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1 **An overview of molecular events occurring in human trophoblast fusion**

2

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5

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22

23 **Abstract**

24 During human placentation, mononuclear cytotrophoblasts fuse to form a multinucleated syncytia
25 ensuring hormonal production and nutrient exchanges between the maternal and fetal circulation.
26 Syncytia formation is essential for the maintenance of pregnancy and for fetal growth. The trophoblast
27 cell fusion process first requires the acquisition of cell fusion properties, then cells set up plasma
28 membrane protein macrocomplexes and fusogen machinery that trigger cell-cell fusion. Numerous
29 proteins have been shown to be directly involved in the initiation of trophoblast cell fusion. These
30 proteins must expressed at the right time and in the right place to trigger cell-cell fusion. In this
31 review, we describe the role of certain fusogenic protein macrocomplexes that form the scaffold for
32 the fusogen machinery underlying human trophoblastic-lipid mixing and merging of cell contents that
33 lead to cell fusion in physiological conditions.

34

35 **Overview**

36 Cell fusion processes are essential for fertilization, fetal and placental development, skeletal muscle
37 formation and bone homeostasis [1-4]. Recently, cell fusion was also shown to play a role in
38 metastasis [5]. Cell fusion involves processes different from those involved in the fusion of vesicles to
39 the plasma membrane, in terms of the necessary proteins, macrocomplexes and cellular signaling
40 pathways. However, the mechanical properties and biophysics of membrane lipids show certain
41 similarities. The purpose of this review is not to provide a list of all proteins and signaling pathways
42 involved in trophoblast fusion (in human primary cell cultures and cell lines), as these have already
43 been reviewed [6]. Instead, we propose to describe for the first time molecular events underlying the
44 main steps of the fusion process observed in the physiological model of cultured human primary
45 trophoblasts, as supported by biochemical and molecular biology experiments.

46 Cell fusion and syncytia formation involves the mixing of plasma membrane components and cell
47 contents between two or more cells. Two different types of cell fusion can be distinguished. In
48 homokaryon formation, during placentation, skeletal formation and bone resorption for example,
49 homotypic cells (cells of similar lineages) fuse together, while in heterokaryon formation (during
50 fertilization and metastasis for example), heterotypic cells (cells of various origins) fuse together. Cell
51 fusion processes occurring in a variety of biological contexts share many steps that are tightly
52 regulated in space and time [7]. As described by Aguilar et al., these processes can be separated into
53 three main steps [7, 8]. The first "competence" stage involves a loss of proliferative activity, followed
54 by induction and differentiation into fusion-competent cells. The second, "commitment" stage is
55 characterized by cell migration, recognition of fusion partners and cell-cell adhesion, which initiate
56 gap junction communication leading to synchronization and exchange of fusogenic signals. This
57 triggers the organization of protein complexes necessary to promote cell-cell fusion, with apposition
58 of the outer lipid monolayers of the two cell membranes before the opening of a fusion pore and
59 progression to full fusion with mixing of cellular contents [9]. Exclusively and in some specific
60 conditions, somatic cells can remain competent for a new round of fusion, generating giant syncytia
61 and/or ensuring regeneration, as observed in muscle and placenta for instance.

62

63 **Introduction**

64 Human embryo implantation requires placentation, a process in which fetal cytotrophoblasts invade
65 the maternal endometrium to form an interphase with the maternal circulation, ensuring effective
66 exchange of gases and nutrients [10]. During human implantation, the blastocyst is composed of a
67 trophoectoderm and embryoblast. The trophoectoderm (of trophoblastic origin) undergoes intercellular
68 fusion to generate primitive syncytia, which promote implantation of the embryo into the maternal
69 endometrium. Two weeks after conception, villi containing cytotrophoblasts and syncytiotrophoblast
70 appear. Throughout human pregnancy, syncytia are maintained by continuous fusion of
71 cytotrophoblasts with overlying syncytiotrophoblasts, in a regenerative process. These multinucleated
72 syncytia in contact with maternal blood control all feto-maternal exchanges and produce and secrete
73 pregnancy-specific hormones [11, 12]. The cytotrophoblast plays an essential role during human
74 pregnancy, through its ability to differentiate into syncytia. These syncytia allow feto-maternal
75 exchanges necessary for fetal growth. For ethical reasons, studies of primitive syncytia are not allowed
76 in certain countries. Thus, the molecular mechanisms underlying cell fusion processes presented in
77 this review reflect syncytial repair processes and the fusion of mononuclear cytotrophoblasts into an
78 overlying syncytiotrophoblast.

79

80 **Human trophoblast fusion models**

81 The fusion process observed in the human placenta is reproducible *in vitro* by using purified
82 cytotrophoblasts (Fig. 1), which aggregate and then fuse to form non proliferative, multinucleated,
83 hormonally active syncytiotrophoblasts [13]. Villous explants containing the whole villous structure
84 and cell types are often used to study placental physiology. This model has the advantages of closely
85 matching human placental physiology but is limited for intensive biochemistry and molecular biology
86 studies. Taking this into account, and in view of our own work on human trophoblast cell fusion, we
87 believe that human primary trophoblast culture remains the most suitable and robust model for
88 studying human trophoblast cell fusion, syncytiotrophoblast formation and regeneration. Studies of
89 primary human trophoblasts require validation of cell purity by means of immunolocalization [14].
90 Trophoblast fusion is quantified in fusion assays, where fusion indices are calculated as the ratio of the

91 number of nuclei in the syncytia divided by the total number of nuclei. A syncytium is defined as at
92 least three nuclei surrounded by a cell membrane, as shown by discontinuous desmoplakin
93 immunostaining [15, 16]. Choriocarcinoma cell lines (the trophoblast-like cell lines BeWo, JAR and
94 JEG3) are commonly used to study human placental functions. However, only BeWo cells are able to
95 fuse and form syncytia in a cAMP-driven process. Interestingly, JEG3 cells could recover a cell fusion
96 capacity under cAMP stimulation by overexpressing connexin-43 gap junction protein (Cx43) or
97 cadherin-11 [17, 18]. We consider that BeWo cells should only be used with care to study trophoblast
98 cell fusion. Indeed, they are transformed carcinoma-like cells missing some trophoblast functions [19].
99 Moreover, BeWo cells fuse after activation of the cAMP signalling pathway. Stimulation by non
100 physiologic agents such as forskolin or cAMP analogs cause uncontrollable and profound
101 modifications of gene and protein expression, which lead directly or indirectly to fusion. Finally, a
102 recent study showed a very weak correlation between gene expression in human cytotrophoblasts and
103 BeWo cells [20]. BeWo cells thus represent a good fusion model but cannot serve as a physiologic
104 model of fusion in the human placenta.

105

106 **Competence stage**

107 Not all cells are fusion-competent. Competent cells commit to fusion in a space- and time-regulated
108 manner. The competence stage is the first step in the complex fusion process. Cells must first exit the
109 cell cycle, which is incompatible with cell fusion. Cytotrophoblasts purified from human placenta are
110 non proliferative [13]. A recent study showed that contacts between the syncytiotrophoblast and
111 cytotrophoblasts are necessary to maintain cytotrophoblasts in a proliferative state [21]. Methods used
112 to purify cells from human placenta probably trap competent cytotrophoblasts, due to their extraction
113 from an environment rich in hormones and other molecules with high potency for competence
114 induction, such as hCG. As observed in other models, satellite cells located close to syncytia are also
115 non proliferative until the syncytium triggers the regeneration process. The fusion-competent stage is
116 characterized by a succession of complex processes which initiate the fusion procedure, including cell
117 migration and morphological changes, and also secretion and response to extracellular signals such as
118 growth factors, cytokines and hormones. Many of these factors, of maternal, placental or fetal origin,

119 have been reported to influence human trophoblast cell fusion: EGF/EGF-receptor (epidermal growth
120 factor; [22]), GM-CFS (granulocyte-macrophage colony-stimulating factor; [23]), LIF (leukemia
121 inhibitory factor; [24]) and PL74 (MIC-1, macrophage inhibitory cytokine 1; [25]) all promote
122 trophoblast fusion. However, the molecular mechanisms underlying trophoblast fusion induced by
123 these factors remain to be deciphered. Interestingly, we recently found that activin-A, secreted by
124 mesenchymal cells in chorionic villi, promotes fusion in a Smad-dependent manner [26], revealing the
125 first paracrine effect of mesenchymal cells inducing trophoblast syncytialization. At a certain time or
126 in a specific location, the syncytium itself controls the rate of fusion and regeneration process [21].
127 This capacity could be acquired by sensing of the composition of the local syncytial environment
128 (chemicals, proteins and hemodynamics) and thereby exert control over cytotrophoblasts in a
129 secretion-dependent manner. In a recent a study of human placental explants, authors demonstrated
130 that, in the absence of a syncytiotrophoblast, the cytotrophoblast is non proliferative and initiates
131 intercellular fusion [21]. More experiments are needed to decipher the underlying molecular
132 mechanisms. Only TGF β (transforming growth factor- β) and TNF α (tumor necrosis factor- α) have
133 been shown to slow or inhibit human trophoblast cell fusion [27, 28]. Interestingly, TGF β is expressed
134 by syncytiotrophoblasts, and this could support negative control of fusion by the syncytium on
135 cytotrophoblasts [29].

136 Human chorionic gonadotrophin (hCG) produced by the syncytiotrophoblast also promotes
137 syncytialization in an autocrine-paracrine manner [15, 30, 31]. Interestingly, hCG is secreted early in
138 human embryonic development by embryonic trophoblast cells and subsequently by placental
139 syncytiotrophoblast cells [32] and could serve as the trigger of primitive syncytium formation as well
140 as a positive regulator of regeneration [32]. Human CG binds to LH/CG-R (Fig. 2) and induces
141 intracellular cAMP production in trophoblasts [15, 30, 31, 33, 34]. Subsequently, cell fusion is
142 triggered by activation of protein kinase A (PKA), leading to phosphorylation and/or an increase in
143 specific gene expression of fusogenic proteins such as syncytins and also cadherin and connexin, two
144 proteins involved in the commitment stage [35-38]. In an autocrine-paracrine loop, hCG induction of
145 the cAMP signaling pathway promotes hCG production and secretion by trophoblasts [15]. In human
146 trophoblasts, PKA phosphorylates the CREB transcription factor (cAMP Response Element-Binding

147 Protein), which associates with CBP (CREB-binding protein) and P300 to increase the transcription of
148 competence or fusogenic genes (*i.e.* hCG, GCM1, Cx43 and syncytins) [39, 40]. Moreover, induction
149 of cAMP/PKA signaling activity promotes association between the transcription factors GCM1 and
150 CBP, thereby enhancing the transcription of fusogenic genes such as those encoding Cx43 and
151 syncytins [17, 40]. However, the first fusogenic signal (or the initiator of the competence stage)
152 remains to be identified.

153

154 **Commitment stage**

155 The commitment stage begins right after the competence stage and is characterized by cell-cell
156 adhesion and communication processes that lead to the activation, expression, exposure or assembly of
157 the fusogenic machinery. Adherens junctions, tight junctions and gap junctions have been shown to
158 trigger the commitment of primary cells, followed by their fusion.

159

160 - Adherens junctions: E-cadherin and cadherin-11

161 Cadherins are transmembrane proteins involved in cell-cell adhesion by promoting the formation of
162 adherens junctions. The extracellular N-terminal domain of cadherin triggers cell-cell adhesion by
163 clustering with homotypic and heterotypic cadherins present on neighboring cells (Fig. 3). Specific
164 antibodies against the extracellular domain of E-cadherin have been shown to block human
165 trophoblast syncytialization by disrupting the aggregation of mononuclear cytotrophoblasts [35].
166 Moreover, in the same study, the authors reported that in physiologic conditions, E-cadherin protein
167 expression diminishes during cell fusion, supporting a direct role in the cellular adhesion step of the
168 commitment stage. Surprisingly, human trophoblasts treated with cadherin-11 antisense form cellular
169 aggregates but are unable to undergo terminal fusion. Contrary to E-cadherin, cadherin-11 expression
170 increases during trophoblast cell fusion [18]. These data suggest that cadherins are required for the
171 entire cell fusion process. E-cadherin mediates mononuclear cell aggregation, while cadherin-11 is
172 required for syncytialization. However, the molecular mechanisms underlying the involvement of E-
173 cadherin and cadherin-11 in the regulation of cell fusion remain obscure. Cadherins stabilize
174 neighboring cell membranes by anchoring them to one another and then trigger the membrane

175 apposition step of the cell fusion process. This allows membrane polarization in the future region of
176 fusion and increases the probability of interaction between fusogenic proteins. Interestingly, E-
177 cadherin, in association with the β -catenin signaling complex, mediates the formation of adherens,
178 tight and gap junctions involved in the regulation of human trophoblast fusion [41].

179

180 - Tight junctions: zonula occludens-1 (ZO-1)

181 Zona occludens-1 (ZO-1), located on the intracellular side of the plasma membrane, belongs to the
182 MAGUK (Membrane-Associated GUanylate Kinase) family and was considered to be only a
183 component of tight junctions. However, ZO-1 was also recently described as a component of adherens
184 junctions. Tight junctions are composed of proteins that mediate the junction between the membranes
185 of neighboring cells. In a recent study, we showed that ZO-1 is expressed in cytotrophoblasts both *in*
186 *vitro* and *in vivo* and that its expression decreases during cell fusion. Interestingly, ZO-1 silencing in
187 human trophoblasts blocks both cell fusion and cell adhesion. This lack of cell fusion is associated
188 with a decrease in the expression of connexin-43 (Cx43), a gap junction protein [16]. These data
189 support a role of ZO-1 in trophoblast fusion by targeting Cx43 protein to the right place on the plasma
190 membrane. Like cadherins in adherens junctions, ZO-1 permits cell-cell adhesion in the membrane
191 apposition step and establishes gap junction communication, a prerequisite for cell fusion. A recent
192 study showed that specific silencing of ZO-1 protein expression led to decreased E-cadherin
193 expression [42].

194

195 - Gap junctions: connexin-43 (Cx43)

196 Communication between adjacent cells is mediated through gap junctions, which are composed of
197 connexin protein (Cx). A gap junction connects two adjacent cells through the alignment of two
198 hemichannels, each composed of Cx hexamers. Gap junction channels allow the exchange of small
199 molecules, ions, metabolites and second messengers, facilitating cellular coordination, differentiation
200 and spatial compartmentalization [43-45]. Cx43 is the only gap junction protein expressed by human
201 cyto- and syncytiotrophoblasts lying at the interface between them [14]. Cx43 is involved in the
202 regulation of human trophoblast fusion through the control of cell-cell communication [14, 17, 46,

203 47]. In recent silencing and reconstitution experiments, we examined the molecular mechanisms
204 underlying Cx43 involvement in the control of trophoblast fusion. We demonstrated that ezrin anchors
205 PKA to a molecular complex composed of Cx43 and ZO-1, and showed a direct association between
206 Cx43 and ezrin. Upon a local increase in the intracellular pool of cAMP following hCG stimulation,
207 PKA bound to ezrin activates and phosphorylates Cx43 (Fig. 4). This PKA-dependent phosphorylation
208 promotes the opening of the gap junction and allows the transfer from cell to cell of fusogenic signals
209 leading to trophoblast fusion [15]. These fusogenic signals initiate cellular synchronization, activation
210 of transcription factors (*i.e.* CREB, GCM1) and organization of the fusogenic macrocomplex
211 machinery in the right place and at the right time to trigger cell-cell fusion.

212

213 **Cell-cell fusion**

214 The last step of the fusion process is defined by the merging of the two plasma membranes and mixing
215 of cell contents. It is characterized by the formation of a fusion pore controlled in space and time.
216 Until recently, only syncytin proteins have been described to be truly fusogenic in homokaryon
217 formation. However, much remains to be understood, particularly membrane biophysics.

218

219 - Syncytins: syncytin-1 and syncytin-2

220 The human genome contains up to 8.5% of sequences of retroviral origin, known as human
221 endogenous retroviruses (HERVs), which are composed of *gag*, *pol* and *env* genes [7]. In the viral
222 replicative cycle, the *env* gene codes for the envelope glycoprotein that mediates infection competency
223 and virus-host membrane fusion [7]. In HERV-W family, the *gag* and *pol* genes turned into
224 pseudogenes, while the *env* gene was domesticated to produce syncytin-1 protein [48, 49]. Syncytin-1
225 was the first fusogenic protein shown to control human trophoblast fusion through its retroviral
226 envelope-like properties [50]. Syncytin-1 promotes cell fusion through its interactions with human
227 sodium-dependent neutral amino acid transporter type 1 or 2 (hASCT1, hASCT2), which must be
228 localized to the target-cell membrane [51]. Human ASCT2 is more highly expressed than hASCT1 in
229 placenta, and colocalizes *in vitro* with Cx43 [17, 52, 53]. Syncytin-2, encoded by the HERV-FRD
230 envelope gene, is expressed in villous trophoblasts [54, 55]. Like syncytin-1, syncytin-2 mediates

231 human trophoblast fusion through the major facilitator superfamily domain-containing 2 receptor
232 (MFSD2) [56, 57]. Although controversial, due to the use of different antibodies, syncytin-1 has been
233 detected *in vitro* in human trophoblasts and is maximally expressed at the cellular aggregation step
234 [56, 58], while hASCT2 is restricted to the cytotrophoblast [7]. It is generally agreed that syncytin-2 is
235 expressed at the junction between the cyto- and syncytiotrophoblast, and syncytin-2 mRNA and
236 protein expression increase during the cell fusion process [56, 59]. MFSD2 has been shown to be
237 expressed exclusively by the syncytiotrophoblast [57]. Pötgens et al. have proposed several models for
238 the initiation of trophoblast-membrane fusion and for how syncytin-1 and hASCT2 interconnect to
239 each other in the mononuclear cell/syncytium interphase [60]. They argue that upregulation of one or
240 both proteins (syncytin-1 and hASCT2) is sufficient and necessary to induce trophoblast cell fusion.
241 Interestingly, *syncytin-A* and *-B* (homologs of human syncytin-1 and -2) dual knockout mice present
242 very few syncytiotrophoblast, suggesting the existence of other fusogenic proteins [61]. Recent studies
243 of other fusion models have shown the direct role of syncytin-1 in sperm and egg fusion, osteoclast
244 fusion, and muscle formation [62, 63].

245

246 - Syncytin-1-dependent fusion mechanism

247 This part of the review will focus on the mechanisms of syncytin-1-dependent fusion mechanisms,
248 which are better characterized than those of syncytin-2. Syncytins are glycoproteins (7 N-
249 glycosylations for syncytin-1) composed of an intracellular domain, a transmembrane domain and an
250 extracellular domain (SU). Syncytin-1 displays a disulfide sequence, a furin cleavage site, a fusion
251 peptide and a receptor-binding domain (RBD) (Fig. 5A). Neo-synthesized syncytin-1 is glycosylated
252 in the endoplasmic reticulum (ER) and subjected to a trimerization process in which a leucine zipper-
253 like motif (LX₆LX₆NX₆LX₆L) present in each TM subunit initiates oligomerization. The last
254 maturation step involves cleavage at a consensus site (³¹⁴RNKR⁻³¹⁷) by cellular furin-like
255 endoproteases [7]. The two new cleaved subunits (SU and TM) are linked to each other by a disulfide
256 bond between the ³⁹⁷CX₆CC⁻⁴⁰⁷ motif of the TM and the CΦΦC (¹⁸⁶CX₂C⁻¹⁸⁹) motif of the SU, prior
257 to expression of syncytin-1 at the cell membrane. Directional mutation of the disulfide bond impairs
258 syncytium formation in the human trophoblast [64]. The ¹¹⁵SDGGGX₂DX₂R⁻¹²⁵ motif located in the

259 syncytin-1-SU subunit has been characterized as the syncytin-1 receptor binding domain (RBD) for
260 hASCT2 [64]. RBD recognition of hASCT2 induces a conformational change which leads to
261 dissociation of the SU subunit from the TM subunit by breakage of the disulfide bond (Fig. 5B). This
262 triggers a loop-to-helix movement of the syncytin-1 fusion peptide, which targets the membrane of the
263 neighbor cell at a distance of approximately 100 Å, in an irreversible manner [7]. Located inside the
264 TM subunit encompassing amino acids 320 to 340, the fusion peptide displays high hydrophobicity
265 essential for targeting the opposing cell membrane. Once fusion peptides have seeded into the target
266 membrane, the trimer initiates plasma membrane bending, merging and fusion-pore formation (Fig.
267 5B). The cytoplasmic tail of syncytin-1 is also essential for the regulation of fusion, by stabilizing the
268 trimer complex and playing a role in membrane bending [7].

269

270 - Models of membrane fusion

271 Although never demonstrated during trophoblast fusion a hemifusion intermediate is highly probable,
272 and is supported by homology with virus entry and fusion of artificial lipid bilayers. The syncytin-1
273 fusion peptide anchors the target membrane and induces local bending or a point-like protusion of the
274 membrane bilayer of the neighboring competent cell, which then establishes close proximity and/or
275 contact of the opposing cell outer membrane leaflets (Fig. 5B). Dehydration of the contact site then
276 occurs, reducing the hydration repulsion between the opposing bilayers and outer leaflets, which form
277 a fusion stalk. The fusion stalk allows the outer lipids to mix and expands radially into a hemifusion
278 diagram (HD). The HD is characterized by the separation of the lipid contents by the still-intact inner
279 membrane. Radial expansion of the HD creates a fusion pore, triggering inner leaflet merging and cell
280 content mixing (Fig. 6). Syncytium formation leads to acquisition of highly specific functions and
281 gene and protein expression. The bending process mediated by fusogenic proteins generates local
282 changes in lipid composition by destabilizing the bilayers and facilitating lipid rearrangement prior to
283 HD and fusion pore formation. This phenomenon could explain the observed phosphatidylserine (PS)
284 flip and PS exposure on the outer membrane, considered a prerequisite for BeWo cell fusion [65].
285 Ridell et al described PS exposure as non systematic during human trophoblast fusion but dependent
286 on the nature of the stimuli [66]. Importantly, no leakage of cell contents into the extracellular medium

287 is possible during hemifusion processes [9, 67]. An alternative model of fusion has been proposed
288 with channel-like structures capable of inducing cell fusion. In this model, proteins form a hemi-
289 channel inside the membrane of the competent cell (as in connexin hemi-channel formation) which,
290 after connection to another hemi-channel exposed by the opposing cell, forms a fusion pore [67]. A
291 new fusion model, the stalk-hole fusion model, has recently been proposed on the basis of studies of
292 artificial lipid membranes. This model postulates the formation of an elongated stalk that expands in
293 an asymmetrical ring-like fashion. Stalk elongation promotes hole formation by destabilizing
294 membranes, thus leading to complete fusion [68]. Membrane biophysics studies of human trophoblasts
295 are needed to validate one of these models.

296

297 **Conclusion**

298 Trophoblast fusion and syncytiotrophoblast formation is a complex biological process essential for the
299 maintenance of pregnancy and for fetal growth. Like other cell-cell fusion models, trophoblast fusion
300 is characterized by three distinct stages: competency, commitment and cell-cell fusion. All these
301 stages are tightly regulated in space and time. Many proteins, chemicals and hormones have been
302 shown to be involved in trophoblast cell fusion. Some of the proteins considered here (fusogenic or
303 fusogenic-regulator, such as syncytins and cadherins) are also involved in other fusion models.
304 Clustering of fusogenic proteins or proteins initiating cell fusion at the right time and the right place
305 on the plasma membrane points to the existence of “fusogenic rafts”. These rafts, similar to lipid rafts,
306 could help to localize fusogenic proteins and thereby ensure lipid mixing, fusion pore formation and
307 full fusion. Interestingly, the cAMP signaling pathway modulates the expression of proteins described
308 in this review and appears to play a key role in the regulation/activation of human trophoblast fusion.
309 However, more studies are needed to fully elucidate the mechanisms of trophoblast cell fusion and
310 syncytiotrophoblast regeneration, and thus the pathophysiology of human placental development. Any
311 alteration of syncytial formation and regeneration during pregnancy will affect fetal growth and the
312 outcome of the pregnancy. Anomalies of villous trophoblast differentiation and cell fusion can lead to
313 severe placental abnormalities, such as a decrease in the villous volume and surface area, both of
314 which are severely compromised in intrauterine growth restriction (IUGR) and preeclampsia [69].

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321 Author contributions: GP wrote the paper and did artistic work. PG commented on the text
322 and figures; all the authors read and commented on the drafts and approved the final version.

323

324 **CONFLICT OF INTEREST**

325 The authors declare that they have no conflict of interest.

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509

510 **FIGURE LEGENDS**

511 **Figure 1: Model of cultured villous trophoblasts purified from human placenta.** (left panel)

512 Schematic view of human chorionic villi. VCT for villous cytotrophoblast, ST for syncytiotrophoblast
513 and EVT for extravillous trophoblast. (Upper right panel) Model of trophoblast fusion.
514 Cytotrophoblasts (CT) aggregate after 24 to 48 h of culture, and fuse into a syncytiotrophoblast (ST)
515 after 72 h. (Lower right panels) Human trophoblast stained at 24 h and 72 h of culture for desmoplakin
516 (magenta) and nuclei (DAPI, cyan). Scale bar: 15 μ m.

517

518 **Figure 2: Cyclic AMP signaling pathway activated by hCG stimulation of human trophoblasts.**

519 Human chorionic gonadotropin (hCG) binds to the LH-CG receptor, a G protein-coupled receptor
520 (GPCR), which activates adenylyl cyclase in its proximity and generates pools of cAMP. Particular
521 GPCRs are confined to specific domains of the cell membrane, in association with intracellular
522 organelles or cytoskeletal constituents. The subcellular structures may harbor specific isozymes of
523 protein kinase A (PKA), that are localized in the vicinity of the receptor and the cyclase. Four
524 molecules of cAMP (red filled circles) bind to PKA regulatory subunits, which release and activate
525 PKA catalytic subunits (PKA C; yellow filled circles). The PKA C subunit phosphorylates a large
526 spectrum of targets and activates proteins or transcription factors such as CREB (cAMP response
527 element binding protein) and/or GCM1 (chorion-specific transcription factor GCMA), which promote
528 transcription activity of fusogenic genes through CBP (CREB binding protein) anchoring.

529

530 **Figure 3: Commitment stage promoted by adherens junctions.** (left panel) Representation of two

531 cells prior to the commitment stage. (right panel) Schematic depiction of the commitment stage
532 mediated through cadherin clustering. Cadherins are transmembrane proteins which ensure cell-cell
533 adhesion by the formation of adherens junctions through their extracellular N-terminal domain. The
534 intracellular domain connects the C-terminal part of cadherin to the cytoskeleton by catenin complex
535 anchoring.

536

537 **Figure 4: Human trophoblast gap junction.** In the resting state (left panel) Cx43 and ZO-1
538 compartmentalize with a pool of PKA anchored by ezrin bound to Cx43. Upon hCG stimulation (right
539 panel), cAMP increases locally and activates PKA, leading to spatiotemporally controlled
540 phosphorylation of Cx43 that increases communication through the gap junction and the transfer of
541 fusogenic signals. Ezrin is represented in purple; Cx43 in dark grey; ZO-1 in green; PKA regulatory
542 subunits in orange; PKA catalytic (C) subunits in yellow; cAMP in pink circle and phosphorylation in
543 blue circle.

544

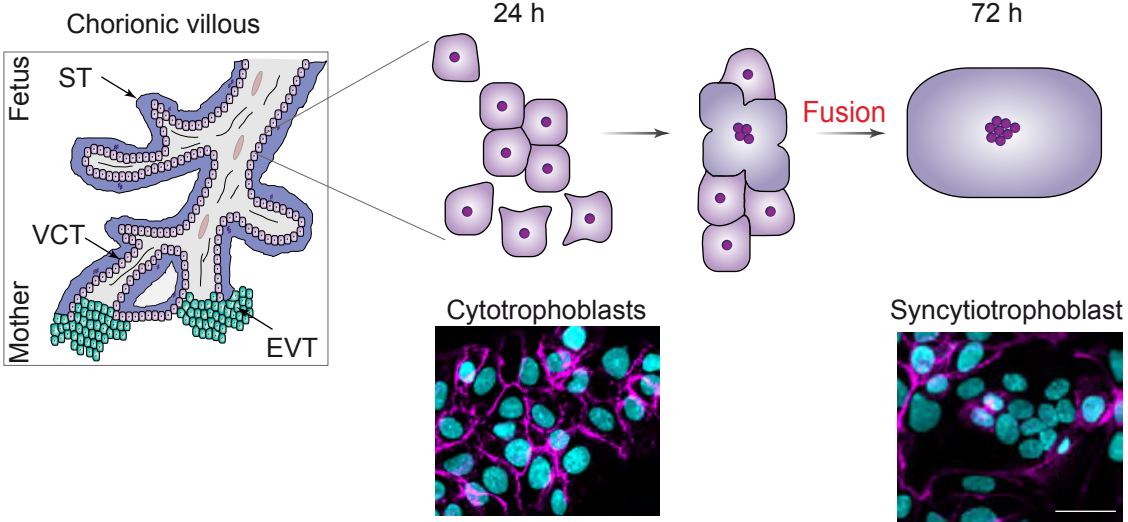
545 **Figure 5: Schematic depiction of trophoblast cell fusion mediated through syncytin-1.** (A)
546 Representation of syncytin-1 structure. SU, surface unit; TM, transmembrane unit. Syncytin-1
547 extracellular domain represented in light gray, furin cleavage site (RNKR) in red, fusion peptide
548 domain (ILPFVIGAGVVLGALGTGIGGI) in purple, plasma membrane domain in green and
549 intracellular domain in blue. The Y indicates N-glycosylation sites. The SDGGGX₂DX₂R motif
550 characterizes the RBD (receptor binding domain). The disulfide bond is formed between the CX₆CC
551 motif of the TM domain and the CΦΦC motif of the SU domain. (B) Schematic depiction of syncytin-
552 1 initiating cell fusion. a) Resting stage, b) RBD of syncytin-1 (dark grey) bound to hASCT2 (dark
553 blue), c) SU domains are removed from the trimer and release the fusion peptide (purple), d) The
554 fusion peptide harpoons the target plasma membrane, e) Plasma membrane bending is initiated.

555

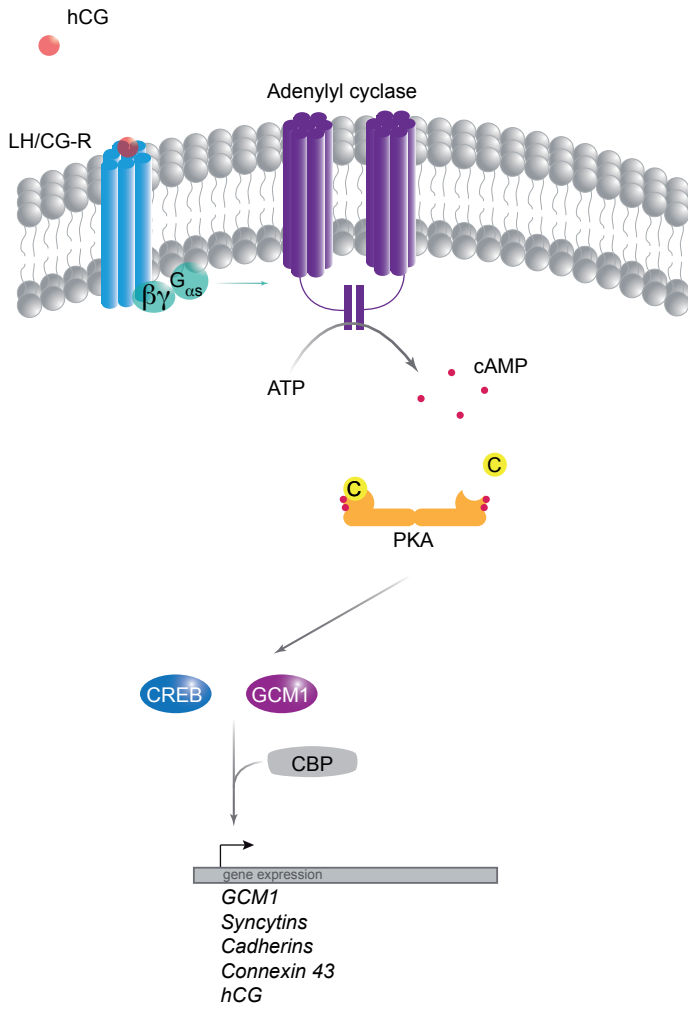
556 **Figure 6: Schematic representation of the successive stages of membrane fusion through**
557 **hemifusion.** For simplicity, no proteins are represented in this scheme. a) Cell membrane apposition at
558 a distance of about 100 Å, b) Membrane bending, c) Stalk formation with mixing of cellular outer
559 lipid leaflets, d) stalk progression to hemifusion diagram (HD), e) fusion pore formation.

560

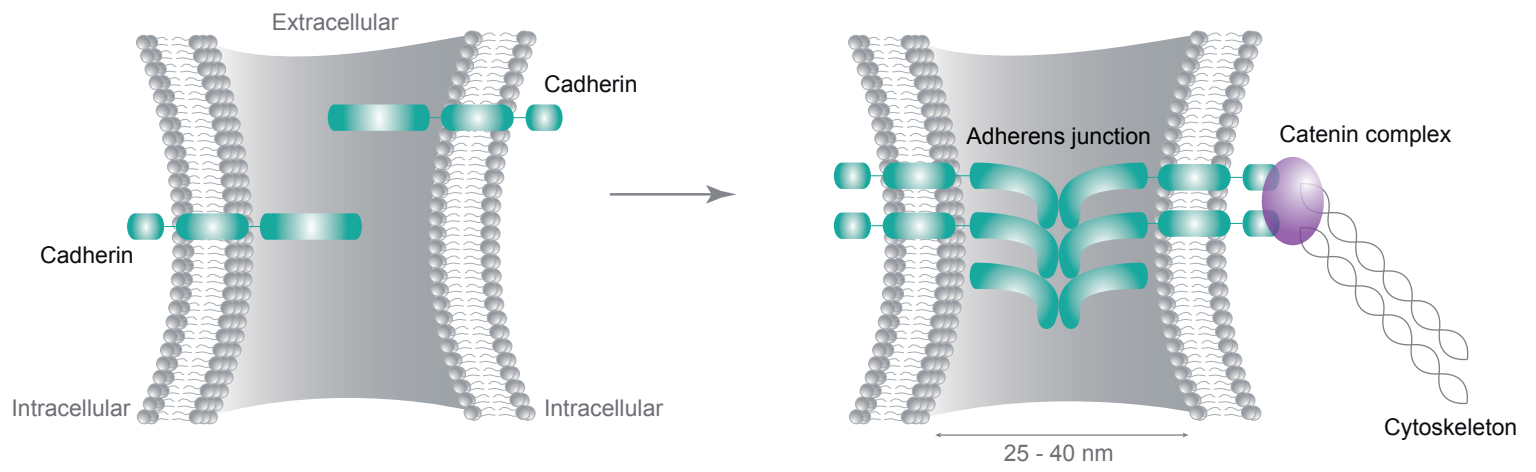
Gerbaud et al, Figure 1



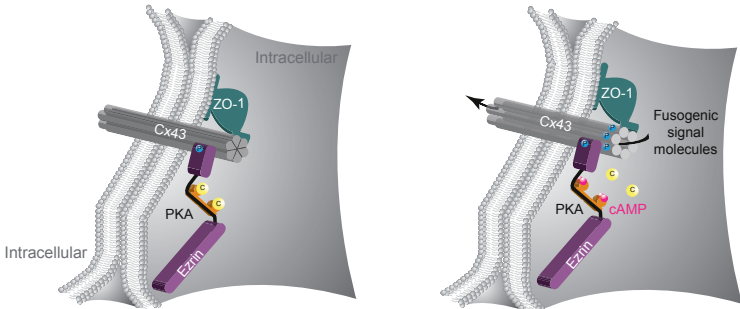
Gerbaud et al, Figure 2



Gerbaud et al, Figure 3

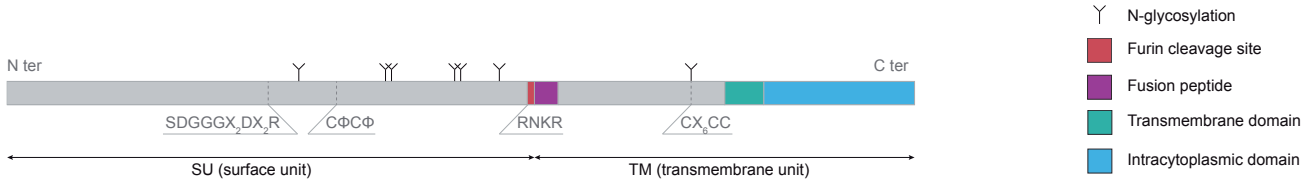


Gerbaud et al, Figure 4

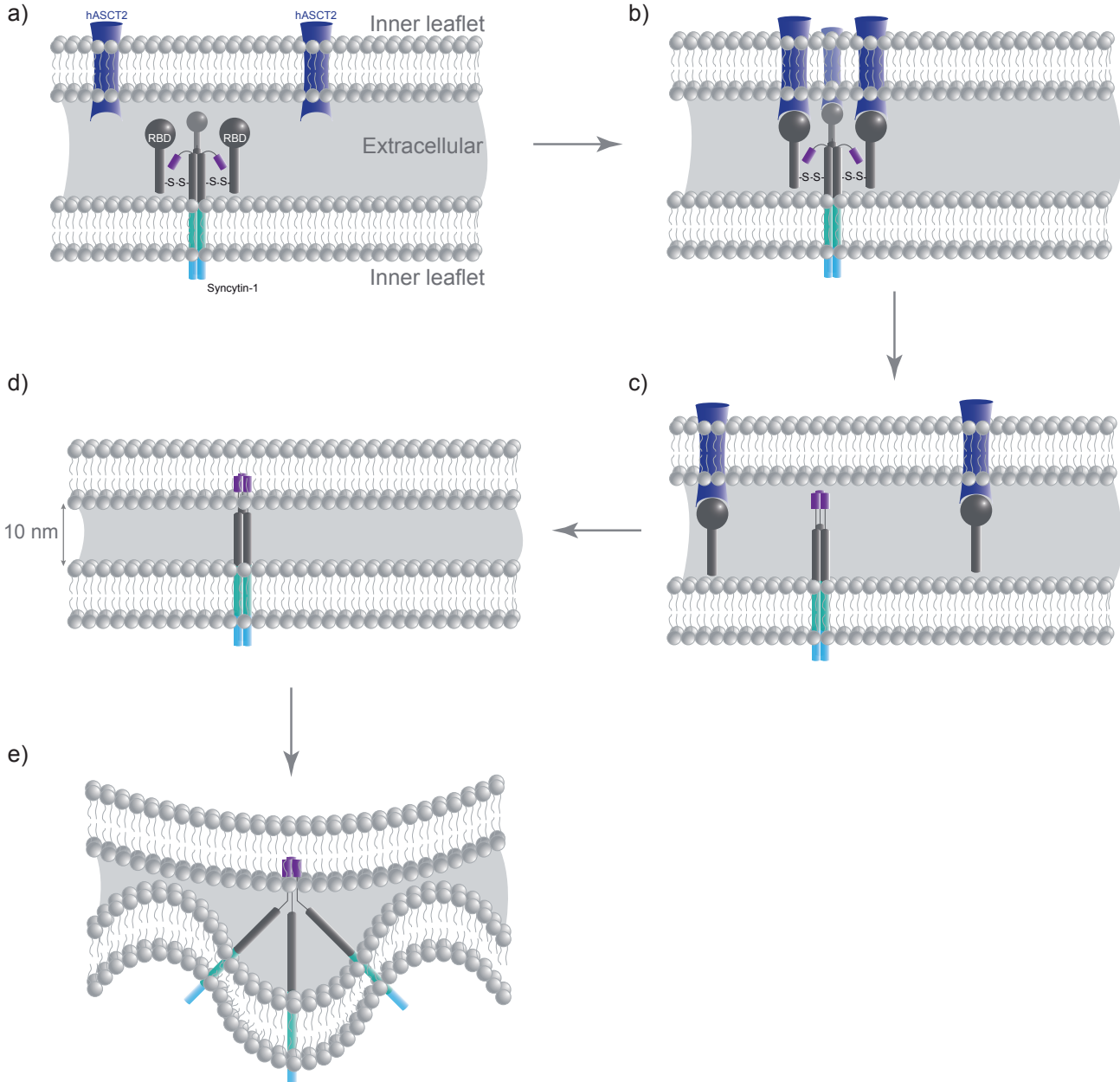


Gerbaud et al, Figure 5

A

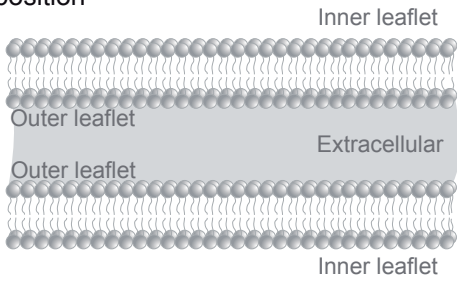


B

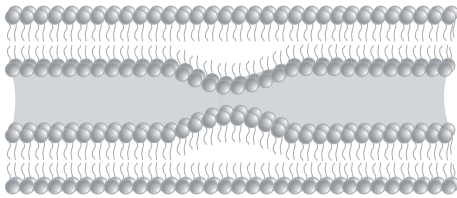


Gerbaud et al, Figure 6

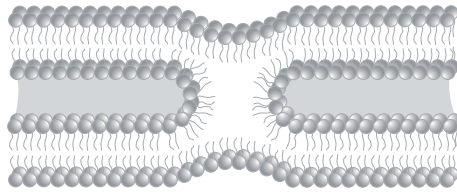
a) Apposition



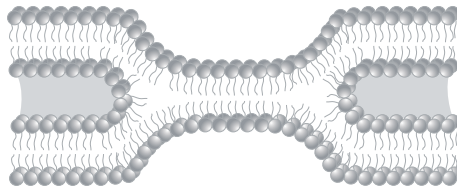
b) Bending



c) Stalk



d) Hemifusion Diagram (HD)



e) Fusion pore

