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1

2 # Serum albumin maintains Wnt water-solubility and activity

3

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21 **Summary**

22 Wnt proteins regulate adult tissue homeostasis and repair by driving stem cell self-
23 renewal and differentiation. High-performance Wnt preparations have enormous
24 therapeutic potential, especially alongside various stem cell technologies. Currently, most
25 of these Wnt preparations contain FBS or the detergent CHAPS to maintain Wnt solubility
26 and activity. Recently, afamin was identified as a serum factor that solubilizes Wnt3a in
27 conditioned media (CM), obviating the requirement for animal sera. Here, we report
28 serum albumin (SA) is required for afamin-mediated solubilization of Wnt3a in CM.
29 Moreover, SA-mediated solubilization of purified Wnt3a in tubes does not require afamin.
30 This means conventional CHAPS-Wnt3a preparations can be modified into SA-purified
31 Wnt3a (SA-pWnt3a) preparations by exchanging CHAPS for SA through dialysis. SA-
32 pWnt3a preparations effectively promote the growth of human stem cell organoids. These
33 data suggest SA as a physiological factor for maintaining Wnt3a activity in therapeutic
34 applications.

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35 **Introduction**

36 Wnt signaling plays important roles in the regulation of a variety of biological processes
37 from development to tissue homeostasis[1]. The activation of Wnt/β-catenin signaling
38 holds enormous therapeutic potential for many disease conditions in which tissue
39 regeneration or the maintenance of tissue homeostasis would be beneficial[2,3], but there
40 is at least one key hurdle that must be resolved before Wnts can be developed as
41 therapeutic proteins. Wnts are water-insoluble in their active form due to palmitoylation,
42 a post-translational lipid modification[4]. This modification, however, is required for the
43 maintenance of Wnt ligand activity because it facilitates the binding of Wnt to Frizzled
44 (FZD), the receptor for secreted Wnts[5]. Consequently, the formation of an active and
45 soluble Wnt preparation has thus far proven elusive. Additional molecules that help
46 solubilize Wnts in their active acylated forms are necessary. Animal sera (e.g., fetal bovine
47 serum or FBS) can maintain the activity and solubility of Wnt proteins[6], but the use of
48 animal serum in therapeutic applications can be hazardous for human patients. This is
49 because it can provoke immunological reactions and even lead to pathogen transmission[7-
50 9]. To minimize the contaminants from animal sera that have various biological functions,
51 a method for purifying Wnts in their active form was developed[4]. In this method, Wnt
52 proteins are purified from serum-containing Wnt-conditioned media (CM) using a
53 detergent such as CHAPS during a multi-step column chromatography protocol[4]. The
54 detergent, which remains in the resulting Wnt preparation, can be toxic to cells even at
55 low concentrations[10]. Diluting the CHAPS to a point where it no longer affects cell viability,
56 however, leads to a significant loss of Wnt activity. A Wnt3a-containing CM or a purified
57 Wnt3a, solubilized and stabilized by a clearly defined physiological factor(s), is thus
58 strongly preferred to a detergent- or animal sera-solubilized Wnt3a preparation.

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59

60 Recently, many groups have become interested in developing a serum-free active Wnt
61 preparation, even for *in vitro* use, due to the increasingly widespread employment of adult
62 stem cell-derived human organoid culture technologies in biomedical research[11]. Since
63 adult stem cell-derived organoid culture techniques were first established with intestinal
64 cells about 10 years ago[12], Wnt signaling has emerged as the most important *in vivo* niche
65 signaling component for the maintenance of adult stem cells *in vitro*[13,14]. Using this
66 technology, 3D *in vitro* tissue structures that mimic the structural and functional
67 properties of *in vivo* organs can be maintained, passaged, scaled-up, and even stored.
68 Wnt3a was the first member of the Wnts added to culture media to support the long-term
69 *in vitro* proliferation and self-renewal of adult stem cells in organoids[13,15,16]. Typically, this
70 Wnt3a is obtained from the CM produced by a Wnt3a-expressing cell line in the presence
71 of FBS[13]. But unknown components in FBS can have undesirable effects on stem cells,
72 reducing the consistency and performance of this type of Wnt3a formulation[17]. To
73 overcome this limitation, several groups have tried to identify the most critical
74 component(s) in sera that solubilize Wnt and maintain its activity[18,19]. Recently, the
75 Takagi group discovered that afamin (AFM), a member of the albumin superfamily of
76 binding proteins, is a serum component that binds and solubilizes Wnts[19]. By co-
77 expressing Wnt and AFM in the same cell line, soluble but active Wnts can easily be
78 prepared in the absence of animal sera in the form of CM (Wnt3a-AFM CM). Moreover,
79 Wnt3a-AFM CM supports the growth and expansion of human intestinal organoids for
80 more than 18 passages[19]. The structure of AFM has been identified by both glycan and
81 pocket analysis[20]. AFM forms a stable complex with Wnt3a that could improve its
82 solubility by accepting the hydrophobic palmitoleic tail of a Wnt serine residue into a

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83 hydrophobic binding pocket on its own surface[20].

84

85 In this study, we found that AFM does not effectively solubilize Wnt3a alone. We have
86 identified serum albumin (SA) as an essential co-factor necessary for Wnt3a
87 solubilization in the production of FBS-free Wnt3a-AFM CM. We also found SA can
88 effectively solubilize and stabilize purified Wnt3a *in vitro* in the absence of AFM. The
89 effective concentration of SA in these two conditions (CM or purified Wnt3a) is far below
90 the normal level present in human plasma or interstitial fluid[21]. In adult stem cell-derived
91 human organoid cultures, both the Wnt3a-AFM CM produced in the presence of SA and
92 the purified active Wnt3a maintained with SA can effectively promote organoid formation.
93 Thus, in this study, we found SA can potently solubilize and stabilize Wnts, making it a
94 simple, cost-effective, and efficient method for producing active Wnt preparations that
95 are more compatible with both *in vitro* and *in vivo* use cases.

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96 **Results**

97 **SA is required for the Wnt ligand activity of CM produced by L-Wnt3a-AFM cells**

98 When the albumin family protein AFM was found to solubilize Wnt3a and maintain its
99 activity in solution[19], we sought to better uncover the mechanism by which this occurs
100 and determine whether it is influenced by other CM components. To confirm the
101 conclusions reached by Mihara et al.[19] regarding the role of AFM, we performed a
102 TOPflash assay (Wnt/β-catenin pathway reporter assay) using CM from cells expressing
103 Wnt3a alone (L-Wnt3a cells) or cells co-expressing Wnt3a and AFM (L-Wnt3a-AFM cells)
104 (Figure 1A). Not only did we find significant differences in the amount of Wnt3a in each
105 CM, but the Wnt ligand activities of each CM depended on the presence of AFM (Figure
106 1B). Thus, we confirmed the reported role of AFM in this Wnt3a CM preparation.

107

108 Next, we asked whether other components in the CM are required for AFM to produce its
109 effect. We first tested the effect of SA, another member of the albumin superfamily of
110 binding proteins and the most abundant plasma protein, on the Wnt ligand activity of
111 Wnt3a-AFM CM. After adding varying amounts of bovine serum albumin (BSA) to Wnt3a-
112 AFM CM, we measured the resulting TOPflash activity. We found that increases in BSA
113 lead to a proportional rise in Wnt/β-catenin activity, implying that BSA further enhances
114 Wnt3a activity (Figure 1C). We then compared the activity of Wnt3a-AFM CM in two
115 different media: advanced DMEM (AdDMEM)/F12, which contains 0.4 mg/mL BSA and
116 DMEM/F12, which is BSA-deficient (see Figure 1 – Figure supplement 1). As expected,
117 Wnt ligand activity was significantly lower in DMEM/F12 (Figure 1D). Next, we further
118 confirmed the requirement of BSA by showing that the addition of BSA to DMEM/F12-

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119 based CM restores its Wnt ligand activity (Figure 1D). This indicates BSA is a potent
120 enhancer of Wnt3a activity in conjunction with AFM. This effect of BSA is unrelated to its
121 potential actions on cell viability or proliferation of L-Wnt3a-AFM cells (see Figure 1-
122 Figure supplement 2). To determine whether this effect of SA is ubiquitous across species,
123 we performed the same test with recombinant human serum albumin (rHSA) derived
124 from rice. We found that, like BSA, rHSA produced an enhancement of Wnt ligand activity
125 (Figure 1D). This result also indicates that the effect of BSA in the TOPflash assay is not
126 due to its contaminating proteins or lipids. We next investigated the specificity of the role
127 of SA in this phenomenon, asking whether other abundant blood-based proteins, such as
128 Immunoglobulin-G (IgG), could give the similar result. After performing a TOPflash assay
129 comparing Wnt3a activity between BSA+ and IgG+ CM, we found that IgG had no effect on
130 Wnt3a activity (Figure 1E). Thus, the phenomenon is specific to BSA.

131

132 We next asked whether BSA exerts its effect by increasing the amount of secreted AFM in
133 the CM. We found relatively consistent levels of AFM in the CMs and cell lysates regardless
134 of their BSA status (Figure 1F). This means that BSA has no effect on the amount of AFM
135 synthesized or secreted and that more AFM cannot substitute for the presence of BSA.
136 This was further confirmed when we increased the concentration (up to 40 μg/mL) of
137 exogenous AFM in the absence of BSA and found that it had little effect on Wnt3a activity
138 compared to 1 μg/mL AFM in the presence of AdDMEM/F12 (Figure 1G). Together, these
139 data demonstrate that while AFM in the media is certainly required for Wnt3a activity in
140 CM, so is the presence of BSA.

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141 **SA specifically requires AFM to support the Wnt ligand activity of Wnt3a-AFM CM**

142 After establishing that BSA is necessary for supporting Wnt3a activity in CM, we asked
143 whether higher concentration BSA (5 mg/mL) can maintain Wnt3a activity in Wnt3a CM
144 even in the absence of AFM. To this end, we compared two cell types: L-Wnt3a-AFM cells,
145 which co-secrete Wnt3a and AFM, and L-Wnt3a cells, which secrete Wnt3a alone. When
146 we added exogenous BSA (5 mg/mL) to the DMEM/F12 media in which each cell line was
147 cultured to produce CM, only the resulting Wnt3a-AFM CM showed a dramatic increase in
148 TOPflash activity (Figure 2A). This clear evidence that AFM is essential for BSA to
149 maintain Wnt3a activity in CM led us to further ask whether this capacity is specific to
150 AFM or whether another member of the albumin superfamily of binding proteins, such as
151 vitamin D binding protein (VDBP) or α-fetoprotein (AFP), could take its place. When we
152 compared the TOPflash activities of CMs produced after adding each candidate protein
153 exogenously along with BSA to the cultured L-Wnt3a cells, we found that neither VBDP
154 nor AFP combined with BSA improved Wnt3a activity when compared to BSA alone or
155 BSA combined with exogenous AFM (Figure 2B). These data suggest AFM is the only other
156 member of the albumin superfamily of binding proteins that is effective for supporting
157 Wnt3a activity in combination with BSA in CM.

158

159 **SA prevents aggregation of Wnt3a protein in Wnt3a-AFM CM**

160 Next, we investigated the mechanisms by which SA increases Wnt3a activity in Wnt3a-
161 AFM CM. First, we did not observe any difference in the Wnt3a protein levels of cell lysates
162 from L-Wnt3a-AFM cells cultured in BSA+ or BSA- media (Figure 3A). From these data,
163 we inferred that BSA does not affect the Wnt3a secretory pool. Next, we asked whether

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164 BSA affects the secretion of Wnt3a by adding the protein synthesis inhibitor
165 cycloheximide (CHX) to the media of L-Wnt3a-AFM cells and measuring cellular changes
166 in Wnt3a protein levels for up to 24 hrs in the absence or presence of BSA. BSA did not
167 affect the rate of Wnt3a loss in cell lysates (Figure 3B). Rather than changes in Wnt3a
168 production, the reduction in intracellular Wnt3a seems to be due to a change in protein
169 secretion. This is because addition of the Wnt secretion inhibitor IWP-2 produced a near
170 full recovery (Figure 3B). These findings suggest BSA does not affect the typical route by
171 which Wnt3a is secreted. Wnt is normally, at least in part, secreted in exosomes[22]. We
172 found, however, that BSA did not alter the amount of Wnt3a in exosomes derived from
173 Wnt3a-AFM cells (see Figure 3 – Figure supplement 1).

174

175 We next asked how BSA affects the way Wnt3a's activity changes after it is released into
176 the CM. Wnt rapidly loses its activity upon its addition to serum- or detergent-free
177 solutions[10,18]. Thus, we looked for any changes in the relative Wnt3a protein level or the
178 TOPflash activity of purified Wnt3a in DMEM/F12 maintained in tubes over 48 hrs at 37°C.
179 Interestingly, although we found TOPflash activity fell rapidly in the absence of BSA, the
180 levels of purified Wnt3a protein did not change over the course of 48 hrs (Figure 3C).
181 Based on this, we hypothesized that BSA supports the long-term maintenance of Wnt3a
182 activity by preventing protein aggregation. To investigate the hypothesis that BSA
183 increases Wnt3a solubility or conformational stability, we performed a protein complex
184 size fractionation, dividing CM with or without BSA into 13 fractions. In the presence of
185 BSA, Wnt3a remained in a monomeric or oligomeric form, with bands present only in the
186 low-density fractions (Figure 3D). In the absence of BSA, however, the Wnt3a bands
187 appeared only in the high-density fractions, indicating the presence of aggregated,

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188 inactive Wnt3a. Using TOPflash assays to measure the activity of each fraction, we found
189 the highest Wnt3a activity in the low-density fractions of BSA+ CM containing soluble
190 Wnt3a (Figure 3D). These findings suggest BSA solubilizes and stabilizes active Wnt3a in
191 Wnt3a-AFM CM by preventing its aggregation. AFM is known to form a complex with
192 Wnt3a[19]. To determine whether BSA also binds Wnt3a to prevent its aggregation, we used
193 Wnt3a antibodies to perform an immunoprecipitation of Wnt3a-AFM CM supplemented
194 with BSA (5 mg/mL). We found that while AFM interacts with Wnt3a, BSA does not
195 (Figure 3E). These data suggest a direct interaction between BSA and Wnt3a is not
196 required for BSA to maintain Wnt3a in its active form.

197

198 **Wnt3a-AFM CM needs BSA to support the growth and expansion of human adult**
199 **stem cell organoids**

200 We compared the effects of Wnt3a-AFM (+BSA) CM, Wnt3a-AFM (-BSA) CM, and Wnt3a-
201 FBS CM on the growth and passaging of human colon and stomach organoids. For both
202 types of organoids, we found that only Wnt3a-AFM (+BSA) CM, not Wnt3a-AFM (-BSA)
203 CM supported a growth rate comparable to that of Wnt3a-FBS CM (Figure 4A and 4D).
204 Organoids cultured in Wnt3a-AFM (-BSA) CM, which is deficient in BSA, showed a cell
205 growth plateau after the first passage (Figure 4A and 4D). In an immunohistochemistry
206 experiment using the proliferation marker Ki67, we found more proliferating cells in
207 organoids grown in Wnt3a-AFM (+BSA) CM than in those grown in the BSA-deficient CM
208 (Figure 4B and 4E). In a real-time RT-PCR experiment, we also found significantly higher
209 expression of the Wnt target genes *LGR5* and *AXIN2* in both types of organoids in the
210 presence of both BSA and AFM (Figure 4C and 4F). Together, these data indicate BSA is

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211 essential for the CM made from L-Wnt3a-AFM cells to support the growth and expansion
212 of adult stem cell organoids. The role of BSA in these preparations seems to maintain
213 Wnt3a in its active form.

214

215 **SA can solubilize and stabilize purified Wnt3a in the absence of AFM**

216 To directly demonstrate that interactions between Wnt3a, AFM, and BSA are sufficient for
217 the solubilization and stabilization of active Wnt3a, we performed an *in vitro* solubility
218 assay. First, we spun down various combinations of purified Wnt3a with AFM or BSA in
219 solution (incubated for 4 hrs before centrifugation) in a centrifuge to produce a pellet and
220 a supernatant (Figure 5A). We then observed on western blots that Wnt3a was primarily
221 soluble and in the supernatant in the presence of BSA, while it mainly appeared in an
222 insoluble form in the pellet in the absence of BSA (Figure 5B). rHSA also showed a similar
223 effect (see Figure 5 – Figure supplement 1). The addition of AFM did not do much to
224 maintain more of the purified Wnt3a in its soluble form than BSA alone (Figure 5B).
225 Moreover, a fractionation experiment with purified Wnt3a solution demonstrated the
226 occurrence of aggregation even in the presence of AFM (Figure 5C, left panel). It was only
227 in the presence of BSA that Wnt3a was present in its soluble monomeric or oligomeric
228 states (Figure 5C, left panel). We also confirmed the Wnt ligand activity of the soluble
229 Wnt3a via a TOPflash assay of each fraction (Figure 5C, right panel).

230

231 The purified Wnt3a preparation we used in *in vitro* solubility assays contained trace
232 amounts of CHAPS because we used CHAPS-purified Wnt3a proteins (R&D systems).
233 Wnt3a would become insoluble as the CHAPS was diluted to a concentration at which

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234 CHAPS alone could not efficiently prevent Wnt3a aggregation (see Figure 5 – Figure
235 supplement 2). It is notable that, even in trace amounts of CHAPS (0.001 %), BSA was able
236 to maintain Wnt3a in a soluble form without the addition of AFM (see Figure 5 – Figure
237 supplement 2). To further determine whether BSA contributes to the process of
238 solubilizing Wnt3a, we added BSA after Wnt3a aggregation had already occurred.
239 Compared to 1% CHAPS which efficiently solubilized Wnt3a[4], we found on western blot
240 that a lower concentration of CHAPS (0.001 %) allowed for significant Wnt3a aggregation
241 (Figure 5D). The addition of BSA (5 mg/ml, 4 hrs after Wnt3a aggregation in 0.001 %
242 CHAPS), however, restored Wnt3a solubility even at a lower CHAPS concentration (Figure
243 5D). This suggests BSA itself can solubilize Wnt3a. We also asked whether BSA affects the
244 thermostability of Wnt3a because a previous study found that purified Wnt3a with
245 CHAPS was unstable at 37°C[23]. We found purified Wnt3a was nearly equally soluble at
246 both 4°C and 37°C in the presence of BSA, indicating that the effect of BSA on Wnt3a
247 activity is unrelated to thermostability (Figure 5E).

248

249 Finally, we examined the effects of BSA on other members of the Wnt protein family,
250 seeking to determine the prevalence of its role in protein solubilization. On western blot,
251 we observed a clear shift of mouse Wnt3a, human Wnt3, and human Wnt5a into the
252 supernatant in the presence of BSA (Figure 5F). This suggests multiple members of the
253 Wnt protein family may similarly interact with BSA, presenting opportunities for
254 expanding the methods outlined in this study to other Wnt family proteins. Ultimately,
255 our data indicate BSA effectively maintains purified Wnt3a in its soluble and active form
256 without the help of AFM.

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257

258 **A new method for preparing purified, active Wnt3a in the absence of CHAPS**

259 Based on the results above, we have developed a new method for maintaining purified
260 Wnt3a in its active form after removing CHAPS. This new method can be used in both *in*
261 *vitro* organoid culture applications and in the development of safe therapeutic agents.
262 Typically, purified Wnt3a protein preparations contain some CHAPS (0.5–1%), which
263 maintains Wnt3a in an active form. When the CHAPS is diluted, however, Wnt3a loses its
264 solubility and activity (Figure 6A). Because we found that BSA can solubilize and stabilize
265 purified Wnt3a, we planned to dialyze CHAPS from the active Wnt3a solution containing
266 both Wnt3a and BSA (Figure 6B). A purified Wnt3a-BSA solution (pWnt3a-BSA) that
267 remains soluble and active even after the removal of CHAPS would be an ideal Wnt3a
268 preparation. To this end, we placed purified Wnt3a proteins containing CHAPS in a 10 kDa
269 dialysis cassette along with BSA (5 mg/mL) and performed dialysis against standard
270 DMEM/F12 media. Since both Wnt3a and BSA have molecular weights larger than 10 kDa
271 (37.5 kDa and 66.5 kDa, respectively), they remain within the dialysis cup while the 0.6
272 kDa CHAPS diffuses out into the surrounding media. In contrast to the surface of the low
273 protein binding tubes (LoBind) we used in other experiments of this study, the dialysis
274 membranes adsorbed much of the Wnt3a in the absence of BSA (Figure 6C). Fortunately,
275 the addition of BSA recovered much of this lost Wnt3a. BSA is known to prevent
276 nonspecific protein adsorption to polymer surfaces. Moreover, when we performed a
277 western blot following dialysis, we saw a clear distinction in the partitioning of Wnt3a
278 into the supernatant only in the presence of BSA (Figure 6D). These findings indicate that
279 BSA both maintains protein solubility and prevents the adsorption of Wnt3a to the
280 membrane. These effects seem to be specific to BSA because the same concentration of

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281 IgG (5 mg/mL) failed to prevent the adsorption and aggregation of Wnt3a (Figure 6C, D).
282 We also observed that BSA can maintain Wnt3a in its soluble form over time with little
283 change in the amount present in the soluble fraction (Figure 6E, F). To confirm the long-
284 term durability of Wnt3a activity in this preparation, we measured the TOPflash activities
285 of Wnt3a in BSA- and BSA+ solutions stored at 4°C for the indicated number of weeks. We
286 found that purified Wnt3a solubilized and stabilized by BSA remained active for at least
287 2 wks when stored at 4°C (Figure 6G). We also found that pWnt3a-BSA solution
288 maintained its activity after a freeze-thaw cycle, suggesting mid- to large-scale production
289 of BSA-stabilized Wnt3a and its storage in freezers is feasible (Figure 6H). We also tested
290 the functional validity of the pWnt3a-BSA solution by examining the cooperative
291 interaction between purified Wnt3a and R-spondin. Indeed, we found co-administration
292 of R-spondin and pWnt3a-BSA markedly potentiated canonical Wnt/β-catenin signaling
293 (Figure 6I). This suggests pWnt3a-BSA is compatible with R-spondin, which is critical for
294 the amplification of Wnt signaling[24]. Last, we found pWnt3a-BSA is at least equal to or
295 more efficient than Wnt3a-FBS CM in supporting the growth, expansion, and
296 development of human colon organoids (Figure 6J and 6K). We also confirmed that rHSA
297 similarly supports the growth and expansion of these organoids (see Figure 6 – Figure
298 supplement 1). Together, we present for the first time, a new method for producing
299 purified Wnt3a that remains active in solution, using BSA or even HSA instead of CHAPS
300 as a solubilizing agent.

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301 **Discussion**

302 Mihara et al. (2016) found AFM binds Wnt3a in a 1:1 ratio in the CM from Expi293F cells
303 co-expressing AFM and Wnt3a, maintaining Wnt3a in a soluble state in CM. In this study,
304 our recognition of a difference between the ability of basal media such as DMEM/F12 and
305 AdDMEM/F12 to solubilize the Wnt3a-AFM complex led us to identify SA as an additional
306 important factor in serum that can improve the yield and performance of active Wnt3a
307 CM preparations. This does not mean AFM is not essential in making serum-free Wnt3a
308 CM. Rather, we found that although SA alone has minimal effects on solubilization or
309 stabilization of Wnt3a in Wnt3a CM production, we saw a large increase in Wnt3a activity
310 with the addition of both BSA and AFM. From their data, we inferred that Mihara et al.[19]
311 likely used AdDMEM/F12 factory-formulated to contain 0.4 mg/mL of BSA. They showed
312 on a protein gel stained with Oriole fluorescent stain that purified Wnt3a-AFM from the
313 eluted Wnt3a-AFM CM fractions was contaminated by BSA. We think this covertly
314 contributed to their identification of AFM as the serum factor that solubilizes and protects
315 Wnt3a. After comparing the activity of all the members of the albumin superfamily
316 proteins, including SA, AFM, α-fetoprotein, and vitamin D binding protein, Mihara et al.[19]
317 concluded that SA is not involved in maintaining active Wnt3a. This finding is consistent
318 with our data showing that BSA alone cannot efficiently solubilize Wnt3a in the process
319 of CM production. Because SA indeed requires AFM to maintain active Wnt3a secreted
320 from cells, it is highly possible AFM has a distinct role from BSA. Naschberger et al.[20]
321 revealed how afamin forms a stable complex with Wnt proteins and Mihara et al.[19]
322 showed that Wnt3a is nearly absent in CM when AFM is removed. However, AFM does not
323 effectively stabilize purified Wnt3a in tubes[10]. Considering these results, the role of AFM
324 may require complex formation between AFM and Wnt3a during the process of its

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325 synthesis, transport, or secretion. In contrast, according to our results and consistent with
326 a previous report[19], SA does not affect Wnt3a secretion. Further studies on possible
327 mechanisms by which AFM functions in the Wnt secretory pathway may clarify the well-
328 known Porcupine and Wntless-mediated Wnt secretion pathway.

329

330 A previous study using Wnt-responsive luciferase reporter cells found that heparan
331 sulfate proteoglycan (HSPG) rather than BSA stabilized purified Wnt3a protein activity[18].
332 This result is inconsistent with our current data showing that BSA can solubilize and
333 stabilize purified Wnt3a. We found that pWnt3a-BSA efficiently increases Wnt/β-catenin
334 pathway reporter expression and promotes the growth of adult stem cell organoids. The
335 discrepancy appears to come from the concentrations of BSA tested in each study. We
336 used 0.4 mg/mL or 5 mg/mL BSA, whereas the previous study only tested concentrations
337 up to 30 μg/mL. Considering the typical concentrations of BSA in the blood (30–50
338 mg/mL) and interstitial fluids (around 30% of that in the plasma), the concentrations of
339 BSA we used are more physiologically relevant and are unlikely to cause any cytotoxicity.

340

341 The structural modeling of the albumin-Wnt3a complex has not yet been successful. This
342 is because the albumin binding pocket, although deep and branched, is narrower and its
343 entrance region presents charged and polar residues[20]. This discourages the entry of
344 hydrophobic palmitoleic O-acylation moieties, resulting in a much-reduced probability of
345 direct interaction between SA and Wnt3a. Our immunoprecipitation results also indicates
346 that SA does not participate in any protein-protein interactions with Wnt3a the way AFM
347 dose. Instead, SA may work indirectly by protecting afamin from taking forms with lower

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348 Wnt3a binding affinities. This is unlikely, however, because purified Wnt3a can be
349 solubilized and stabilized by SA alone, even in the absence of AFM. It is also unlikely that
350 BSA binds AFM directly because there is a clear separation of SA from the Wnt3a-AFM
351 complex when they are run through a size exclusion chromatography column[19]. On the
352 molecular level, albumin does not have a strong chaperone-like activity[25]. It is possible
353 that Wnt3a stabilization occurs via weak nonspecific interactions with albumin that
354 become physiologically relevant at higher albumin concentrations. Further studies will
355 be necessary to clarify the exact mechanism by which SA solubilizes Wnt3a and whether
356 its function applies only to the Wnts.

357

358 Wnt preparations hold promise as additives to the media used for stem cell cultures,
359 especially adult stem cell-derived human organoid cultures[15]. Such organoids have
360 enormous diagnostic and therapeutic potential in precision medicine[11]. Our identification
361 of the most abundant serum protein (35–50 mg/mL)[21] as a critical factor that supports
362 the prolonged maintenance of active Wnts is encouraging because it provides a
363 solubilization and stabilization strategy that is both physiological and robust. SA has a
364 long half-life of 21 days[26], suggesting that it will prove to be a durable Wnt stabilizer.
365 Recombinant human SA produced in rice gives an equivalent, or even superior,
366 performance in stabilizing the Wnt3a-AFM complex. This will make it even easier to
367 produce safe Wnt3a at the high yields. We expect the use of purified Wnt3a preparations
368 to be far preferable to CM in organoid cultures. pWnt3a-BSA has less animal cell-derived
369 contamination, which is beneficial when culturing cells as sensitive as stem cells. It is also
370 now possible to add measurable amounts of Wnt3a into cultures with minimal variability,
371 permitting more precise comparisons of stem cell and organoid culture results from

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372 different laboratories. We have not yet tested the long-term effects of pWnt3a-BSA on
373 organoid cultures, but it seems likely that even more advantages can be found, such as the
374 maintenance of stemness and improvements to differentiation or maturation potential.

375

376 SA is cheaper and seems to act via a more "physiological" mechanism than other currently
377 known Wnt stabilizers, such as heparan sulfate proteoglycan (HSPG), secreted Frizzled-
378 related protein (sFRP), and liposomes[10,18,27]. Although HSPG is also a physiological
379 component of FBS, it is too expensive for producing HSPG-stabilized Wnt3a preparations
380 of practical use. sFRP both promotes and suppresses Wnt/β-catenin signaling, depending
381 on the context[28]. Liposomes mixed with purified Wnt3a are an effective stabilizer of
382 Wnt3a[10], but they are costly to produce and it is technically demanding to prepare
383 liposomal Wnt in standard biology labs. Moreover, liposomes sometimes undergo leakage
384 of their encapsulated molecules and suffer from phospholipid oxidation, limiting their
385 effectiveness. Wnt3a preparations stabilized with SA overcome all the drawbacks of these
386 other Wnt stabilizers. And perhaps most important, the SA-based preparation of Wnt3a
387 we propose here is simpler to make and more physiological, seemingly mimicking how
388 human organs stabilize their extracellular Wnts.

389

390 In this study, we identified SA as an essential and physiological factor that maintain Wnt3a
391 in its soluble and active form. The use of SA in preparing Wnt3a-containing CM or purified
392 Wnt3a will greatly contribute to higher-yield production and purification of active Wnt3a
393 and other Wnts. This will pave the way for the optimization of Wnt preparations for
394 various therapeutic uses and as a culture additive in producing high-quality adult stem

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395 cell-derived human organoids.

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396 ## Experimental procedures

397 ### Reagents and consumables

398 Bovine serum albumin (BSA), recombinant human albumin (rhALB), and
399 immunoglobulin G (IgG) were purchased from Sigma-Aldrich (St. Louis, MO, USA).
400 Protein LoBind tubes were purchased from Eppendorf (Hamburg, Germany). Human
401 Wnt3a, Mouse Wnt3a and human Wnt5a proteins were purchased from R&D systems
402 (Minneapolis, MN, USA). Human Wnt3 and afamin protein were purchased from Origene
403 (Rockville, MD, USA) and R&D systems, respectively.

404

405 ### Cell culture

406 HEK293 STF (ATCC, CRL-3249), Wnt3a-producing L cells (L-Wnt3a cells), and L-Wnt3a
407 cells transduced with pLVX-EF1α-human afamin-IRES-blasticidin-S deaminase (BSD) (L-
408 Wnt3a-AFM cells) were grown in Dulbecco's Modified Eagle's medium (DMEM, Hyclone)
409 supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine
410 serum (FBS, Hyclone). Cells were maintained in a 5% CO_2/95% air incubator with a
411 humidified environment at 37°C. The L-Wnt3a cells were a kind gift from Hans Clevers.

412

413 ### Plasmid construction

414 To generate the lentiviral human AFM expression plasmid, pLVX-EF1α-IRES-BSD was first
415 generated by modifying pLVX-EF1α-IRES-Puro (Clontech). Then, the human AFM CDS was
416 amplified by PCR from pCR-BluntII-TOPO-AFM (MHS6278-211689548; Dharmacon,
417 Lafayette, CO, USA) with the primers listed in Table 1. The product was digested with XbaI

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418 and inserted into the XbaI site of pLVX-EF1α-IRES-BSD. Primer used for constructing the
419 AFM expression plasmid is as described below.

420

Name	Sequences
hAfamin U1	TATAATCTAGAGCCACCATGGTGAAACTACTAAAACTTACAGG
hAfamin L1	TATATTCTAGATTCAGTTGCCAATTTTTGGAC

421

422 **Generation of L-Wnt3a-AFM cells by lentiviral transduction**

423 HEK293T cells (human embryonic kidney cells) were seeded on 6-well plates pre-coated
424 with poly-L-lysine (PLL) at a density of 600,000 cells/well and incubated for 24 hrs. The
425 cells were transfected with 3 μg of a 4:3:1 mixture of lentiviral human AFM expression
426 plasmid, packaging plasmid (psPAX2, Addgene 12260), and envelope expression plasmid
427 (pMD2.G, Addgene 12259) in the presence of polyethyleneimine. The cells were refreshed
428 with 3 mL of growth medium 16 hrs after transfection and further incubated for 36 hrs.
429 Then, the media containing the lentiviruses were harvested and centrifuged at 3,000 rpm
430 for 3 min to eliminate cell debris. The supernatants were collected and stored at −80 °C
431 until use.

432 One day before transduction, L-Wnt3a cells were plated on 24-well plates at a density of
433 50,000 cells/well and grown for 24 hrs. The culture medium was replaced with 400 μL of
434 a 1:1 mixture of the lentivirus-containing medium and fresh culture medium
435 supplemented with polybrene at a final concentration of 4 μg/mL. After 15 hrs, the cells
436 were refreshed with 500 μL of culture medium and incubated for an additional 72 hrs. L-
437 Wnt3a-AFM cells were generated by selection with 2 μg/mL puromycin until a parallel

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438 culture of L-Wnt3a cells died.

439

440 **Production of Wnt CM**

441 L-Wnt3a-AFM or L-Wnt3a cells were plated at a density of 150,000 cells per 24-well plate
442 or 4,500,000 cells per 100-mm plate and incubated for 48 hrs. Then, the cells were
443 washed twice with phosphate-buffered saline (PBS), refreshed with the indicated
444 medium (500 μL for the 24-well plates; 12 mL for the 100-mm plates), and incubated for
445 5 days. The CM was harvested and centrifuged at 1,000 rpm for 2 min. The supernatants
446 were cleared through a 0.45 μm syringe filter and stored at 4 °C.

447

448 **Luciferase reporter assay to measure Wnt activity**

449 HEK293 STF cells were seeded on PLL-coated 96-well opaque plates at a density of
450 25,000 cells/well and grown for 24 hrs. Then, each well was treated with 100 μL of fresh
451 culture medium combined with 50 μL of CM to assay for 24 hrs. After aspiration, 100 μL
452 of the 1x steady-Glo reagent (Steady-Glo® Luciferase Assay System, E2520; Promega,
453 Madison, WI, USA) was added to each well. The luminescence of each well was measured
454 using an Infinite 200 PRO plate reader (Tecan).

455

456 **MTT assay for assessing cell viability**

457 The growth medium was removed from the cells and 1 mg/mL 3-(4,5-Dimethylthiazol-2-
458 yl)-2,5-diphenyltetrazolium bromide (MTT) dissolved in growth medium was added to
459 each well of interest. The cells with the MTT solution were incubated in 5% CO_2/95% air

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460 at 37°C for 2 hrs. Then, after aspirating the MTT solution, solvent solutions (10% sodium
461 dodecyl sulphate and 25% dimethyl formamide, pH 4.7) were added to each well and
462 incubated overnight. The solutions were then transferred to a 96-well plate and the
463 absorbance at 590 nm was measured.

464

465 **Immunoblot**

466 CM samples were harvested and centrifuged at 1,000 rpm for 2 min. Then, the
467 supernatants were mixed with 5× Laemmli sample buffer and boiled for 10 min. For cell
468 lysate samples, the cells were placed on ice and washed twice with cold PBS containing
469 100 mg/L $CaCl_2$ and 100 mg/L $MgCl_2$. The cells were lysed with cold RIPA lysis buffer
470 containing 10 mM Tris-Cl (pH 8.0), 1% Triton X-100, 140 mM NaCl, 1 mM
471 ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate, and 1× cOmplete
472 Protease Inhibitor Cocktail (Roche, Basel, Switzerland). The lysates were clarified by
473 centrifugation at 13,000 rpm at 4 °C for 10 min. The protein concentration was measured
474 using a bicinchoninic acid (BCA) assay. Samples with identical concentrations were added
475 to 5× Laemmli sample buffer and boiled for 10 min. For purified Wnt proteins, samples
476 were mixed with 5x Laemmli sample buffer and boiled for 10 min. The denatured samples
477 in Laemmli buffer were separated by 12% sodium dodecyl sulfate polyacrylamide gel
478 electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Whatman,
479 Dassel, Germany). The membranes were blocked with 5% skim milk in PBS containing
480 0.05% Tween 20 (PBST). Primary antibodies (anti-mouse Wnt3a [2391; Cell Signaling
481 Technology, Danvers, MA, USA], anti-human AFM [sc-373849; Santa Cruz Biotechnology,
482 Santa Cruz, CA, USA], anti-human albumin [ab207327; Abcam, Cambridge, UK] and anti-

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483 β-actin Ab [sc-1651; Santa Cruz Biotechnology]) were used at a 1:1,000 dilution in
484 blocking solution. Secondary antibody conjugated to horseradish peroxidase was used at
485 a 1:5,000 dilution in skim milk. Immunoblot images generated with enhanced
486 chemiluminescence (ECL) solution (Thermo Fisher Scientific) were captured using a
487 Fusion Solo 4M (Vilber Lourmat, Eberhardzell, Germany) camera. The band intensities
488 were analyzed using ImageJ software.

489

490 **Evaluation of Wnt stability**

491 The indicated Wnt3a CM (Wnt3a-AFM CM with or without BSA, Wnt3a-FBS CM) or
492 purified Wnt3a was added to Protein LoBind tubes and further incubated for 6, 12, 24, or
493 48 hrs in 5% CO_2/95% air at 37°C. Immunoblotting with anti-Wnt3a antibodies and
494 TOPflash assays were conducted to assess Wnt3a protein levels and Wnt3a ligand
495 activities at the indicated time of incubation.

496

497 **Sucrose density gradient centrifugation**

498 Sucrose density gradient centrifugation was conducted as previously described, with
499 minor modifications. One milliliter each of 20%, 15%, 10%, and 5% sucrose solutions was
500 layered in a 5 mL polyallomer tube (326819, Beckman Coulter) from bottom to top. Then,
501 1 mL of the indicated Wnt preparation (Wnt3a CM or purified Wnt3a) was loaded on the
502 top of the sucrose gradient. The tubes with their samples were centrifuged at 150,000 x
503 g at 4°C for 5 hrs. The centrifugation was performed with an Optima MAX-XP
504 ultracentrifuge using an MLS 50 rotor (Beckman Coulter). Then, 400-μL fractions were

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505 collected from top to bottom and stored at 4°C. Each fraction was analyzed for Wnt
506 activity via TOPflash assays and for Wnt protein amount via immunoblotting using anti-
507 Wnt3a antibodies.

508

509 **Solubility assays on purified Wnt**

510 Solubility assays were conducted as previously described[29], with minor modifications.
511 Wnt proteins (500 ng/mL) were incubated in serum-free media containing vehicle, AFM,
512 or BSA at 37°C. After incubation for the indicated time, the solutions were centrifuged at
513 27,000 g for 1 hr at 4°C. The supernatants were collected and mixed with 5× Laemmli
514 sample buffer, and the pellets were dissolved with 1× Laemmli sample buffer. The samples
515 were then subjected to immunoblotting, and the amount of Wnt protein in each fraction
516 was detected using Wnt subtype-specific antibodies.

517

518 **Exosome purification**

519 Exosomes were purified by differential centrifugation as previously described [22]. Briefly,
520 Wnt3a CMs harvested from L cells or L-Wnt3a-AFM cells with or without BSA were
521 subjected to sequential centrifugation steps of 300 g, 2,000 g, and 10,000 g for 10 min, 10
522 min, and 30 min, respectively, before pelleting the exosomes at 100,000 g in a SW41Ti
523 swinging bucket rotor for 3 hrs (Beckman). Supernatants (SNΔ) were collected, mixed
524 with 5x Laemmli sample buffer, and incubated at 37°C for 10 min. For the exosomes
525 (P100), samples were mixed with 1x Laemmli sample buffer and incubated at 37°C for 10
526 min. Then, the samples were analyzed by immunoblotting using anti-mouse Wnt3a, anti-

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527 GPR177 (17950-1-AP; Proteintech, IL, USA), or anti-TSG101 (ab125011, Abcam)
528 antibodies.
529

530 **Dialysis**

531 Purified Wnt3a (500 ng/mL) and 1% CHAPS were loaded into Slide-A-Lyzer Dialysis
532 Device (10 kDa MWCO) with or without AFM or BSA. Dialysis against DMEM/F12 was
533 performed for 48 hrs at 4 °C. During dialysis, the old dialysate (DMEM/F12) was
534 discarded and replaced with fresh DMEM/F12 every 6 or 12 hrs. The samples were then
535 transferred to new 1.5 mL LoBind tubes for further assays.
536

537 **Human organoids culture**

538 Human normal stomach and colon tissue samples were obtained from patients who
539 underwent gastrectomies, colectomies or colonoscopies. All samples were collected after
540 obtaining informed consent from the patients before their participation in the study. The
541 use of donor materials for research purposes was approved by the Institutional Review
542 Board (IRB) of Yonsei University Health System (4-2012-0859 and 4-2017-0106). The
543 tissue samples were minced, washed with ice-cold DPBS, and then incubated with a gentle
544 cell dissociation reagent (Stemcell technologies) at room temperature for 30 min to
545 release gastric glands or colonic crypts. Isolated gastric glands were washed with ice-cold
546 DPBS, suspended in 25 μL of Matrigel, and seeded into 48-well plates. After 15 minutes
547 for solidification of the Matrigel at 37°C, human stomach organoid culture medium was
548 added to each well. Human stomach organoid culture medium contains advanced

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549 DMEM/F12 supplemented with 10 mM HEPES, 2 mM GlutaMax and 1x antibiotics-
550 antimycotics (all from Gibco) with the following additional factors: 2% B-27 supplement
551 (Gibco), 1 mM N-acetylcysteine (Sigma), 50 ng/ml EGF (Peprotech), 150 ng/ml noggin
552 (Peprotech), 10% R-spondin1 CM (produced using HA-R-Spondin-1-Fc 293T cells,
553 Trevigen), 200 ng/ml FGF10 (Peprotech), 10 nM gastrin-I (Sigma), 2 μM A-8301 (Tocris),
554 and 50% Wnt3a CM for stomach organoids; 2% B-27 supplement, 1 mM N-acetylcysteine,
555 50 ng/ml EGF, 100 ng/ml noggin, 10% R-spondin1 CM, 10 nM gastrin-I, 500 nM A-8301,
556 10 μM SB202190 (Tocris), and 50% Wnt3a CM for colon organoids. The medium was
557 replaced every 3 days, and the organoids were split every 7 days.

558

559 **Organoid growth assay**

560 To assess the growth of organoids treated with each Wnt3a CM preparation, the organoids
561 were digested with TrypLE (Gibco) at 37°C for 5–10 minutes until they were dissociated
562 into single cells. Cells were washed with DMEM supplemented with 1% fetal bovine
563 serum and 1% penicillin-streptomycin (Gibco) and plated on 24-well plates at a density
564 of 5,000 cells/well in 40 μL of Matrigel. After 10 days of growth, the organoids were
565 digested down to single cells using TrypLE and re-plated at the same density, while the
566 cell numbers for each well were counted. The cumulative number of cells at each passage
567 was calculated using the following equation: number of cells in the previous passage x the
568 number of cells in the current passage / the number of cells plated in the current passage.

569

570 **Quantitative real-time RT-PCR**

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571 RNA extraction from organoids was conducted using the RNeasy Mini Kit (Qiagen)
572 followed by cDNA synthesis with Superscript IV (Invitrogen) according to the
573 manufacturer's instructions. Then, cDNAs were amplified using SYBR Green Master mix
574 (Applied Biosystems) on a StepOne Real-Time PCR system (Applied Biosystems). β-actin
575 was used as an internal control and the expression level of each gene was normalized to
576 the level of β-actin. The PCR primers used were as follows: 5'-
577 AGGTCTGGTGTGTTGCTGAG-3' and 5'-GTGAAGACGCTGA GGTTGGA-3' for *LGR5* and 5'-
578 GCTGCGCTTTGATAAGGTCC-3' and 5'-GCTCATCTGAACCTCCTCT CTTT-3' for *AXIN2*.

579

580 **Immunostaining**

581 Organoids were fixed with 4% paraformaldehyde for 30 min at room temperature. Fixed
582 organoids were embedded in Histogel (Thermo scientific) and further processed into
583 paraffin blocks. Immunostaining was performed according to standard immunostaining
584 protocols. Anti-Ki67 (1:200, Cell Signaling Technology #9449) was used, and fluorescent
585 images were taken on an LSM 780 confocal microscope (Zeiss). Ki67-positive cells were
586 counted and expressed as a percentage of DAPI-positive cells.

587

588 **Quantification and statistical analysis**

589 The data were presented as means ± SEM. All statistical analyses were performed using
590 GraphPad Prism 5 software. Data obtained from the luciferase reporter assays, MTT
591 assays, immunoblots, quantitative real-time RT-PCR experiments, organoid growth assays,
592 and immunostaining experiments were analyzed using the Student's t test, ratio paired t

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593 test, one-way Analysis of Variance (ANOVA), two-way ANOVA or repeated measures two-
594 way ANOVA techniques with post-hoc Bonferroni corrections for multiple comparisons.
595 p-values < 0.05 were considered statistically significant. Additional details and the p-
596 values for statistical significance are described in the figure legends.

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605 **Author contributions**

606 J.L. and C.H.K. conceived and designed experiments. J.H.Y., S.S.K., Y.O.J., J.C., J.H., and J.Y.L.
607 performed the experiments. J.H.Y., S.S.K., J.C., I.K., J.L., Y.O.J., and C.H.K. analyzed the data.
608 J.C. and T.K. contributed reagents or materials. J.H.Y., S.S.K., J.C., I.K., J.L., Y.O.J., and C.H.K.
609 wrote the paper.

610 **Data availability**

611 All data generated or analyzed for this study are included in the manuscript and
612 supporting files; source data files have been provided for Figure 1-6.

613

614 **Declaration of interests**

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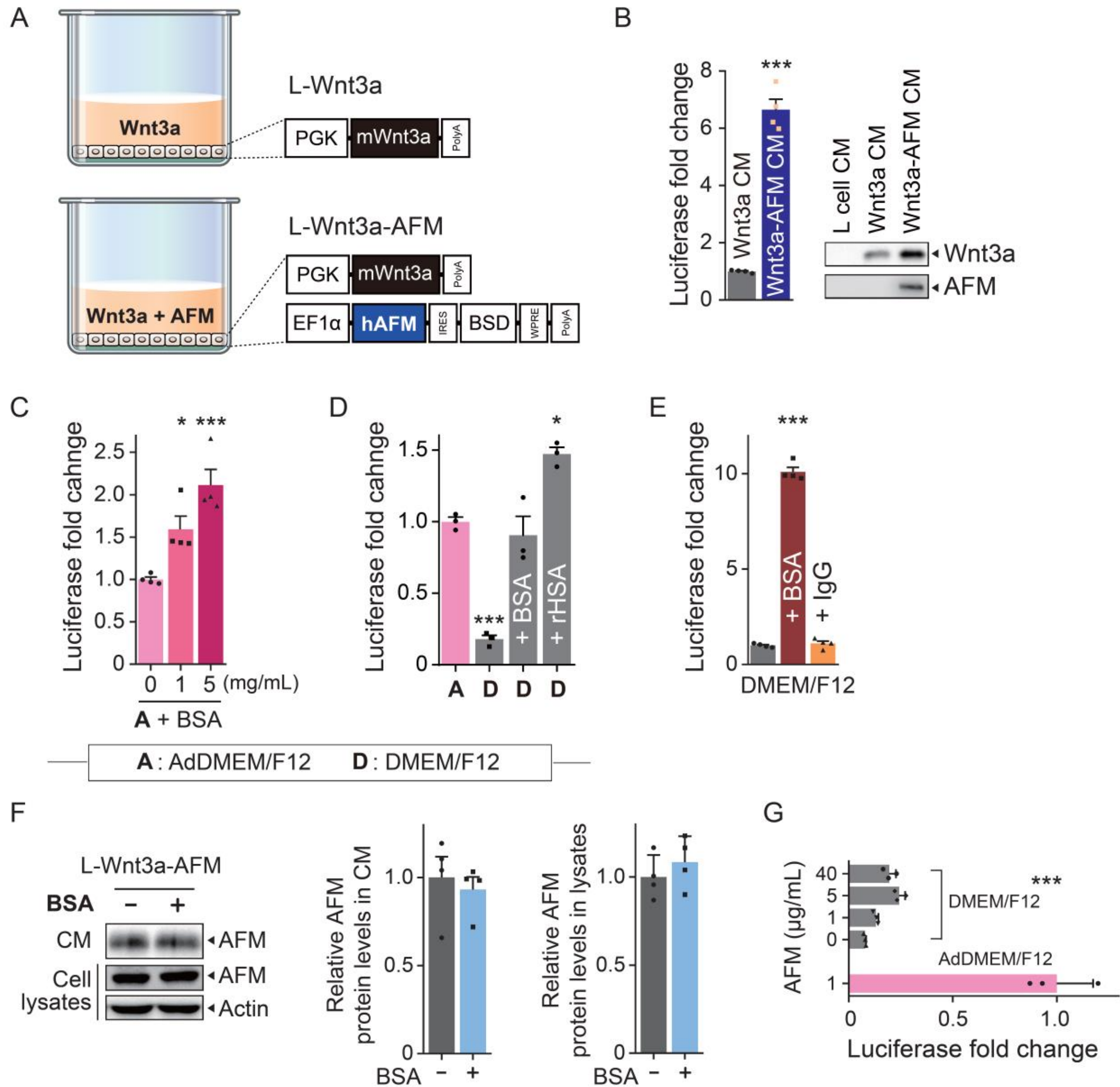

688
689 **Figure 1. SA is required to maintain the Wnt ligand activity of Wnt3a-AFM CM**. (A) L-
690 Wnt3a cells and L-Wnt3a cells transduced with lentivirus harboring pLVX-EF1α-human AFM-
691 IRES-blasticidin-S deaminase (L-Wnt3a-AFM cells) were used to produce Wnt3a CM and
692 Wnt3a-AFM CM, respectively. The media were conditioned by each cell line for 5 days. (B)
693 TOPflash assays (luciferase reporter assays of the Wnt/β-catenin signaling pathway) were
694 performed with both Wnt3a CM and Wnt3a-AFM CM to compare their Wnt3a ligand activities.
695 The luciferase activity with Wnt3a CM stimulation was set to 1 (left). Along with L cell CM,
696 which was used as a negative control, Wnt3a CM and Wnt3a-AFM CM were subjected to anti-
697 Wnt3a and anti-AFM immunoblotting to compare their Wnt3a and AFM levels (right). ***$P <$
698 0.001, unpaired Student's *t* test, compared to Wnt3a CM. (C) The effects of increasing
699 amounts of BSA on the Wnt3a ligand activity of Wnt3a-AFM CM were measured via TOPflash.
700 AdDMEM/F12: advanced DMEM/F12. *$P < 0.05$ and ***$P < 0.001$ compared to A + BSA 0
701 CM; one-way ANOVA followed by post-hoc Bonferroni corrections for multiple comparisons.
702 The luciferase activity when stimulated by Wnt3a CM produced in AdDMEM/F12 was set to 1.
703 (D) TOPflash assays were used to measure the luciferase fold changes of Wnt3a-AFM CM
704 produced in DMEM/F12 compared to Wnt3a-AFM CM produced in AdDMEM/F12.
705 AdDMEM/F12 contains 0.4 mg/ml of BSA, whereas DMEM/F12 lacks BSA. Adding BSA (0.4
706 mg/mL) or rHSA (0.4 mg/mL) to Wnt3a-AFM CM (in DMEM/F12) restored Wnt3a ligand
707 activity. rHSA: recombinant human serum albumin. The luciferase activity when stimulated by

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708 Wnt3a-AFM CM produced in AdDMEM/F12 was set to 1. **P* < 0.05, ****P* < 0.001 compared to
709 A (Wnt3a-AFM CM produced in AdDMEM/F12); one-way ANOVA followed by post-hoc
710 Bonferroni corrections for multiple comparisons. (E) Comparison of Wnt3a protein activity
711 resulting from adding either BSA (5 mg/mL) or IgG (5 mg/mL) to Wnt3a-AFM CM (in
712 DMEM/F12). The luciferase activity of Wnt3a-AFM CM (in DMEM/F12) was used as a control
713 and set to 1 on the Y-axis. IgG: Immunoglobulin-G. ****P* < 0.001 compared to the control; one-
714 way ANOVA followed by post-hoc Bonferroni corrections for multiple comparisons. (F) L-
715 Wnt3a-AFM cells were grown in DMEM/F12 media for 5 days in the presence or absence of
716 5 mg/mL BSA. AFM protein levels in both CM and cell lysates were measured using
717 immunoblotting (left). Relative protein levels according to their immunoblotting band intensities
718 are indicated in the bar graphs (right). (G) L-Wnt3a cells were grown in DMEM/F12 media for
719 5 days with varying concentrations of added AFM. TOPflash assays were performed to
720 measure the Wnt ligand activity of Wnt3a (+AFM) CM. The TOPflash activity of Wnt3a CM
721 made in AdDMEM/F12 supplemented with 1 μg/mL AFM was used as a control and set to 1
722 on the Y-axis. ****P* < 0.001 compared to the control, one-way ANOVA followed by post-hoc
723 Bonferroni corrections for multiple comparisons. The data (n=3 or 4 biological replicates) are
724 presented as means ± SEM.
725
726 **Figure supplement 1.** Formulation comparison between DMEM/F12 and AdDMEM/F12
727
728 **Figure supplement 2.** MTT assays in L-Wnt3a-AFM cells
729 L-Wnt3a-AFM cells were cultured in the indicated media for 5 days and then MTT assays
730 were performed to measure cell viability or cell proliferation. (Left) BSA (1 or 5 mg/mL) was
731 added to AdDMEM/F12 for 5 days. (Right) L-Wnt3a-AFM cells were cultured in
732 AdDMEM/F12 or DMEM/F12.

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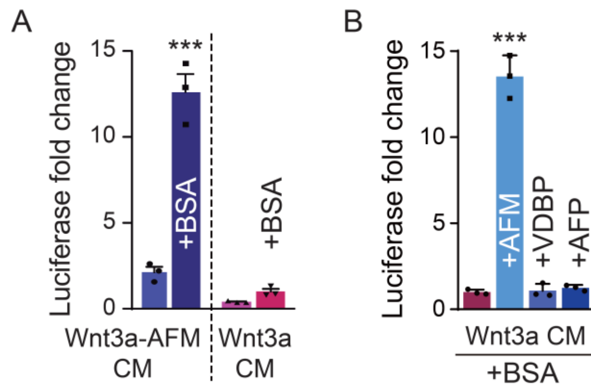

733
734 **Figure 2. AFM is the only albumin superfamily protein member required for BSA's**
735 **maintenance of Wnt3a activity in CM.** (A) The effect of exogenous BSA (5 mg/mL) on the
736 Wnt3a ligand activity of Wnt3a-AFM CM (in DMEM/F12) (left) and Wnt3a CM (in DMEM/F12)
737 (right) as measured via TOPflash assays. The luciferase activity when stimulated with Wnt3a
738 (BSA+) CM produced in DMEM/F12 was set to 1. ***$P < 0.001$, unpaired Student's *t* test,
739 compared to Wnt3a (BSA+) CM. (B) The effect of exogenous co-administration of BSA (5
740 mg/mL) with AFM (5 μg/mL), VDBP (50 μg/mL), or AFP (10 ng/mL) on the Wnt3a ligand
741 activities of Wnt3a CM (in DMEM/F12). BSA in combination with AFM, VDBP, or AFP was
742 added to the final DMEM/F12 media change for L-Wnt3a cells when producing CM. The
743 resulting CMs were subjected to TOPflash assays to measure their Wnt3a ligand activities.
744 The luciferase activity when stimulated with Wnt3a (BSA+) CM was set to 1 on Y-axis. ***$P <$
745 0.001 compared to Wnt3a (BSA+) CM; one-way ANOVA followed by post-hoc Bonferroni
746 corrections for multiple comparisons. The data (n=3) are presented as means ± SEM.

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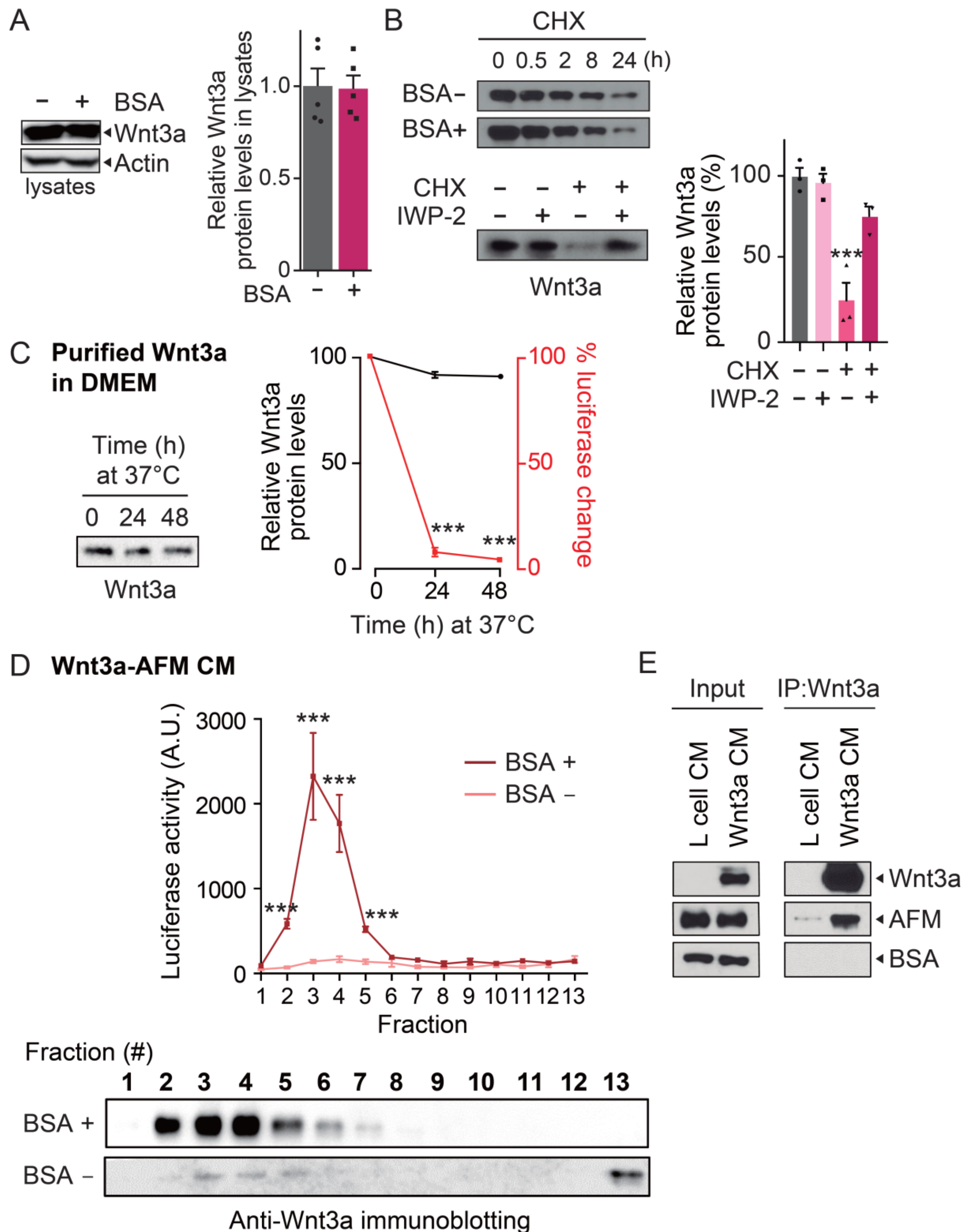

747
748 **Figure 3. SA solubilizes and stabilizes the Wnt3a-AFM complex.** (A) Wnt3a protein levels
749 in the presence or absence of additional BSA (5 mg/mL) were measured via immunoblotting
750 for Wnt3a in cell lysates (left). BSA was added to the media bathing L-Wnt3a-AFM cells for 5
751 days. The relative Wnt3a protein levels according to immunoblotting band intensities are
752 indicated in bar graphs (right) and compared to the amount of Wnt3a in lysates of L-Wnt3a-
753 AFM cells cultured in DMEM/F12 (BSA-) set to 1. Wnt3a-AFM (BSA-) CM via an unpaired
754 Student's *t* test. (B) The protein synthesis inhibitor cycloheximide (CHX, 5 μg/mL) was added
755 to L-Wnt3a-AFM cells with or without BSA (5 mg/mL) for the indicated times. Changes in

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756 intracellular Wnt3a protein levels over time were monitored using anti-Wnt3a antibodies
757 (upper). In the absence of new protein synthesis, BSA did not affect Wnt3a levels. CHX (5
758 µg/mL) and IWP-2 (5 µM), which is an inhibitor of Wnt processing and secretion, were added
759 to L-Wnt3a-AFM cells for 15 hrs. The decrease in Wnt3a levels in the presence of CHX can
760 be almost fully attributed due to the secretion of Wnt3a because IWP-2 treatment rescued the
761 loss of Wnt3a protein caused by CHX treatment (lower). ****P* < 0.001 compared to the CHX-,
762 IWP-2- control, one-way ANOVA followed by post-hoc Bonferroni corrections for multiple
763 comparisons. (C) Purified Wnt3a protein in DMEM/F12 was incubated for the indicated times
764 at 37°C and changes in Wnt3a protein levels were measured via immunoblotting (left).
765 Changes in Wnt3a protein levels and TOPflash activities are depicted in black and red,
766 respectively, in a line graph (right). ****P* < 0.001 compared to the zero-time control, one-way
767 ANOVA followed by post-hoc Bonferroni corrections for multiple comparisons. (D) Wnt3a-AFM
768 CM produced in the presence and absence of BSA were subjected to sucrose density gradient
769 centrifugation and separated into 13 fractions. The lower numbers indicate the lower-density
770 fractions containing soluble, monomeric or oligomeric Wnt3a; the higher numbers indicate the
771 higher-density fractions containing aggregated, insoluble Wnt3a. Wnt3a ligand activity was
772 measured in each of the 13 fractions using TOPflash assays (upper). The Wnt3a protein level
773 in each fraction was measured via anti-Wnt3a immunoblotting (lower). ****P* < 0.001 compared
774 to Wnt3a-AFM (BSA-) CM at each fraction, repeated two-way ANOVA followed by post-hoc
775 Bonferroni corrections for multiple comparisons. (E) L cell CM and Wnt3a CM were produced
776 in cultures of L cells and L-Wnt3a cells which were incubated in DMEM/F12 supplemented
777 with AFM 5 µg/mL and BSA 5 mg/mL for immunoprecipitation. L cell CM and Wnt3a CM were
778 subjected to western blotting using anti-Wnt3a, anti-AFM, and anti-albumin antibodies as 2 %
779 input controls. After immunoprecipitating L cell CM and Wnt3a CM with an anti-Wnt3a
780 antibody, the immunoprecipitated proteins were subjected to western blotting using the same
781 sets of antibodies. The data (n = 3–5) are presented as means ± SEM.
782
783 **Figure Supplement 1.** BSA does not affect Wnt3a secretion on exosomes

784 (A) Exosomes were purified from CM produced in cultures of L-Wnt3a-AFM, L-Wnt3a, or L
785 cells (as a negative control) with or without BSA (5 mg/mL). Differential centrifugation of CM
786 separated it into $SN_{\triangle}$ fractions and P100 exosome fraction. Western blotting for the
787 exosome marker TSG101 confirmed that P100 was enriched with exosomes. Wnt3a was
788 present in P100 pellets of L-Wnt3a or L-Wnt3a-AFM CM (based on DMEM/F12) along with
789 its cargo protein GPR177 (Wntless). BSA did not affect the amount of Wnt3a and GPR177 in
790 exosomes. (B) A quantification of Wnt3a and GPR177 in exosomes is depicted in bar
791 graphs.

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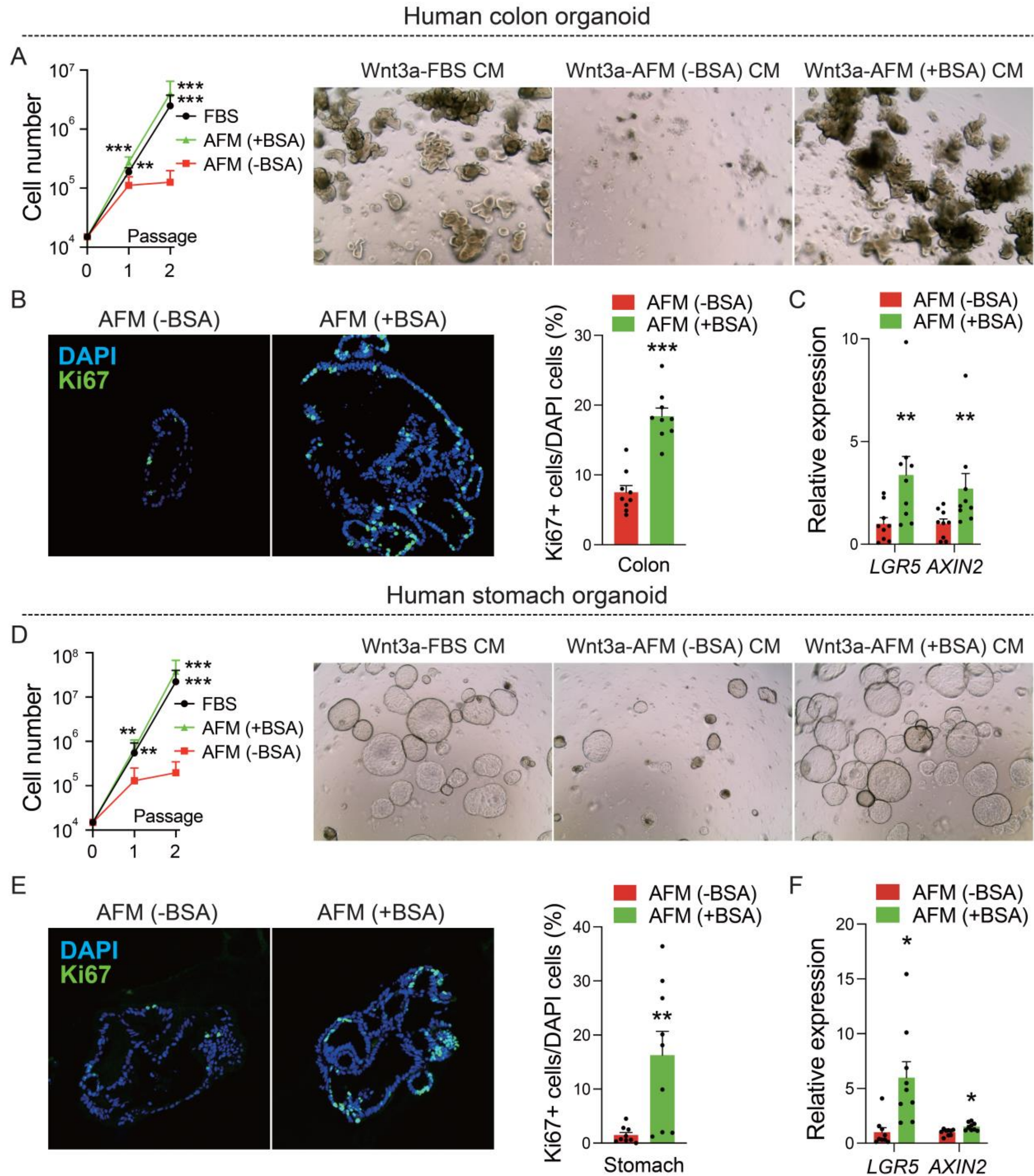

792
793 **Figure 4. Wnt3a-AFM CM needs BSA to support the growth and expansion of human adult stem cell organoids.** (A, D) Growth rate of human colon or stomach organoids in the following conditioned media: Wnt3a-FBS CM, Wnt3a-AFM (-BSA) CM, or Wnt3a-AFM (+BSA) CM. The cells in each well were counted after each passage and the cumulative number of cells at each passage was calculated by the equation in the methods section (left). Image of organoids after 10 days of culture in the indicated conditioned media (right). ***P* < 0.01 and ****P* < 0.001 compared to Wnt3a-AFM (-BSA) CM, repeated measures two-way ANOVA with log-transformed data followed by post-hoc Bonferroni corrections for multiple comparisons. (n=3 biological replicates) (B, E) Human colon or stomach organoids were immuno-stained with the proliferation marker Ki67. Confocal images of fixed organoids processed into paraffin blocks and then immuno-stained with Ki67 (green) and DAPI (blue) (left). The proportion of Ki67+ cells among the DAPI+ cells was calculated and indicated in a graph (right). ***P* < 0.01

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805 and ****P* < 0.001, unpaired Student's *t* test, compared to Wnt3a-AFM (-BSA) CM. (n=9
806 biological replicates) (C, F) Quantitative real-time RT-PCR was performed on cDNA
807 synthesized from human colon or stomach organoids cultured in the indicated CM. Expression
808 levels of the Wnt target genes *LGR5* and *AXIN2* were compared. The amount of mRNA (for
809 both *LGR5* and *AXIN2*) when stimulated with Wnt3a-AFM (-BSA) was set to 1 on the Y-axis.
810 **P* < 0.05 and ***P* < 0.01, unpaired Student's *t* test, compared to Wnt3a-AFM (-BSA) CM. (n=9
811 biological replicates) The data are presented as means ± SEM.

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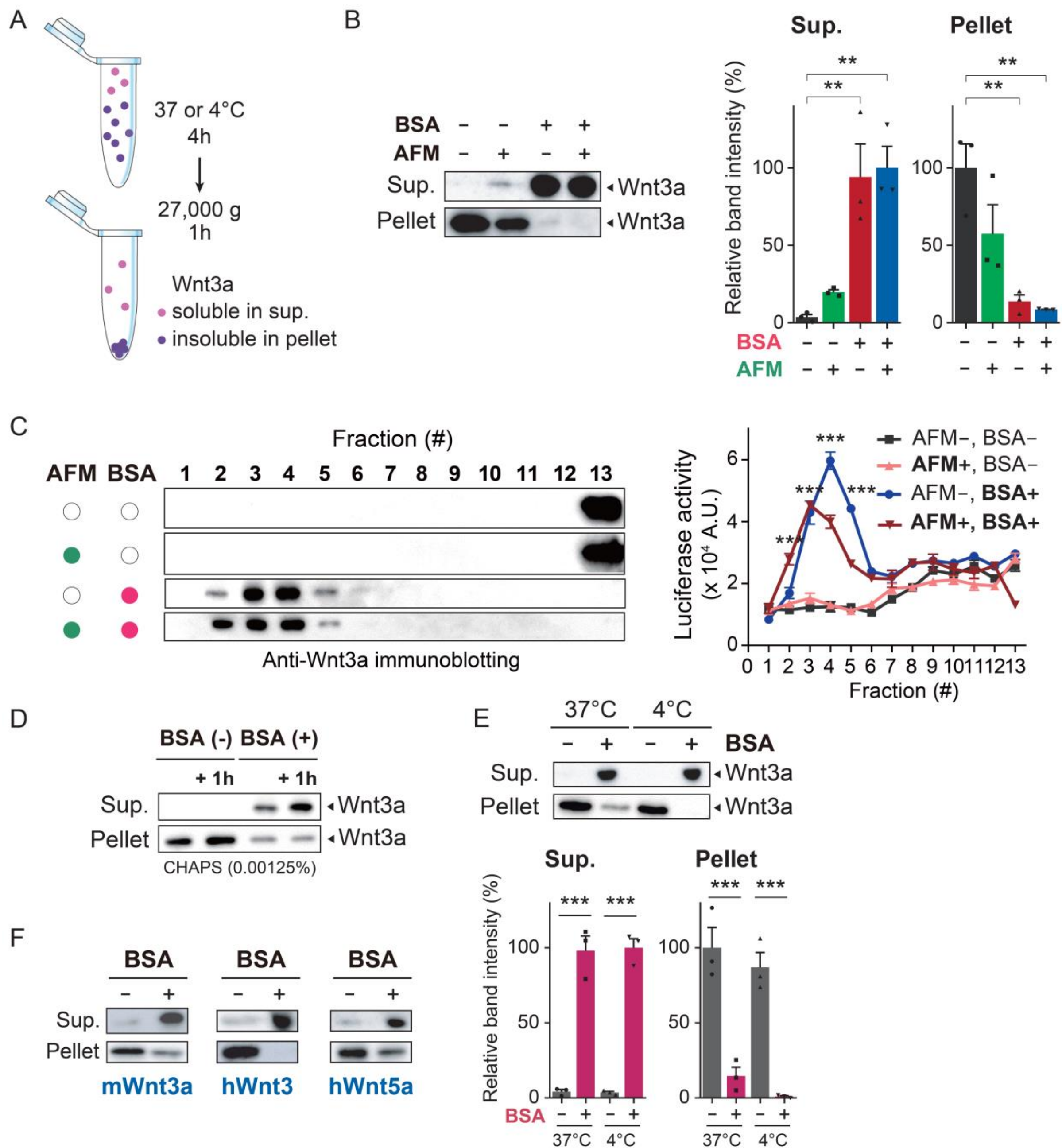

812
813 **Figure 5. SA solubilizes and stabilizes purified Wnt3a in the absence of AFM.** (A) Purified
814 Wnt3a proteins were incubated for 4 hrs in serum-free media (DMEM/F12) containing vehicle,
815 AFM, or BSA at either 4°C or 37°C. Then, the solutions were centrifuged at 27,000 g for 1 hr
816 at 4°C to separate soluble and insoluble Wnt3a. (B) Purified Wnt3a (200 μg/mL with 0.5%
817 CHAPS) was added to a final concentration of 500 ng/mL in DMEM/F12 containing vehicle,
818 BSA (5 mg/mL), or AFM (5 μg/mL). Then, the partitioning of Wnt3a into soluble or insoluble
819 fractions was examined via anti-Wnt3a immunoblotting (left). Relative Wnt3a protein levels in
820 supernatants and pellets are depicted in bar graphs (right). ***P* < 0.01 compared to BSA-
821 /AFM- control, one-way ANOVA followed by post-hoc Bonferroni corrections for multiple
822 comparisons. (C) Purified Wnt3a solution was subjected to sucrose density gradient
823 centrifugation as in Figure 3D. The effects of adding BSA and/or AFM were examined. The
824 Wnt3a ligand activity of each fraction was measured via TOPflash assays. ****P* < 0.001
825 compared to the BSA-/AFM- control at each fraction, repeated two-way ANOVA followed by
826 post-hoc Bonferroni corrections for multiple comparisons. (D) Purified Wnt3a (200 μg/mL with

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827 0.5% CHAPS) was added to a final concentration of 500 ng/mL in 1 mL of DMEM/F12. BSA
828 was added after aggregating the purified Wnt3a for 4 hrs. The solution was subjected to
829 centrifugation to separate the supernatant and the pellet just after the addition of BSA, or after
830 a 1 hr incubation with BSA. (E) The effect on Wnt3a solubility of adding BSA to purified Wnt3a
831 solution at different temperatures was measured via anti-Wnt3a immunoblotting (upper).
832 Relative Wnt3a protein levels are quantified and depicted in a graph (lower). $^{***}P < 0.001$
833 compared to the BSA- control at each temperature, two-way ANOVA followed by post-hoc
834 Bonferroni corrections for multiple comparisons. (F) BSA was added to solutions of multiple
835 members of the Wnt protein family: mouse Wnt3a (mWnt3a), human Wnt3 (hWnt3a), and
836 human Wnt5a (hWnt5a). Protein solubility was examined via a solubility assay as in (A) and
837 the presence of Wnt proteins in the supernatant or in the pellet was determined via anti-Wnt3a
838 immunoblotting. The data (n=3 or 4) are presented as means ± SEM.
839
840 **Figure supplement 1.** rHSA and BSA show similar effects on Wn3a solubility.

841 Purified Wnt3a (200 µg/mL with 0.5% CHAPS) was added to a final concentration of 500
842 ng/mL in DMEM/F12 containing vehicle, BSA (5 mg/mL), or rHSA (5 mg/mL). Then, the
843 partitioning of Wnt3a into soluble or insoluble fractions was examined via anti-Wnt3a
844 immunoblotting (left). Relative Wnt3a protein levels in supernatants and pellets are depicted
845 in bar graphs (right). Data ($n = 3$) are presented as means ± SEM. $^{***}P < 0.001$ compared to
846 BSA-, rHSA- control.
847
848 **Figure supplement 2.** BSA efficiently solubilizes Wnt3a even in trace amounts of CHAPS

849 Solubility assays were performed with purified Wnt3a co-incubated with different
850 concentrations of CHAPS in the presence or absence of BSA (5 mg/mL). Supernatants
851 (Sup.) and pellets were subjected to western blotting using anti-Wnt3a antibodies.

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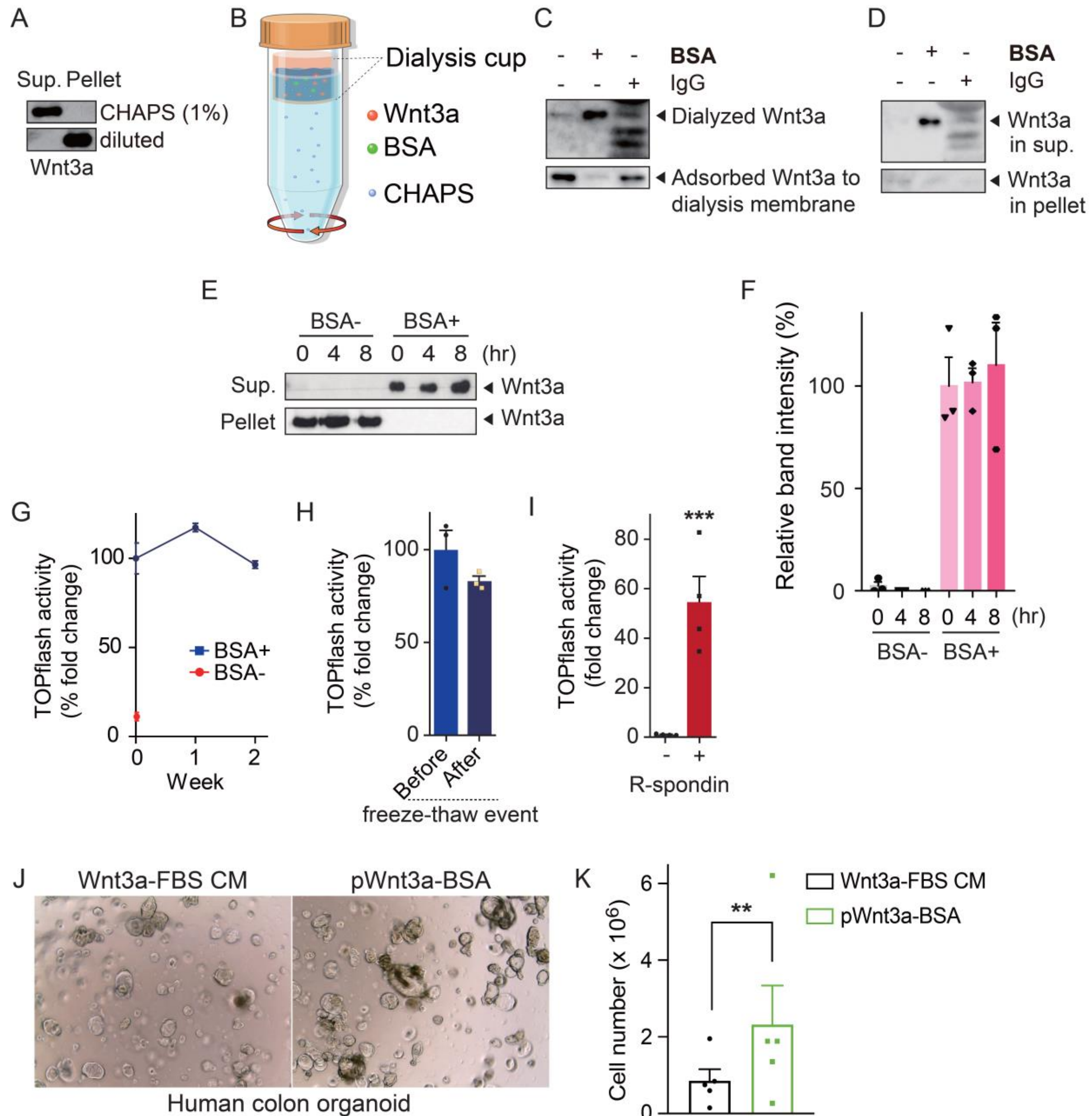

852

853 **Figure 6. A new method for solubilizing and maintaining purified Wnt3a in its active**
854 **form in the absence of CHAPS.** (A) Adding 1% CHAPS to a purified Wnt3a protein solution
855 maintains Wnt3a solubility. A solubility assay was performed on CHAPS-supplemented (1 %)
856 and CHAPS-diluted (0.001 %) purified Wnt3a solutions as seen in Figure 5A. The protein that
857 remained in the resulting supernatants and pellets was evaluated via anti-Wnt3a
858 immunoblotting. (B) Purified Wnt3a protein (200 µg/mL) containing 0.5% CHAPS was diluted
859 in 2 mL of DMEM/F12 media supplemented with 5 mg/mL BSA. Then, 2 mL of the diluted
860 purified Wnt3a was placed in a 10 kDa cut-off dialysis device. Dialysis was performed against
861 DMEM/F12 media at 4°C for 48 hrs. (C) The effect of adding BSA (5 mg/mL) or IgG (5 mg/mL)
862 to Wnt3a solutions before dialysis on the adsorption of Wnt3a to a dialysis membrane. Wnt3a
863 proteins adsorbed to the dialysis membrane were recovered using 1X Laemmli sample buffer.
864 (D) The effect of adding BSA or IgG to Wnt3a sample solutions before dialysis on their
865 partitioning into supernatants or pellets in a solubility assay of the dialyzed samples. Anti-
866 Wnt3a immunoblotting was performed with supernatants and pellets. (E, F) Purified Wnt3a
867 (200 µg/mL with 0.5% CHAPS) was added to a final concentration of 500 ng/mL to DMEM/F12
868 containing vehicle or BSA (5 mg/mL) and incubated for the indicated times at 37°C. Then, the

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869 partitioning of Wnt3a into soluble or insoluble fractions was examined via anti-Wnt3a
870 immunoblotting. Relative Wnt3a protein levels in supernatants are depicted in bar graphs. The
871 band intensity of BSA+ 0 hr was set to 100. $^{***}P < 0.001$, one-way ANOVA followed by post-
872 hoc Bonferroni corrections for multiple comparisons, compared to corresponding BSA- time
873 control. (G) Changes in TOPflash activities of purified Wnt3a dialyzed in the presence or
874 absence of BSA (5 mg/mL), after being stored at 4°C for indicated wks, were monitored for 2
875 wks. (H) Activities of dialyzed purified Wnt3a-BSA solutions (pWnt3a-BSA, 1 μg/mL) were
876 tested via TOPflash assays after a freeze-thaw cycle. (I) Wnt ligand activities of pWnt3a-BSA
877 solutions (1 μg/mL) measured via TOPflash assays after adding R-spondin1. The luciferase
878 activity when stimulated with pWnt3a-BSA solution was set to 1. $^{***}P < 0.001$, unpaired
879 Student's *t* test, compared to solutions without R-spondin1. (J) Images of human colon
880 organoids after 10 days of culture in indicated media. (K) Growth rate of human colon
881 organoids in indicated media. Cell numbers of each well were counted and the cumulative
882 number of cells after passage 2 was calculated by the equation mentioned in the method. $^{**}P$
883 < 0.01, ratio paired *t* test compared to Wnt3a-FBS CM. Data (n=3-5 biological replicates) are
884 presented as means ± SEM.
885
886

887 **Figure supplement 1.** rHSA and BSA show similar effects on growth and expansion of human
888 colon organoids

889 Growth rate of human colon organoids in indicated media (Wnt3a-FBS CM or pWnt3a-
890 rHSA). Cell numbers of each well were counted and the cumulative number of cells after
891 passage 2 was calculated by the equation mentioned in the Method. $^{**}P < 0.01$, ratio paired
892 *t* test compared to Wnt3a-FBS CM. Data ($n = 5$ biological replicates) are presented as
893 means ± SEM.

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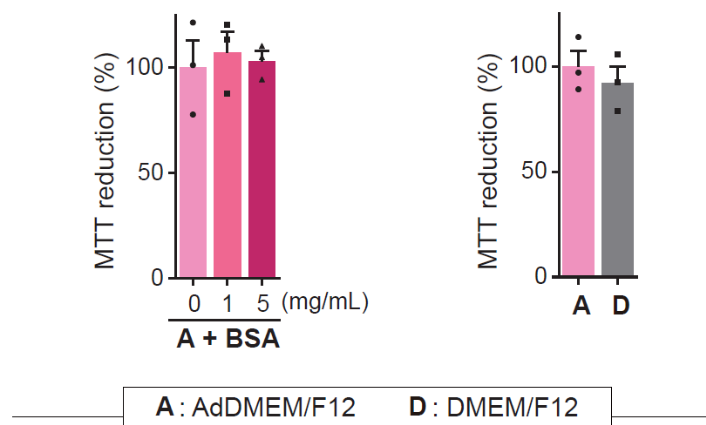
Components*	$C_{DMEM/F12}$ (mg/L)	$C_{Advanced\ DMEM/F12}$ (mg/L)
AlbuMAX® II	-	400
Insulin Recombinant Full Chain	-	10
Human Transferrin (Holo)	-	7.5
Ascorbic Acid Phosphate	-	2.5
Glutathione, Monosodium	-	1
Sodium Selenite	-	0.005
Ammonium Metavanadate	-	0.0003
Manganous Chloride	-	0.00005
L-Glutamine	365	- **

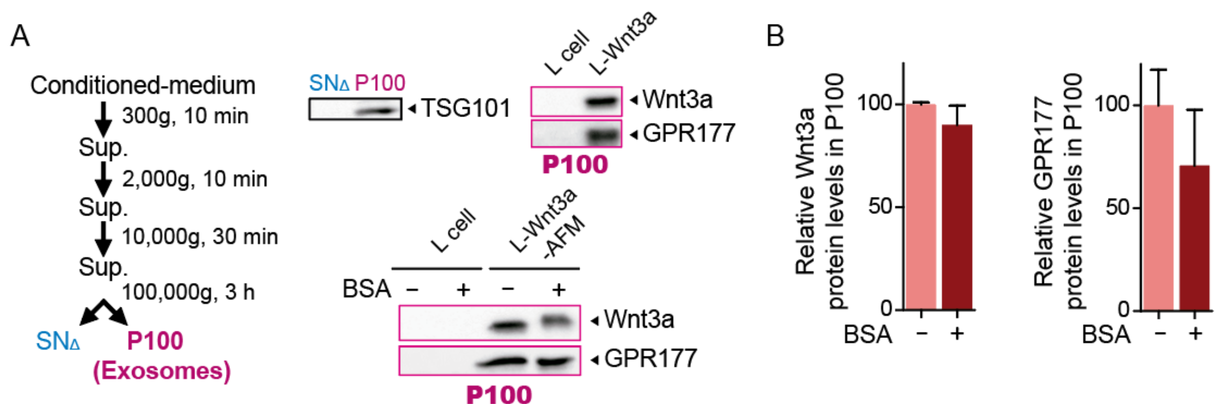
894 *The identical components in both media are not presented.
895 **The same amount of L-glutamine was added when used in experiments
896
897 **Figure 1 – Figure supplement 1. Formulation comparison between DMEM/F12**
898 **and AdDMEM/F12**
899



900

901 **Figure 1- Figure supplement 2. MTT assays in L-Wnt3a-AFM cells**

902



903

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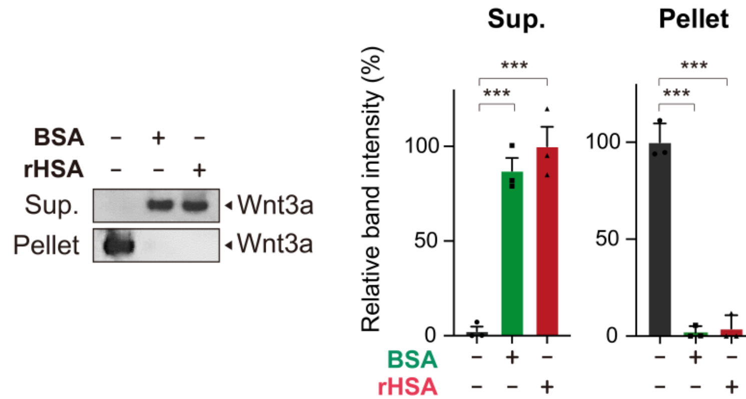
904 **Figure 3 – Figure supplement 1. BSA does not affect Wnt3a secretion on exosomes**

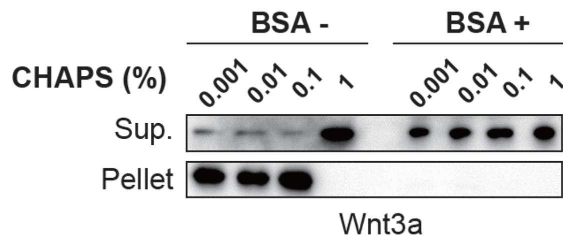
905



906

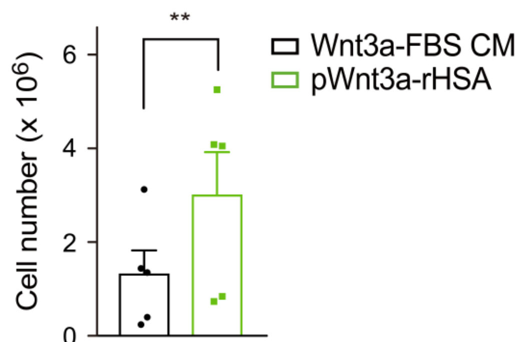
907 **Figure 5 – Figure supplement 1. rHSA and BSA show similar effects on Wnt3a**
908 **solubility**

909



910

911 **Figure 5 – Figure supplement 2. BSA efficiently solubilizes Wnt3a even in trace**
912 **amounts of CHAPS**



913

914 **Figure 6 – Figure supplement 1. rHSA and BSA show similar effects on growth and**
915 **expansion of human colon organoids**

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916 **Source data – Figure 1-Source data 1. Raw data related to Figure 1B-1G; Original**
917 **blots for Figure 1F**

918 **Source data – Figure 2-Source data 1. Raw data related to Figure 2A, B**

919 **Source data – Figure 3-Source data 1. Raw data related to Figure 3A-D; Original blots**
920 **for Figure 3A-E**

921 **Source data – Figure 4-Souce data 1. Raw data related to Figure 4A-F**

922 **Source data – Figure 5-Source data 1. Raw data related to Figure 5B, 5C and 5E;**
923 **Original blots for Figure 5B-F**

924 **Source data – Figure 6-Source data 1. Raw data related to Figure 6F-I and 6K;**
925 **Original blots for Figure 6A, 6C-E**