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REVIEW

Role of connexin 43 in different forms of intercellular communication – gap junctions, extracellular vesicles and tunnelling nanotubes

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ABSTRACT

Communication is important to ensure the correct and efficient flow of information, which is required to sustain active social networks. A fine-tuned communication between cells is vital to maintain the homeostasis and function of multicellular or unicellular organisms in a community environment. Although there are different levels of complexity, intercellular communication, in prokaryotes to mammalians, can occur through secreted molecules (either soluble or encapsulated in vesicles), tubular structures connecting close cells or intercellular channels that link the cytoplasm of adjacent cells. In mammals, these different types of communication serve different purposes, may involve distinct factors and are mediated by extracellular vesicles, tunnelling nanotubes or gap junctions. Recent studies have shown that connexin 43 (Cx43, also known as GJA1), a transmembrane protein initially described as a gap junction protein, participates in all these forms of communication; this emphasizes the concept of adopting strategies to maximize the potential of available resources by reutilizing the same factor in different scenarios. In this Review, we provide an overview of the most recent advances regarding the role of Cx43 in intercellular communication mediated by extracellular vesicles, tunnelling nanotubes and gap junctions.

KEY WORDS: Connexin 43, GJA1, Extracellular vesicles, Gap junction, Intercellular communication, Tunnelling nanotube

Introduction

The existence of highly organized and differentiated tissues and organs in multicellular organisms largely relies on a complex and intricate network of specialized cells that are devoted to particular biological functions. To work as a whole and respond in a synchronized and integrated manner, the different cells and tissues within an organism have to establish efficient communication strategies that allow the exchange of biological information. This crosstalk is essential to disseminate signals and transfer cellular components, including metabolites and organelles, allowing a syncytial behaviour, not only in basal conditions, but also in response to external stimuli. Given the importance of a fine-tuned intercellular communication for the homeostasis of a healthy organism, its deregulation has been extensively associated with the development of several pathologies, such as cancer, cardiovascular diseases and age-related disorders (Aasen et al., 2016; Bang et al., 2015; Grek et al., 2014; López-Otín et al., 2013; Pitt et al., 2016; Rustom, 2016).

In this Review, we aim at providing a comprehensive and critical perspective of some of the different strategies cells have developed to communicate and outline how the same protein, connexin 43 (Cx43, also known as GJA1), can participate in diverse forms of intercellular communication.

Cx43

A total of 21 different connexin genes have been identified in the human genome, each coding for a transmembrane protein with the same topology, namely four α-helical transmembrane domains (TM1–TM4), two extracellular loops (EL1 and EL2), a cytoplasmic loop (CL) between TM2 and TM3, and cytoplasmic N-terminal (NT) and C-terminal (CT) domains (Fig. 1A); however, of these, Cx43 is the most widely expressed and the one that has been studied in most detail (Kelly et al., 2015; Leybaert et al., 2017; Molica et al., 2014). The association of six identical (homomeric channels) or different (heteromeric channels) connexin proteins leads to the formation of a hexameric structure called hemichannel (‘connexon’) (Laird, 2006; Pfenniger et al., 2011). These hemichannels are transported to the plasma membrane, where they can dock with similar entities present in closely opposed membranes, forming gap junction channels (Fig. 1B). Tens to thousands of connexin channels accumulate in well-defined membrane domains called gap junction plaques, where direct exchange of ions, small metabolites, second messengers, microRNAs (miRNAs) and linear peptides between the cytoplasms of connected cells occurs, providing a simple method to synchronize responses in multicellular organisms (Aucher et al., 2013; Katakowski et al., 2010; Lemcke et al., 2015; Leybaert et al., 2017; Neijssen et al., 2007). Remarkably, the gap junction plaques are only internalized into one of the connected cells as vesicle structures called annular gap junctions, which comprise the junctional membranes of the two adjacent cells. Besides mediating cell–cell communication, undocked connexin channels, or hemichannels, thus allow the communication between the intracellular and the extracellular milieu, thereby playing an important role in paracrine communication during disease (Begandt et al., 2017; Belousov et al., 2017; Lemcke et al., 2015; Leybaert et al., 2017; Neijssen et al., 2007; Retamal et al., 2015).

Although Cx43 was initially described as a protein required for gap junction-mediated intercellular communication, the way we consider this protein has dramatically changed in the past few years. Indeed, recent pioneer studies have demonstrated that Cx43 can also mediate communication between non-opposed cells, namely through tunnelling nanotubes (TNTs) and extracellular vesicles (Soares et al., 2015; Wang et al., 2010). While TNTs sustain

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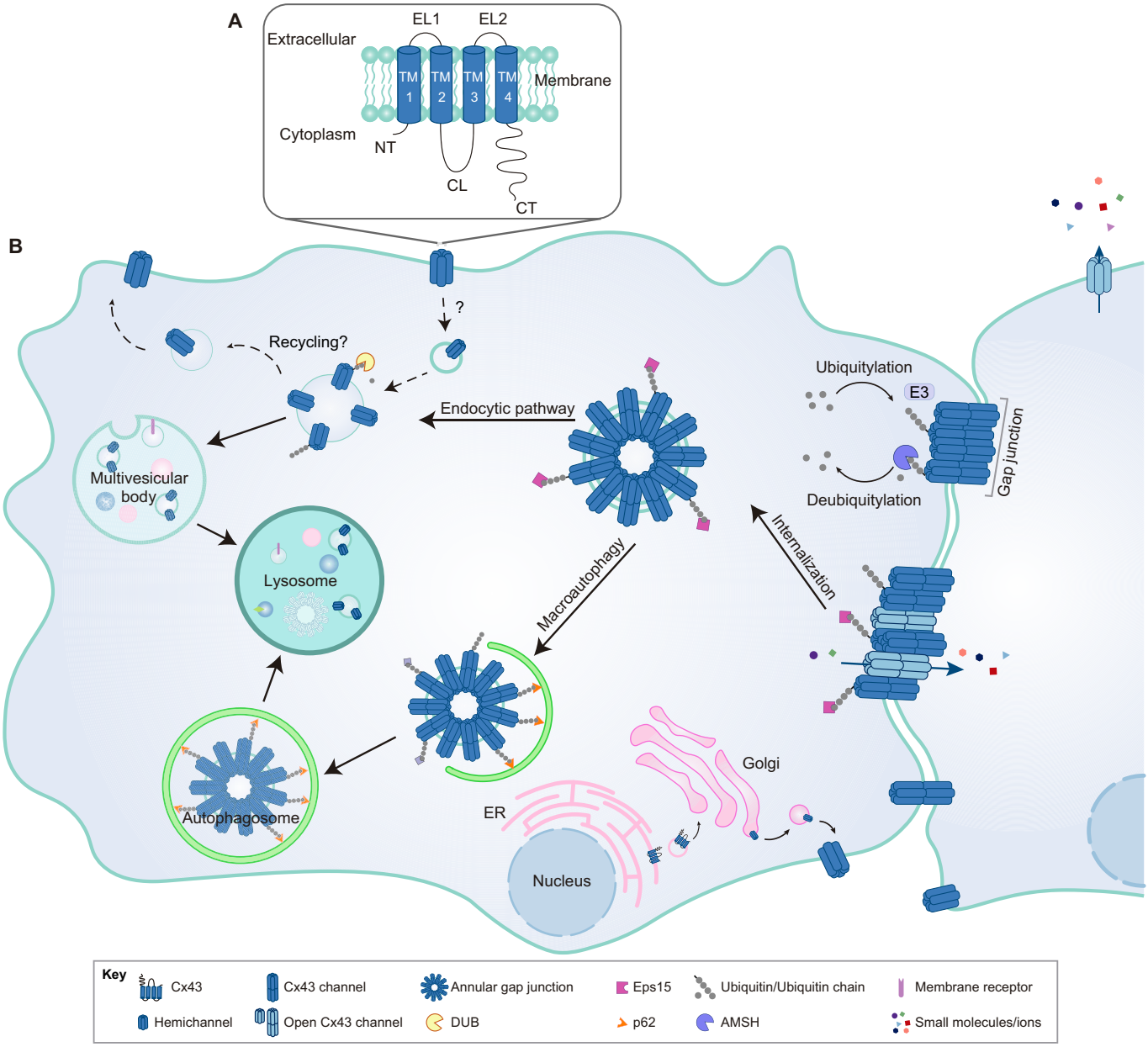

Fig. 1. The life cycle of Cx43. (A) Illustrated here is the characteristic topology of Cx43; it comprises four transmembrane domains (TMs), two extracellular loop (EL) domains, one cytoplasmic loop (CL) and a cytoplasmic N-terminus (NT) and C-terminus (CT). (B) Schematic illustration of Cx43 trafficking. Cx43 folds in the ER and after oligomerization in the Golgi, traffics to the plasma membrane where it might function as a hemichannel, exchanging small molecules with the extracellular milieu, or dock with a hemichannel from the neighbouring cell to form a gap junction. Ubiquitylation of Cx43 at the plasma membrane, mediated by the balance between an E3 ligase (Nedd4) and DUB activity (AMSH), signals the internalization of the protein, through a process that requires the endocytic adaptor Eps15. The internalization of Cx43 can occur through entire or partial removal of gap junction plaques, with the resulting structures called annular gap junctions, or, more likely, by hemichannel endocytosis. Once internalized, Cx43 can be either sorted to the endocytic pathway into a multivesicular body and then the lysosome, or recognized by autophagy adaptors such as p62 (also known as SQSTM1) and directed to a nascent autophagosome, which both result in its degradation. Cx43 deubiquitylation may rescue the protein from lysosomal degradation, allowing its recycling back to the plasma membrane.

communication between physically connected cells through membrane tubular structures, extracellular vesicles allow the transfer of information between separated cells, ensuring communication over long distances.

Recent data provide further support to the emerging concept that connexins have additional roles that go beyond cellular communication and are independent of their channel function, namely, regulation of gene transcription, interaction with cell growth and cell death modulators and mechanical roles (Esseltine and Laird, 2016; Leo-Macias et al., 2016; Martins-Marques et al., 2015a).

Below, we explore the idea that Cx43 participates in different forms of intercellular communication, likely as part of an evolutionary strategy to maximize the resources available by perfecting the use of Cx43 as a communication protein.

The role of Cx43 in gap junction-mediated intercellular communication

Intercellular communication through gap junctions allows not only the rapid exchange of ions, which is required for action potential conduction and efficient spreading of signalling molecules, but

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also participates in slower physiological processes, such as cell growth and development (Belousov et al., 2017; Berthoud and Ngezahayo, 2017; Jagger and Forge, 2015; Lilly et al., 2016; Meens et al., 2015; Merrifield and Laird, 2016; Wei et al., 2004). For instance, Cx43-mediated intercellular communication has been described in immune-mediated processes, where gap junctions participate in the immunological synapse and promote the transfer of viral- and tumour-derived peptides and miRNA, allowing further stimulation and modulation of immune responses (Aucher et al., 2013; Bermudez-Fajardo et al., 2007; Mendoza-Naranjo et al., 2007; Neijssen et al., 2005).

Although initially considered a rather non-specific mechanism whereby molecules of less than 1 kDa could freely cross the channel, several recent studies have shown that connexin-mediated communication is a highly regulated and selective process. Such fine-tuning includes the tissue- and cell-type-specific expression of different (combinations of) connexins with unique properties with regard to their permeability, gating and ability to interact with regulatory binding partners.

In addition to channel composition, connexin-mediated intercellular communication can also be modulated by a highly orchestrated activity of post-translational modifications (PTMs), including phosphorylation, acetylation, nitrosylation, sumoylation and ubiquitylation (Laird et al., 2015). The effect of these PTMs can either be direct, by affecting channel gating, or indirect, by regulating folding, trafficking, accretion, docking and degradation of the Cx43 channel (reviewed in Axelsen et al., 2013; D'Hondt et al., 2013; Pogoda et al., 2016). Indeed, phosphorylation of Cx43 not only modulates channel activity, but also regulates every stage of the Cx43 life cycle (see Box 1) (Lampe and Lau, 2004; Solan and Lampe, 2014). Apart from phosphorylation, ubiquitylation has recently gained increasing attention as an important regulator of Cx43-mediated intercellular communication. The protein neuronal precursor cell-expressed developmentally down-regulated 4 (Nedd4) was the first ubiquitin protein ligase identified to catalyse the attachment of ubiquitin moieties to Cx43, which induces its internalization and further lysosomal degradation mediated by the endocytic pathway or autophagy (Bejarano et al., 2012; Leithe, 2016; Leykauf et al., 2006; Ribeiro-Rodrigues et al., 2014, 2015; Spagnol et al., 2016) (Fig. 1B). In accordance, impairing Nedd4-mediated ubiquitylation of Cx43 results in the accumulation of gap junctions at the plasma membrane with a concomitant increase of intercellular communication (Leykauf et al., 2006; Girão et al., 2009, Totland et al., 2017). Ubiquitylation of Cx43 has also been linked to proteasome-dependent quality control mechanisms in the context of endoplasmic reticulum (ER)-associated protein degradation (ERAD) (Kopanic et al., 2015; VanSlyke and Musil, 2002). It is worth noting that the amount of ubiquitin moieties bound to Cx43 depends on the balance between ubiquitylation, catalysed by E3 ligases, and deubiquitylation, mediated by deubiquitylating enzymes (DUBs). We have previously shown that deubiquitylation of Cx43 by the protein AMSH (associated molecule with the SH3 domain of STAM, also known as STAMBP) stabilizes gap junctions at the plasma membrane (Ribeiro-Rodrigues et al., 2014). However, other studies using ubiquitylation-resistant forms of Cx43 have suggested that direct ubiquitylation of Cx43 is not necessary to regulate protein stability. Indeed, by using mutated forms of Cx43, where lysine residues were replaced by arginine residues, it was shown that proteasomal degradation of ER-derived Cx43 is mediated by Cx43-interacting protein of 75 kDa (CIP75, also known as UBQLN4) and that the role of Cx43 ubiquitylation is through its effect on regulatory molecules such as Akt proteins (Dunn et al., 2012; Su et al., 2010). Interestingly, despite the similarity to ubiquitin, the attachment of small ubiquitin-like modifier (SUMO) to Cx43 results in its stabilization at the plasma membrane, with the consequent enhancement of gap junction-mediated intercellular communication (GJIC), as measured by an increased dye transfer between neighbouring cells (Kjenseth et al., 2012). In an analogous manner to ubiquitylation, acetylation of Cx43 has been shown to regulate the subcellular localization of Cx43, and to promote its dissociation from gap junctions, with a resulting impairment of GJIC (Colussi et al., 2011). Because ubiquitylation, sumoylation and acetylation all occur at lysine residues, it is likely that a competition for the same residues can contribute to the crosstalk between PTMs that is crucial for regulating Cx43 localization and trafficking and its roles in intercellular communication.

A number of mutations in Cx43 have been identified that not only impact on gap junction plaque formation, but also directly affect the activity of gap junction channels (for a recent review see Molica et al., 2014). Indeed, mutations in the N-terminal domain of Cx43 (G2V, D3N, L7V, L11P, Y17S and P18S) or in its cytoplasmic loop (I130T, K124E and G138R), which render the channels non-functional, have been associated with oculodentodigital dysplasia (ODDD) (Kelly et al., 2016; Laird, 2014; Roscoe et al., 2005; Seki et al., 2004; Shao et al., 2012).

Box 1. Post-translational regulation of Cx43

Post-translational regulation of Cx43 has a critical impact on GJIC, either through direct control of channel activity, or by modulating protein–protein interactions and Cx43 localization. Phosphorylation is by far the most well-studied PTM, with several studies demonstrating its instrumental role upon regulation of channel gating, trafficking, assembly/disassembly and degradation of gap junction channels (Lampe and Lau, 2004; Popolo et al., 2013; Takens-Kwak and Jongsma, 1992). At the C-terminus of Cx43, 21 phosphorylation sites have been described (19 serine residues and two tyrosine residues) that are regulated by the action of more than ten kinases and phosphatases, including protein kinase C (PKC), protein kinase A (PKA), mitogen-activated protein kinases (MAPKs), Src kinases and protein phosphatase 2A (PP2A) (Axelsen et al., 2013; Solan and Lampe, 2014). Specific phosphorylation and dephosphorylation events occur in a well-balanced manner throughout the Cx43 lifecycle. For example, phosphorylation at S364 and/or S365 regulates its trafficking to or within the plasma membrane, thereby contributing to increased GJIC, whereas phosphorylation of S368 results in Cx43 internalization and downregulation of GJIC (Leithe and Rivedal, 2004; Solan and Lampe, 2014). Under pathological conditions (i.e. during myocardial ischaemia or wound healing), it has been suggested that the phosphorylation of the residues S373 followed by S279 and S282 simultaneously, and Y247 induces an acute increase in gap junction size, followed by a rapid internalization of Cx43 and downregulation of GJIC (Dunn and Lampe, 2014; Solan and Lampe, 2014). In fact, myocardial ischaemia constitutes a paradigmatic example for the dramatic alteration of the dynamics of Cx43 phosphorylation, and so affects channel conductance and localization. During ischaemia, the phosphorylation of S325, S328 and/or S330, which is restricted to the intercalated disc, is lost (Lampe et al., 2006). Additionally, dephosphorylation of S365 (the GJIC 'gatekeeper') takes place, enabling subsequent phosphorylation of S368 by PKC, which negatively impacts on electrical coupling between cardiomyocytes (Bao et al., 2004; Kwak et al., 1995; Lampe et al., 2000; Morel et al., 2012). More recently, it was demonstrated that ischaemia-induced phosphorylation of Cx43 S373 creates a so-called mode-1 binding domain for 14-3-3 proteins, which drives the internalization of Cx43 (Smyth et al., 2014).

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The role of GJIC in the heart

Gap junction-mediated electric and metabolic coupling is required to ensure the homeostasis in various organs and tissues. This is especially important in the heart, where the fine-tuned connexin-dependent cell–cell communication through gap junctions has been shown to be essential to ensure a coordinated contraction and pumping activity (see for instance Beyer et al., 1987; Revel and Karnovsky, 1967; Yeager and Gilula, 1992). Therefore, an impairment in GJIC has been implicated in the development of electric conduction defects that underlie several cardiac disorders (Severs, 2009; Severs et al., 2004; Stroemlund et al., 2015).

The most abundantly expressed connexin in the heart is Cx43. Although its presence has been also reported within subsarcolemmal mitochondrial membranes, in atrial and ventricular cardiomyocytes, Cx43 is mainly localized at specialized plasma membrane domains enriched in adhesion proteins, called intercalated discs (Fig. 2) (Agullo-Pascual et al., 2014, 2013; Boengler et al., 2009). Such a polarized distribution of gap junctions allows the rapid and efficient anisotropic propagation of the electrical impulse that is generated in the cardiomyocytes that are localized at the sinoatrial and atrioventricular nodes, which owing to the intrinsic depolarization activity, are considered the main pacemakers of the heart (Gros and Jongsma, 1996). The action potential generated in the nodes travels through low-resistance gap junction channels throughout the entire cardiac muscle, thereby allowing the heart to work as a functional syncytium. Interestingly, gap junction-mediated action potential propagation is not restricted to cardiomyocytes. Strikingly, it was recently demonstrated that the formation of Cx43 gap junction channels between cardiomyocytes and tissue-resident macrophages facilitates electrical conduction through the distal atrioventricular node (Hulsmans et al., 2017). Furthermore, a new concept emerging in the field is that the coordinated propagation of action potential in the heart relies not solely on gap junctions, but also on the profile of proteins found within the Cx43-scaffold at the intercalated disc, the so-called ‘cardiac connexome’ (Leo-Macias et al., 2016). In fact, a specific pool of undocked connexons that are localized to the periphery of a gap junction plaque (‘the perinexus’), rather than contributing to gap junction formation, serves to maintain interactions with a network of proteins, such as the desmosomal protein plakophilin-2, the cytoskeletal adaptor protein ankyrin-G and the pore-forming subunit of the cardiac Na^+ channel $Na_v1.5$ (formed from protein SCN5A) (Rhett and Gourdie, 2012; Sato et al., 2011). Interestingly, Cx43 expression has been suggested to be necessary for the full activity of Na^+ currents and for the accumulation of $Na_v1.5$ at the cardiac intercalated disc (Jansen et al., 2012; Lübkemeier et al., 2013). The disturbance of electrical coupling, which has been implicated in various cardiac disorders, including heart failure and arrhythmogenesis, appears to be

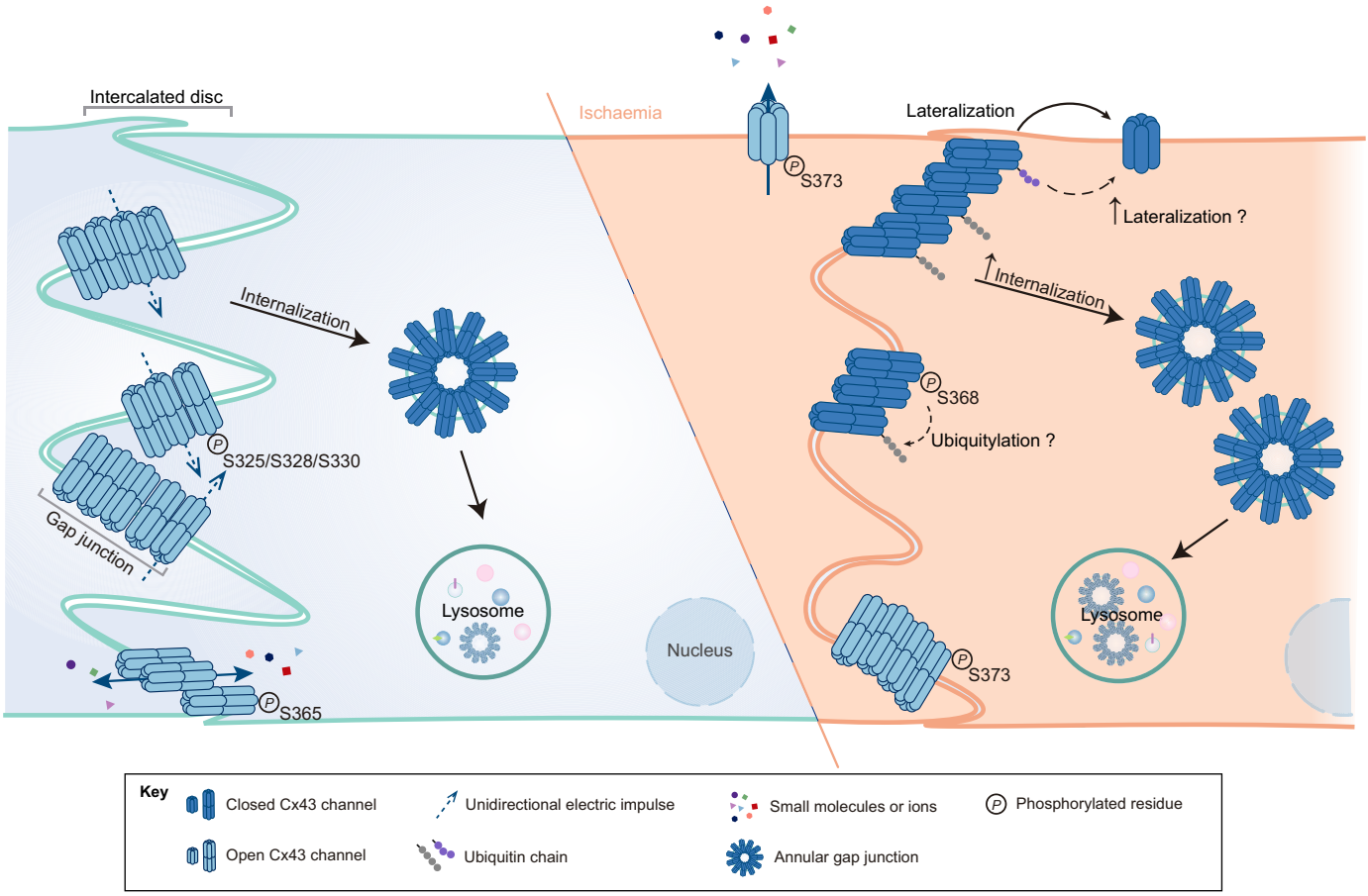

Fig. 2. Cardiac Cx43 and its function in health and disease. The physiological arrangement of gap junctions at the intercalated discs allows the anisotropic propagation of electrical currents and small metabolites and ions between adjacent cardiomyocytes (left panel). During myocardial ischaemia (shown on the right), there is a dramatic alteration of the PTMs of Cx43, including dephosphorylation of S325, S328, S330 and S365, and phosphorylation of S368 and S373, as well as Cx43 ubiquitylation. These PTMs impact on Cx43 channel gating and permeability, likely preceding the lateralization of gap junctions or Cx43 degradation, thereby contributing to arrhythmogenesis. Opening of Cx43 hemichannels, which are usually closed under basal conditions (see left panel), is also promoted during ischaemia (shown on the right).

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modulated, at least in part, by Cx43 expression and distribution, as well as the gating properties of the Cx43 channels (Duffy, 2012; Severs et al., 2004; Stroemlund et al., 2015). For instance, defects of electrical conductance in cardiac ischaemia, which is associated with an increase in cytosolic Ca^{2+} concentration, a decrease of intracellular pH and changes in the activity of different protein kinases and phosphatases, have been ascribed to the closure and internalization of gap junction channels, the appearance of Cx43 at the lateral membrane of cardiomyocytes ('lateralization') and the opening of non-opposed Cx43 hemichannels (Fig. 2) (Beardslee et al., 2000; De Vuyst et al., 2009; Johansen et al., 2011). In addition to ischaemia, Cx43 lateralization has been implicated in other pathological cardiac conditions associated with a decrease in electrical coupling (Desplantez et al., 2012; Jansen et al., 2012). Although the mechanisms underlying lateralization of Cx43 remain largely unknown, it has been suggested that Cx43 ubiquitylation might be a trigger for the removal of gap junction channels from the intercalated disc, which can result in Cx43 degradation and/or redistribution to the lateral membranes (Fig. 2) (Martins-Marques et al., 2015b,c). However, one should keep in mind that arrhythmias have multifactorial origins (i.e. perturbations in gap junction coupling, membrane excitability and tissue architecture), and the alteration of Cx43 gap junction channels during ischaemia cannot solely explain the development of all types of arrhythmias (Severs, 2009).

Besides their primary role in electrical propagation, Cx43 gap junction channels allow the intercellular spread of small metabolites, which can be either protective or detrimental, thus directing cells towards survival or death (Belousov et al., 2017; Fu, 2017; García-Dorado et al., 2004). For instance, during myocardial ischaemia and/or reperfusion, gap junctions have been proposed to mediate the progression of cell injury from the infarct zone to the myocardium at a distance (García-Dorado et al., 2004; Severs et al., 2004) by modulating the propagation of noxious metabolites. In accordance with this notion, gap junction inhibitors, such as heptanol, halothane and 18-alpha-glycyrrhetinic acid, have been reported to protect against reperfusion-induced cell death (Garcia-Dorado et al., 1997; Schlack et al., 1997).

The role of Cx43 hemichannels in paracrine communication

Although undocked connexin hemichannels were initially viewed as a mere intermediate stage of entities that are in transit to their final destination – the gap junction plaque – emerging evidence suggests that these structures have an active role in various pathological conditions, including inflammation, liver, lung and heart disease, wound healing, ischaemia–reperfusion lesion and atherosclerosis (for a review see Willebrords et al., 2017). In contrast to gap junction channels, which are typically open under physiological states, hemichannels are usually closed and open only in pathological conditions, such as the presence of disease-associated connexin mutations, membrane depolarization, mechanical stimulation, inflammation, low pH, phosphorylation, oxidative stress, high intracellular Ca^{2+} and ischaemia–reperfusion (Batra et al., 2014; Castellano and Eugenin, 2014; De Vuyst et al., 2009; Johansen et al., 2011; Leybaert et al., 2017; Retamal et al., 2015, 2007). The opening of these hemichannels allows the exchange of ions and metabolites between the cytosol and extracellular milieu. Indeed, it has been reported that cell swelling and Ca^{2+} overload associated with ischaemia–reperfusion injury is caused by the diffusion of Na^+ and K^+ between the cytoplasmic and extracellular compartments owing to uncontrolled opening of hemichannels (Fiori et al., 2012; Schalper et al., 2010). Moreover, the loss of important metabolites, such as ATP, glutamate, lactate and prostaglandins, through hemichannels has been implicated in acute inflammatory responses of the central nervous system (CNS) (Willebrords et al., 2016). Interestingly, mutations in genes encoding for connexins that cause a gain-of-function in hemichannels have been associated with cataract or deafness (Retamal et al., 2015; Sáez and Leybaert, 2014). Not surprisingly, the pharmacological modulation of hemichannels has been shown to have great therapeutic potential. Indeed, the use of the specific hemichannel-inhibiting peptide Gap19, which is derived from the cytoplasmic loop of Cx43, has been demonstrated to protect cardiomyocytes from volume overload and cell death, and reduce infarct size following ischaemia and/or reperfusion in mice (Wang et al., 2013). Furthermore, mimetic peptides of the external Cx43 domains have been reported to alleviate inflammation and neuronal damage by attenuating damage after spinal cord injury, ischaemia and ischaemia–reperfusion insults (Chen et al., 2015, 2013; Danesh-Meyer et al., 2012; O'Carroll et al., 2013).

Therefore, although 50 years have passed since the first structural description of gap junctions in the heart and liver (Revel and Karnovsky, 1967), followed by the molecular identification of their connexin building blocks shortly thereafter (Goodenough, 1974), the functions ascribed to connexins still increase year-after-year. Of particular interest in this regard are the newly described roles Cx43 might have in TNTs and extracellular vesicles, as discussed below.

The role of Cx43 in intercellular communication through tunnelling nanotubes

First described in 2004, TNTs are highly dynamic thin (with a diameter of 50 to 700 nm) and long (up to 100 µm) membrane structures that permit the transfer of diverse materials, including miRNAs, proteins, endocytic vesicles, lysosomes, vesicles derived from the ER and Golgi complex, prion proteins and virus, through connected cells (Fig. 3B) (Eugenin et al., 2009; Gousset et al., 2009; Koyanagi et al., 2005; Onfelt et al., 2006; Rustom et al., 2004; Sowinski et al., 2008). Although there is no strict definition of TNTs, there is consensus that these structures are characterized by the presence of F-actin and the fact that they are not attached to the substrate. In addition to actin, microtubules have also been shown to be present in thicker tubular TNT structures (Onfelt et al., 2006; Wang et al., 2012; Wang and Gerdes, 2015). Their short-lived nature, fragility and their sensitiveness to light exposure, mechanical stresses and chemical fixation, together with the lack of specific markers for their identification and/or detection, has thus far hindered the detailed study of TNTs (for reviews, see Abounit and Zurzolo, 2012; Gerdes et al., 2013; Sisakhtnezhad and Khosravi, 2015). Despite the limitations, TNTs have been observed in many *in vitro* cell systems, including fibroblasts, epithelial, immune, neural and cardiac cells, suggesting that these structures occur frequently. The biological relevance of TNTs and TNT-like structures has been demonstrated in various processes, including embryogenesis, stem cell differentiation, wound healing, resistance to stress, immune defence, spread of pathogens and cancer progression (Arkwright et al., 2010; Caneparo et al., 2011; Chinnery et al., 2008; Cselenyák et al., 2010; Demontis and Dahmann, 2007; Eugenin et al., 2009; Kadiu et al., 2009; Koyanagi et al., 2005; Kumar et al., 2017; Lou et al., 2012; Pyrgaki et al., 2010; Quinn et al., 2016; Roberts et al., 2015; Salas-Vidal and Lomeli, 2004; Sherer et al., 2007; Sowinski et al., 2008; Teddy and Kulesa, 2004; Wang and Gerdes, 2015). Two mechanisms have been proposed for TNT formation: the establishment of a membrane bridge when two previously interacting cells depart from each other,

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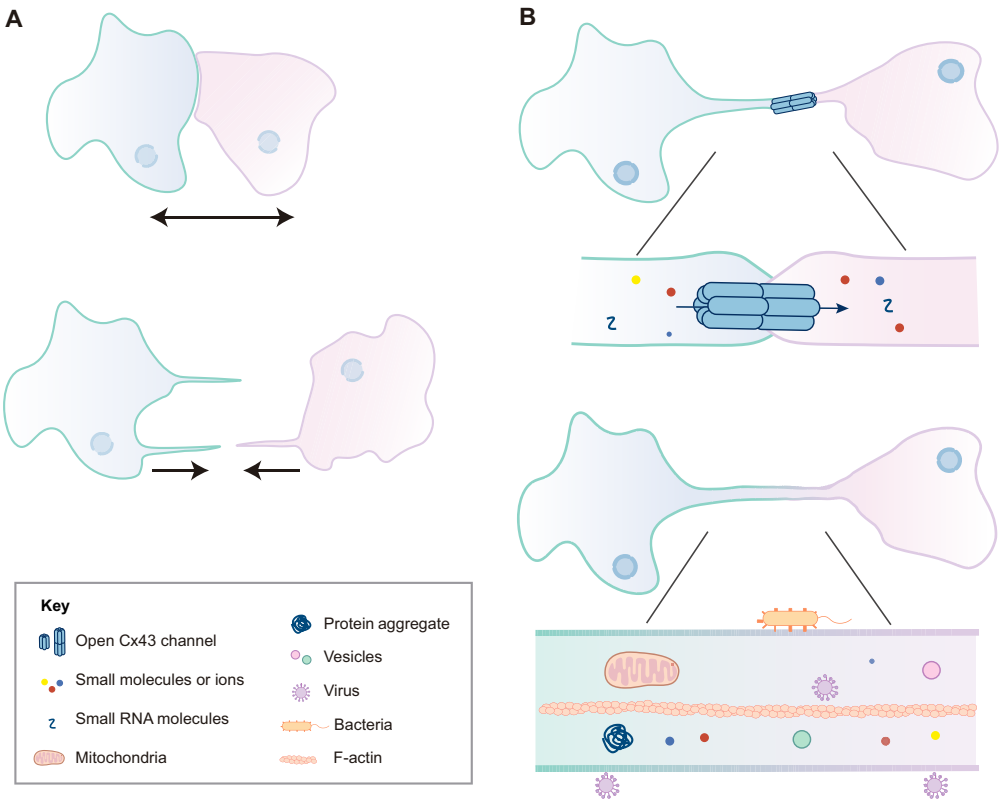

Fig. 3. Overview of TNT formation and cargo transfer. (A) Models for TNT biogenesis; in the cell dislodgment model (upper scheme), two cells that have been previously in contact migrate away from each other, extending the membrane and leading to TNT formation. In the actin-driven protrusion model (lower scheme), one or both cells extend a protrusion in the direction of the other cell until physical contact is established and a TNT is formed. (B) Depending on the specific properties of a TNT, different cargoes can be transferred; for instance, Cx43-coupled TNTs allow the intercellular propagation of small molecules (e.g. small dyes, small interfering RNA), ions and electrical currents from one cell to the other (upper scheme). In the case of open-ended TNTs, organelles (e.g. mitochondria, lipid droplets), vesicles (Golgi- and ER-derived vesicles, early endosomes and lysosomes), protein aggregates and pathogens (e.g. virus, bacteria) can be transferred from one cell to the other (lower scheme).

in a process called cell dislodgment, or the direct outgrowth of an actin-driven protrusion that can fuse either with the cell surface or with a similar protrusion from another cell (Fig. 3A) (Gerdes et al., 2013; Onfelt et al., 2006; Rustom et al., 2004; Wang et al., 2010; Watkins and Salter, 2005). It is plausible that the mechanisms and signals underlying TNT formation are specific to the cell type and/or stimuli. For example, it has been suggested that TNT formation through cell dislodgment is associated more with motile cells, such as lymphocytes or macrophages. Studies carried out in human natural killer (NK) cells showed that the ends of TNTs are localized in areas with high density ligand-receptor-based cell–cell contacts, suggesting that TNT formation can be driven by protein-mediated cell–cell interactions (Chauveau et al., 2010). Similarly, connexin-mediated cell junctions have been associated with TNTs. In this case, it has been suggested that connexons present in the tip of the growing filopodia dock with connexons in the target cell (Abounit and Zurzolo, 2012; Wang and Gerdes, 2012). Moreover, interaction of Cx43 with adhesion factors such as N-cadherin can also contribute to an increase in formation and/or stabilization of TNTs. In addition to protein interaction-mediated mechanisms, it has been speculated that TNT growth can be guided by chemical gradients (Abounit and Zurzolo, 2012; Sun et al., 2012).

Not surprisingly, cytoskeleton regulators, such as N-WASP, Rac1, ezrin and Cdc42, have been linked to TNTs (Arkwright et al., 2010; Hanna et al., 2017; Hase et al., 2009; Lachambre et al., 2014). However, and despite structural similarities, a recent study revealed that TNT formation and TNT-mediated vesicle transfer is negatively regulated by the filopodia-promoting complex Cdc42–IRSp53–VASP (IRSp53 is also known as BAIAP2), suggesting that the formation of filopodia and TNTs involves different mechanisms that may depend on the cell or stimulus (Delage et al., 2016). Although TNT formation occurs under normal physiological conditions, it can also be triggered by pro-inflammatory stimuli, viral infection, prion-like proteins and oxidative stress (Costanzo et al., 2013; Eugenin et al., 2009; Kadiu et al., 2009; Wang et al., 2011; Zhu et al., 2005). One of the main functions ascribed to TNTs is to mediate intercellular electrical coupling and Ca^{2+} flux (Wang and Gerdes, 2012; Watkins and Salter, 2005; Wittig et al., 2012). The finding that Cx43 is frequently present at one end of a TNT, together with the observation that electrical coupling between TNT-connected cells is inhibited in cells that lack gap junctions or when their function has been blocked, suggest that Cx43 is required for TNT-mediated electrical coupling (Lock et al., 2016; Smith et al., 2011; Wang et al., 2010; Wittig et al., 2012). In agreement, it has been shown that depolarization signals inflicted by mechanical stimulation can diffuse through Cx43-containing TNTs, which results in the activation of low-threshold voltage-gated Ca^{2+} channels in non-stimulated cells (Wang et al., 2010). Moreover, it has been proposed that this signalling process relies on gap junction-coupled TNT-mediated diffusion of inositol trisphosphate (IP_3) and low levels of Ca^{2+} that can trigger Ca^{2+} release from ER stores in the responding cell (Lock et al., 2016). Apart from Ca^{2+} and IP_3, it remains controversial which other molecules can spread through Cx43-containing TNTs. Indeed, although there is evidence for an absence of dye coupling and small conductance in Cx43-containing TNTs when compared with regular gap junctions (Wang et al., 2010), other studies have reported that Cx43-containing TNTs are permissive to the passage of small molecules, including small interfering RNAs and dyes such as Lucifer Yellow, but exclude larger molecules such as Cascade Blue (Fig. 3B) (Antanavičiūtė et al., 2014; Wang et al., 2010; Wittig et al., 2012). These observations support the idea that the presence of Cx43 in TNTs can confer selectivity to TNT-mediated intercellular communication, similar to what is observed for Cx43-containing gap junction channels at the plasma membrane (Rimkutė et al., 2016; Wittig et al., 2012). Cx43-containing TNTs have been implicated in

various biological and physiological processes, including Ca^{2+} signalling and electrical coupling between immature neurons and astrocytes during their maturation process (Wang et al., 2012). Moreover, it has been suggested that Cx43 has an important role in TNT-mediated coupling during neural migration and differentiation (Wang et al., 2012). The presence of Cx43 has been also described in actin-based tubular intercellular bridges formed during mitotic rounding, called mitotic nanotubes. In this scenario, TNT-like structures ensure the transfer of cytoplasmic components, including endocytic vesicles, between mitotic cells and adjacent cells, because GJIC is lost in this phase of the cell cycle (Fykerud et al., 2016). Functional Cx43 channels have also been observed on membranous tunnelling tubes that are larger than TNTs (with a diameter of greater than 1 mm). These Cx43-containing tunnelling tubes have been implicated in cell communication over longer distances mediating the passage of small molecules, including double-stranded small interfering RNA (Antanavičiūtė et al., 2014). An *in vivo* study demonstrated that structures with characteristics similar to TNTs called 'tumour microtubes' are required to maintain Ca^{2+} homeostasis in an astrocytoma cell network (Osswald et al., 2015). A reduced synchronicity of intercellular Ca^{2+} waves and a decrease of tumour microtubes in cells upon Cx43 knockdown suggests that Cx43 can play a role in the formation, maintenance of function of these structures. More importantly, disruption of tubular-mediated intercellular communication upon Cx43 deficiency leads to tumour reduction and improved survival (Osswald et al., 2015). Therefore, based on the increasing evidence for the importance of TNTs and their existence *in vivo*, it is conceivable that, similar to their role at the plasma membrane, the presence of Cx43 channels in TNTs could constitute a cellular strategy to regulate intercellular communication and contribute to the maintenance of homeostasis in an organism (Chinnery et al., 2008; He et al., 2011; Osswald et al., 2015).

The role of Cx43 in intercellular communication by extracellular vesicles

Extracellular vesicles can be found ubiquitously throughout an organism, where they either support cell–cell communication within an organ or travel in circulation to elicit biological responses in more distant organs and tissues. For instance, it has been demonstrated that extracellular vesicles can mediate crosstalk between the heart and the brain (Bang et al., 2014; Frühbeis et al., 2013), as well as contribute to the protective effects of remote ischemic preconditioning, in which short cycles of ischaemia and reperfusion applied to a distant organ can confer protection against cardiac ischaemia (Giricz et al., 2014).

With regard to their subcellular origin, communicating extracellular vesicles can be divided into (1) exosomes (50–200 nm in diameter), which are secreted after fusion of multivesicular bodies with the cell surface, and (2) microvesicles (100–1000 nm), which are formed by direct outward budding of the plasma membrane. Given the lack of specific markers to distinguish the subsets of vesicles and available techniques to isolate pure preparations, the International Society for Extracellular Vesicles (ISEV) recommended the indiscriminate use of the term 'extracellular vesicles' (Lötvall et al., 2014).

Although initially considered to be a random process, recent evidence indicates that extracellular vesicle-mediated flow of information is highly specific. However, the mechanisms and signals underlying such a selectivity are not fully understood. The unique repertoire of proteins on the membrane of extracellular vesicles may dictate the tropism of such vesicles, and/or modulate the way they interact with recipient cells to unload their content or to elicit a response. For example, a complex of transforming growth factor β (TGF-β) with β-glycan at the extracellular vesicles surface can trigger SMAD-dependent juxtacrine signalling in cells that contain the receptor (Webber et al., 2010). The presence of adhesion factors including tetraspanins at the vesicle membrane is important for their tethering, before fusion or endocytosis of its cargo into acceptor cells (Fig. 4) (Edgar et al., 2016; Nazarenko et al., 2010; Rana et al., 2012). Furthermore, the pathogenesis of tumour metastasis constitutes a paradigmatic example of selective long-distance communication mediated by extracellular vesicles (Costa-Silva et al., 2015; Liu et al., 2016). Accordingly, it was shown that extracellular vesicles derived from pancreatic cell lines, which metastasize primarily to the liver, follow the organotropism of their parental cells by communicating specifically with Kupffer cells within the liver, a mechanism that is driven by expression of specific integrins in both the extracellular vesicles and target cells (Costa-Silva et al., 2015; Hoshino et al., 2015).

Recently, several proteomic studies have identified the presence of some connexins in extracellular vesicles, namely Cx43, Cx45 (also known as GJC1) and Cx32 (also known as GJB1) that are secreted by a variety of cell types, including melanoma cells, neuroblastoma cells, ovarian and prostate cancer cells (Keerthikumar et al., 2015; Kharaziha et al., 2015; Lazar et al., 2015; Liang et al., 2013). Given their known roles in cell–cell communication, these findings point to a new role for connexin channels in mediating the interaction of extracellular vesicles with target cells and/or the delivery of small molecules and miRNAs, which could constitute alternative mechanisms of information processing in target cells, including the escape from lysosomal degradation.

Supporting this model, our recent study demonstrated that Cx43 assembles into functional channels at the surface of extracellular vesicles and, more importantly, facilitates the release of their intraluminal contents (Soares et al., 2015). Indeed, the presence of Cx43 in these vesicles increases the efficiency of luciferin unloading into luciferase-expressing recipient cells. Moreover, the passage of luciferin through Cx43-containing channels formed by the docking between extracellular vesicles and target cells can be impaired upon Cx43-channel blockade, either chemically by using Cx43-mimetic peptides, or genetically upon expression of a Cx43 mutant (Cx43S368D) that mimics a constitutively phosphorylated form of Cx43, which is likely in a closed state (Soares et al., 2015). Our findings thus revealed a new mechanism for rapid and efficient cell–cell communication through extracellular vesicles that is facilitated by the docking of Cx43 hemichannels (Fig. 4).

Reflecting the fact that Cx43 is widely expressed throughout the organism, we found that Cx43 is present in extracellular vesicles that are secreted by a number of different cell types, including retinal epithelial cells, endothelial cells, cardiac cell lines and organotypic heart cultures, suggesting that release of Cx43-containing extracellular vesicles is a more general phenomenon (Soares et al., 2015). Assuming that other connexins could have similar roles in extracellular vesicle-mediated communication, and taking into account their differential tissue expression and permeability properties, it is conceivable that the presence of connexins in extracellular vesicles constitutes an additional strategy to confer specificity and/or tropism to the vesicles.

The presence of Cx43 in extracellular vesicles could also be exploited therapeutically. For instance, it has been recently demonstrated that incorporation of doxorubicin in giant plasma membrane-derived vesicles containing Cx43 substantially increased its therapeutically effective dose (LD50) *in vitro* by more than an

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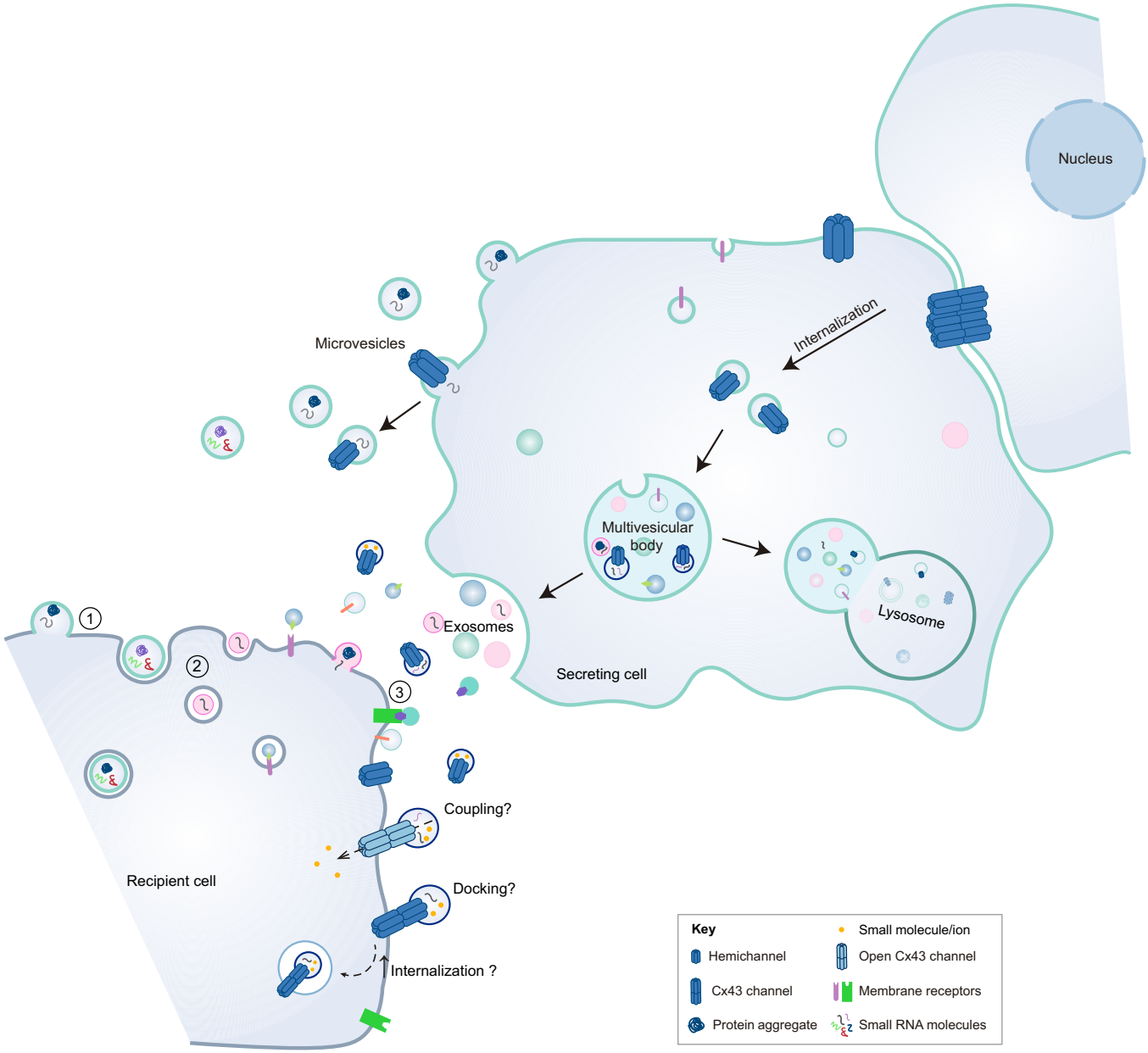

Fig. 4. Extracellular vesicle biogenesis and the role of Cx43 in vesicle-mediated intercellular communication. Maturation of endocytic compartments includes the formation of intraluminal vesicles, which mature into multivesicular bodies; these can either fuse with the lysosome, sending their content to degradation, or with the plasma membrane, thereby releasing small vesicles, called exosomes, into the extracellular space. Additionally, microvesicles can originate from the plasma membrane by outward budding. In the recipient cells, exosomes and microvesicles can either fuse (1), be endocytosed (2), or dock with the plasma membrane (3), thereby triggering a response. The presence of Cx43 at the membrane of microvesicles and exosomes may facilitate their docking and/or allow a faster release of extracellular vesicles content into the target cell.

order of magnitude when compared with that seen upon treatment by the free drug (Gadok et al., 2016). Contrary to our initial hypothesis, results from our lab have shown that the presence of Cx43 in extracellular vesicles loaded with doxorubicin did not significantly improved its anti-tumour effect. However, using a subcutaneous mouse tumour model, we demonstrated that transport of doxorubicin within Cx43-containing extracellular vesicles not only preserved the anti-tumour properties of doxorubicin, but also partially alleviated the cardiotoxicity of the drug (Martins-Marques et al., 2016). It is thus conceivable that Cx43-positive extracellular vesicles preferentially communicate with tumour cells rather than with cardiac cells, suggesting that Cx43 can confer specificity to cell-type targeting. It is plausible that tissues with a high density of fully assembled gap junctions and with reduced levels of free undocked Cx43, such as the heart, are less prone to interact and communicate with extracellular vesicles that contain Cx43 channels. However, further studies are clearly required to elucidate in depth the role of Cx43 in this mode of intercellular communication.

A more recent study has demonstrated that the selectivity of Cx43-mediated release of vesicle content can be enhanced upon surface expression of single-domain antibodies against model receptors that target specific cell populations (Gadok et al., 2017). In this work, the inclusion of single-domain antibodies against GFP in Cx43-containing plasma membrane-derived vesicles was used to increase the vesicle targeting to cells overexpressing GFP at their surface. Following cell–vesicle interaction, Cx43 channels mediate

the release of soluble dyes as demonstrated by the use of gap junction blockers such as carbenoxolone, which significantly inhibited the process (Gadok et al., 2017). The authors suggested that a strategy that combines the presence of antibodies, to increase the targeting, and Cx43, to facilitate the release of vesicular cargo, might be successfully used for the targeted delivery of doxorubicin and likely other therapeutic compounds to specific cell types and/or tissues (Gadok et al., 2017).

Conclusions and future perspectives

Multicellular organisms resort to different communication strategies to ensure an efficient exchange of information between cells. Although typically addressed as independent and isolated mechanisms, it is conceivable that nature has evolved in a way to develop approaches that allow the maximization of the resources available, namely by engaging the same factor in different tasks. In this Review, we explore the idea that Cx43 serves multiple purposes in different forms of intercellular communication. Although initially described as gap-junction-channel-forming protein, recent findings have demonstrated that Cx43 assists in other types of intercellular communication, including in TNTs and extracellular vesicles. While our knowledge about gap junctions and the role of Cx43 is relatively solid, our understanding of its role in TNTs and extracellular vesicles is rather preliminary. It will be important to establish the signals and mechanisms that divert Cx43 from traditional gap junctions to these forms of communication. It is plausible that the short half-life of Cx43 (approximately one to three hours) makes it suitable to participate in dynamic and highly regulated processes, which require a rapid readjustment of the total levels of the protein. For example, in circumstances where a rapid response is needed to re-establish Cx43 levels or enhance intercellular communication, the accumulation of Cx43 can be quickly attained by inhibiting the degradation. Furthermore, as it is a short-lived protein, Cx43 is synthetized at high rates, which allows intercellular communication to be restored in a short period, after a transient insult or stimuli.

In addition, it will be important to establish whether the selectivity and gating of Cx43 channels is determined by their localization in gap junctions, TNTs or extracellular vesicles. It is conceivable that depending on the molecular environment and context, the molecules that are able to cross the channels could vary. Similarly, there is a clear link between deregulation of GJIC and disease development, but the pathophysiological implications of disturbed Cx43-dependent channel activity in TNTs or extracellular vesicles remain to be clarified. Because it is clear that several different strategies are used, a better understanding of how Cx43 is regulated in these different scenarios is fundamental for the design of innovative strategies to target Cx43 in any disease-associated contexts.

In addition to their role in communication, mounting evidence suggests that Cx43 also has channel-independent functions, including in the modulation of cell adhesion, differentiation, proliferation and gene transcription, which is thought to be largely determined by its interacting partners. Accordingly, the concept of non-canonical roles for Cx43 is emerging, and further studies are required to fully unveil the biological importance of these processes, as well as their involvement in human diseases.

Competing interests

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