Plasmodesmata-like intercellular connections by plant remorin in animal cells

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Summary

Plasmodesmata (PD) are crucial structures for intercellular communication in multicellular plants with remorins being their crucial plant-specific structural and functional constituents. The PD biogenesis is an intriguing but poorly understood process. By expressing an *Arabidopsis* remorin protein in mammalian cells, we have reconstituted a PD-like filamentous structure, termed remorin filament (RF), connecting neighboring cells physically and physiologically. Notably, RFs are capable of transporting macromolecules intercellularly, in a way similar to plant PD. With further super-resolution microscopic analysis and biochemical characterization, we found that RFs are also composed of actin filaments, forming the core skeleton structure, aligned with the remorin protein. This unique heterologous filamentous structure might explain the molecular mechanism for remorin function as well as PD construction. Furthermore, remorin protein exhibits a specific distribution manner in the plasma membrane in mammalian cells, representing a lipid nanodomain, depending on its lipid modification status. Our studies not only provide crucial insights into the mechanism of PD biogenesis, but also uncovers unsuspected fundamental mechanistic and evolutionary links between intercellular communication systems of plants and animals.

Significance

Remorin is rising as a crucial lipid microdomain marker, as well as an essential component of plasmodesmata in plants. However, the biological role of remorin in plants is elusive. With a heterologous system, we found that remorin expression is able to form intercellular filamentous structure, namely remorin filament (RF), connecting neighboring mammalian cells functionally. By employing multiple approaches, we tested the functionality of RFs, as well as investigated their structures. RFs highly resemble plant plasmodesmata, in many ways, such as its morphology, molecular constitutes, and its ability to transport macromolecules intercellularly. Our study provides novel insights into the biogenesis of plasmodesmata and uncovers fundamental evolutionary links in molecular construction of intercellular connections in both plants and animals.

Introduction

In a multicellular organism, cells need to exchange both substances (such as proteins, sugars and nuclear acids) and information (such as hormones and secondary messengers) to coordinate with each other for normal cell activity and coordinated tissue development. Cell-to-cell communication is essential in both plants and animals; however, the involved subcellular structures and underlying molecular machineries are fairly diverse (1, 2), presumably owing to the fact of independent evolution of multicellularity in these two major eukaryotic groups and very different cellular structures and tissue morphology. The vesicle systems of plants and animals transport biomolecules/materials to neighboring cells through phagocytosis, pinocytosis, or receptor-mediated endocytosis. A complex system specific for plants, plasmodesmata (PD), executes more efficient intercellular communication, enabling plants to have a tightly controlled symplasmic network crossing the apoplast, whereas animal cells without complication of cell walls can rely on direct membrane connections.

PD are nanotubes connecting neighboring cells in plants and given their absence in animals it is generally considered to be one of the major differences between animal and plant tissues (1–3). Previous studies showed that remorin is a plant-specific protein located at the PD that marks the lipid microdomain or nanodomain, it has also been reported to be related to the function of plant PD (4–9). In rice (*Oryza sativa*), *GRAIN SETTING DEFECT1* (*GSD1*), encoding a remorin protein, regulates PD conductance to participate in the grain setting process (5). GSD1 functions through interacting with OsACTIN1 and OsPDCB1 (PLASMODESMATA CALLOSE BINDING PROTEIN1) (5). A recent study reveals that *Arabidopsis* remorin proteins, REM1.2 and REM1.3, are crucial regulators for plasma membrane nanodomain assembly to control PD closure, in response to a defense hormone salicylic acid (SA) (9). Based on these observations, remorin might be a key factor for PD biogenesis. However, due to the essential physiological function of the plant PD system, it is difficult to verify the molecular role of remorin in PD function with the plant system.

Here, we investigated the effects of heterologous expression of remorin in mammalian cells. This leads to the formation of PD-like structures connecting cells and allowing exchange of material. Our study shows that a single plant protein, remorin, is sufficient to induce functional PD-like intercellular connections in animal cells providing crucial insights not only into the

91 mechanism of PD biogenesis but also into the evolutionary commonalities in intercellular
92 communication between animals and plants.

93 **Results**

94

95 **Deficiency of *REMORINs* leads to PD morphological abnormality in *Arabidopsis***

96 In *Arabidopsis*, the remorin family comprises 16 members, of which Remorin1.2 (REM1.2,
97 AT3G61260) and Remorin1.3 (REM1.3, AT2G45820) show high similarities and were
98 reported to be localized at the PD (4, 6, 7, 9). To study the role of remorin in PD formation
99 and function, we obtained a T-DNA insertional mutant for *rem1.3* and a CRISPR-CAS9 mutant
100 for *rem1.2*, and generated also a double mutant *rem1.2 rem1.3* (Fig. S1A, B). Notably, the
101 *rem1.2 rem1.3* double mutant exhibited attenuated growth (Fig. S1C), indicating that REM1.2
102 and REM1.3 are essential for plant growth. This is in line with the previous notion that the
103 *rem1.2 rem1.3* double mutant also showed attenuated growth at the seedling stage (9). We
104 firstly tested the potential involvement of PD in the remorin function. PD play an essential
105 role in transporting different molecules, including carbohydrates in plant cells (1, 2). Notably,
106 51% of the leaves of the *rem1.2 rem1.3* had a significantly positive starch accumulation
107 whereas undetectable in WT (Fig. S1D), indicating its deficiency in starch unloading. A further
108 test for the PD permeability with a fluorescent dye (Fig. S1E), FDA (fluorescein diacetate) (5,
109 10) confirmed that, FDA was pre-infiltrated into plants and then bleached by a strong laser
110 exposure. Intriguingly, the *rem1.2 rem1.3* plants recovered more slowly than WT (WT, 70%;
111 *rem1.2 rem1.3*, 30% at 290 s).

112 Furthermore, detailed analysis with TEM (transmission electron microscopy) indicated that
113 the number of PD was significantly decreased in *remorin* mutants (Fig. S1F; WT, 5.4; *rem1.3-*
114 *1*, 4.0; *rem1.2-1*, 2.1; *rem1.2 rem1.3*, 1.8). However, several dark osmiophilic globules (~ 50
115 nm in size) were observed in the *rem1.3* and *rem1.2* cells, with a greater number in the *rem1.2*
116 *rem1.3* cells (Fig. S1F), which is probably due to PD dysfunction (9, 11). In consistent with
117 the notion that REM1.2 and REM1.3 also play a crucial role in the closure of PD (5, 9), all
118 these defects support the essential function of remorin in PD.

119

120 **Filamentous intercellular structures by expressing remorin in mammalian cells**

121 In line with the notion that remorin localizes to PD and regulates PD aperture (9), our
122 observations support that the requirement of remorin for PD function. To gain additional
123 insights into remorin function, we transfected eGFP-tagged *REM1.3* into the mammalian cells.
124 We hypothesize that this heterologous system might be helpful to understand the exact cellular
125 function of remorin, for the relatively less complicated background and the absence of all other
126 plant-specific factors.

127 Introducing eGFP-REM1.3 into Cos7 cells, confocal laser scanning microscopy (CLSM)
128 observation revealed eGFP-REM1.3 distribution in some filamentous structures (termed
129 remorin filaments, RFs) connecting neighboring cells (Fig 1A). Interestingly, both ends of the
130 RFs were swelling, with positive staining of the endoplasmic reticulum (ER) marker protein
131 calnexin (12) accumulating at both ends. This became particularly apparent when the cell
132 density is low. A schematic diagram of RFs across two animal cells (Fig 1A right) is a typical
133 structural feature for plant PD that also included ER structures more prominent at the endos
134 (9). Therefore, we hypothesize that these RFs possibly assemble into structures similar to plant
135 PD. To directly observe their biogenesis, a time-lapse imaging was obtained using a video
136 microscope. The results clearly show how the RFs bud out of the vesicles and grow by gradual
137 elongation (Fig 1B).

138 The main function of PD in plants is to communicate through signals and biomolecules
139 between cells (1–3, 13). To confirm that remorin expression in animal cells can create
140 functional cell-to-cell connections, we infected primary myocardial cells, using the eGFP-
141 REM1.3 adenovirus. Because primary myocardial cells have a rhythmic impulse, it is possible
142 to demonstrate that two cells or whole cell populations are connected if they have coordinated
143 rhythm. In line with this, simultaneous video analysis showed that two cells or cell populations
144 linked by RFs share a synchronous beat rhythm revealing that ions pass through the two groups
145 of cardiomyocytes through the RFs consistent with the functional intercellular connections
146 compared to the control (free eGFP) cells (Fig 1C and D).

147 These experiments reveled that expression of plant remorin in different types of animal
148 cells produce RF cell-to-cell connections similar to plant PD.

149

150 **Parallels between remorin-induced connections in animal cells and plasmodesmata**

151 To characterize the similarity between RFs and the plant PD and to explore their biogenesis,
152 we performed a series of microscopic observations and biochemical experiments. RFs have a
153 diameter of approximately 100-150 nm, one-tenth to half of a cell length, and are usually
154 branched (Fig 1E, 4A-B and Table S1). Co-stained with different markers, RFs stained positive
155 for phalloidin (actin filaments, AFs) and negative for α-tubulin (microtubule, MT) markers,
156 vimentin (intermediate filament) and Fascin (filopodial markers), suggesting that RFs are AF-
157 based (Fig 1E). Cells treated with cytochalasin B, an AF-depolymerizing reagent, exhibited
158 collapsed RFs (Fig 1F), confirming that RFs not only contain AFs and also that AFs are
159 essential for RF integrity. These characteristics are very similar to plant PD, which was also
160 found to be associated with enriched actin filaments (1, 3, 14–16). Indeed, these observations
161 may also help elucidate the role of actin filaments in PD function, here via interacting with the
162 remorin protein.

163 To further explore the analogy between the RFs in animal cells and PD in plants, we
164 analyzed the basic biochemical properties of remorin in animal cells. Remorin was previously
165 reported to localize at certain lipid microdomains (4, 5, 7). In consistent with the previous
166 results, remorin isoforms (REM1.2, REM1.4, and REM1.1) can be expressed in mammalian
167 cells and is associated with the lipid microdomains through sucrose-density gradient
168 centrifugation (Fig S2A). Remorin is also reported to be post-translationally modified by
169 palmitoylation (7). We employed an acyl-biotin exchange assay to detect the palmitoylation
170 of REM1.3. The results showed that wild-type REM1.3 expressed in mammalian cells can be
171 palmitoylated (Fig S2B). These results showed that heterologously expressed REM1.3 in
172 mammalian cells presents the same subcellular localization and biochemical modification as
173 in plant cells.

174 These experiments reveled that plant remorin-induced RF cell-to-cell connections in animal
175 cells are actin-based like PSs and are structurally and biochemically similar to plant PD.

176

177 **Remorin-induced functional intercellular connections in mammalian cells**

178 To test whether heterologous expression of remorin in animal Cos7 cells can result in
179 communication/transport of macromolecules between cells to mimic the PD function. A set of
180 highly sensitive detection systems based on Cre-LoxP was designed (Fig 2A). The lmlg (LoxP-
181 mCherry-multiple stop codons-LoxP) element is an expression box consisting of LoxP,

182 mCherry, multiple stop codon sites, LoxP and GFP proteins in series. In the absence of Cre
183 recombinase, only mCherry was expressed (red); in the presence of Cre recombinase, the
184 mCherry and multi-stop codon sites in the middle of LoxP were excised, leading to expression
185 of the GFP protein (green). The Cre recombinase was transfected into donor cells. If the
186 proteins between the donor and recipient cells could communicate with each other, receptor
187 cells with green fluorescence were expected to be visualized.

188 Analysis by fluorescence microscopy and flow cytometry showed that donor cells
189 expressing remorin and an ER-localized Cre recombinase enabled the receptor cells to exhibit
190 GFP fluorescence (Fig 2B and C), while non-ER-localized Cre recombinase or a lack of
191 remorin expression in the donor cells exhibited no detectable signal (Fig 2B and C). This result
192 confirms that remorin expression in the donor cells can transmit Cre recombinase to the
193 receptor cell (Fig 2B and C). Next, by DNA staining, we observed that RFs were able to
194 transport DNA between cells (Fig 2D).

195 These observations collectively show that RFs indeed represent functional intercellular
196 connections capable of transmitting not only electrical signals, but also proteins and DNA
197 between animal cells.

198

199 **Role of different remorin domains in mammalian cells and plants**

200 To study the biogenesis process of RFs, we investigated how the primary structure of REM1.3
201 contributes to RF formation in mammalian cells. We expressed various truncated REM1.3s
202 (Fig 3A and S5), and observed RF formation and lipid microdomain localization to gain insight
203 into the contribution of each domain as summarized in Table S2.

204 Lack of the N-terminus of REM1.3 (CT) does not obviously affect the remorin localization
205 pattern (Fig 3B and S6). On the other hand, when we truncated most of the N-terminus
206 containing the coiled-coil domain (T) or truncated only four amino acids in the C-terminus (1-
207 186), the distribution pattern of REM1.3 significantly changed into a disperse eGFP signal.
208 Lipid microdomain localization of these two truncated versions was also abolished (Fig 3D
209 and S7), indicating that the coiled-coil domain and the four amino acids in the C-terminus are
210 essential for the formation of RFs. Consistently, a CC domain deletion mutation (ΔCC) could
211 not form RFs (Fig 3A-C).

212 To study the contribution of the TLD domain (aa 150-186), we linked the four amino acids
213 of the C-terminus directly to the N-terminus with the coiled-coil domain (NCP). This
214 truncation showed a disperse distribution but did not affect the protein lipid microdomain
215 localization. Further deletion of the N-terminal amino acids 1-76 (CP) showed a lipid
216 microdomain distribution but, notably, also small uniform punctate distribution in cells.
217 Phalloidin staining demonstrated that those special structures are adjacent to, but not
218 overlapping with, the F-actin dots (Fig 3B). These results suggest that the TLD domain
219 contributes to the formation of the long RF helix and retains the ability to associate with actin
220 oligomers as an initiative structure of RFs. The observation that the CP version cannot form
221 shaped structures also indicated that the N-terminus 1-76 of REM1.3 is an inhibitory domain
222 for RF formation (Fig 3B).

223 The lack of coiled-coil domain, mutation of the cysteine at the C-terminal anchor or
224 deletion of the TCHP-like domain (TLD) impairs RF biogenesis. Removing the N-terminal
225 domain from the TCHP-like-domain-lacking REM1.3 can still form the initiatory structure of
226 RFs next to F-actin but results in RFs extending to a lesser degree. This indicates that the
227 coiled-coil domains interact with F-actin and form an RF organization center near the cell
228 membrane, which is responsible for RF initiation. The docking data based on Remorin's
229 predicted structure and the F-actin crystal structure (http://hexserver.loria.fr/) also support the
230 above notion (Fig 3C). So TCHP-like domain is likely to be responsible for the extension of
231 RFs.

232 To further study the molecular mechanism of remorin binding to lipid microdomains and
233 their contributions to the formation of RFs, we mutated cysteine 187 or 189 to alanine (Ala,
234 A) or aspartic acid (Asp, D) separately or together. The distribution of REM1.3 changed to
235 inclusion bodies in the cytoplasm (Fig S6), and the proteins disappeared from the lipid
236 microdomains (Fig 3D and S7). This corresponded to our results from the palmitoylation study
237 (Fig S3B). We also linked the four amino acids CGCF of the C-terminus of REM1.3 (aa 187-
238 190) directly into the tail of eGFP; ~30% eGFP proteins were partially associated with the
239 lipid microdomains (Fig 3D and S7) but showed a dispersed localization (Fig S6), which
240 suggests that those four amino acids are sufficient to recruit the protein onto the lipid
241 microdomains.

242 To test whether that the knowledge obtained from animal cells is also relevant in plants, we

243 transformed different truncated versions of *REM1.3* driven by a CaMV35S promoter into WT
244 *Arabidopsis*. The results showed that the full-length (FL) protein and the C-terminus (CT)
245 localized at the plasma membrane (PM) without any nuclear signals (Fig S8). In contrast, the
246 CP truncation (aa 77-149+187-190) showed uniform punctate structures (Fig S8), which is
247 consistent to our observations in mammalian cells (Fig S8). Further TEM observation revealed
248 that the FL, CT and CP, led to the dark staining bodies (osmiophilic globules) as observed in
249 the *rem1.2 rem1.3* cells (Fig S1 and S8), which might be due to the ectopic overexpression of
250 *REM1.3*.

251

252 **Deep structures of RFs by super-resolution and electron microscopy**

253 To determine the fine structure of plant-remorin induced RF in aminal cells, we use
254 structured-illumination microscopy (SIM), a super-resolution technique evaluated the detailed
255 structure of RFs by eGFP-REM1.3. Refined images (Fig 4A) indicate that the two eGFP-
256 remorin parallel spiral helices twin AFs with an external diameter of 103.34±5.95 nm and a
257 pitch of 140.11±27.21 nm, which is further confirmed by quantification analysis (Table S1).
258 We also used another super-resolution microscopy with a different principle from SIM,
259 stochastic optical reconstruction microscopy (STORM). Both N-Storm (Fig 4B and S4) and
260 C-Storm (Fig 4C) images showed that eGFP-REM1.3-labelled RFs were spiral helices,
261 consistent with the SIM results. These observations revealed striking similarities between RFs
262 and PD structures.

263 Moreover, a comprehensive transmission electron microscopy (TEM), which allows us to
264 combine fluorescence imaging and TEM in the same sample by using a fluorescein and nano-
265 gold particle double-labeled secondary antibody, clearly demonstrated that the fluorescence
266 signal of RFs was observed as gold-particle-positive helical structures on TEM (Fig 4D). This
267 further confirmed the spiral structure of RFs determined by super-resolution microscopy.

268 In summary, these super-resolution and ultrastructure studies revealed that the functional,
269 plant remorin-induced intercellular connections in animal cells show striking ultrastructural
270 similarities to plant plasmodesmata.

271

272

Discussion

Plant remorin induces functional intercellular connections in animal cells

Previous studies established that plant specific remorins are detected at and functionally important constituents of PD (6, 12, 17). Using a genetic approach, we provided further evidence that remorin regulates the connectivity of the PD confirming remorins as crucial structural and functional components of PD intercellular connections in plants.

Unexpectedly, expressing remorin in animal cell cultures induced functional, PD-like intercellular connections that are clearly distinct from the previously reported well known filamentous cell surface structures, such as cilia, flagella or intermediate filaments (18); therefore we called them RFs.

RFs perserve a high degree of similarity with PD in function. Transporting proteins in the endoplasmic reticulum system is an important functional feature of plant PD. Our experiments show that RFs can not only connect two cardiac muscle cells, and communicate signals between cardiac muscle cell populations and keep them in synchronous rhythm. In addition, RFs can also communicate the biological macromolecules in two animal cells. In our system, RFs enable the provider cells to transport the Cre protein existing in the endoplasmic reticulum to the recipient cells, cut the LoxP motif, and make fluorescence protein expression. An ER-distributed protein is indeed transferred from donor cells to acceptor cells in the presence of remorin, which is very similar to the classic function of PD (1–3).

Structural similarity between plant PDs and RFs in animals

Here we identified RFs are assembled from remorin, i.e., a quadruple actin-remorin helix (double actin helices × double remorin helices) skeleton for RFs (Fig 4E). Although RFs formed by remorin consists of AFs, it is different from filopodia and tunneling-nanotubes (19). RFs do not have filopodia or tunneling-nanotube marker proteins (3, 19, 20). Moreover, RFs are swollen at both ends, which is different from the classic structure of tunneling-nanotubes, but it is very similar to the structure of a plasmodesma (3). This suggests that a single remorin protein is sufficient for the initiation and maintenance of the core PD structure.

The similar results obtained from both plant and mammalian cells are mutually supportive; for instance, the REM1.3(CP) mutated version exhibited similar behavior in both animal and plant cells linking the cytoskeleton to the cell membrane and stretching it into a filamentous form. Based on these observations, we propose a hypothesized model for PD biogenesis (Fig 4F). Remorin binds to cell membrane through lipid modification, forming a local membrane nanodomain. Meanwhile, remorin also binds the actin filaments, perhaps guiding their direction of extension. This dual function of remorin organizes a local filament, providing the force to remodel the membrane to form a tubular PD structure.

Evolutionary relation of plant plasmodesmata and animal migrasomes

Migrasome is a newly discovered filamentous structure of animal cells (19, 20). It is related to the cell movement and plays a role in intercellular communication in animal cells. Firstly, migrasome has spherical protrusions on the filaments, and our RFs also have this feature. Second, migrasome has been shown to be encapsulated into cytoplasmic components and released outside the cell (19), and we have observed this phenomenon in RFs, which encapsulates intracellular substances such as DNA and endoplasmic reticulum. Finally, the biochemical properties of RFs are similar to migrasomes, which are rich in Actin and are related to lipid microdomains. Those factors suggest RFs are structurally similar to migrasome (Fig S9). However, biogenesis of migrasome seems to depend on cell movement and division (19, 20), while RFs actively protrude out of cells. Therefore, we hypothesize that remorin may be a factor for multicellular organisms to actively form intercellular connections. This can also be confirmed by the molecular evolution of remorin itself, which firstly appears in multicellular plants above algae, suggesting its causal relationship to multicellular communication. Because animals have evolved a highly developed circulatory system, their permissive capacity between cells is weaker than that of plants. Therefore, the biological mechanism of similar structures is slightly degraded and passive.

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Author contributions

Z. W., S. T. proposed the initial idea of exogenous Remorin in animal cells. Z. W., S. T., K. L and H. X. designed the experiments. Z. W., S. T. and T. L. performed most of the experiments and data analysis. Z. W., T. L. carried out substances transport experiment in animal cells. Z. W., S. T., J.F., K. L and H. X. wrote the manuscript.

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388

389

390 **Figure Legends**

391 **Fig 1. PD-like structure formed in animal cells transfected with eGFP-REM1.3.**

392 (A) PD-like structure formed in animal Cos7 cells transfected with eGFP-REM1.3 and stained
393 for ER with calnexin antibody (red: Calnexin; green: REM1.3; blue: DNA; scale bar, 10 μm).
394 The right portion shows PD-like structure pattern on animal cells (red: endoplasmic reticulum;
395 green: cell body; blue: cell nucleus).

396 (B) Image subtraction of GFP-REM1.3-transfected Cos7 cells by video microscope (gray:
397 REM1.3; scale bar, 10 μm). The upper right panel shows the pattern diagram of remorin
398 budding out the vesicle.

399 (C) Mouse cardiac myocytes infected with eGFP-REM1.3 or eGFP control vector adenovirus.
400 Connections are two or more cells or cell groups linked by filamentous structures with
401 collaborative impulse. The pulsatile displacement of both cell clusters (red: A; blue: B) was
402 recorded on the red and blue lines on Image Pro Plus software, respectively. (green: REM1.3
403 or eGFP-control; gray: bright field; scale bar, 200 μm).

404 (D) Statistical analysis of cell populations or cells that are connected together (Numbers of
405 connectors per field ± SD).

406 (E) HEK293 cells transfected with eGFP-REM1.3 (green: REM1.3; red: phalloidin-stained F-
407 actin; scale bar, 10 μm).

408 (F) F-actin depolymerization prompted collapse of RFs. eGFP-REM1.3-transfected HEK-293
409 cells were treated with the depolymerization reagent cytochalasin B (1 μg/mL) for 15 min
410 (green: REM1.3; red: phalloidin-stained F-actin; blue: DNA; DMSO: solvent control; CBB:
411 cytochalasin B, scale bar, 10 μm).

412

413 **Fig 2. Exogenously expressed remorin enables animal cell-cell material transport.**

414 (A-C) Co-expression of remorin and endoplasmic-reticulum-located CRE recombinase (ERs-
415 Cre) in donor cells, expression of "lmlg" (LoxP-mCherry-multiple stop codons-LoxP)
416 elements in acceptor cells. The lmlg element is an expression box consisting of LoxP, mCherry,
417 multiple stop codon sites, LoxP and GFP proteins in series. In the absence of Cre recombinase,
418 only mCherry was expressed (red); in the presence of Cre recombinase, the mCherry and

419 multi-stop codon sites in the middle of LoxP were excised, leading to expression of the GFP
420 protein (green). The Cre recombinase was transfected into donor cells. If the proteins between
421 the donor and recipient cells could communicate with/transport to each other, the lmlg element
422 is cleaved by CRE, mCherry (mCr) and multiple stop codons are excised, and GFP begins to
423 express, receptor cells with green fluorescence were expected to be visualized. After
424 transfection for 48 hours, 1:1 mixed cells are cultured for 48 hours before observation by
425 microscope (green: GFP; red: mCherry; blue: DNA: Scale bar, 100 μm), double-positive cell
426 statistics conducted by flow cytometry with a bar chart [% Numbers of GFP positive / (GFP
427 positive + mCherry positive) cells ± SD].

428 (D) PD-like structure transports DNA from cell. Animal cells transfected with eGFP-REM1.3
429 (green: REM1.3; red: phalloidin stained F-Actin; pink: Calnexin; blue: DNA stained by
430 diaminophenindole, DAPI; scale bar, 10 μm)

431

432 **Fig 3. Remorin forms PD-like RFs in mammalian cells.**

433 (A) Remorin truncated mutation pattern diagram, the number represents the amino acid
434 position, C represents cysteine.

435 (B) Remorin truncated mutant-transfected in HEK293 cells (green: REM1.3; red: phalloidin-
436 stained F-actin; blue: DNA; scale bar, 10 μm).

437 (C) Predicted structures of REM1.3 protein docking to actin oligomer from PDB (1ATN, 1J6Z,
438 3EL2 and 4A7N).

439 (D) Western blot analysis of the extracts of lipid microdomains (Arrow indicates) from
440 truncated or mutated eGFP-remorin-transfected HEK-293 cells, a. FL, b. C2A, c. delete 77-
441 149 (ΔCC), d. 1-149 and 186-190 (NCP), e. 77-149 and 186-190 (CP), f. 77-190 (CT). The
442 upper panel indicates the sucrose gradient. IN is an unisolated start sample.

443

444 **Fig 4. Ultrastructural analysis of RFs**

445 (A) SIM image of eGFP-REM1.3-transfected HEK-293 cell (green: REM1.3; red: phalloidin-
446 stained F-actin; blue: DNA; scale bar, 10 μm).

(B) N-STORM images of eGFP-Remorin and Actin-mCherry co-transfected HEK-293 cell stained by activator-reporter paired secondary antibodies (green: 405-647; red: Cy3-647; scale bar, 10 μm).

(C) C-STORM images eGFP-Remorin-transfected HEK-293 stained by a GFP antibody and ALEXA-647-labeled secondary antibody (green: REM1.3; red: phalloidin-stained F-actin; scale bar, 100 nm).

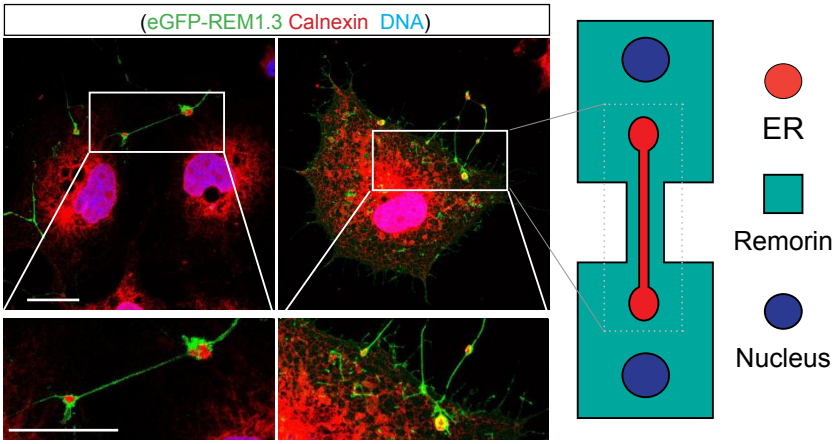
(D) Comprehensive TEM images of RFs. Fluorescein and nano-gold particle double-labeled secondary antibodies were used to stain RFs in HEK293 cells. Fluorescence signal was collected by confocal microscopy and then analyzed by TEM (green: REM1.3; gray: TEM).

(E) A structural cartoon of RFs. RFs are single- or multiple-microfilament structures formed by remorin on the surface of mammalian cells. As seen from the thumbnail on the right, the green remorin and red actin filaments form a double helix. Remorin interacts with the lipid microdomains on the cell membrane (PM) due to its palmitoylation. Therefore, we speculate that the lipid microdomains (Lipid MD) on the surface of the RFs are also helically distributed. The RFs' head has remorin's congregation, presumably the center responsible for extending the RFs. On the left is an enlarged fine structure model.

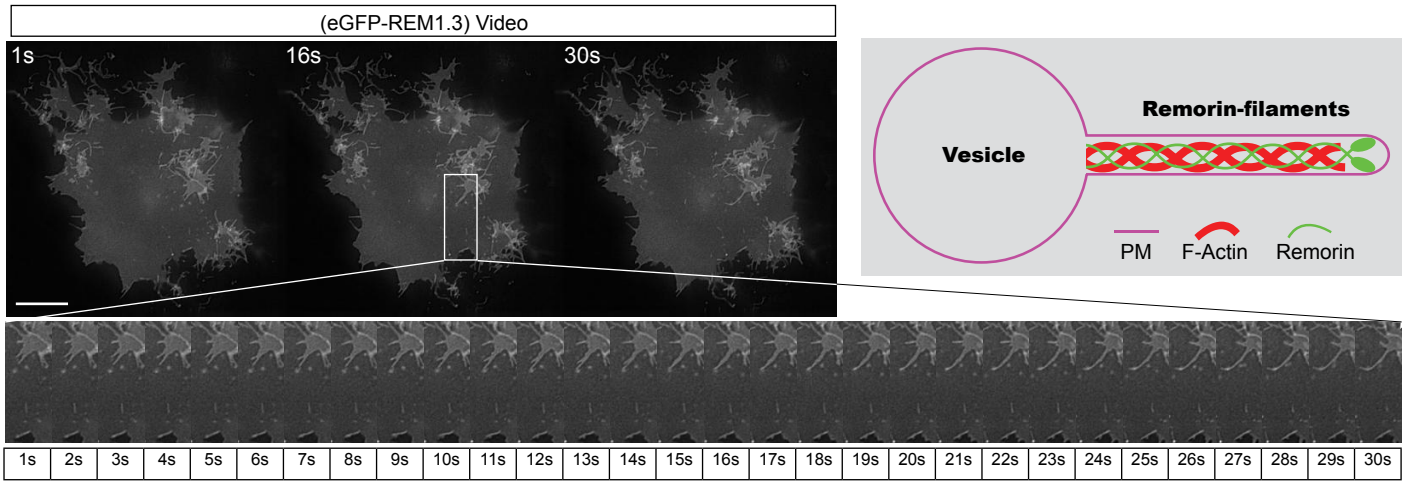
(F) A schematic diagram of RFs biogenesis. Remorin modification by palmitoyltransferase is the first step. The second step is remorin and cholesterol-rich cell membrane forming lipid microdomains. In the third step, remorin and lipid microdomains form multimers with actin. In the fourth step, as the RFs polymerize, they begin to extend further to form long RFs.

FIG 1

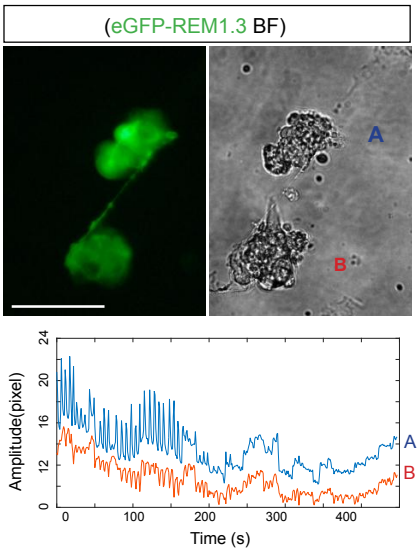
A


(eGFP-REM1.3 Calnexin DNA)
ER
Remorin
Nucleus

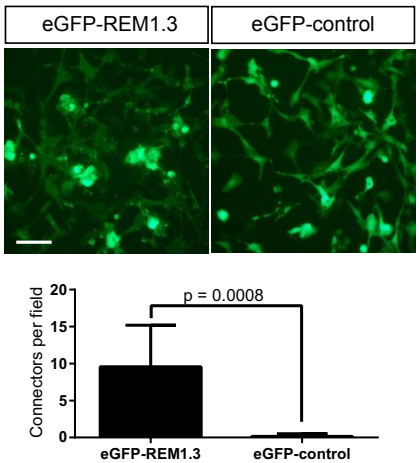
B


(eGFP-REM1.3) Video
1s
16s
30s
Vesicle
Remorin-filaments
PM
F-Actin
Remorin
1s 2s 3s 4s 5s 6s 7s 8s 9s 10s 11s 12s 13s 14s 15s 16s 17s 18s 19s 20s 21s 22s 23s 24s 25s 26s 27s 28s 29s 30s

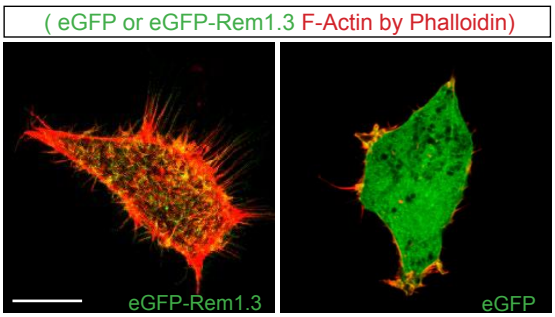
C


(eGFP-REM1.3 BF)
A
B
Amplitude(pixel)
0
12
16
20
24
0
100
200
300
400
Time (s)
A
B

D


eGFP-REM1.3
eGFP-control
p = 0.0008
Connectors per field
0
5
10
15
20
eGFP-REM1.3
eGFP-control

E


(eGFP or eGFP-Rem1.3 F-Actin by Phalloidin)
eGFP-Rem1.3
eGFP

F

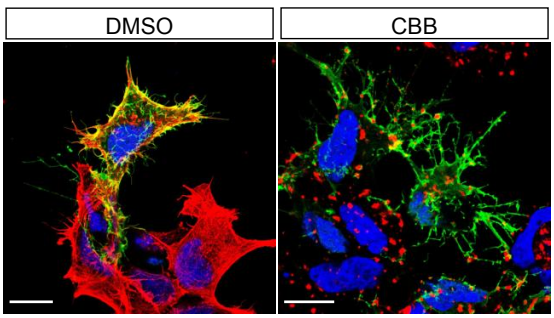
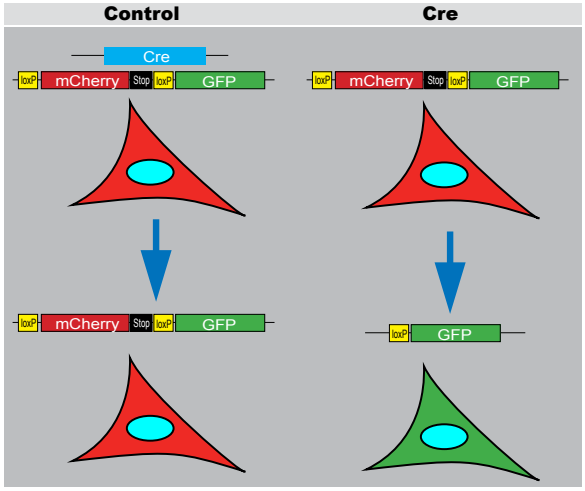
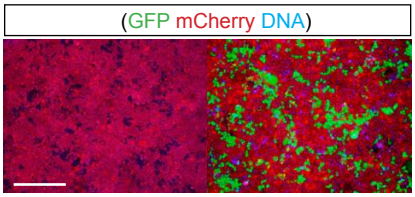

DMSO
CBB

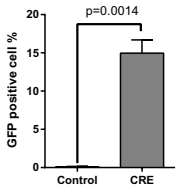
FIG 2

A

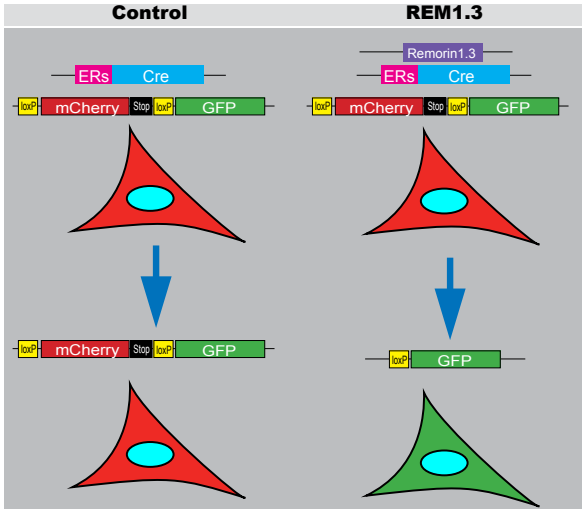


	Control		Cre	
	Donor	Receptor	Donor	Receptor
Imlg	-	-	-	+
Cre	-	+	-	+

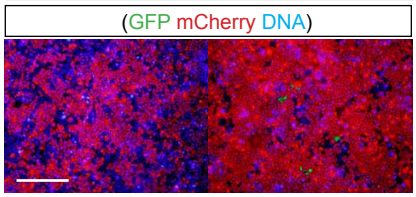


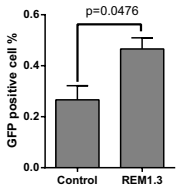


B

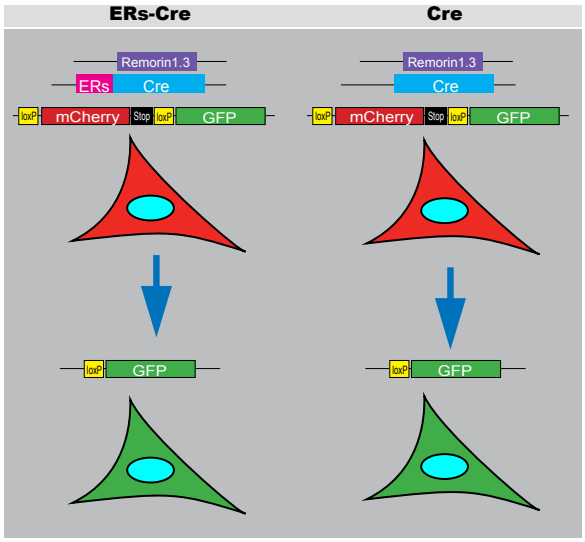


	Control		Cre	
	Donor	Receptor	Donor	Receptor
REM	-	-	+	-
Imlg	-	+	-	+
Cre	+	-	+	-

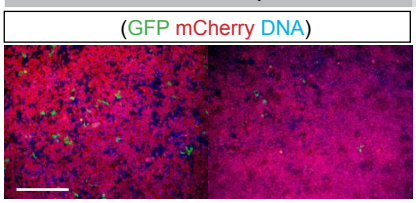


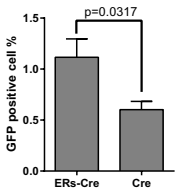


C



	ERs-Cre		Cre	
	Donor	Receptor	Donor	Receptor
REM	+	-	+	-
Imlg	-	+	-	+
ERs-Cre	+	-	-	-
Cre	-	-	+	-





D

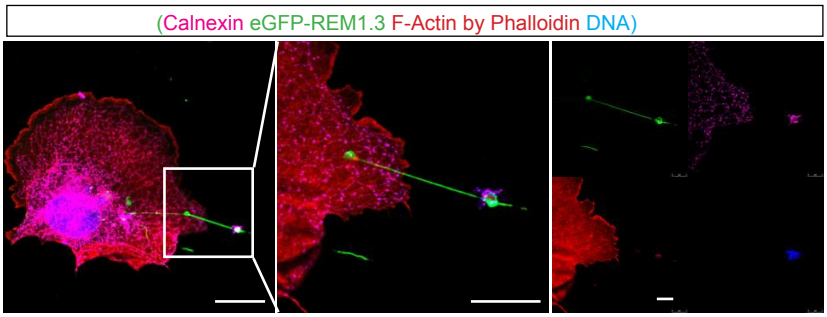
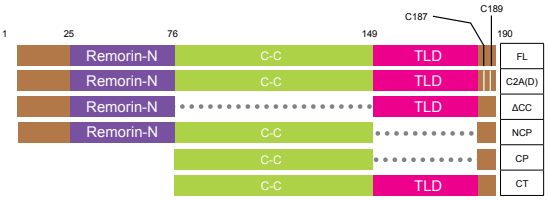
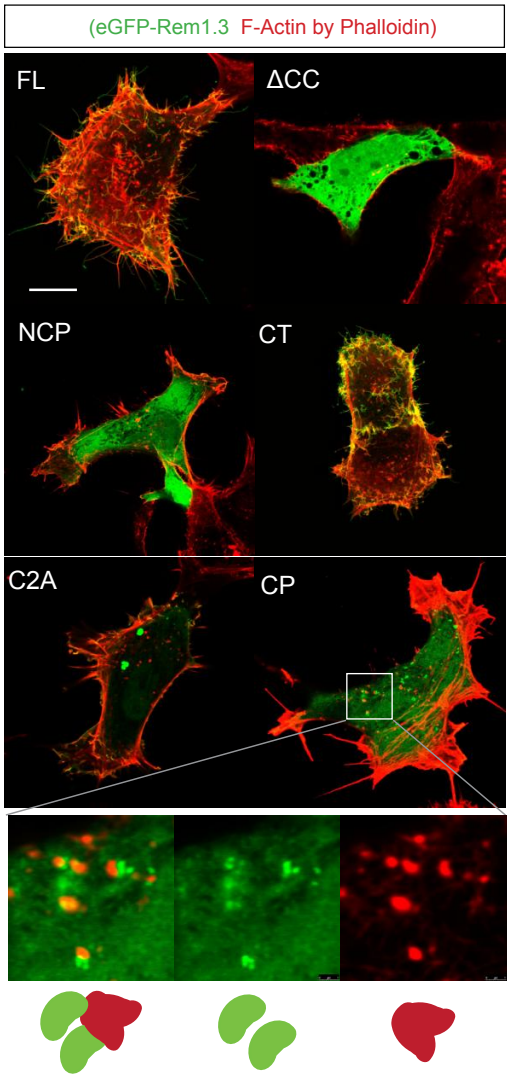


FIG 3

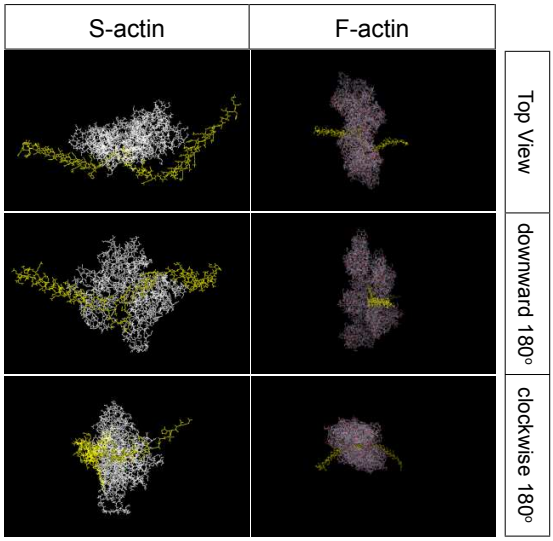
A


C187
C189
1
25
76
149
190
Remorin-N
C-C
TLD
FL
C2A(D)
ΔCC
NCP
CP
CT

B


(eGFP-Rem1.3 F-Actin by Phalloidin)
FL
ΔCC
NCP
CT
C2A
CP

C


S-actin
F-actin
Top View
downward 180°
clockwise 180°

D

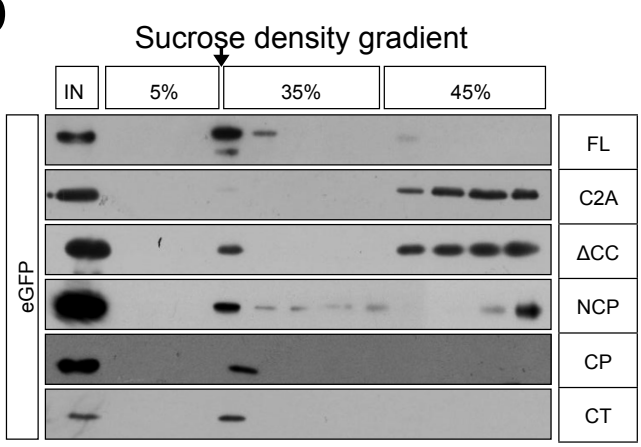
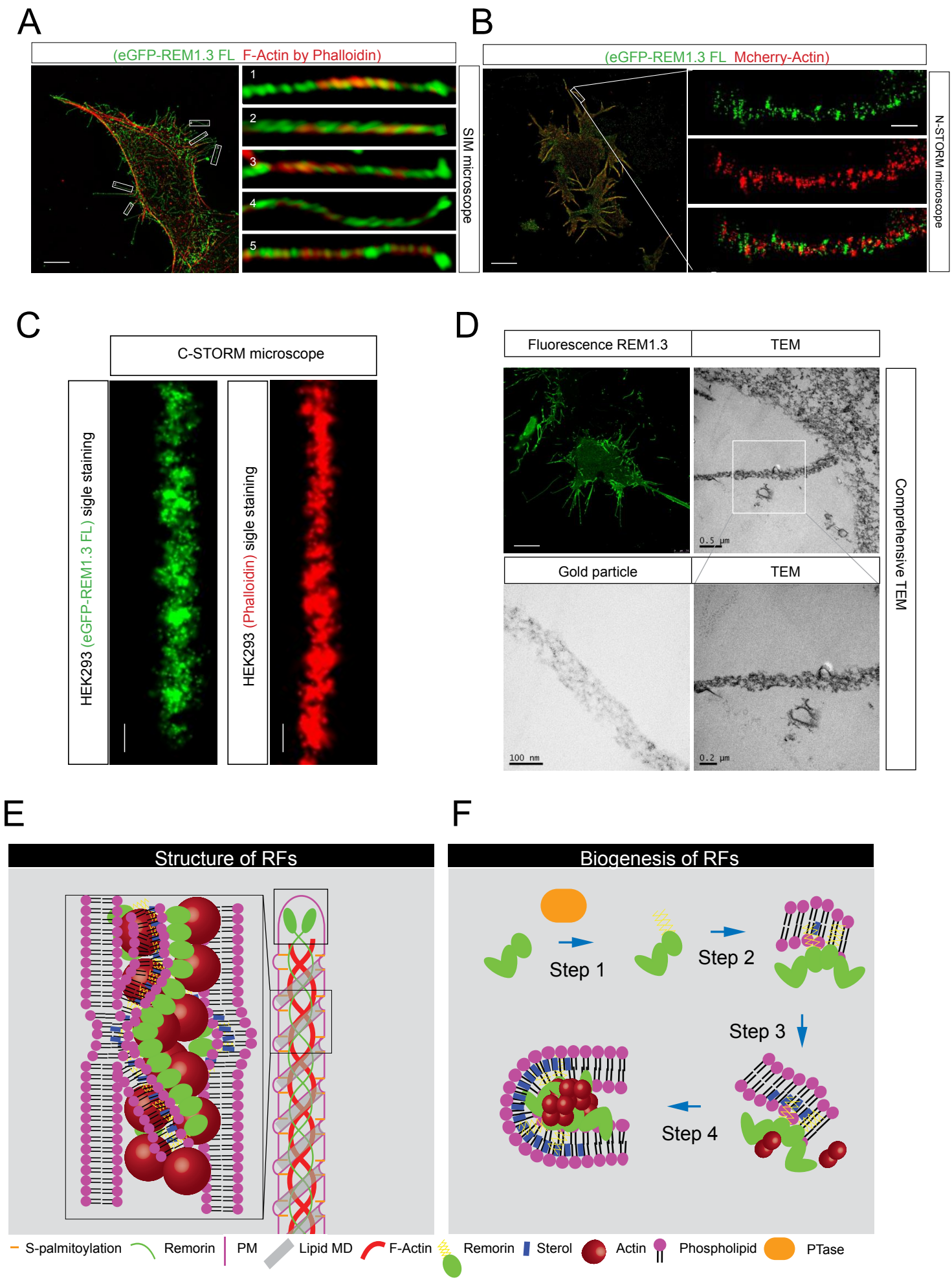

Sucrose density gradient
IN
5%
35%
45%
eGFP
FL
C2A
ΔCC
NCP
CP
CT

FIG 4


A
(eGFP-REM1.3 FL F-Actin by Phalloidin)
1
2
3
4
5
SIM microscope
B
(eGFP-REM1.3 FL Mcherry-Actin)
N-STORM microscope
C
C-STORM microscope
HEK293 (eGFP-REM1.3 FL) sigle staining
HEK293 (Phalloidin) sigle staining
D
Fluorescence REM1.3
TEM
0.5 μm
Gold particle
TEM
100 nm
0.2 μm
Comprehensive TEM
E
Structure of RFs
F
Biogenesis of RFs
Step 1
Step 2
Step 3
Step 4
S-palmitoylation
Remorin
PM
Lipid MD
F-Actin
Remorin
Sterol
Actin
Phospholipid
PTase