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

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## **Abstract**

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

## Overview

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

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

## Introduction

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

## Human trophoblast fusion models

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

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

### **Competence stage**

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

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

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

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

### **Commitment stage**

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

#### **- Adherens junctions: E-cadherin and cadherin-11**

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



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

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

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

#### - Gap junctions: connexin-43 (Cx43)

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

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

### **Cell-cell fusion**

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

#### **- Syncytins: syncytin-1 and syncytin-2**

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

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

#### - Syncytin-1-dependent fusion mechanism

This part of the review will focus on the mechanisms of syncytin-1-dependent fusion mechanisms, which are better characterized than those of syncytin-2. Syncytins are glycoproteins (7 N-glycosylations for syncytin-1) composed of an intracellular domain, a transmembrane domain and an extracellular domain (SU). Syncytin-1 displays a disulfide sequence, a furin cleavage site, a fusion peptide and a receptor-binding domain (RBD) (Fig. 5A). Neo-synthesized syncytin-1 is glycosylated in the endoplasmic reticulum (ER) and subjected to a trimerization process in which a leucine zipper-like motif (LX<sub>6</sub>LX<sub>6</sub>NX<sub>6</sub>LX<sub>6</sub>L) present in each TM subunit initiates oligomerization. The last maturation step involves cleavage at a consensus site (<sup>314</sup>RNKR<sup>317</sup>) by cellular furin-like endoproteases [7]. The two new cleaved subunits (SU and TM) are linked to each other by a disulfide bond between the <sup>397</sup>CX<sub>6</sub>CC<sup>407</sup> motif of the TM and the CΦΦC (<sup>186</sup>CX<sub>2</sub>C<sup>189</sup>) motif of the SU, prior to expression of syncytin-1 at the cell membrane. Directional mutation of the disulfide bond impairs syncytium formation in the human trophoblast [64]. The <sup>115</sup>SDGGGX<sub>2</sub>DX<sub>2</sub>R<sup>125</sup> motif located in the

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

#### - Models of membrane fusion

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

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

## **Conclusion**

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

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323

324    **CONFLICT OF INTEREST**

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

## 326 REFERENCES

- 327 [1] Wakelam M. The fusion of myoblasts. *Biochem J.* 1985;15:1-12.
- 328 [2] Oren-Suissa M and Podbilewicz B. Cell fusion during development. *Trends Cell Biol.*
- 329 2007;17(11):537-46.
- 330 [3] Midgley A, Pierce G, Denau G and Gosling J. Morphogenesis of syncytiotrophoblast in vivo: an
- 331 autoradiographic demonstration. *Science.* 1963;141:350-1.
- 332 [4] Zamboni Zallone A, Teti A and Primavera M. Monocytes from circulating blood fuse in vitro
- 333 with purified osteoclasts in primary culture. *J Cell Sci.* 1984;66:335-42.
- 334 [5] Lu X and Kang Y. Cell fusion as a hidden force in tumor progression. *Cancer Res.*
- 335 2009;69(22):8536-9.
- 336 [6] Huppertz B and Gauster M. Mechanisms regulating human trophoblast fusion. 2011; (Ed.) Larsson
- 337 LG. pp. 203-17. Springer.
- 338 [7] Pérot P, Montgiraud C, Lavillette D and Mallet F. A comparative portrait of retroviral fusogens
- 339 and syncytins. 2011; (Ed.) Larsson LI. pp. 63-115. Springer.
- 340 [8] Aguilar PS, Baylies MK, Fleissner A, Helming L, Inoue N, Podbilewicz B, Wang H and Wong M.
- 341 Genetic basis of cell-cell fusion mechanisms. *Trends in genetics : TIG.* 2013;29(7):427-37.
- 342 [9] Chernomordik LV and Kozlov MM. Membrane hemifusion: crossing a chasm in two leaps. *Cell.*
- 343 2005;123(3):375-82.
- 344 [10] Benirschke K and Kaufmann P. Pathology of the human placenta. 2000; New-York.: Springer-
- 345 Verlag. 22-70 pp. pp.
- 346 [11] Eaton B and Contractor S. In vitro assessment of trophoblast receptors and placental transport
- 347 mechanisms. 1993; (Ed.) Redman CW, Sargent IL and Starkey PM. pp. 471-503. London:
- 348 Blackwell Scientific Publication.
- 349 [12] Ogren L and Talamantes F. The placenta as an endocrine organ: polypeptides. 1994; (Ed.) Knobil
- 350 E and Neill J. pp. 875-945. New-York: Raven Press.
- 351 [13] Kliman H, Nestler J, Sermasi E, Sanger J and Strauss III J. Purification, characterization , and *in*
- 352 *vitro* differentiation of cytotrophoblasts from human term placentae. *Endocrinology.*
- 353 1986;118:1567-82.
- 354 [14] Cronier L, Defamie N, Dupays L, Theveniau-Ruissy M, Goffin F, Pointis G and Malassine A.
- 355 Connexin expression and gap junctional intercellular communication in human first trimester
- 356 trophoblast. *Mol Hum Reprod.* 2002;8(11):1005-13.
- 357 [15] Pidoux G, Gerbaud P, Dompierre J, Lygren B, Solstad T, Evain-Brion D and Tasken K. A PKA-
- 358 ezrin-connexin 43 signaling complex controls gap junction communication and thereby
- 359 trophoblast cell fusion. *J Cell Sci.* 2014;127(19):4172-85.
- 360 [16] Pidoux G, Gerbaud P, Gnidehou S, Grynberg M, Geneau G, Guibourdenche J, Carette D, Cronier
- 361 L, Evain-Brion D, Malassine A and Frendo JL. ZO-1 is involved in trophoblastic cell
- 362 differentiation in human placenta. *Am J Physiol Cell Physiol.* 2010;298(6):C1517-26.
- 363 [17] Dunk CE, Gellhaus A, Drewlo S, Baczyk D, Potgens AJ, Winterhager E, Kingdom JC and Lye
- 364 SJ. The molecular role of connexin 43 in human trophoblast cell fusion. *Biol Reprod.*
- 365 2012;86(4):115.
- 366 [18] Getsios S and MacCalman CD. Cadherin-11 modulates the terminal differentiation and fusion of
- 367 human trophoblastic cells in vitro. *Dev Biol.* 2003;257(1):41-54.
- 368 [19] King A, Thomas L and Bischof P. Cell culture models of trophoblast II: trophoblast cell lines--a
- 369 workshop report. *Placenta.* 2000;21 Suppl A:S113-9.
- 370 [20] Bilban M, Tauber S, Haslinger P, Pollheimer J, Saleh L, Pehamberger H, Wagner O and Knofler
- 371 M. Trophoblast invasion: assessment of cellular models using gene expression signatures.
- 372 *Placenta.* 2010;31(11):989-96.
- 373 [21] Forbes K, Westwood M, Baker PN and Aplin JD. Insulin-like growth factor I and II regulate the
- 374 life cycle of trophoblast in the developing human placenta. *Am J Physiol Cell Physiol.*
- 375 2008;294(6):C1313-22.
- 376 [22] Morrish DW, Bhardwaj D, Dabbagh LK, Marusyk H and Siy O. Epidermal growth factor induces
- 377 differentiation and secretion of human chorionic gonadotropin and placental lactogen in normal
- 378 human placenta. *J Clin Endocrinol Metab.* 1987;65(6):1282-90.

- [23] Garcia-Lloret MI, Morrish DW, Wegmann TG, Honore L, Turner AR and Guilbert LJ. Demonstration of functional cytokine-placental interactions: CSF-1 and GM-CSF stimulate human cytotrophoblast differentiation and peptide hormone secretion. *Experimental cell research*. 1994;214(1):46-54.
- [24] Yang M, Lei ZM and Rao Ch V. The central role of human chorionic gonadotropin in the formation of human placental syncytium. *Endocrinology*. 2003;144(3):1108-20.
- [25] Li H, Dakour J, Guilbert LJ, Winkler-Lowen B, Lyall F and Morrish DW. PL74, a novel member of the transforming growth factor-beta superfamily, is overexpressed in preeclampsia and causes apoptosis in trophoblast cells. *J Clin Endocrinol Metab*. 2005;90(5):3045-53.
- [26] Gerbaud P, Pidoux G, Guibourdenche J, Pathirage N, Costa JM, Badet J, Frendo JL, Murthi P and Evain-Brion D. Mesenchymal activin-A overcomes defective human trisomy 21 trophoblast fusion. *Endocrinology*. 2011;152(12):5017-28.
- [27] Morrish DW, Bhardwaj D and Paras MT. Transforming growth factor beta 1 inhibits placental differentiation and human chorionic gonadotropin and human placental lactogen secretion. *Endocrinology*. 1991;129(1):22-6.
- [28] Leisser C, Saleh L, Haider S, Husslein H, Sonderegger S and Knofler M. Tumour necrosis factor-alpha impairs chorionic gonadotrophin beta-subunit expression and cell fusion of human villous cytotrophoblast. *Mol Hum Reprod*. 2006;12(10):601-9.
- [29] Lysiak JJ, Hunt J, Pringle GA and Lala PK. Localization of transforming growth factor beta and its natural inhibitor decorin in the human placenta and decidua throughout gestation. *Placenta*. 1995;16(3):221-31.
- [30] Shi QJ, Lei ZM, Rao CV and Lin J. Novel role of human chorionic gonadotropin in differentiation of human cytotrophoblasts. *Endocrinology*. 1993;132(3):1387-95.
- [31] Pidoux G, Gerbaud P, Tsatsaris V, Marpeau O, Ferreira F, Meduri G, Guibourdenche J, Badet J, Evain-Brion D and Frendo JL. Biochemical characterization and modulation of LH/CG-receptor during human trophoblast differentiation. *J Cell Physiol*. 2007;212(1):26-35.
- [32] Seshagiri PB, Terasawa E and Hearn JP. The secretion of gonadotrophin-releasing hormone by peri-implantation embryos of the rhesus monkey: comparison with the secretion of chorionic gonadotrophin. *Hum Reprod*. 1994;9(7):1300-7.
- [33] Keryer G, Alsat E, Tasken K and Evain-Brion D. Cyclic AMP-dependent protein kinases and human trophoblast cell differentiation in vitro. *J Cell Sci*. 1998;111 ( Pt 7):995-1004.
- [34] Pidoux G, Gerbaud P, Marpeau O, Guibourdenche J, Ferreira F, Badet J, Evain-Brion D and Frendo JL. Human placental development is impaired by abnormal human chorionic gonadotropin signaling in trisomy 21 pregnancies. *Endocrinology*. 2007;148(11):5403-13.
- [35] Coutifaris C, Kao LC, Sehdev HM, Chin U, Babalola GO, Blaschuk OW and Strauss JF, 3rd. E-cadherin expression during the differentiation of human trophoblasts. *Development*. 1991;113(3):767-77.
- [36] Knerr I, Schubert SW, Wich C, Amann K, Aigner T, Vogler T, Jung R, Dotsch J, Rascher W and Hashemolhosseini S. Stimulation of GCMA and syncytin via cAMP mediated PKA signaling in human trophoblastic cells under normoxic and hypoxic conditions. *FEBS Lett*. 2005;579(18):3991-8.
- [37] Chen CP, Chen LF, Yang SR, Chen CY, Ko CC, Chang GD and Chen H. Functional characterization of the human placental fusogenic membrane protein syncytin 2. *Biol Reprod*. 2008;79(5):815-23.
- [38] Darrow BJ, Fast VG, Kleber AG, Beyer EC and Saffitz JE. Functional and structural assessment of intercellular communication. Increased conduction velocity and enhanced connexin expression in dibutyryl cAMP-treated cultured cardiac myocytes. *Circ Res*. 1996;79(2):174-83.
- [39] Ul Hussain M. Transcriptional regulation of the connexin gene. 2014; (Ed.) Ul Hussain M. pp. 17-23. Springer.
- [40] Chen H and Cheong ML. Syncytins: Molecular aspects. 2011; (Ed.) Larsson LG. pp. 117-37. Springer.
- [41] Getsios S, Chen GT and MacCalman CD. alpha-, beta-, gamma-catenin, and p120(CTN) expression during the terminal differentiation and fusion of human mononucleate cytotrophoblasts in vitro and in vivo. *Molecular reproduction and development*. 2001;59(2):168-77.



- [42] Fanning AS and Anderson JM. Zonula occludens-1 and -2 are cytosolic scaffolds that regulate the assembly of cellular junctions. *Annals of the New York Academy of Sciences*. 2009;1165:113-20.
- [43] Saez JC, Berthoud VM, Moreno AP and Spray DC. Gap junctions. Multiplicity of controls in differentiated and undifferentiated cells and possible functional implications. *Adv Second Messenger Phosphoprotein Res*. 1993;27:163-98.
- [44] Bruzzone R, White TW and Paul DL. Connections with connexins: the molecular basis of direct intercellular signaling. *Eur J Biochem*. 1996;238(1):1-27.
- [45] Willecke K, Eiberger J, Degen J, Eckardt D, Romualdi A, Guldenagel M, Deutsch U and Sohl G. Structural and functional diversity of connexin genes in the mouse and human genome. *Biol Chem*. 2002;383(5):725-37.
- [46] Frendo JL, Cronier L, Bertin G, Guibourdenche J, Vidaud M, Evain-Brion D and Malassine A. Involvement of connexin 43 in human trophoblast cell fusion and differentiation. *J Cell Sci*. 2003;116(Pt 16):3413-21.
- [47] Cronier L, Frendo JL, Defamie N, Pidoux G, Bertin G, Guibourdenche J, Pointis G and Malassine A. Requirement of gap junctional intercellular communication for human villous trophoblast differentiation. *Biol Reprod*. 2003;69(5):1472-80.
- [48] Mi S, Lee X, Li XP, Veldman GM, Finnerty H, Racie L, LaVallie E, Tang XY, Edouard P, Howes S, Keith JC and McCoy JM. Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature*. 2000;403(6771):785-9.
- [49] Blond JL, Lavillette D, Cheynet V, Bouton O, Oriol G, Chapel-Fernandes S, Mandrand B, Mallet F and Cosset FL. An envelope glycoprotein of the human endogenous retrovirus HERV-W is expressed in the human placenta and fuses cells expressing the type D mammalian retrovirus receptor. *Journal of virology*. 2000;74(7):3321-9.
- [50] Frendo JL, Olivier D, Cheynet V, Blond JL, Bouton O, Vidaud M, Rabreau M, Evain-Brion D and Mallet F. Direct involvement of HERV-W Env glycoprotein in human trophoblast cell fusion and differentiation. *Mol Cell Biol*. 2003;23(10):3566-74.
- [51] Lavillette D, Marin M, Ruggieri A, Mallet F, Cosset FL and Kabat D. The envelope glycoprotein of human endogenous retrovirus type W uses a divergent family of amino acid transporters/cell surface receptors. *Journal of virology*. 2002;76(13):6442-52.
- [52] Jansson T. Amino acid transporters in the human placenta. *Pediatric research*. 2001;49(2):141-7.
- [53] Cariappa R, Heath-Monnig E and Smith CH. Isoforms of amino acid transporters in placental syncytiotrophoblast: plasma membrane localization and potential role in maternal/fetal transport. *Placenta*. 2003;24(7):713-26.
- [54] Blaise S, de Parseval N, Benit L and Heidmann T. Genomewide screening for fusogenic human endogenous retrovirus envelopes identifies syncytin 2, a gene conserved on primate evolution. *Proc Natl Acad Sci U S A*. 2003;100(22):13013-8.
- [55] Malassine A, Frendo JL, Blaise S, Handschuh K, Gerbaud P, Tsatsaris V, Heidmann T and Evain-Brion D. Human endogenous retrovirus-FRD envelope protein (syncytin 2) expression in normal and trisomy 21-affected placenta. *Retrovirology*. 2008;5:6.
- [56] Vargas A, Moreau J, Landry S, LeBellego F, Toufaily C, Rassart E, Lafond J and Barbeau B. Syncytin-2 plays an important role in the fusion of human trophoblast cells. *J Mol Biol*. 2009;392(2):301-18.
- [57] Esnault C, Priet S, Ribet D, Vernochet C, Bruls T, Lavialle C, Weissenbach J and Heidmann T. A placenta-specific receptor for the fusogenic, endogenous retrovirus-derived, human syncytin-2. *Proc Natl Acad Sci U S A*. 2008;105(45):17532-7.
- [58] Malassine A, Pidoux G, Gerbaud P, Frendo JL and Evain-Brion D. Human trophoblast in trisomy 21: a model for cell-cell fusion dynamic investigation. *Adv Exp Med Biol*. 2011;714:103-12.
- [59] Malassine A, Blaise S, Handschuh K, Lalucque H, Dupressoir A, Evain-Brion D and Heidmann T. Expression of the fusogenic HERV-FRD Env glycoprotein (syncytin 2) in human placenta is restricted to villous cytotrophoblastic cells. *Placenta*. 2007;28(2-3):185-91.
- [60] Potgens AJ, Drewlo S, Kokozidou M and Kaufmann P. Syncytin: the major regulator of trophoblast fusion? Recent developments and hypotheses on its action. *Hum Reprod Update*. 2004;10(6):487-96.

- [61] Dupressoir A, Vernochet C, Harper F, Guegan J, Dessen P, Pierron G and Heidmann T. A pair of co-opted retroviral envelope syncytin genes is required for formation of the two-layered murine placental syncytiotrophoblast. *Proc Natl Acad Sci U S A*. 2011;108(46):E1164-73.
- [62] Bjerregaard B, Talts JF and Larsson LI. The endogenous envelope protein syncytin is involved in myoblast fusion. 2011; (Ed.) Larsson LI. pp. 267-75. Springer.
- [63] Soe K, Andersen TL, Hobolt-Pedersen AS, Bjerregaard B, Larsson LI and Delaisse JM. Involvement of human endogenous retroviral syncytin-1 in human osteoclast fusion. *Bone*. 2011;48(4):837-46.
- [64] Cheynet V, Oriol G and Mallet F. Identification of the hASCT2-binding domain of the Env ERVWE1/syncytin-1 fusogenic glycoprotein. *Retrovirology*. 2006;3:41.
- [65] Lyden TW, Ng AK and Rote NS. Modulation of phosphatidylserine epitope expression by BeWo cells during forskolin treatment. *Placenta*. 1993;14(2):177-86.
- [66] Riddell MR, Winkler-Lowen B, Jiang Y, Davidge ST and Guilbert LJ. Pleiotropic actions of forskolin result in phosphatidylserine exposure in primary trophoblasts. *PloS one*. 2013;8(12):e81273.
- [67] Chernomordik LV and Kozlov MM. Mechanics of membrane fusion. *Nature structural & molecular biology*. 2008;15(7):675-83.
- [68] Muller M and Schick M. An alternate path for fusion and its exploration by field-theoretic means. *Current topics in membranes*. 2011;68:295-323.
- [69] Huppertz B and Kingdom JC. Apoptosis in the trophoblast--role of apoptosis in placental morphogenesis. *J Soc Gynecol Investig*. 2004;11(6):353-62.

## FIGURE LEGENDS

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

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

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

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

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

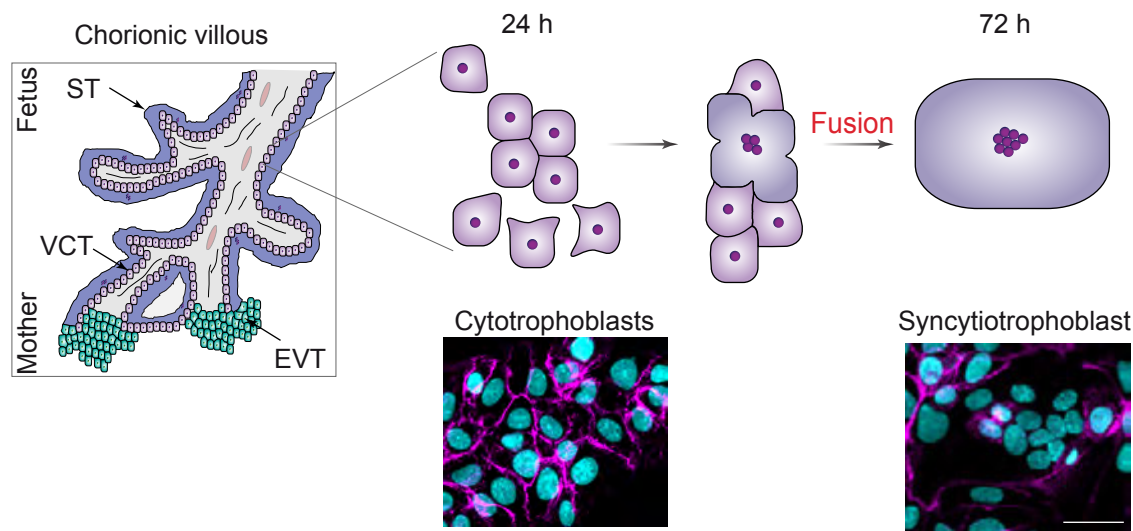
cells prior to the commitment stage. (right panel) Schematic depiction of the commitment stage mediated through cadherin clustering. Cadherins are transmembrane proteins which ensure cell-cell adhesion by the formation of adherens junctions through their extracellular N-terminal domain. The intracellular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

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

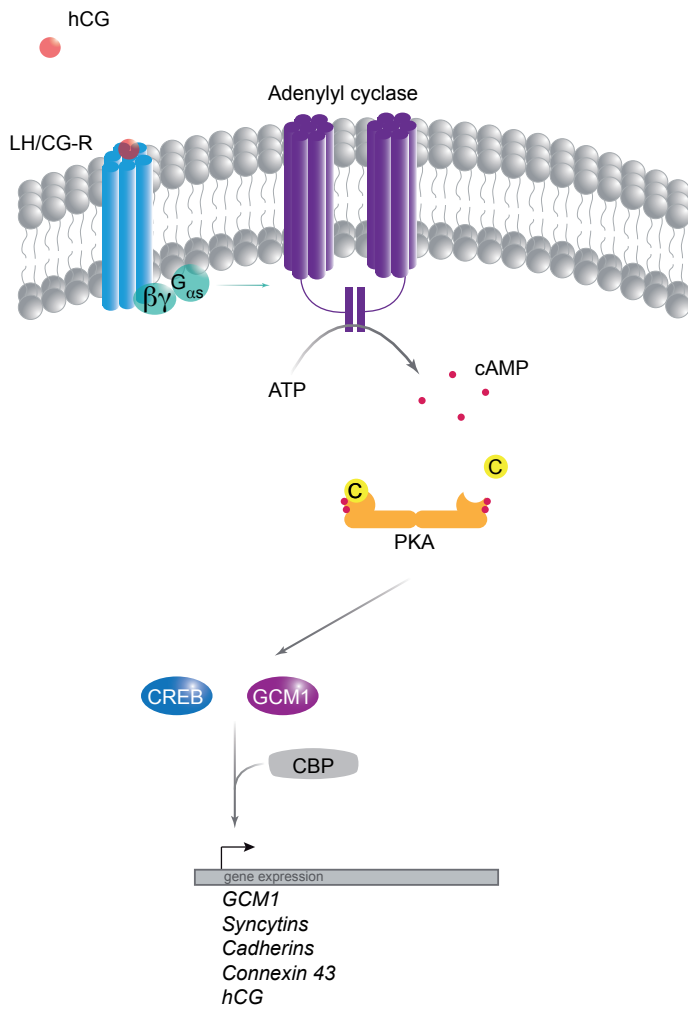
**Figure 5: Schematic depiction of trophoblast cell fusion mediated through syncytin-1.** (A) Representation of syncytin-1 structure. SU, surface unit; TM, transmembrane unit. Syncytin-1 extracellular domain represented in light gray, furin cleavage site (RNKR) in red, fusion peptide domain (ILPFVIGAGVLGALGTGIGGI) in purple, plasma membrane domain in green and intracellular domain in blue. The Y indicates N-glycosylation sites. The SDGGGX<sub>2</sub>DX<sub>2</sub>R motif characterizes the RBD (receptor binding domain). The disulfide bond is formed between the CX<sub>6</sub>CC motif of the TM domain and the CΦC motif of the SU domain. (B) Schematic depiction of syncytin-1 initiating cell fusion. a) Resting stage, b) RBD of syncytin-1 (dark grey) bound to hASCT2 (dark blue), c) SU domains are removed from the trimer and release the fusion peptide (purple), d) The fusion peptide harpoons the target plasma membrane, e) Plasma membrane bending is initiated.

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

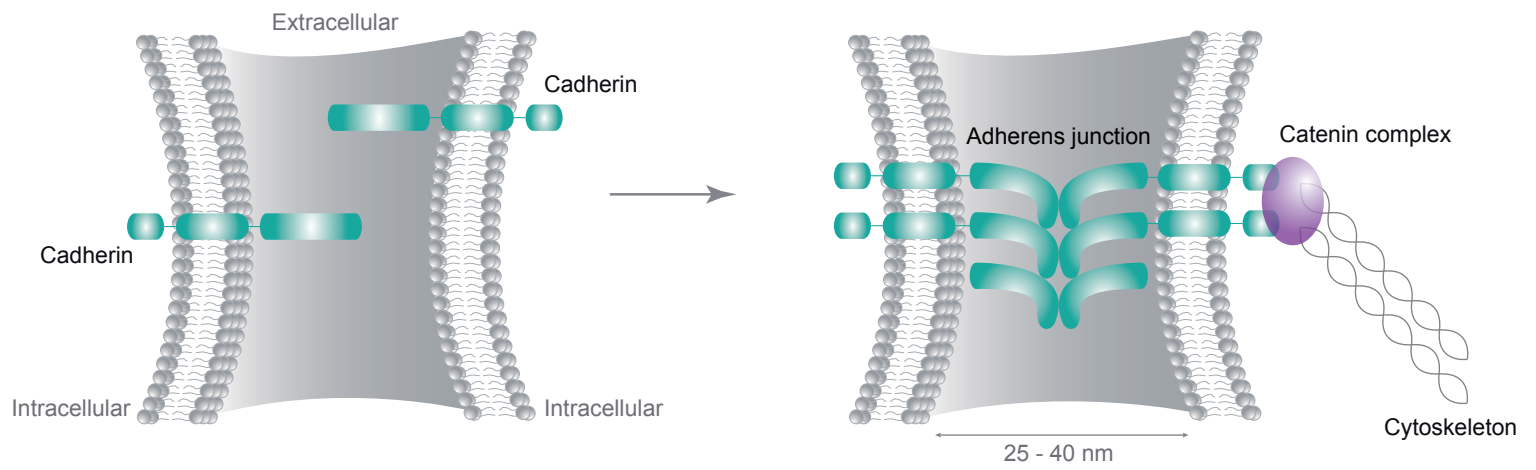
Gerbaud et al, Figure 1



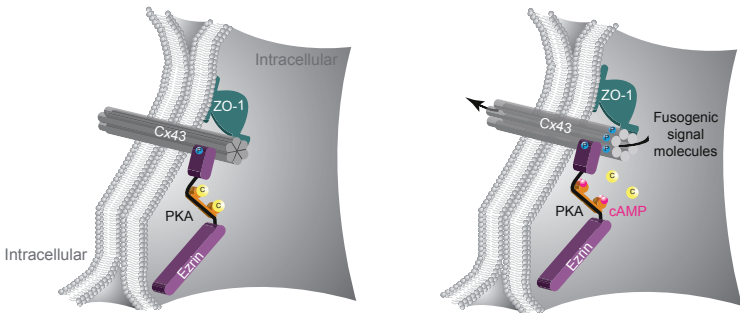
Gerbaud et al, Figure 2



Gerbaud et al, Figure 3

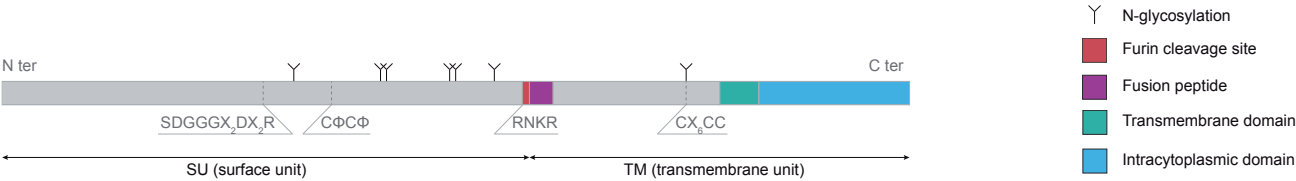


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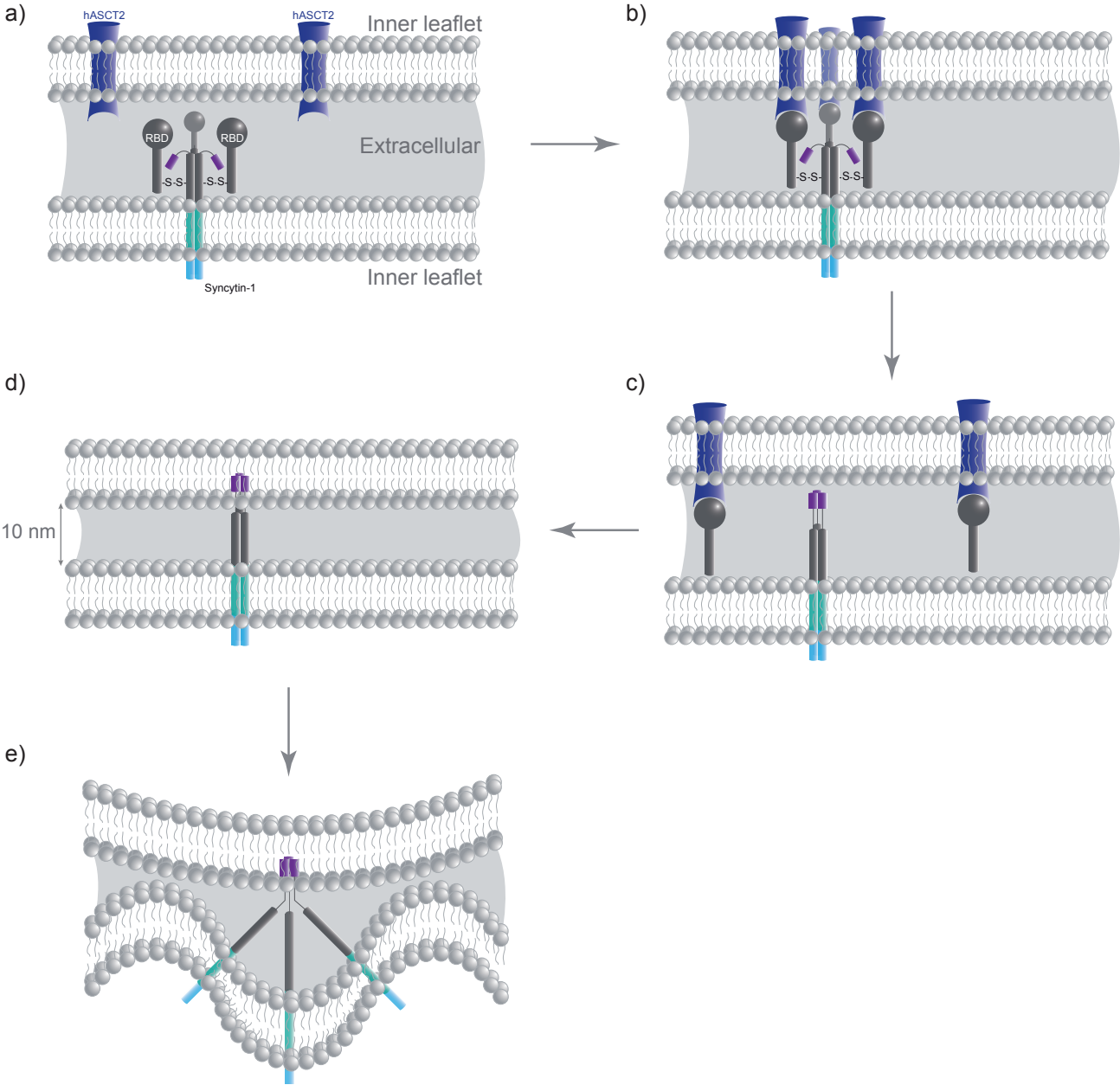




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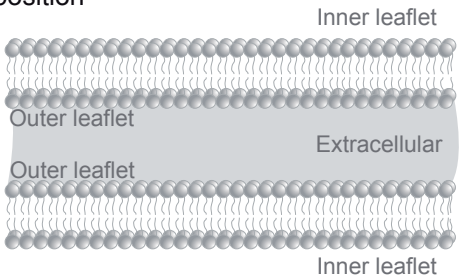


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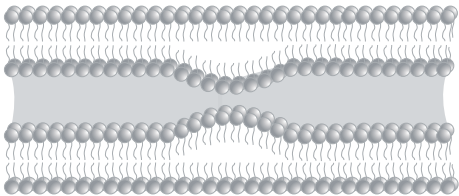


Gerbaud et al, Figure 6

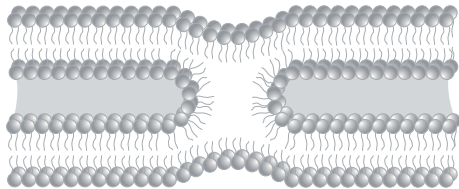
a) Apposition



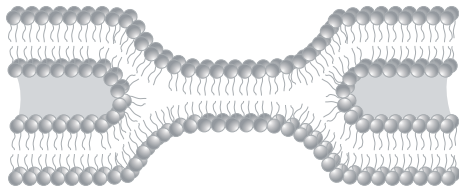
b) Bending



c) Stalk



d) Hemifusion Diagram (HD)



e) Fusion pore

