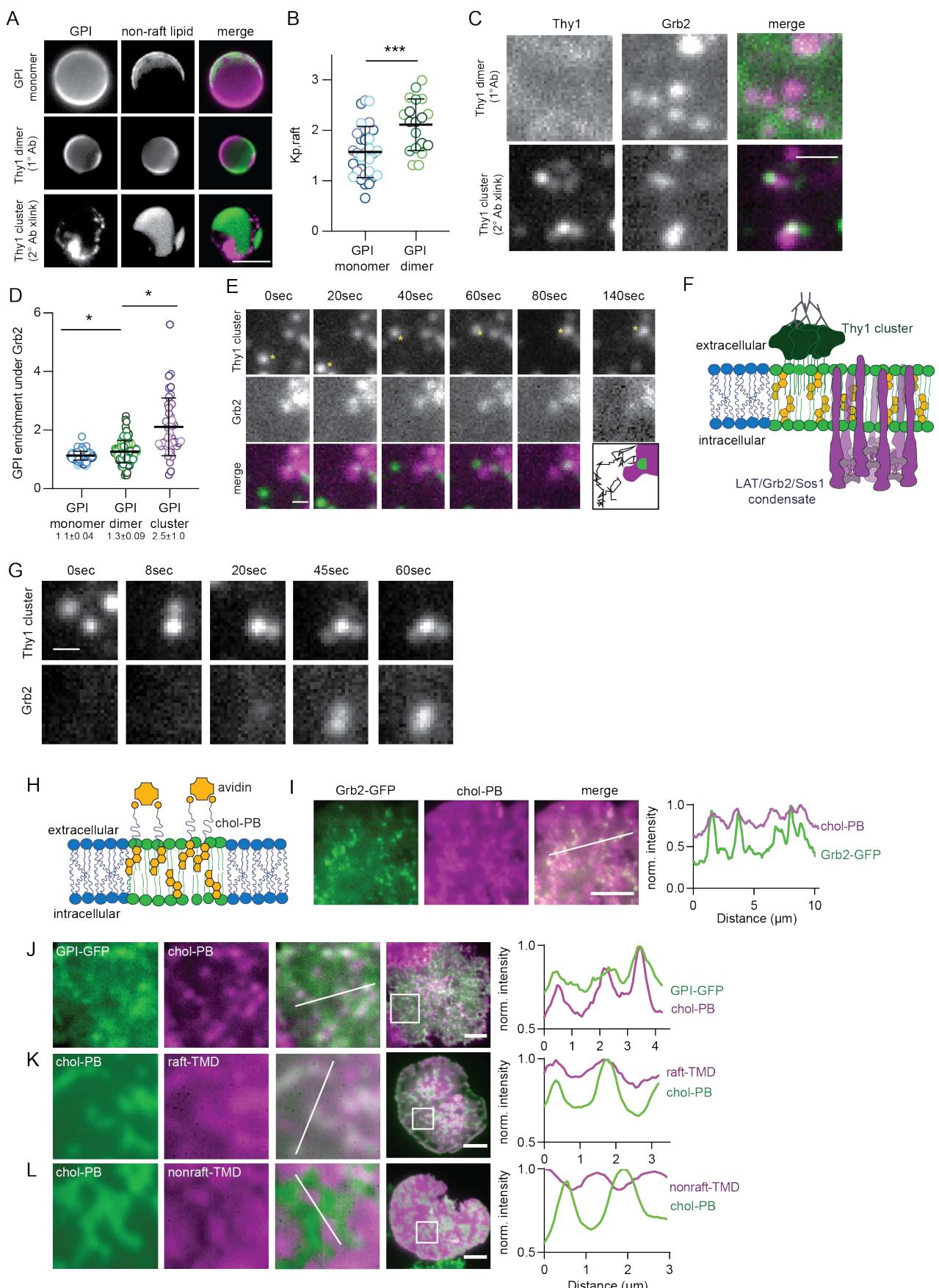
199 Consistent with our prediction that enhancing raft affinity would amplify colocalization with condensates,
200 dimerization of Thy1 by primary antibody significantly increased enrichment under Grb2 condensates generated
201 by TCR activation compared to monomeric GPI-AP (Fig 3C-D). Further crosslinking by secondary antibody
202 produced Thy1 clusters that were strongly enriched in areas juxtaposed to Grb2 condensates (Fig 3C-D). In live
203 cells, these Thy1 clusters rapidly diffused on the extracellular surface of the PM (especially early in the
204 activation time course) and were often observed stopping underneath Grb2 condensates (Fig 3E, Supplementary
205 Movie 1). We conclude that the multiplicity of saturated acyl chains (previously estimated at ~20 proteins/cluster
206 (48)) in these GPI-AP clusters enhances their affinity for transbilayer ordered membrane domains (1), which
207 tend to be associated with, and immobilized by, Grb2 condensates (Fig 3F). The recruitment of ordered domains

208 by LAT/Grb2/Sos1 condensates is directly analogous to our observations in reconstituted membranes (Fig 1C).
209 Correspondingly, after TCR activation we also observed that membrane regions marked by Thy1 clusters could
210 nucleate Grb2 condensate formation (Fig 3G, Supplementary Movie 2). We hypothesize that these nascent
211 condensates were induced by enrichment of LAT in membrane domains (26, 27) (as in the reconstitution
212 experiment in Fig 1G), suggesting that cytoplasmic conditions are poised such that protein condensation can be
213 induced by localized protein concentration via lipid domains.

214 Following a similar strategy of enhancing raft affinity via oligomerization, we evaluated the effects of
215 crosslinking the prototypical raft lipid, cholesterol (8). Cholesterol was modified with a biotinylated PEG-spacer
216 (Fig 3H), relying on a strategy validated in design of other raft probes (49, 50). Labeling and crosslinking with
217 fluorescent avidin (Av647/Av488) confirmed that oligomerized cholesterol-PEG-biotin (chol-PB) enriches in
218 the ordered phase of GPMVs more strongly than monomeric chol-PEG-FITC (Table S1 and Fig S11). In PMs
219 of activated Jurkat T-cells, avidin-labeled chol-PB enriched in micron-sized domains (Fig 3I-L). Strikingly,
220 Grb2 and LAT condensates were found exclusively overlying these cholesterol-rich regions (Fig 3I, S12), as
221 small foci distributed throughout relatively larger membrane domains.

222 Direct microscopic observations of cholesterol-rich domains in live cells were surprising, as raft domains have
223 largely evaded unambiguous microscopic detection (8). These domains were initially observed by TIRF but
224 were also visible by epifluorescence (Fig S13) and confocal (Fig S14) imaging and were not membrane
225 accumulations or large invaginations/deformations (Fig S13-15). Most importantly, chol-PB domains
226 selectively recruited raft markers, including GPI-GFP (Fig 3J), glycolipid-binding cholera toxin B (CTxB) (Fig
227 S16A), and Thy1 clusters (Fig S17), all of which enrich in rafts due to saturated acyl chains (Table S1). Chol-
228 PB domains also enriched raft-TMD and excluded nonraft-TMD (Fig 3K-L). Another non-raft TMD, from the
229 immune cell phosphatase CD45 (Table S1), was also robustly excluded from chol-PB domains (Fig S16B).
230 Broadly similar selective domains were also observed in Jurkat cells plated on fluid synthetic supported bilayers
231 (Fig S18). Thus, mesoscopic raft domains are strongly coupled to Grb2/LAT condensates, revealing cooperative

232 templating between protein condensates and membrane domains in cells, directly analogous to reconstituted
 233 systems (Fig 1A-D).



234
 235 **Figure 3 – Mutual templating between Grb2 condensates and raft-like membrane domains. (A)** Monomeric

236 GPI AP (GPI-GFP) is subtly enriched in raft phase of phase separated GPMVs. Dimerization of the endogenous
237 GPI-AP Thy1 increases raft preference. Further oligomerization of Thy1 via secondary antibodies leads to
238 exclusively raft-associated clusters. Scale bar is 5 μ m. (B) Quantification of the partitioning coefficient ($K_{p,raft}$)
239 showed that antibody dimerized Thy1 has a higher raft affinity than non-crosslinked GPI-AP. (C) Recruitment of
240 oligomerized Thy1 to Grb2 condensates. Top: overlapping of Grb2 condensates with primary antibody dimerized
241 Thy1. Bottom: overlapping of Grb2 condensates with Thy1 clusters induced by secondary antibody crosslinking.
242 Scale bar is 1 μ m. (D) Quantification of GPI-AP enrichment under Grb2 condensates enhanced by antibody
243 crosslinking. Data points represent individual cells from at least three independent experiments; *p<0.05. (E)
244 Time-lapse of live cell imaging showing capture/immobilization of a Thy1 cluster by Grb2 condensate. Scale bar
245 is 0.5 μ m. (F) Schematic of coupling between clustered Thy1 and LAT/Grb2/Sos1 condensate mediated by
246 ordered membrane domain. (G) Time series showing formation of Grb2 condensate above an immobilized Thy1
247 cluster. Scale bar = 1 μ m. (H) Schematic of cholesterol-PB oligomerization and labeling. Cells are labeled with
248 chol-PB, then fluorescent avidin, before plating on OKT3-coated coverslips. (I) TIRF images of Grb2
249 condensates overlying micron-sized cholesterol-rich domains. Plot shows normalized fluorescence intensity
250 along the line trace shown in white. (J-L) TIRF imaging reveals the colocalization of cholesterol-rich domains
251 with raft markers GPI-GFP and raft-TMD, and exclusion of nonraft-TMD from chol-rich domains. Scale bars are
252 5 μ m. Plots show normalized fluorescence intensity along the line traces.

253

254 **Condensates stabilize cell membrane domains.** Reconstituted condensates stabilize membrane domains *in vitro*
255 (Fig 1E-F, S6). We hypothesized that the microscopic raft domains revealed by chol-PB were potentiated by
256 condensation of raft-associated proteins (i.e. LAT) induced by T-cell activation. Consistently, micron-scale
257 cholesterol-rich patterns were only observable in activated Jurkat T-cells (i.e. in presence of condensates),
258 whereas cells plated on a non-activating (ICAM1-coated) surface were more laterally homogeneous (Fig S19A).
259 The distribution of pixel intensities confirmed that OKT3 patterns were bimodal whereas ICAM1 were normally
260 distributed (Fig S19B); quantification by coefficient of variation (CoV) of chol-PB intensity revealed significant
261 differences between cell populations (Fig S19B-inset), and there were robust spatial autocorrelations in chol-
262 PB intensity in activated Jurkat T-cells, compared to smaller scale and amplitude autocorrelations on ICAM1
263 (Fig S20). Thus, membrane-associated condensates reorganize the PM in live cells, enhancing the propensity to

264 form large cholesterol-rich lipid domains (Fig 4H).

265 We further explored this effect by analyzing the dynamics of antibody-crosslinked Thy1 clusters as reporters of
266 membrane organization, with immobile clusters reflecting the presence of underlying ordered membrane
267 domains (Fig 3E-G, as previously described (48, 51)). Thy1 clusters were notably less dynamic in the presence
268 of LAT/Grb2 condensates (i.e. in Jurkat T-cells activated by OKT3), stalling frequently and for long periods,
269 compared to more mobile clusters in the absence of condensates on ICAM1-coated coverslips (Fig 4A, S21).
270 The majority of Thy1 clusters in activated Jurkats were stalled for >80% of any individual track (minimum track
271 length = 10 sec), whereas most clusters on ICAM1-plated cells were either mobile throughout the tracking or
272 exhibited short, intermittent stalls (Fig 4B, S21,-Supplementary Movies 3&4). These observations suggest that
273 induction of condensates stabilized ordered membrane domains analogous to previous demonstrations of
274 crosslinking-induced domains in model membranes (25, 37, 46) or actin-asters associated with GPI-rich
275 domains in cells (43). This induction and recruitment of membrane domains by LAT/Grb2/Sos1 condensates
276 may explain previous reports of selective enrichment of membrane raft markers around activated immune
277 receptors (46, 52, 53).

278

279 **Cell membrane domains potentiate condensates.** Since lipid domains and protein condensates are coupled in
280 purified systems and living cells, we next tested whether perturbing membrane domains would affect
281 LAT/Grb2/Sos1 condensates in activated T-cells. Membrane domains can be disrupted by inhibiting synthesis
282 of raft-forming lipids using a combination of myriocin to inhibit sphingolipid synthesis and Zaragozic acid (ZA)
283 to inhibit cholesterol synthesis. This treatment disrupts raft-like nanodomains in cells (54, 55) and inhibits
284 membrane phase separation in isolated GPMVs (56) without off-target effects on cellular phospholipid
285 composition (54). Treatment with myriocin+ZA significantly reduced condensate density in Jurkat T-cells
286 activated by OKT3 (Fig 4C).

287 Conversely, crosslinking of raft components has been shown to promote and stabilize membrane domains (25,

288 48, 57) (Fig 1E-F). Consistently, crosslinking Thy1 with antibodies stabilized membrane domains in GPMV
289 experiments, indicated by increased lipid phase separation temperature (T_{misc}) (Fig 4D, Fig 4H right).
290 Remarkably, stabilizing cell membrane domains without any other activating stimulus was sufficient to produce
291 cytoplasmic protein condensates. Plating Jurkat cells labeled with anti-Thy1 antibodies onto 2°Ab coated
292 coverslips to crosslink the GPI-anchored protein induced Grb2 condensate formation (Fig 4E). No condensates
293 were observed in absence of GPI crosslinking (on 1°Ab-coated coverslips) nor in the absence of LAT (i.e. LAT-
294 deficient Jurkat line, JCam2.5). Condensates induced by GPI-AP crosslinking were able to activate MAPK
295 signaling, revealed by immunostaining against phosphorylated ERK (pERK) (Fig 4F-G). These results suggest
296 that stabilizing membrane domains can induce condensate formation and downstream T-cell activation, even in
297 the absence TCR, mediated by LAT coupling between membrane domains and condensates. Several groups
298 have previously reported that crosslinking GPI-APs or raft glycolipids can activate T-cells without TCR ligation
299 (39, 48, 58, 59). Here, we show that LAT (as a transmembrane link) is necessary and suggest a mechanism for
300 these puzzling findings, i.e. that stabilizing rafts potentiates protein condensates (Fig 4H) that facilitate T-cell
301 signaling (21).

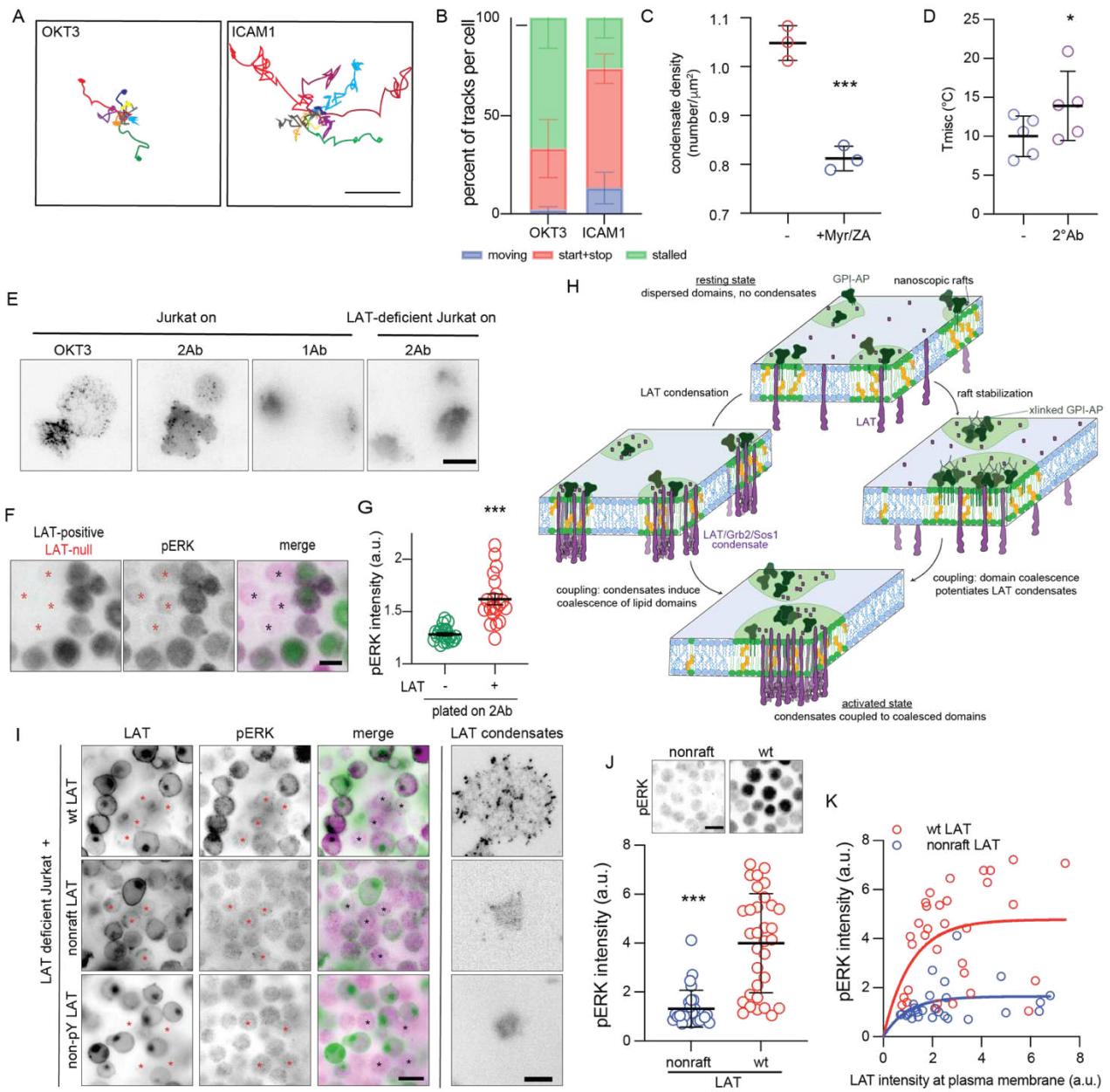
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303 ***Uncoupling of membrane domains from protein condensates abrogates T-cell activation.*** Our observations
304 suggest that coupling to membrane domains underlies the formation and location of protein condensates.
305 Condensates have been previously implicated in T-cell signaling and activation (18, 21). Therefore, we
306 hypothesized that coupling between domains and condensates might be important in T-cell function. We tested
307 this hypothesis by uncoupling domains from condensates via two mutations of their critical linker LAT: (1)
308 replacing the native LAT TMD with non-raft TMD (22 Leu)(26, 27)(Table S1) to create ‘nonraft LAT’ that still
309 interacts with Grb2 but does not partition to raft domains or (2) mutating LAT’s 3 Tyr residues to Ala to create
310 non-pY LAT, which can partition to rafts but cannot interact with Grb2. Fluorescence-tagged versions of these
311 mutants (or wild-type LAT) were stably introduced into LAT-deficient Jurkat T-cells, and their capacity to

312 facilitate T-cell activation was monitored by immunostaining against phosphorylated ERK (pERK) after plating
313 on OKT3. JCam2.5 cells stably re-expressing wt-LAT showed strong pERK staining compared to the LAT-
314 deficient negative controls (selected negative cells marked by asterisks in Fig 4I). In contrast, neither nonraft-
315 LAT nor non-pY LAT showed pERK activation above LAT-deficient cells (Fig 4I and S22). Consistently, LAT-
316 containing condensates were only observed in wt-LAT cells (Fig 4I, right). Cells expressing wt-LAT had ~4-
317 fold higher pERK signal than those expressing nonraft-LAT (Fig 4J). Importantly, these results were
318 independent of PM LAT expression (Fig 4K) and even LAT phosphorylation (Fig S23), all of which were similar
319 between wt-LAT and nonraft-LAT expressing cells. Thus, we conclude that coupling of membrane domains and
320 cytoplasmic condensates via LAT is essential for activating signaling downstream of TCR ligation.

321

322 Synthesizing these observations, we find strong coupling of cytoplasmic protein condensates with lateral
323 membrane domains in reconstituted models and in living Jurkat T-cells (Fig 4H). LAT condensates recruit
324 specific lipids and proteins to their adjacent membrane by interactions between the LAT transmembrane domain
325 and raft components (26, 27). LAT condensation stabilizes microscopic membrane domains (Fig 1E, 3H-L, 4A-
326 B, S16, S19, 4H left), consistent with prevailing models of mammalian PMs containing dynamic nanodomains
327 poised for coalescence by external inputs (60). Correspondingly, raft domains can nucleate and potentiate
328 cytoplasmic condensates (Fig 1G, 3G, 4H right). Uncoupling LAT from rafts abrogates condensation and ERK
329 activation downstream of TCR ligation. Therefore, we conclude that protein phase separation is
330 thermodynamically and mechanistically coupled to lateral phase separation of membrane lipids to regulate the
331 functional organization underlying immune cell signal transduction.



332

333 **Fig 4 – Protein condensates potentiate membrane domains.** (A) 8 representative >10 sec tracks of Thy1
 334 clusters from a cell plated on either OKT3 (left) or ICAM1 (right). (B) Trajectories of 2° antibody-crosslinked
 335 Thy1 clusters were calculated through single particle tracking (30-100 tracks/cell; five cells/condition). Each
 336 track was classified as either mobile (<20% of the track time stalled), start-stop (21-79% stalled), or stalled (≥80%
 337 stalled). Shown are percentages (mean +/- st.dev.) of each class of track across individual cells. (C) Inhibition
 338 of raft-forming lipids perturbs Grb2 condensate formation. Grb2-mScarlet-expressing Jurkat T-cells were
 339 incubated with 25 μM myriocin and 5 μM Zaragozic acid for 3 days to deplete cells of lipids necessary for raft
 340 formation (e.g. sphingomyelin and cholesterol, respectively) as previously described (54). Cells were then plated
 341 on OKT3-coated coverslips for 10 minutes at 37°C, fixed, then imaged via TIRF microscopy. Shown is
 342 condensate density for 3 experiments with >5 cells/experiments. ***p<0.001 for differences between
 343 experiments. (D) T_{misc} is higher in GPMVs with 2°-antibody-crosslinked Thy1, indicating increased raft stability.
 344 (E) Grb2-scarlet transfected cells imaged via TIRF 20 min after plating. 2°Ab-coated coverslips crosslink 1°Ab-

345 labeled endogenous Thy1, which is sufficient to induce condensates in Jurkat T-cells, but not in LAT-deficient
346 cells (JCam2.5). Scale bar = 10 μ m. (F) LAT-deficient cells (marked with asterisks) were mixed with LAT-positive
347 Jurkat T-cells (labeled with Grb2-mScarlet) and cell activation induced by Thy1 crosslinking by 2°Ab-coated
348 coverslips was examined by pERK immunostaining. Thy1 crosslinking induced pERK, but not in LAT-deficient
349 cells. Scale bar = 10 μ m. LAT-deficient cells served as internal negative control for IF staining and imaging. (G)
350 Quantification of pERK activation by Thy1 clustering in LAT-deficient JCam2.5 cells (green) versus LAT-
351 expressing Jurkat cells (red). (H) Schematic model of condensate-domain coupling. (left) Clustering LAT by
352 intracellular condensates enhances LAT recruitment into membrane domains and promotes their coalescence.
353 (right) Clustering raft components stabilizes membrane domains to potentiate LAT condensation. (bottom) Both
354 processes result in an activated state where membrane-associated condensates template and stabilize raft-like
355 membrane domains. (I) Coupling of protein condensates and lipid domains through LAT is necessary for pERK
356 activation. LAT-deficient JCam2.5 cells were mixed with cells stably repleted with either wt, non-raft, or non-pY
357 LAT and plated on OKT3 for 10 mins. Only wt-LAT repleted cells formed LAT condensates and had pERK
358 staining above LAT-deficient negative controls (selected LAT-deficient cells marked with asterisks). Scale bar =
359 15 μ m (left) and 5 μ m (right). (J) Quantification of pERK in raft versus non-raft LAT repleted cells. Scale bar =
360 15 μ m. (J) pERK intensity as a function on LAT on the PM. Mean \pm SD of individual cell quantifications shown
361 in F, I-J for one representative experiment. Three independent experiments were performed with similar results.

362

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371

372 **References**

373
374 1. K. Simons, W. L. Vaz, Model systems, lipid rafts, and cell membranes. *Annu Rev Biophys Biomol Struct* **33**, 269-

- 375 295 (2004).
- 376 2. E. London, How principles of domain formation in model membranes may explain ambiguities concerning lipid
- 377 raft formation in cells. *Biochim Biophys Acta* **1746**, 203-220 (2005).
- 378 3. S. L. Veatch, S. L. Keller, Seeing spots: complex phase behavior in simple membranes. *Biochim Biophys Acta*
- 379 **1746**, 172-185 (2005).
- 380 4. E. Sezgin *et al.*, Elucidating membrane structure and protein behavior using giant plasma membrane vesicles. *Nat*
- 381 *Protoc* **7**, 1042-1051 (2012).
- 382 5. K. R. Levental, I. Levental, Giant plasma membrane vesicles: models for understanding membrane organization.
- 383 *Current topics in membranes* **75**, 25-57 (2015).
- 384 6. S. P. Rayermann, G. E. Rayermann, C. E. Cornell, A. J. Merz, S. L. Keller, Hallmarks of reversible separation of
- 385 living, unperturbed cell membranes into two liquid phases. *Biophys J* **113**, 2425-2432 (2017).
- 386 7. A. Toulmay, W. A. Prinz, Direct imaging reveals stable, micrometer-scale lipid domains that segregate proteins in
- 387 live cells. *J Cell Biol* **202**, 35-44 (2013).
- 388 8. I. Levental, K. R. Levental, F. A. Heberle, Lipid rafts: controversies resolved, mysteries remain. *Trends in cell*
- 389 *biology* **30**, 341-353 (2020).
- 390 9. Y. Shin, C. P. Brangwynne, Liquid phase condensation in cell physiology and disease. *Science* **357**, (2017).
- 391 10. S. F. Banani, H. O. Lee, A. A. Hyman, M. K. Rosen, Biomolecular condensates: organizers of cellular biochemistry.
- 392 *Nat Rev Mol Cell Biol* **18**, 285-298 (2017).
- 393 11. A. A. Hyman, K. Simons, Cell biology. Beyond oil and water--phase transitions in cells. *Science* **337**, 1047-1049
- 394 (2012).
- 395 12. C. P. Brangwynne *et al.*, Germline P granules are liquid droplets that localize by controlled
- 396 dissolution/condensation. *Science* **324**, 1729-1732 (2009).
- 397 13. M. Zeng *et al.*, Reconstituted Postsynaptic Density as a Molecular Platform for Understanding Synapse Formation
- 398 and Plasticity. *Cell* **174**, 1172-1187 e1116 (2018).
- 399 14. D. Milovanovic, Y. Wu, X. Bian, P. De Camilli, A liquid phase of synapsin and lipid vesicles. *Science* **361**, 604-
- 400 607 (2018).
- 401 15. Y. Shin *et al.*, Liquid Nuclear Condensates Mechanically Sense and Restructure the Genome. *Cell* **175**, 1481-1491
- 402 e1413 (2018).
- 403 16. B. A. Gibson *et al.*, Organization of Chromatin by Intrinsic and Regulated Phase Separation. *Cell* **179**, 470-484
- 404 e421 (2019).
- 405 17. S. Banjade, M. K. Rosen, Phase transitions of multivalent proteins can promote clustering of membrane receptors.
- 406 *eLife* **3**, (2014).
- 407 18. X. Su *et al.*, Phase separation of signaling molecules promotes T cell receptor signal transduction. *Science* **352**,
- 408 595-599 (2016).
- 409 19. J. A. Ditlev *et al.*, A composition-dependent molecular clutch between T cell signaling condensates and actin.
- 410 *eLife* **8**, (2019).
- 411 20. W. Zhang, J. Sloan-Lancaster, J. Kitchen, R. P. Trible, L. E. Samelson, LAT: the ZAP-70 tyrosine kinase substrate
- 412 that links T cell receptor to cellular activation. *Cell* **92**, 83-92 (1998).
- 413 21. W. Y. C. Huang *et al.*, A molecular assembly phase transition and kinetic proofreading modulate Ras activation
- 414 by SOS. *Science* **363**, 1098-1103 (2019).
- 415 22. W. T. Snead, A. S. Gladfelter, The Control Centers of Biomolecular Phase Separation: How Membrane Surfaces,
- 416 PTMs, and Active Processes Regulate Condensation. *Molecular cell* **76**, 295-305 (2019).
- 417 23. M. L. Dustin, J. Muller, CELL SIGNALING. Liquidity in immune cell signaling. *Science* **352**, 516-517 (2016).
- 418 24. D. Lingwood, J. Ries, P. Schwille, K. Simons, Plasma membranes are poised for activation of raft phase
- 419 coalescence at physiological temperature. *Proc Natl Acad Sci U S A* **105**, 10005-10010 (2008).
- 420 25. A. T. Hammond *et al.*, Crosslinking a lipid raft component triggers liquid ordered-liquid disordered phase
- 421 separation in model plasma membranes. *Proc Natl Acad Sci U S A* **102**, 6320-6325 (2005).
- 422 26. B. B. Diaz-Rohrer, K. R. Levental, K. Simons, I. Levental, Membrane raft association is a determinant of plasma
- 423 membrane localization. *Proc Natl Acad Sci U S A* **111**, 8500-8505 (2014).

- 424 27. J. H. Lorent *et al.*, Structural determinants and functional consequences of protein affinity for membrane rafts.
425 *Nature communications* **8**, 1219 (2017).
- 426 28. H. Shogomori *et al.*, Palmitoylation and intracellular domain interactions both contribute to raft targeting of linker
427 for activation of T cells. *J Biol Chem* **280**, 18931-18942 (2005).
- 428 29. W. Zhang, R. P. Trible, L. E. Samelson, LAT palmitoylation: its essential role in membrane microdomain targeting
429 and tyrosine phosphorylation during T cell activation. *Immunity* **9**, 239-246 (1998).
- 430 30. M. Sundh, S. Svedhem, D. S. Sutherland, Influence of phase separating lipids on supported lipid bilayer formation
431 at SiO₂ surfaces. *Phys Chem Chem Phys* **12**, 453-460 (2010).
- 432 31. J. A. Goodchild, D. L. Walsh, S. D. Connell, Nanoscale substrate roughness hinders domain formation in
433 supported lipid bilayers. *Langmuir* **35**, 15352-15363 (2019).
- 434 32. D. Beckers, D. Urbancic, E. Sezgin, Impact of Nanoscale Hindrances on the Relationship between Lipid Packing
435 and Diffusion in Model Membranes. *J Phys Chem B* **124**, 1487-1494 (2020).
- 436 33. W. F. Zeno, K. E. Johnson, D. Y. Sasaki, S. H. Risbud, M. L. Longo, Dynamics of Crowding-Induced Mixing in
437 Phase Separated Lipid Bilayers. *J Phys Chem B* **120**, 11180-11190 (2016).
- 438 34. J. C. Stachowiak, C. C. Hayden, D. Y. Sasaki, Steric confinement of proteins on lipid membranes can drive
439 curvature and tubulation. *Proc Natl Acad Sci U S A* **107**, 7781-7786 (2010).
- 440 35. M. Rouches, S. L. Veatch, B. B. Machta, Surface densities prewet a near-critical membrane. *Proc Natl Acad Sci
441 U S A* **118**, (2021).
- 442 36. I. Levental, D. Lingwood, M. Grzybek, U. Coskun, K. Simons, Palmitoylation regulates raft affinity for the
443 majority of integral raft proteins. *Proc Natl Acad Sci U S A* **107**, 22050-22054 (2010).
- 444 37. J. Zhao, J. Wu, S. L. Veatch, Adhesion stabilizes robust lipid heterogeneity in supercritical membranes at
445 physiological temperature. *Biophys J* **104**, 825-834 (2013).
- 446 38. M. Rouches, S. Veatch, B. Machta, Surface Densities Prewet a Near-Critical Membrane. *bioRxiv*,
447 2021.2002.2017.431700 (2021).
- 448 39. J. K. Chung *et al.*, Coupled membrane lipid miscibility and phosphotyrosine-driven protein condensation phase
449 transitions. *Biophys J* **120**, 1257-1265 (2021).
- 450 40. A. Honigmann *et al.*, A lipid bound actin meshwork organizes liquid phase separation in model membranes. *eLife*
451 **3**, e01671 (2014).
- 452 41. A. P. Liu, D. A. Fletcher, Actin polymerization serves as a membrane domain switch in model lipid bilayers.
453 *Biophys J* **91**, 4064-4070 (2006).
- 454 42. K. Gowrishankar *et al.*, Active remodeling of cortical actin regulates spatiotemporal organization of cell surface
455 molecules. *Cell* **149**, 1353-1367 (2012).
- 456 43. R. Raghupathy *et al.*, Transbilayer lipid interactions mediate nanoclustering of lipid-anchored proteins. *Cell* **161**,
457 581-594 (2015).
- 458 44. I. H. Lee, M. Y. Imanaka, E. H. Modahl, A. P. Torres-Ocampo, Lipid Raft Phase Modulation by Membrane-
459 Anchored Proteins with Inherent Phase Separation Properties. *ACS Omega* **4**, 6551-6559 (2019).
- 460 45. J. Yi, L. Balagopalan, T. Nguyen, K. M. McIntire, L. E. Samelson, TCR microclusters form spatially segregated
461 domains and sequentially assemble in calcium-dependent kinetic steps. *Nature communications* **10**, 277 (2019).
- 462 46. M. B. Stone, S. A. Shelby, M. F. Nunez, K. Wisser, S. L. Veatch, Protein sorting by lipid phase-like domains
463 supports emergent signaling function in B lymphocyte plasma membranes. *eLife* **6**, (2017).
- 464 47. S. M. Haeryfar, D. W. Hoskin, Thy-1: more than a mouse pan-T cell marker. *J Immunol* **173**, 3581-3588 (2004).
- 465 48. I. Koyama-Honda *et al.*, High-speed single-molecule imaging reveals signal transduction by induced transbilayer
466 raft phases. *J Cell Biol* **219**, (2020).
- 467 49. N. Momin *et al.*, Designing lipids for selective partitioning into liquid ordered membrane domains. *Soft Matter*
468 **11**, 3241-3250 (2015).
- 469 50. A. Honigmann, V. Mueller, S. W. Hell, C. Eggeling, STED microscopy detects and quantifies liquid phase
470 separation in lipid membranes using a new far-red emitting fluorescent phosphoglycerolipid analogue. *Faraday
471 discussions* **161**, 77-89; discussion 113-150 (2013).
- 472 51. N. Komura *et al.*, Raft-based interactions of gangliosides with a GPI-anchored receptor. *Nat Chem Biol* **12**, 402-

- 473 410 (2016).
- 474 52. K. Gaus, E. Chklovskiaia, B. Fazekas de St Groth, W. Jessup, T. Harder, Condensation of the plasma membrane at
475 the site of T lymphocyte activation. *J Cell Biol* **171**, 121-131 (2005).
- 476 53. K. Simons, D. Toomre, Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1**, 31-39 (2000).
- 477 54. R. Lasserre *et al.*, Raft nanodomains contribute to Akt/PKB plasma membrane recruitment and activation. *Nat
478 Chem Biol* **4**, 538-547 (2008).
- 479 55. C. M. Blouin *et al.*, Glycosylation-Dependent IFN-gammaR Partitioning in Lipid and Actin Nanodomains Is
480 Critical for JAK Activation. *Cell* **166**, 920-934 (2016).
- 481 56. K. R. Levental *et al.*, omega-3 polyunsaturated fatty acids direct differentiation of the membrane phenotype in
482 mesenchymal stem cells to potentiate osteogenesis. *Science advances* **3**, eaao1193 (2017).
- 483 57. T. Harder, P. Scheiffele, P. Verkade, K. Simons, Lipid domain structure of the plasma membrane revealed by
484 patching of membrane components. *J Cell Biol* **141**, 929-942 (1998).
- 485 58. S. Hiscox, M. B. Hallett, B. P. Morgan, C. W. van den Berg, GPI-anchored GFP signals Ca²⁺ but is
486 homogeneously distributed on the cell surface. *Biochem Biophys Res Commun* **293**, 714-721 (2002).
- 487 59. P. W. Janes, S. C. Ley, A. I. Magee, Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor.
488 *J Cell Biol* **147**, 447-461 (1999).
- 489 60. L. J. Pike, Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *J Lipid Res* **47**,
490 1597-1598 (2006).
- 491
- 492

493 **FIGURE LEGENDS**

494 **Figure 1 - *in vitro* coupling between protein condensates and membrane domains.** (A) pLAT is uniformly
495 distributed in Lo domains in a phase separated membrane when conjugated to DSIDA. Addition of Grb2/Sos1
496 produces protein condensates exclusively on top of Lo domains, i.e. completely excluded from Ld domains
497 (marked by trDHPE). (B) Lo domains enriching pLAT are present at 23°C and disperse at 45°C, then reappear
498 randomly after cooling. (C) Condensates recruit nascent Lo domains. LAT/Grb2/Sos1 condensates form in small
499 Lo domains in majority-Ld membranes. Lipid domains are dissolved by increasing temperature above the
500 miscibility transition threshold (~37°C for this composition, see Supp Methods for detailed lipid compositions);
501 pLAT condensates were not notably affected at these temperatures. Cooling membranes below the transition
502 temperature induces Lo domain formation exclusively beneath protein condensates. (D) pLAT was bound to
503 both phases by 2% DP-NTA and 1% DO-NTA in the bilayer, yielding ~2-fold Lo enrichment of LAT. Adding
504 Grb2/Sos1 produces condensates exclusively in the Lo phase (labeled by naphthopyrene, blue). (E) Protein
505 condensates can induce lipid phase separation in GUVs. (F) Protein condensates enhance lipid phase
506 separation, evidenced by significantly increased T_{misc} (temperature at which 50% of GUVs show lipid phase
507 separation). (G) Lipid phase separation facilitates protein condensation. Low concentrations of pLAT (20 nM),
508 Grb2 (100 nM), and Sos1 (100 nM) do not produce pLAT condensates on DOPC membrane (left panel),
509 whereas the same protein mixture undergoes Lo-confined condensation on phase separated membranes (right
510 panel). Scale bars are A/C/D/E/G = 5 μm , B = 2 μm .

511

512 **Figure 2 - *in situ* recruitment of raft marker proteins to Grb2 condensates in cells.** (A) Schematic of LAT
513 condensate formation in activated Jurkats: T-cell receptor (TCR) clustering by OKT3 induces signaling that
514 leads to phosphorylation of LAT and multivalent recruitment of Grb2/Sos1 to produce liquid condensates. (B)
515 TIRF imaging of Grb2-rich condensates formed in OKT3-activated Jurkats, but not in non-activated cells (BSA-
516 or ICAM1-coated coverslip) Scale bar is 5 μm . (C-E) TIRF images of Grb2 condensates relative to GPI-GFP,
517 raft-TMD, and nonraft-TMD. Scale bar is 5 μm . Enlarged images of white squares demonstrate recruitment of
518 raft-TMD / GPI-GFP and exclusion of nonraft-TMD under Grb2 condensates. Scale bar is 0.5 μm . (right) line
519 scans showing probe enrichment under Grb2 condensates. (F) Quantification of relative enrichment in cells
520 imaged at room temperature (imaging at 37°C gave similar results, Fig S10). Each point represents the mean
521 enrichment (>1) or depletion (<1) of probes under Grb2 condensates relative to adjacent region for individual
522 cells across 3 independent experiments. Each cell included >10 Grb2 condensates. *** $p<0.001$, ** $p<0.01$,
523 * $p<0.05$ for difference from 1 (no enrichment/depletion) of means of individual cells.

524

525 **Figure 3 – Mutual templating between Grb2 condensates and raft-like membrane domains.** (A) Monomeric
526 GPI AP (GPI-GFP) is subtly enriched in raft phase of phase separated GPMVs. Dimerization of the endogenous
527 GPI-AP Thy1 increases raft preference. Further oligomerization of Thy1 via secondary antibodies leads to
528 exclusively raft-associated clusters. Scale bar is 5 μ m. (B) Quantification of the partitioning coefficient ($K_{p,raft}$)
529 showed that antibody dimerized Thy1 has a higher raft affinity than non-crosslinked GPI-AP. (C) Recruitment of
530 oligomerized Thy1 to Grb2 condensates. Top: overlapping of Grb2 condensates with primary antibody dimerized
531 Thy1. Bottom: overlapping of Grb2 condensates with Thy1 clusters induced by secondary antibody crosslinking.
532 Scale bar is 1 μ m. (D) Quantification of GPI-AP enrichment under Grb2 condensates enhanced by antibody
533 crosslinking. Data points represent individual cells from at least three independent experiments; *p<0.05. (E)
534 Time-lapse of live cell imaging showing capture/immobilization of a Thy1 cluster by Grb2 condensate. Scale bar
535 is 0.5 μ m. (F) Schematic of coupling between clustered Thy1 and LAT/Grb2/Sos1 condensate mediated by
536 ordered membrane domain. (G) Time series showing formation of Grb2 condensate above an immobilized Thy1
537 cluster. Scale bar is 1 μ m. (H) Schematic of cholesterol-PB oligomerization and labeling. Cells are labeled with
538 chol-PB, then fluorescent avidin, before plating on OKT3-coated coverslips. (I) TIRF images of Grb2
539 condensates overlying micron-sized cholesterol-rich domains. Plot shows normalized fluorescence intensity
540 along the line trace shown in white. (J-L) TIRF imaging reveals the colocalization of cholesterol-rich domains
541 with raft markers GPI-GFP and raft-TMD, and exclusion of nonraft-TMD from chol-rich domains. Scale bars are
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543

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