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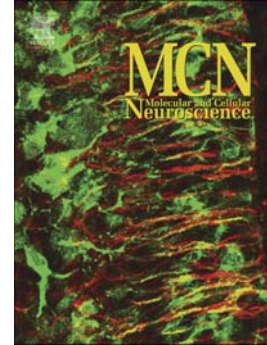
Cell-cell communication mediated by the CAR subgroup of immunoglobulin cell adhesion molecules in health and disease

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Abstract

The immunoglobulin superfamily represents a diverse set of cell-cell contact proteins and includes well-studied members such as NCAM1, DSCAM, L1 or the contactins which are strongly expressed in the nervous system. In this review we put our focus on the biological function of a less understood subgroup of Ig-like proteins composed of CAR (coxsackievirus and adenovirus receptor), CLMP (CAR-like membrane protein) and BT-IgSF (brain and testis specific immunoglobulin superfamily). The CAR-related proteins are type I transmembrane proteins containing an N-terminal variable (V-type) and a membrane proximal constant (C2-type) Ig domain in their extracellular region which are implicated in homotypic adhesion. They are highly expressed during embryonic development in a variety of tissues including the nervous system whereby in adult stages the protein level of CAR and CLMP decreases, only BT-IgSF expression increases within age. CAR-related proteins are concentrated at specialized cell-cell communication sites such as gap or tight junctions and are present at the plasma membrane in larger protein complexes. Considerable progress has been made on the molecular structure and interactions of CAR while research on CLMP and BT-IgSF is at an early stage. Studies on mouse mutants revealed biological functions of CAR in the heart and for CLMP in the gastrointestinal and urogenital systems. Furthermore, CAR and BT-IgSF appear to regulate synaptic function in the hippocampus.

Structural features of CAR-related cell adhesion molecules

The CAR-related proteins form a subgroup within the larger subgroup of CTX (the cortical thymocyte marker in *Xenopus*) of Ig-like cell adhesion molecules (Chretien et al., 1998; Du Pasquier and Chrétien, 1996). Besides CAR, CLMP, BT-IgSF and ESAM (endothelial cell-selective adhesion molecule) are assigned to this CAR subgroup (Schreiber et al., 2014). These are type I transmembrane glycoproteins sharing an identical overall domain organization including two extracellular Ig domains, a transmembrane segment and a cytoplasmic tail. The N-terminal located domain belongs to the V-subtype of Ig domains (D1) which is connected to the membrane proximal Ig domain of the C2 subtype (D2) by a short junction which might create flexibility in the extracellular part of the protein. Some diversity between these members is seen in the cytoplasmic domain, but all three contain PDZ binding motifs at their C-termini (Raschperger et al., 2004). Up to date, only for CAR a three-dimensional structure of the extracellular domains has been solved by crystallography (Patzke et al., 2010; van Raaij et al., 2000; Verdino et al., 2010). Due to the high similarities to CAR at the amino acid level a potential protein structure can be predicted for the extracellular part of CLMP and BT-IgSF as shown in Figure 1. Beside these structural similarities all three proteins mediate homotypic cell aggregation and for CAR and CLMP a homophilic binding mode has been demonstrated (Harada et al., 2005; Honda et al., 2000; Patzke et al., 2010; Raschperger et al., 2004).

The coxsackie- and adenovirus receptor

Initially CAR was identified as a receptor for subtypes of adenoviruses and group B coxsackieviruses by Bergelson et al. (1997) and Tomko et al. (1997) whereby CAR acts as attachment site for adenoviruses and for binding and entry in the case of coxsackieviruses (Bergelson et al., 1997; Bewley, 1999; He et al., 2001; Martino et al., 2000; Roelvink et al., 1999; Salinas et al., 2014; Tomko

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et al., 1997). The murine and human *Car* genes are composed of 8 exons and are located on the chromosome 16 and chromosome 21 (21q21.1), respectively, from which different isoforms are generated by alternative splicing (Bergelson et al., 1997; Bowles et al., 1999; Chen et al., 2003; Excoffon et al., 2010). Three transmembrane forms differing in the cytoplasmic segment of murine CAR are known of which the two dominating forms contain class I PDZ binding motifs (Chen et al., 2003; Excoffon et al., 2010). In humans two transmembrane forms have been described and in addition three soluble forms have been predicted at the transcript level which, however, have not been confirmed at the protein level (for further details and literature on the different isoforms of CAR see the recent reviews by Excoffon et al. 2014 and Loustalot et al. 2015). CAR orthologues were also found in rats, pigs, dogs, chick and zebrafish and revealed a highly conserved amino acid sequence in particular in the cytoplasmic domain (Coyne and Bergelson, 2005; Fechner et al., 1999; Patzke et al., 2010; Petrella et al., 2002; Thoelen et al., 2001).

In crystals the full extracellular region of CAR forms U-shaped homodimers through the binding of their N-terminally located Ig domains which is reminiscent to JAM-A homodimers (Kostrewa et al., 2001; Patzke et al., 2010; Prota et al., 2003; Verdino et al., 2010). D1 and D2 associate in a head-to-tail manner and form a rod-like, dumbbell-shaped structure whose protrusions are formed by the two globular Ig domains (Figure 1). A linker segment of five residues tethers the extracellular Ig domains of CAR to the plasma membrane. The resolution of this extracellular CAR protein structure allowed to define an interface of amino acid residues with a size of 684 $Å^2$ per monomer which is implicated in homodimer formation. Interestingly, CAR-CAR homodimer or CAR-JAM-L heterodimer formation and binding of the adenovirus fiber knob to CAR in crystals is mediated by overlapping amino acid residues within the D1 domain. The fiber knob is the homotrimeric protein of the adenovirus capsid which binds CAR on the host cell surface for infection (Howitt et al., 2003; Law and Davidson, 2005; Patzke et al., 2010; van Raaij et al., 2000; Verdino et al., 2010). For coxsackievirus B3 attachment also the D1 domain is required, however compared to adenovirus binding different amino acid residues are involved (Figure 2) (He et al., 2001). The membrane-proximal C2-like Ig domain (D2) might not be necessary for correct adenovirus binding or homodimer formation (Carson, 2001; van Raaij et al., 2000), however, in polarized airway epithelial

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cells the complete extracellular domain was required for efficient adenovirus binding and infection (Excoffon et al., 2005). Furthermore, in biochemical binding and adhesion studies Patzke et al. (2010) showed an interaction between D1 and D2 domains suggesting that both Ig like domains are implicated in homophilic interactions which can also be deduced from molecular docking simulations (Figure 2). The combined adhesion, binding and structural data on CAR suggest that additional arrangements of CAR molecules other than that observed in the crystal might be proposed. In a hypothetical model trans-homophilic interactions might be initiated by CAR monomers from opposing cells via the observed D1-D1 interface. CAR mediated adhesion could then be further strengthened by a change in the conformation which relocates the Ig domains in a manner in which they bind reciprocally by forming D1-D2 interfaces in a linear arrangement (Patzke et al., 2010). The binding affinity of the fiber knob of the adenovirus to CAR is 100 to 1000-fold higher compared to homodimer formation probably to ensure correct attachment of the virus for proper cell entry (Kirby et al., 2000; Patzke et al., 2010; van Raaij et al., 2000). The trimeric fiber knob binds up to three D1 domains of CAR molecules which most likely are attached to the same plasma membrane (Bewley, 1999; Freimuth et al., 2008; Roelvink et al., 1999). Consistently, the binding of the fiber knob to CAR on the cell surface disrupts the formation of cell-cell contacts which might also allow the virus to cross tissue barriers (Patzke et al., 2010; Walters et al., 2002).

Extra- and intracellular molecular interactions of CAR

In addition to homophilic interactions, heterophilic binding of CAR was observed with different extracellular matrix proteins like fibronectin, agrin or tenascin-R in biochemical assays which appear to be mediated by D2 of CAR. However the biological significance of these interactions are currently unknown. Other heterophilic interactions were defined with the junctional adhesion molecules JAM-L and JAM–C (Figure 2) (Mirza et al., 2006; Patzke et al., 2010; Verdino et al., 2010; Witherden et al., 2010; Zen et al., 2005). The interaction between CAR on keratinocytes and JAM-L on $\gamma\delta$ T cell in the skin regulates signals for neutrophil transepithelial migration across tight junctions. It induces co-stimulation, cytokine production and activation of the MAP kinase pathway via

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recruitment of the phosphoinositide-3-kinase (PI3K) to a JAM-L intracellular sequence motif (Luissint et al., 2008; Verdino et al., 2010; Witherden et al., 2010; Zen et al., 2005). Direct binding of CAR to integrin adhesion receptors has so far not been demonstrated, however several reports have described a close association of CAR with specific integrins on cardiomyocytes and CAR mediated activation of MAPK resulted in an increased activation of β1 and β3 integrins (Morton et al., 2013; Noutsias et al., 2001). Furthermore, CAR co-operates with integrins to enable efficient virus entry into the cell suggesting a crosstalk between both receptor types (Bai et al., 1994; Davison et al., 1997; Li et al., 2001; Uchio et al., 2007; Wickham et al., 1993).

The cytoplasmic tails of CAR isoforms include two distinct class I PDZ (PSD-95/Disc-large/ZO-1) binding motifs. Deletion of the PDZ binding segment of CAR resulted in altered cell adhesion and cell growth in airway epithelia cells suggesting CAR interactions with different intracellular signaling molecules (Excoffon et al., 2004). Consistently, several partners were identified that bind to the PDZ motifs of human or murine CAR including the tight junction protein ZO-1 (Zona occludens-1), MUPP-1 (Multi-PDZ domain protein-1), MAGI-1b (Membrane associated guanylate kinase, WW and PDZ domain containing 1b), PICK-1 (Protein interacting with C kinase 1), the synaptic scaffolding protein PSD-95 (postsynaptic density protein 95) and LNX (Ligand-of-Numb protein-X) and LNX2 (Cohen et al., 2001; Coyne et al., 2004; Kolawole et al., 2012; Mirza et al., 2005; Raschperger et al., 2006; Sollerbrant et al., 2003). Overall, these interactions with intracellular proteins indicate that CAR is present at the plasma membrane in larger protein complexes. Colocalisation of CAR with ZO-1 suggested that CAR itself is involved in the formation of tight junctions and therefore promotes cell-cell contacts probably together with the multi-PDZ domain protein MUPP1 (Cohen et al., 2001; Coyne et al., 2004). Indeed, the integrity of cell-cell contact sites was reduced in CAR knockout mouse models, after inhibition of CAR by a soluble form of CAR or after fiber knob binding (Cohen et al., 2001; Lim et al., 2008; Patzke et al., 2010; Walters et al., 2002). In growth cones CAR seems to associate with actin filaments to modulate the cytoskeletal organization during growth and migration (Huang et al., 2007). Furthermore, CAR is able to affect the trafficking of E-cadherin and ASIC3, a H^+-gated cation channel implicated in mechanosensation (Excoffon et al., 2012). E-cadherin trafficking is dependent on phosphorylation of CAR and may

serve to stabilize cell-cell adhesion in human epithelial cells (Hussain et al., 2011; Morton et al., 2013). The interaction of CAR with scaffolding proteins such as PSD-95 might be one option of CAR to modulate intracellular signaling cascades which might be one of the subjects of future investigations.

The expression of CAR and its involvement during neuronal development

Extensive studies on the timing and pattern of CAR expression have been performed which indicated that CAR is tightly regulated in many organs like brain, heart, lung, pancreas or kidney. In general, at embryonic and early postnatal stages CAR is expressed abundantly, whereas expression in the mature mouse is more restricted in several organs such as heart, pancreas or testis. CAR is also found at specialized cell-cell contact sites, e. g. at tight junctions of epithelial cells (Cohen et al., 2001) or at the neuromuscular junction (Shaw et al., 2004). In mouse CAR expression starts at embryonic day (E) 6.5 in the ectoderm, the primary decidua and the uterine epithelium (Hotta et al., 2003). From E8.5 on CAR is localized in the neural tube and from E10.5 on throughout the brain including cerebral cortex, cerebellum, brainstem, retina and olfactory bulb (Honda et al., 2000; Patzke et al., 2010; Persson et al., 2006; Venkatraman et al., 2006). In primary cultivated neurons CAR is found in the growth cone, neurites and cell bodies where it is localized at cell-cell contacts (Honda et al., 2000; Hotta et al., 2003; Patzke et al., 2010). From developmental stage P10 on the expression of CAR is strongly downregulated (Honda et al., 2000; Patzke et al., 2010). In adult stages it is detectable in the posterior corpus callosum, between layers IV and V and layer I of the cerebral cortex. In the hippocampus CAR is found on axons of the entorhinal cortex and mossy fibers. Biochemical fractionation and immunohistochemical studies indicated a localization of CAR in the presynapse of excitatory and inhibitory neurons (Zussy et al., 2016).

The strong expression of CAR at developmental stages suggests that CAR mainly regulates processes during neuronal development. However, no gross morphological anomalies in the brain were observed in conditional CAR knockouts. Only minor differences in the organization of the dentate gyrus were detected between wild-type and CAR mutants (Zussy et al., 2016) which, however,

might be the cause for a loss of hippocampal synaptic plasticity observed only in CAR-deficient females but not in males. This surprising finding is further supported by the downregulation of a number of pre- and postsynaptic proteins in conditional knockout females (Zussy et al., 2016).

In behavioral tests analyzing locomotor or sensory function in conditional CAR mutants, in which CAR expression is abolished from postnatal stages on, CAR knockout mice only significant changes were observed during the transfer arousal test. CAR-deficient mice showed a significant lower tendency to freeze (46% of the tested animals in contrast to 80% of wild type animals) (Pazirandeh et al., 2011). In contrast when CAR is inactivated in the brain from embryonic stages on by Nestin-Cre impaired behavior in the elevated pulse maze, the Y maze and Morris water maze was detected reflecting altered spatial working memory and anxiety. It might be hypothesized that the impaired neurogenesis that was observed in the adult CAR-deficient hippocampus contributes to these behavioral deficits (Zussy et al., 2016).

There are still many open questions about the physiological function of CAR in the adult brain and even more in the embryonic brain. Interestingly, the CAR level appears to be reduced in hippocampus of inflammatory brains as shown for patients at the early phase of late-onset Alzheimer disease suggesting for CAR a potential involvement in neuronal diseases (Zussy et al., 2016).

CAR is essential for heart development and electrical conduction in the mature heart

Constitutive CAR KO mice revealed embryonic lethality around embryonic day 11.5 to 13.5 due to malformations of the heart and hemorrhage (Asher et al., 2005; Chen et al., 2006; Dorner et al., 2005). CAR-deficient hearts revealed enlarged endocardial cushions and only one atrioventricular canal instead of two. In addition, an engorgement of cardinal veins and thickening of the ventricular and atrial wall was reported (Asher et al., 2005; Dorner et al., 2005). This thickening of cardiac tissue was related to increased proliferation in CAR KO embryos as an increased expression of the proliferation marker Ki67 was detected by Chen et al. (2006). However this result was not supported by proliferation assays using BrdU incorporation (Dorner et al. 2005). These distinct observations on cell proliferation in the CAR knockout heart might be explained by the different stages that were

studied (E10.5 versus E11.5). CAR deficient cardiomyocytes also showed highly disorganized myofibrils, thinner myofibers and impaired intercellular junctions between neighbouring cardiomyocytes (Chen et al., 2006; Dorner et al., 2005).

In the adult heart, CAR expression is strongly downregulated in comparison to embryonic hearts and CAR is mainly found at the intercalated discs where it colocalizes to connexins and ZO-1 (Lim et al., 2008; Matthäus et al., 2014; Shaw et al., 2004). Cardiac specific deletion of CAR after birth resulted in mice that are viable and which did not reveal overall morphological abnormalities in the heart. However, different stages of atrioventricular (AV) heart blocks up to a complete AV block were measured (Lim et al., 2008; Lisewski et al., 2008; Pazirandeh et al., 2011). CAR deficient hearts showed disrupted intercellular contacts in the myocardium in which an altered localization of connexins, β-catenin and ZO-1 preceded cardiac dysfunction. These mislocalizations probably caused the observed impairment of electrical conduction through gap junctions in the mature CAR KO hearts. In contrast, overexpression of CAR in the heart causes disorganization and degeneration of cardiomyocytes, disrupted adherens junctions, nuclear translocation of β-catenin and cardiomyopathy (Caruso et al., 2010).

As a receptor for group B coxsackieviruses CAR is implicated in virus-induced myocarditis (Freiberg et al., 2014). CAR itself initiates a strong immune response via the inflammatory mitogen-ativated protein kinase (MAPK) after virus binding and cell entry and even further, CAR expression is upregulated during myocarditis (Ito et al., 2000; Yuen et al., 2011). Surprisingly CAR also gets re-expressed in adult hearts of patients suffering from dilated cardiomyopathy or after infarction (Fechner, 2003; Noutsias et al., 2001). Up to date the cellular mechanism why and how CAR gets re-expressed during cardiac disease remains to be determined.

In conclusion, in the embryonic and mature heart CAR is required for the generation of intact cell-cell contact sites including the localization of connexins within the intercalated discs. Absence of CAR at these sites results in a defective communication through gap- and/or tight junctions. Increased CAR expressions in heart failure suggest a remodeling function at cardiac cell-cell contact sites.

CAR in pancreas, kidney, testis and the lymphatic system

CAR is also detectable in several other organs such as pancreas, testis, kidney and the intestine (Coyne and Bergelson, 2005; Mena et al., 2000; Pazirandeh et al., 2011; Tomko et al., 2000; Zanone et al., 2007). Zanone et al. (2007) showed CAR expression in human pancreatic islet endothelial cells whereby coxsackievirus B infection even further increased CAR expression and induced pancreatitis. Pazirandeh et al. (2011) demonstrated in conditional CAR KO mouse enlarged intestines and atrophy of exocrine pancreas.

Although, CAR is also found in germ cells and Sertoli cells of the testes conditional testicular depletion of CAR revealed no severe impairments in the fertility and the blood-testis-barrier (Mirza et al., 2006; Sultana et al., 2014; Tomko et al., 2000; Wang et al., 2007). No influences of CAR absence were observed during the development of podocytes (Schell et al., 2015). These epithelial cells are located in the Bowman's capsule of the kidney and CAR expression is found already at E15 during murine embryogenesis until adulthood. However, specific deletion of CAR in podocytes did not reveal any impairment in structure and function of the renal filter barrier (Schell et al., 2015).

During embryonic development of lymphatic vessels CAR is strongly expressed in endothelial cells of the early lymphatic system followed by a decrease after E16.5 (Mirza et al., 2012; Vigl et al., 2009). Consistently, CAR deficient embryos revealed impaired lymphatic endothelial cell adhesion which impaired the separation of blood and lymphatic vessels. This might contribute to the above mentioned hemorrhage and embryonic death (Mirza et al., 2012; Vigl et al., 2009). These results showed that CAR is also essential for correct development of the lymphatic system during embryogenesis.

A potential role of CAR in cancer growth

Due to its high binding affinity to adenoviruses CAR became in focus for gene therapeutic studies. Therefore, CAR expression was compared in a multitude of cancer cells: in parotid glands, colon cancers, prostate cancers or bladder cancer cells CAR expression is downregulated (Loustalot et al., 2015; Okegawa et al., 2001; Reeh et al., 2013) while human cancer cell lines like the cervical cell

lines HeLa and SiHa, the glioblastoma cancer line U87MG or the ovarian cancer cell line OV-UL-2 showed increased CAR expression (Brüning and Runnebaum, 2003). In comparison to healthy patients, a significant increased CAR level was found in specimens from patients suffering from basalioma, lung, ovarian, urinary bladder cancer and in neuroblastoma (Reeh et al., 2013). Downregulation of CAR in cancer cells is related to activation of Raf/MEK/ERK pathway and TGF-beta signaling as well as loss of alpha-catenin and increased proliferation and migration (Brüning and Runnebaum, 2003; Loustalot et al., 2015). However, up to date it is still unknown whether CAR itself is involved in the progression of cancer development.

In conclusion, a high diversity of intracellular and extracellular binding partners has been defined for CAR. Its involvement in cardiac development and conduction has been clearly demonstrated while its function in the developing nervous system – the organ of strongest CAR expression - is less understood. Furthermore, an overall cellular mechanism for CAR that can be adapted to a wide range of organs and tissues is still lacking. As a cell adhesion molecule CAR in conjunction with intracellular PDZ-domain containing proteins might contribute to the correct formation and regulation of specialized cell-cell contact sites such as gap or tight junctions.

The CAR-like membrane protein (CLMP)

Bioinformatics screening of expressed sequence tag (EST) and genomic databases led to the identification of cDNAs of human and murine origin that showed a high sequence similarity of about 49% at the amino acid level to the IgCAM CAR and a related overall domain organization (Figure 1). Therefore, it was termed CAR-like membrane protein (Raschperger et al., 2004). CLMP was also identified in a screen for upregulated genes in visceral adipose tissue of Otsuka Long-Evans Tokushima fatty (OLETF) rats, a model of type 2 diabetes and termed adipocyte adhesion molecule (ACAM) (Eguchi et al., 2005; Hida et al., 2000). The human and mouse *CLMP* gene consists of seven exons on chromosome band 11q24.1 and 9A5.1, respectively (Raschperger et al., 2004). The promoter site of the murine *Clmp* gene and responsive elements that are required for basal *Clmp* expression in

Sertoli TM4 cells contain binding motifs for the transcription factors GATA-1, GATA-6, Kruppel-like factor 4 (KLF 4), and sex-determining region Y (Sze et al., 2008). In addition, *Clmp* transcript in TM4 cells has been found to be post-transcriptionally negatively regulated by TNF-alpha via the RNA-binding protein tristetraprolin (TTP) and the c-Jun N-terminal kinase (JNK) signaling pathway (Sze et al., 2008).

Expression of CLMP at transcript and protein levels

A wide tissue and organ distribution in humans and mice has been described for CLMP at the level of mRNA transcripts using Northern blotting, quantitative PCR and *in situ* hybridization (Eguchi et al., 2005; Raschperger et al., 2004). Although some discrepancies exist between both studies, strong expression was described in heart, brain, intestine, white adipose tissue and placenta and lower levels in kidney, lung, skeletal muscle and spleen. *In situ* hybridization data (www.genepaint.org) also demonstrated a wide-spread expression of *Clmp* mRNA. In the intestine localization in the outer layers of the gastrointestinal tract at E14.5 has been observed pointing to a localization in smooth muscle cells, neurons and/or interstitial cells of Cajal. In immunohistochemical stainings on human embryonic tissue CLMP was observed in the intestine, in rapidly dividing cells of the nervous system, in the mesenchyme of the frontonasal and mandibular processes and in the dermamyotome. Endodermal derivatives of the foregut, midgut and hindgut, lung, liver, esophagus and trachea were also found to express CLMP (Van der Werf et al., 2012). In addition to tissue sections, endogenous CLMP expression has been detected in several epithelial cell lines (Raschperger et al., 2004; Sze et al., 2008; Van der Werf et al., 2012) as well as in human glioblastoma T98G cells. In transfected cells CLMP co-localizes with the tight junction markers ZO-1 and occludin suggesting that CLMP might be a tight junction molecule. Consistent with a cell adhesive function and a tight junction localization, transfection of MDCK epithelial monolayers with cDNA encoding full-length CLMP resulted in a significant increase of transepithelial electrical resistance (Raschperger et al., 2004). The transfection of CHO cells with a mutated variant of human CLMP, which is unable to integrate into the plasma

membrane but instead localizes in the cytoplasm, leads to mislocalization of ZO-1 with an increased aggregation of ZO-1 in the cytoplasm (Figure 2) (Van der Werf et al., 2012).

Taken together, expression of CLMP protein and its transcript has been investigated in several studies. While transcript expression suggests a more restricted expression of *Clmp* concentrated in brain, heart and adipose tissue; immunological studies indicate a rather ubiquitous expression of CLMP protein with a focus on epithelial cell types (Eguchi et al., 2005; Raschperger et al., 2004; Sze et al., 2008; Van der Werf et al., 2012). However, additional research is required to determine CLMP expression more precisely during murine development and its subcellular localization in order to shed light on the biological function of CLMP.

CLMP mediates homotypic cell adhesion

In order to elucidate the function of CLMP, mainly *in vitro* experiments have been carried out and only recently the role of CLMP was addressed in animal models. Since CLMP is the closest homologue of CAR, its ability to mediate cell adhesion was investigated *in vitro*. Transfection of Chinese hamster ovary (CHO) cells with full-length human CLMP or its rat orthologue induced cell aggregation which was comparable to that of CAR. However, aggregation intensity was not as strong as observed by transfections with the adhesion molecule cadherin1 (also known as E-cadherin) (Eguchi et al., 2005; Raschperger et al., 2004; Van der Werf et al., 2013).

Clmp transcript expression has been reported to be stimulated during the peri-ovulatory period in rats. Peri-ovulatory processes in rat ovaries and ovarian cell cultures were induced by treatment with human chorionic gonadotrophin and shortly afterwards a rise in *Clmp* transcript and protein abundance was detected, which was controlled by signaling pathways dependent on protein kinase A (PKA), phosphoinositide-3-kinase (PI3K), p38 kinase and epidermal growth factor (EGF) receptor (Li et al., 2014). Since cell adhesion is crucial for ovulation during the transition of the pre-ovulatory follicle to the corpus luteum and CLMP expression is increased in this period, it might be hypothesized that CLMP might have a function in these processes.

CLMP is involved in adipocyte function and obesity

When obesity was stimulated in OLETF rats by administration of the hormone pioglitazone, the white adipose tissue of these animals upregulated *Clmp* transcript and CLMP protein as compared to non-treated OLETF rats, suggesting that CLMP might function in the conversion of premature to mature adipocytes (Eguchi et al., 2005). In a recent study Murakami and colleagues demonstrated that adipocyte-specific expression of CLMP in transgenic mice were protected from obesity under a high-fat high-sucrose (HFHS) chow (Murakami et al., 2016). These mice exhibited a significant reduction of body and fat pad weight as well as a significantly improved glucose tolerance and insulin sensitivity. In addition, adipocyte-specific expression of *Clmp* in transgenic CLMP mice under HFHS chow mediated a homotypic adhesion of adipocytes, formed zonula adherens structures and promoted actin polymerization (Murakami et al., 2016). Taken together, these findings indicate a role of CLMP in adipocyte function and obesity.

Mutations in the human CLMP gene cause congenital short-bowel syndrome

The *in vivo* function of CLMP is currently not well defined. In human patients homozygous and compound heterozygous loss-of-function mutations have been detected in a small number of patients with congenital short bowel syndrome (CSBS, OMIM 615237) by a genome-wide scan and homozygosity mapping. The defects in the *CLMP* gene included frameshift, missense, and splice donor site mutations in coding regions and an intronic deletion, which were not present in chromosomes of control individuals. *In silico* and *in vitro* experiments showed that these mutations most likely result in a loss of function of CLMP (Gonnaud et al., 2016; Van der Werf et al., 2012) . CSBS is a rare hereditary gastrointestinal disorder for which no cure is available. Patients with CSBS revealed a very short small intestine with a length of approximately 50 cm at birth while normal humans display a length of 190-280 cm and which is accompanied by intestinal malrotation (Alves et al., 2016; Hamilton et al., 1969; Van der Werf et al., 2015, 2012) .

To determine whether loss-of-function mutations are associated with a short bowel, morpholino knockdown zebrafish models were generated. In zebrafish, two *clmp* transcripts have been identified and one transcript variant was expressed in the zebrafish intestine. Knockdown of this enteric *clmp* variant by splice-blocking or translation-blocking resulted in morpholinos with a significant developmental delay. When compared to wildtype controls, the morphants exhibited a significantly shorter body length associated with a significant reduction of intestinal length, which, however, was proportional to the overall decreased body length. Interestingly, histological analyses revealed the absence of enteric goblet cells in morphants (Van der Werf et al., 2012). Goblet cells are glandular epithelial cells and a typical attribute of the zebrafish midintestine, which is comparable to the human small intestine (Ng et al., 2005). Thus, the authors supposed that the loss of goblet cells as markers for midintestinal epithelial tissue might indicate a lack of small intestine in zebrafish *clmp* morphants (Van der Werf et al., 2012). Based on the reported co-localization of CLMP with epithelial tight junction markers like ZO-1, which is involved in cell proliferation (Matter and Balda, 2007), it was hypothesized that loss-of-function *CLMP* mutations in CSBS patients might be important for proliferative processes of enteric epithelia during intestinal human development (Van der Werf et al., 2012). Further *in vitro* analyses using T84 colonic adenocarcinoma cells transfected with cDNA encoding a missense mutant of CLMP, which had been reported in one of the CSBS patients, could not show any implication of CLMP in key processes of intestinal epithelial development including migration, proliferation, viability and transepithelial electrical resistance (Van der Werf et al., 2013). Thus, the precise function of CLMP in CSBS pathogenesis remains to be elucidated.

In order to further clarify the biological function of CLMP in the living organism, a constitutive *Clmp* knockout mouse model was generated in our laboratory and phenotypic analyses of homozygous CLMP-deficient mice revealed multiple recessive abnormalities in growth, survival, the gastrointestinal tract and the urogenital system (Langhorst et al., in preparation). *Clmp* mutant animals displayed a high rate of mortality during neonatal and early postnatal stages which was accompanied by a delay in body growth. Although the underlying mechanisms leading to compromised survival and growth needs further investigations, it is likely that gastrointestinal malfunctions are involved. For instance, in embryos or in newborn animals an impaired transport of meconium or chyme,

respectively, along the intestine was observed. Consistently, an impaired peristalsis of the intestine was demonstrated in an organ bath. Analogous to the reduction of small intestine length in CSBS patients, the bowel length of surviving adult *Clmp* mutant mice was analyzed, but in contrast to CSBS patients, *Clmp* knockout mice did not display a shortening of the small bowel. Instead, gut malrotation and duodenal dilation were observed. The distinction in gut length might reflect different developmental processes in the length growth of the intestine in humans and mice.

Furthermore, CLMP-deficient mice developed a high degree of bilateral hydronephrosis with onset at a perinatal stage which has not been described so far in human CSBS patients. Hydronephrotic kidneys, characterized by accumulation of urine in renal pelvis and calyces and accompanied by renal parenchymal atrophy, were not caused by physical obstruction of the urinary tract. Instead, ureteral peristalsis, which is essential for propagation of urine from kidneys to urinary bladder, is impaired by the loss of CLMP. In addition, calcium imaging experiments on ureteral explants revealed an absence of organized Ca^{2+} transients in *Clmp* mutants (Langhorst et al., in preparation). It might be of great interest to investigate whether CLMP is also implicated in human congenital anomalies of the urinary tract.

In conclusion, since its discovery the knowledge about CLMP is gradually advancing and demonstrates multiple functions for CLMP in the organism. CLMP has been associated with obesity (Eguchi et al., 2005; Murakami et al., 2016; Van der Werf et al., 2012), the human intestinal disorder CSBS (Van der Werf et al., 2015, 2012) as well as with the peristalsis of the gastrointestinal and urogenital system (Langhorst et al., in preparation). The function of CLMP in the central nervous system where it is highly expressed remains to be determined.

The brain and testes specific immunoglobulin superfamily protein (BT-IgSF)

Human and mouse BT-IgSF were cloned in 2002 (Suzu et al., 2002). Due to its strong expression in brain and testis, it was termed brain and testis specific immunoglobulin superfamily

protein. It was also characterized independently as a gene frequently up-regulated in intestinal-type gastric cancers and designated IgSF11 (Katoh and Katoh, 2003). Alignment of the murine amino acid sequence of BT-IgSF with its closest homologs shows a 29% identity with CAR and ESAM and a 30% identity with CLMP. The murine and human *BT-IgSF* gene are located on chromosome band 16B4 or 3q13.3, respectively, a region that is associated with corpus callosum agenesis in humans (Genuardi et al., 1994; Mackie Ogilvie et al., 1998; Suzu et al., 2002). While the mouse gene consists of 7 exons the human gene is composed of 10 exons which are alternatively spliced into two isoforms: *BT-IgSF/IGSF11-1* and *BT-IgSF/IGSF11-2*. *IGSF11-1* contains exons 1a, 2a, 3a and 4-9 whereas *IGSF11-2* contains exons 1b and exons 4-9. The exon splice variants 1a and 1b lack an N-terminal signal peptide suggesting that *IGSF11-2* is the representative isoform of IGSF11 in humans (Katoh and Katoh, 2003). Human and murine BT-IgSF are highly glycosylated and among all CAR subgroup members, BT-IgSF has the longest cytoplasmic segment of 167 (in mouse) or 169 (in human) amino acid residues (Katoh and Katoh, 2003; Suzu et al., 2002).

BT-IgSF is implicated AMPAR-mediated synaptic transmission and migration of melanophores

As CAR and CLMP, BT-IgSF mediates in transfected cells homophilic cell adhesion in a Ca^{2+}/Mg^2-independent manner which is independent of integrins (Figure 2) (Eom et al., 2012; Harada et al., 2005). In zebrafish BT-IgSF has been described in the spinal cord and adult pigment cells and their precursors at postembryonic stages (Eom et al., 2012). In the mouse brain the corpus callosum, the amygdala and the hippocampus reveal high expression in contrast to weak expression in the colon, adrenal gland and the fetal brain (Suzu et al., 2002). In the hippocampus BT-IgSF appears to be developmentally regulated reaching a peak during adulthood with a strong localization in the dentate gyrus rather than in the CA1 region (Jang et al., 2015). BT-IgSF was detectable in synaptic and postsynaptic fractions of rat brains in particular only at excitatory synapses. BT-IgSF can bind and interact with PSD-95 and AMPARs in a tripartite manner. These molecular interactions and the pattern of expression suggested a pivotal function in the hippocampus. Knockdown of BT-IgSF in cultured neurons suppressed AMPAR clustering on the surface and impaired AMPAR- but not

NMDA-mediated synaptic transmission in CA1 pyramidal synapses in slice cultures. These SC-CA1 synapses showed suppressed long-term potentiation in BT-IgSF knockout mice. However, the DG granule cells showed a reduced amplitude of mEPSCs, which could be rescued by the re-expression of BT-IgSF in the BT-IgSF knockout animals (Jang et al., 2015). These results show that BT-IgSF is implicated in AMPAR-mediated synaptic transmission and plasticity.

On non-neuronal cells a critical role for BT-IgSF in promoting the migration and survival of melanophores and their precursors was demonstrated in zebrafish BT-IgSF mutants (Eom et al., 2012). In zebrafish, melanophores migrate to the hypodermis to form a typical horizontal patterning. *In vivo* imaging revealed that the migration of melanophore precursors with a mutation in the BT-IgSF gene (s*eurat* mutant) was impaired resulting in an irregular spotting pattern.

A potential role of BT-IgSF in cancer

Ailan et al. (2009) showed that *BT-IgSF* is a target gene of AP-2γ, a transcription factor involved in the initiation and progression of breast cancer. The binding of AP-2γ to the *BT-IgSF* gene increases the expression of *BT-IgSF* in breast cancer cell lines (Ailan et al., 2009). Also for other cancer types, such as gastrointestinal and hepatocellular carcinomas, it is known that *BT-IgSF* gets up-regulated. It was even shown that BT-IgSF is able to induce cell growth *in vitro* and further, the inhibition of *BT-IgSF* expression via siRNA in cancer cells leads to a decreased number of transfected cells suggesting that the increased expression of *BT-IgSF* in cancer cells may be important for cancer growth (Watanabe et al., 2005). Both studies suggest *BT-IgSF* as a promising target for cancer therapy.

Conclusions

Although research on the CAR subgroup of IgCAMs is still at an early stage studies on mouse models revealed that these proteins are implicated in a variety of biological processes during developmental stages including synaptic transmission, peristalsis in the gastrointestinal and ureteral

system, formation of the embryonic heart and atrioventricular conductance in the mature heart. Of particular interest are links to hereditary human diseases as it has been found for CLMP. A recurrent picture on the function of this set of proteins that evolves from current studies is their participation in the development or regulation of specialized cell-cell communication sites. However, the detailed molecular mechanism and/or signaling functions of this set of proteins remains to be determined.

Although these three proteins discussed in this article are strongly expressed in the developing (CAR and CLMP) or mature nervous system (BT-IgSF) their function is least understood here. Studies on their interaction with a number of complex scaffolding proteins might be of great interest to begin to understand the function of CAR members in particular in the developing and mature nervous system to modulate synaptic plasticity.

Acknowledgements

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Figure legends

Fig. 1: Members of the CAR subgroup of IgCAMs. The crystal structure of the complete extracellular portion of mouse CAR (Patzke et al., 2010; Verdino et al., 2010) (PDB: 3jz7A) and of the single membrane distal domain D1 (van Raaij et al., 2000) has been solved. For CLMP a protein tertiary structure was calculated on the basis of the mouse CAR template using Phyre2 (Kelly et al., 2015). The extracellular protein structure of BT-IgSF was also deduced from the CAR structure analog to Jang et al. (2015).

Fig. 2: Scheme of molecular interactions of CAR subgroup members. Binding regions of CAR that have been mapped are indicated by vertical lines. The homophilic binding regions for CLMP and BT-IgSF are currently not known but have been provisionally deduced from studies on CAR. For the numerous scaffolding proteins that interact with the C-terminus of CAR please see the text. PM, plasma membrane.
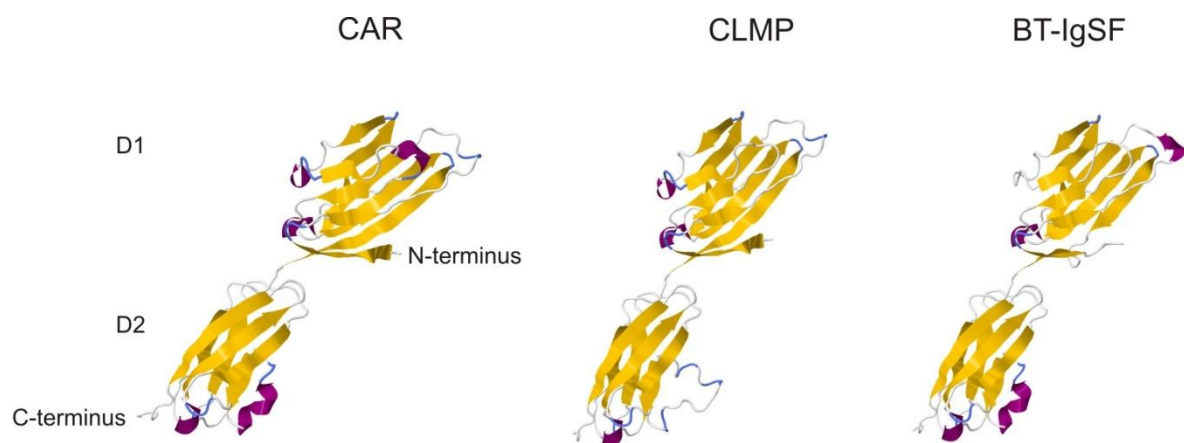
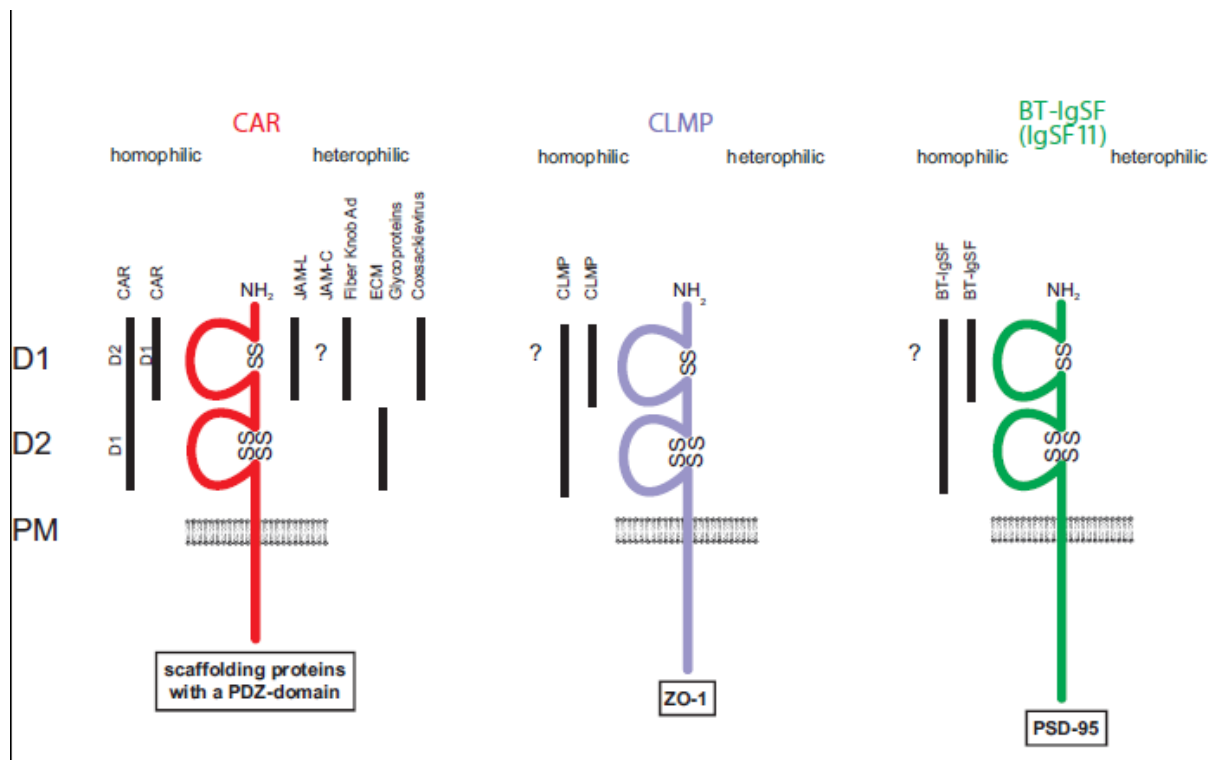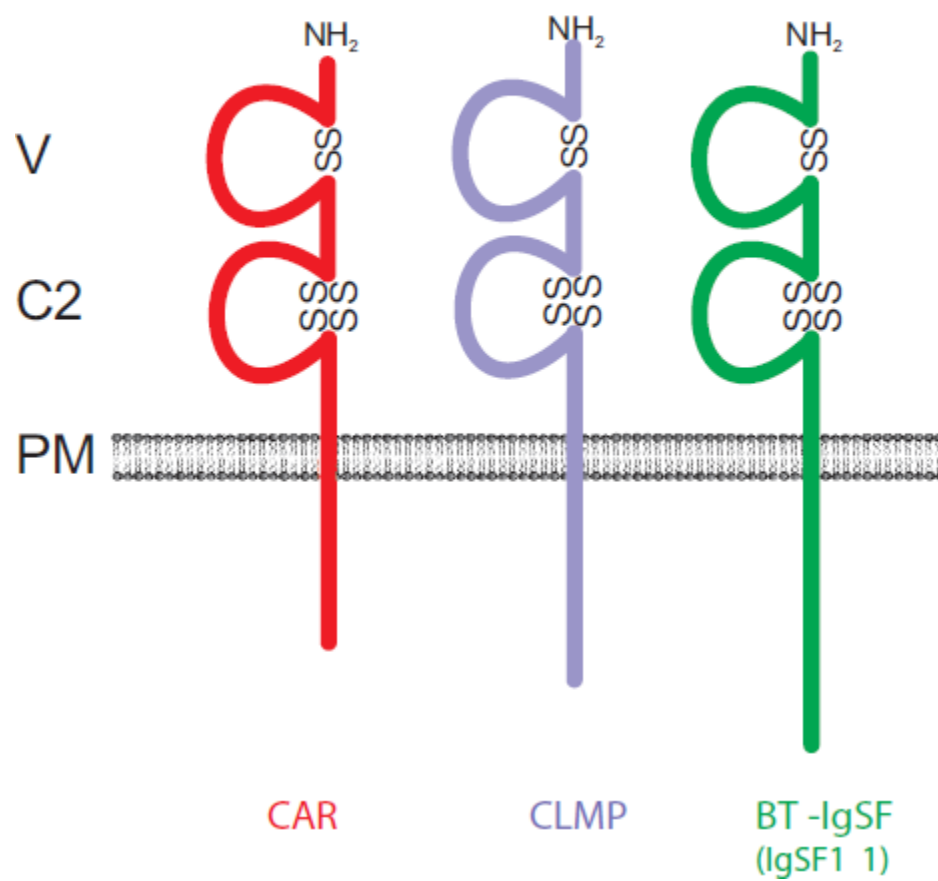CAR   CLMP   BT-IgSF

Figure 1

Figure 2

Graphical abstract

MCN Review Highlight

- Hereditary diseases in the absence of CAR-like cell adhesion molecules