Claudin-4 localization in epithelial ovarian cancer

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ABSTRACT

Claudin-4, a protein with the structure of classic claudins most often found in cell-cell junctions, is frequently overexpressed in epithelial cancers where its localization has not been studied. In this study we aimed to find out where this membrane protein is localized in an ovarian tumor model, OVCAR3 cells, that express high levels of the protein. Immunohistochemical studies showed claudin-4 staining in a perinuclear region, at most plasma membranes and in cytoplasmic puncta. Native claudin-4 did not overlap with phosphorylated claudin-4, which was partially located in focal adhesions. Using claudin-4 BioID technology we confirmed that large amounts of claudin-4 are localized to the Golgi compartment, including in dispersed Golgi in cells where claudin-4 is partially knocked down and in dividing cells. Claudin-4 appears to be present in the vicinity of several types of cell-cell junctions, but there is no evidence that it forms tight junctions in these tumor cells. Both claudin-4, the Golgi marker GM130, and the plasma membrane receptor Notch2 were found in dispersed Golgi in dividing cells. This definition of the cellular architecture of claudin-4 should provide a framework for better understanding of the function of claudin-4 in tumor cells and its molecular interactions.

1. Introduction

High grade serous ovarian cancer (HGSOC) is the most lethal of the tumors of the female reproductive system. In 2022, it is estimated that nearly 20,000 individuals will be newly diagnosed with this cancer and nearly 13,000 will die of the disease (ACS, 2022). Claudin-4 (protein)/CLDN4 (RNA) was identified more than two decades ago as a frequently overexpressed gene in ovarian cancer (Hough et al., 2000). In cultured cells, such as MDCK kidney cells and EPH4 mammary cells, claudin-4 localizes to tight junctions and contributes to transcellular resistance (Baumgartner et al., 2011; Fredriksson et al., 2015). Although claudin-4 is found in a number of normal tissues including bladder, salivary glands (Zhang et al., 2018b) gastrointestinal tract (Schumann et al., 2012), and kidney (Gong and Hou, 2017), it is not entirely clear that it normally functions as a tight junction protein (Piontek et al., 2020). It is also found in damaged lung cells (Schlingmann et al., 2015) and in epithelial cells of the involuting, but not the milk-secreting, mammary gland (Baumgartner et al., 2017). Claudin-4 is often overexpressed in epithelial cancers (Hewitt et al., 2006) including breast (Kolokytha et al., 2014; Szasz et al., 2011), prostate (Landers et al., 2008; Li, 2021), gastric (Soini et al., 2006; Liu and Li, 2020), pancreatic (Li, 2021; Torres et al., 2018), bladder (Kuwada et al., 2015), lung (Li, 2021), colorectal (Fujiwara-Tani et al., 2018; Holzbauer et al., 2013) and ovarian (Hewitt et al., 2006; Hough et al., 2000; Li, 2021). Survival time in ovarian cancer patients is decreased when tumors have high levels of claudin-4 (Yoshida et al., 2011; Martin de la Fuente et al., 2018). Knockdown of claudin-4 has recently been found to sensitize ovarian cancer cells to paclitaxel and PARP inhibitors (Breed et al., 2019; Yamamoto et al., 2022) and targeting of claudin-3 and claudin-4 has been proposed as a chemotherapeutic strategy in ovarian cancer (Uthayanan and El-Bahrawy, 2022). In order to better understand the biology of claudin-4 in epithelial cancers it is necessary to know its localization in tumor cells, particularly since it is not thought to localize to tight junctions.

Claudins are a family of 23 tetraspanin proteins in humans, each with four alpha helices which cross the plasma membrane separated by two extracellular loops and a short intracellular loop (Figure 1A). The extracellular loops are hydrophobic and often interact in trans with loops of similar molecules on adjacent cells to form tight junctions. Claudin-4 has the general structure of classic claudins (Piontek et al., 2020); the N-terminus is cytoplasmic and short, commencing with a methionine-alanine...
sequence. This terminus allows the attachment of biotin ligase for the BioID experiments to be described in this paper. The length of the cytoplasmic carboxy-terminus is variable containing about 27 amino acids depending on species. This segment terminates in the sequence YV as in all classic claudins (Piontek et al., 2020), allowing the membrane molecule to interact with the PDZ domains of submembrane cytoskeletal molecules like ZO1.

Our laboratory has demonstrated that claudin-4 contributes functionally to ovarian tumor cell survival, migration, and overall tumor burden (Hicks et al., 2016). However, the molecular mechanisms involved remain largely unknown. In this paper, we present the results of our studies of claudin-4 localization in an ovarian tumor cell line, OVCAR3, that expresses high levels of claudin-4 compared to most HGSOC cell lines (Breed et al., 2019). We chose OVCAR3 (claudin-4 positive, Log2 TPM Expression \(= 7.25\)) and OVCAR8 (claudin-4 negative, Log2 TPM Expression \(= 0.94\)) cell lines for further study. To determine where claudin-4 is localized in OVCAR3 cells, we performed immunocytochemistry using confocal microscopy. We evaluated claudin-4 localization in clumps of OVCAR3 cells grown on collagen (Figure 1B) using an antibody to the final 22 amino acids of the C-terminus of human claudin-4. A blocking peptide attenuated the signal in OVCAR3 cells verifying the specificity of the antibody used (see Supplementary Material, Figure 1).

Figure 1C shows that the claudin-4 in OVCAR3 cells is clustered in puncta near the nucleus (white arrows). Claudin-4 is also found in isolated cytoplasmic puncta (arrowheads) and in the plasma membrane, possibly associated in part with cell-cell junctions (yellow arrows).

Claudin-4 phosphorylated on tyrosine 208 does not overlap with non-phosphorylated claudin-4. The C-terminus of claudin-4 has known phosphorylation sites that serve to facilitate claudin-4-protein interactions (Figure 2A). We used an antibody to the C-terminal peptide of claudin-4 with phosphorylated tyrosine 208. Dual staining of OVCAR3 cells with the antibody to claudin-4 and the antibody to p(208)-claudin-4 is shown in Figure 2B. Claudin-4 (non-phosphorylated claudin-4, green) and p(208)-claudin-4 (Y208 phosphorylated, red) did not overlap as shown clearly in the images in Figure 2B. Non-phosphorylated

2. Results

Localization of claudin-4 in OVCAR3 cells. Claudin-4 has a short N-sequence, four hydrophobic transmembrane segments, two extracellular loops, and a C-terminus with about 22 amino acids ending at amino acid 209 in humans (Figure 1A). Breed and colleagues (Breed et al., 2019) showed by Western blot that OVCAR3 and several other cell lines (PEO4, OV429, and OVCAR5) express high levels of claudin-4 protein, whereas the protein was not visible in Western blots from OVCAR8, OVCAR4, and DOV13 cells. We chose OVCAR3 (claudin-4 positive, Log2 TPM Expression \(= 7.25\)) and OVCAR8 (claudin-4 negative, Log2 TPM Expression \(= 0.94\)) cell lines for further study. To determine where claudin-4 is localized in OVCAR3 cells, we performed immunocytochemistry using confocal microscopy. We evaluated claudin-4 localization in clumps of OVCAR3 cells grown on collagen (Figure 1B) using an antibody to the C-terminal peptide of claudin-4 with phosphorylated tyrosine 208. Dual staining of OVCAR3 cells with the antibody to claudin-4 and the antibody to p(208)-claudin-4 is shown in Figure 2B. Claudin-4 (non-phosphorylated claudin-4, green) and p(208)-claudin-4 (Y208 phosphorylated, red) did not overlap as shown clearly in the images in Figure 2B. Non-phosphorylated
claudin-4 shows clear perinuclear and cytoplasmic puncta. In contrast, the p(208)-CLDN4 stain is confined to puncta distributed closer to the periphery of the cell as well as some nuclear stain indicating that phosphorylation alters the cellular distribution of claudin-4.

There is less claudin-4 stain in the shCLDN4 knockdown cells as expected (the same cell line as shCLDN4_2 in the Breed paper where claudin-4 was shown to be decreased markedly by Western blot in these cells (Supplementary Figure 4 (Breed et al., 2019)). Interestingly, the stain for p(208)-claudin-4 does not appear to be diminished in shCLDN4 cells examined immunohistochemically. To help determine the localization of the p(208)-claudin-4 puncta, we costained OVCAR3 cells with an antibody to pY397 focal-adhesion kinase (pFAK), finding that it stained some cytoplasmic puncta that overlapped with p-claudin-4 (Figure 2C). Focal adhesions assist in cell mobility suggesting a role for p(208)-claudin-4 in cell migration. If non-phosphorylated claudin-4 plays a role in cell mobility it must be by a different mechanism since p(208)-claudin-4 and native claudin-4 do not overlap (Figure 2B).

**Figure 2.** Phosphorylated claudin-4 does not overlap with non-phosphorylated claudin-4. A. The c-terminus of human claudin-4 has three tyrosine phosphorylation sites at residues 193, 197, and 208 (9 (Van Itallie and Anderson, 2018)). B. Distribution of nuclei (DAPI, blue), non-phosphorylated claudin-4 (green), and claudin phosphorylated at Y-208 (red) in OVCAR3 shCTRL and OVCAR3 shCLDN4 cells. C. Three color view of the distribution of phospho-claudin-4 and focal adhesion kinase (FAK) in OVCAR3 shCTRL cells. Green arrows identify puncta of co-localized p(Y397)-FAK and p-CLDN4. Scale bars: 10 μ.

Claudin-4 accumulates in the Golgi of OVCAR3 cells. The perinuclear puncta observed in Figure 1C suggest claudin-4 accumulation in the Golgi complex. We examined claudin-4 co-localization with a known Golgi-specific protein, GM130 (Figure 3A), and found that the two proteins often co-localized (yellow in the merged image) in a perinuclear compartment. As in Figure 1, claudin-4 is also observed in numerous cytoplasmic puncta as well as at cell borders. When claudin-4 was knocked down in shCLDN4 cells, the Golgi became fragmented, as is often seen in cancer cells (Petrosyan, 2015). Very little claudin-4 could be observed by immunohistochemistry, but what was there also appeared to have a fragmented distribution (Figure 3B).

The area occupied by claudin-4 and GM130 was quantitated using Slidebook software. The data in Table 1 show first that the cross-sectional

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area occupied by cells in which claudin-4 is partially knocked down (2328 \( \mu \text{m}^2 \)) is about double that of the control cells (1270 \( \mu \text{m}^2 \)). In the knockdown cells claudin-4 occupied an average of 329 \( \mu \text{m}^2 \) or about 26% of the total cross-sectional area of the cell, whereas GM130 stain occupied about 79 \( \mu \text{m}^2 \) or only about 6% of the total cell area. However, 85% of the area occupied by GM130 also contained claudin-4 indicating that a majority of Golgi vesicles contain claudin-4. When claudin-4 was knocked down with shRNA (cells labelled shCLDN4), claudin-4 occupied 358 \( \mu \text{m}^2 \) or only about 16% of the area of these larger cells. The area occupied by the Golgi (GM130 marker) nearly doubled to 140 \( \mu \text{m}^2 \) in the shCLDN4 cells, again about 6% of the total area. Although very light in its stain, claudin-4 was still found in about 54% of the fragmented Golgi area. Thus, in both cell types, about 20% of the total claudin-4 protein was associated with the Golgi. These results tell us that claudin-4 has a broad distribution in both shCTRL and shCLDN4 ovarian cancer cells and that the distribution of claudin-4 is not simply a function of the high expression level of this protein in OVCAR3 cells.

The effect of Brefeldin A on CLDN4 distribution. Brefeldin A (BFA) is an antiviral lactone first isolated from the fungus Penicillium Brefeldianum. It inhibits protein transport from the endoplasmic reticulum to
the Golgi leading to dissociation of the Golgi stack into dispersed vesicles particularly associated with mitosis (Colanzi et al., 2013). We treated OVCAR3 cells, with 10 μg/ml BFA for 60 min. The dispersion of the Golgi stack observed after 60 min is shown in Figure 3C, where the Golgi marker GM130 is imaged along with claudin-4. These images along with those of shCLDN4 above indicate that fragmentation does not alter the association of claudin-4 with the Golgi vesicles. In dividing cells the Golgi vesicles are dispersed (Figure 3D) but still contain both claudin-4 and GM130.

Proteins biotinylated by biotin ligase labelled claudin-4 in OVCAR3 and OVCAR8 cells.

The distribution of claudin-4 shown in Figure 1 suggests that the protein is distributed in many of the membrane components of the cell. To verify this localization, we next determined claudin-4 proximity proteins using BioID technology as described in the methods. Two experiments each were carried out in the OVCAR3 and the OVCAR8 cell lines. OVCAR3 and OVCAR8 cells expressing claudin-4-biotin ligase were cultured on collagen I and incubated with biotin. The proximity of claudin-4-biotin ligase to neighboring proteins (within 10–15 nm) leads to their biotinylation. Biotinylated proteins were purified and identified via mass spectrometry. Because the results differed little between the two cell lines, they were combined giving 52 proteins localized near to the N-terminus of claudin-4 (Supplementary Table 1 and Supplementary Tables 2A to 2G). Two carboxylases, ACACA and ACACB, and a scaffold protein, AHNAK, were not significantly biotinylated. These are elongated coiled-coil proteins whose carboxy terminus is anchored to the Golgi membrane. Using immunohistochemistry, both proteins overlapped with claudin-4 (Figures 4B and C). Proteins associated with the trans-Golgi membranes were also biotinylated including APIB1 (Anitei et al., 2010), IGF2R (Brown et al., 2008), and CPD (Thomas et al., 2015). Finally, YES1 (Zhou et al., 2020), a cytoplasmic non-receptor kinase, shuttles between the Golgi and the plasma membrane and was biotinylated. In conclusion claudin-4 is associated with many proteins in the Golgi compartment.

Proteins associated with cell-cell junctions, supplementary Table 2C. Three different types of junctions link epithelial cells to neighboring epithelial cells – tight junctions, adherens junctions, and desmosomes (Figure 5A). The mRNA expression levels for these proteins are available in the TCGA and are shown in Figure 5B. The five first proteins shown in Figure 5B, CLDN4, CLDN3, TJ1P1 (ZO-1), CDH1, and AHNAK, were not significantly biotinylated in the BioID experiment but are shown here to give a measure of their expression in HGSOC. Both claudin-3 and claudin-4 have been found to be overexpressed in HGSOC in a number of laboratories (Zhang et al., 2018a; Rangel et al., 2003; Choi et al., 2007). TJ1P1, also known as ZO-1, is an important component of classic tight junctions providing a cytoplasmic anchor for the YV sequences that terminate classic claudins, including claudin-4 and claudin-3 (Nomme et al., 2015). The facts that it was not biotinylated and its low expression in the TCGA led us to the hypothesis that classic tight junctions may not be formed between HGSOC cells.

LSR (lipolysis stimulated lipoprotein receptor) is a component of the tricellular junctions as well as being a lipid transporter in the plasma membrane (Masuda et al., 2011). It was prominently biotinylated. OCLN is often associated with tight junctions, but it can also be associated with adherens junction proteins (Müller et al., 2005). The junctional protein E-cadherin (CDH1) is heavily associated with cell borders in OVCAR3 cells (Figure 5C) where it likely participates in calcium-dependent cell-cell interactions (Sundfeldt et al., 1997). While CDH1 was not biotinylated in the BioID experiment, its interacting proteins, CTNND1 (Ishiyama et al., 2010), DLG1 (Marziali et al., 2019), and CXADR (Nilchian et al., 2019) were. The heavy staining of DLG1 in OVCAR3 cells (Figure 5C) is consistent with the hypothesis that adherens junctions may be important in cell-cell interactions of ovarian cancer cells.

Because of the orientation of claudin-4 in the membranes, biotinylated membrane proteins must have at least part of their structure on the cytoplasmic side of the membrane compartments where the N-terminus of claudin-4 would be located. Because claudin-4 appears by immunohistochemistry to be heavily represented in the ER and Golgi compartment, we examine data related to these compartments in this text. In addition, claudins are generally thought to be tight junction proteins; we therefore examine the biotinylated proteins associated with cell-cell junctions more closely, finding no clear evidence that claudin-4 acts as a tight junction protein in OVCAR3 cells. Finally, we look at the distribution of a well-studied plasma membrane protein, Notch2, which showed substantial colocalization with CLDN2 in OVCAR3 cells.

BioID proteins associated with the endoplasmic reticulum (ER) and Golgi apparatus (Supplementary Table 2A, Figure 4A). Six proteins associated with the ER-GOLGI complex were significantly biotinylated by biotin ligase bound CLDN4. Two prominent Golgi proteins, GMAP210 (TRIP) (Infante et al., 1999) and GOLGB1 (Giantin) (Linstedt and Hauri, 1993), were biotinylated. These are elongated coiled-coil proteins whose carboxy terminus is anchored to the Golgi membrane. Using immunohistochemistry, both proteins overlapped with claudin-4 (Figures 4B and C). Proteins associated with the trans-Golgi membranes were also biotinylated including APIB1 (Anitei et al., 2010), IGF2R (Brown et al., 2008), and CPD (Thomas et al., 2015). Finally, YES1 (Zhou et al., 2020), a cytoplasmic non-receptor kinase, shuttles between the Golgi and the plasma membrane and was biotinylated. In conclusion claudin-4 is associated with many proteins in the Golgi compartment.

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Members of the protein families that make up desmosomes (Figure 5A (Green et al., 2019b)), the transmembrane cadherin family (desmoglein, DSG2, and desmocollin, DSC2), and the armadillo family protein (plakoglobin, JUP) (Aktary et al., 2017), as well as DLG1, interacted with claudin-4 in the BioID experiment. JUP is thought to be a biomarker for ovarian cancer (Weiland et al., 2020) as well as being active in the Wnt signaling pathway (Aktary et al., 2017). These findings indicate that claudin-4 is present in or near most of the junctional complexes in OVCAR3 ovarian cancer cells but the lack of interaction with TJ1P1 (ZO1) and the low expression of the mRNA for TJ1P1 suggest that claudin-4 is not acting as a tight junction protein in these cancer cells.
Proteins associated with the plasma membrane. Plasma membrane proteins traverse the plasma membrane and interact with elements of external environment of the tumor cells. Supplementary Figure 2 shows the mRNA expression levels of the 23 membrane proteins from the HGSOC TCGA that were identified with claudin-4-BioID. All the biotinylated proteins from OVCAR3 variants are fairly highly expressed in this large ovarian tumor dataset. They fall into three categories: 1) membrane receptors that interact with hormones like IGF2 or EGF or with membrane resident ligands on adjacent cells, 2) adhesion molecules that interact with extracellular matrix proteins, and 3) transporters that

Figure 4. ER, Golgi and endosomal proteins identified in the BioID experiment. A. Diagram showing position of Golgi-related proteins. Adapted from (Modica et al., 2017). B. Merged immunocytochemical images of claudin-4 (green), GMAP210—TRIP11 (red) and nuclei (blue) in OVCAR3 shCTRL cells, OVCAR3 shCLDN4 cells and OVCAR 8 cells. Overlapping CLDN4 and GMAP10 appear yellow. C. Images of claudin-4 (green), GOLGB1—Giantin (red) and nuclei (blue) in OVCAR3 shCTRL cells showing overlap (yellow) between the two molecules. Scale bars: 10 μM.
transfer ions and nutrients into the cell. The biotinylation of all these proteins provides strong evidence that claudin-4 is associated with plasma membranes in OVCAR3 cells. Of these molecules we discuss Notch2 here because it overlaps extensively with claudin-4 by immunohistochemistry. Brief summaries of the actions of the other identified plasma membrane proteins are given in Supplementary Tables 2E-G.

**Notch receptor 2.** Co-culture of ovarian cancer cells and endothelial cells induced increased Notch receptors on the surface of the ovarian cells as well as an increase in the Notch ligand Jagged1 on the endothelial cells (Hoarau-Véchet et al., 2019). The authors suggested that Notch2 mediates interactions between ovarian cancer cells and the endothelium. The mRNA for NOTCH2 is the most highly expressed in the TCGA of the biotinylated receptors in HGSOC cells (supplementary Figure 2). In OVCAR3 cells Notch receptor 2 protein was found at the cell border; this is particularly evident in the CLDN4 knockdown cells (Figure 6A). A significant increase in the area occupied by Notch2 was observed in the CLDN4 knockdown cells and about half the area occupied by NOTCH2 overlapped with the CLDN4 area in both shCTRL and shCLDN4 cells (Figure 6B). Since NOTCH2 mRNA is not significantly increased in shCLDN4 cells (unpublished data), this finding suggests that claudin-4 protein localized to the membrane alters the membrane distribution of Notch2 by an unknown mechanism. Interestingly, claudin-4 and Notch2 are colocalized in vesicles surrounding the nuclei of dividing OVCAR3 cells (Figure 6C). High NOTCH2 expression was correlated with worse overall survival in all ovarian cancers (Zhou et al., 2016). Many putative Notch targeting agents are in study with 70 clinical trials registered in 2020 (Moore et al., 2020) suggesting the importance of studies of the interaction of claudin-4 and Notch2.

### 3. Discussion

Claudins are best known as molecules that promote tight junction closure in normal epithelia, interacting with the scaffold protein ZO-1 (TJP1) in complex ways to promote the transepithelial barrier. Claudin-4 is only sporadically found in normal epithelia but is highly expressed in many epithelial cancers including breast (Kolokytha et al., 2014; Szasz et al., 2011), prostate (Landers et al., 2008; Li, 2021), pancreatic (Li, 2021; Torres et al., 2018), bladder (Kuwada et al., 2015), lung (Li, 2021), gastric (Liu and Li, 2020), colorectal (Holeczbauser et al., 2013) and ovarian (Hewitt et al., 2006; Hough et al., 2000; Li, 2021). In many of these cancers, although claudin-4 appears to be localized in the membranes, the cellular compartments with which it is associated have not been determined.

**Claudin-4 localization.** About 20% of the total area occupied by claudin-4 in OVCAR3 cells localized with the Golgi-associated protein, GM130 even in cells where claudin-4 was partially knocked down (Table 1). Claudin-4 was also associated with cell borders as well as in distinct cytoplasmic puncta (Figure 1). To localize claudin-4 more precisely, we performed a BioID experiment, transducing both OVCAR3 and OVCAR8 cells with the gene for claudin-4 with the biotin ligase gene attached to the N-terminus. We then determined the identity of biotinylated proteins in cultures of these cell lines. OVCAR8 cells express
CLDN4 mRNA at a level less than 1% of the level in OVCAR3 cells, allowing us to determine the effect of endogenous claudin-4. The BioID experiment produced 52 biotinylated proteins of which one (LEMD) is generally localized to the nuclear membrane; six of these are located in the Golgi and endoplasmic reticulum, five in endosomal vesicles, eight at cell-cell junctions, eight with the cytoskeleton, and 23 are plasma membrane proteins. It appears as if, once synthesized, claudin-4 starts down the pathway from the nucleus to the endoplasmic reticulum and Golgi, to endoplasmic vesicles which carry it to the plasma membrane—portions of which are associated with cell-cell junctions and other portions at other cell borders.

The protein neighbors identified in the BioID analysis of claudin-4 provide insight into the structural architecture of the membranes in OVCAR3 cells. Not only were membrane proteins in all cellular compartments (except the mitochondria) biotinylated, many scaffolding proteins adjacent to the membranes were also biotinylated. Scaffolding proteins are often thought to provide a focus for the clustering of functional proteins. Whether the organization of functional proteins depends on expression of claudin-4 is an important question for future investigation. However, previously published findings relating to claudin-4 expression and activity suggest that it plays an important role in the function of tumor cells. Thus, the fraction of apoptotic ovarian tumor cells is altered by the knockdown of claudin-4 (Breed et al., 2019; Hicks et al., 2016). In addition, claudin-4 appears to affect mitotic progression in OVCAR3 cells, perhaps by interaction with tubulin (Breed et al., 2019).

Figure 6. Overlap between Notch2 and claudin-4 in A. Immunohistochemical staining of Notch2 and claudin-4 in OVCAR3 shCNTL and shCLDN4 cells. B. Quantitation of cell area occupied by Notch2 and claudin-4 proteins. Area occupied by Notch2 is significantly increased in shCLDN4 cells (p = 0.0328). C. Overlap of NOTCH2 and claudin-4 in vesicles surrounding dividing nuclei in 4 sections from OVCAR3 cells.
Claudin-4 and AHNAK. It is relevant to discuss AHNAK (Neuroblast differentiation-associated protein, SUSD2), a scaffolding protein highly expressed in OVCAR3 cells; it is often associated with the mesenchymal phenotype in cancers (Sheets et al., 2016). Biotinylation of AHNAK was increased 2.04-fold (log, 0.78) in the presence of claudin-4-biotin ligase, too low to meet our cut-off because AHNAK has a high basal level of biotinylation. However, AHNAK may be an important protein in epithelial cancers since it is associated both with EMT (Sheets et al., 2016) and with microvesicle production (Silva et al., 2016). It is referred to as a “giant” protein because of its large size, about 5500 amino acids (Davis et al., 2014). Further, the functional classification of AHNAK as a scaffold protein is supported by the presence of a PDZ domain within its protein structure, which could interact with the YV sequence at the C-terminus of claudin-4 (Itoh et al., 1999; Zardab et al., 2022). Could it be that the membrane distribution of claudin-4 in OVCAR3 cells is dependent on the scaffolding function of AHNAK?

Claudin-4 and solute carriers. Claudin-4 was found in proximity to several solute carriers, including LAT1 (SLC3A2 and SLC7A5), Na/K ATPase (ATP1A1), and ASC2 (SLC1A5). The interaction and possible regulatory effect of claudin-4 on these solute carriers is intriguing in the context of metabolism and the tumor microenvironment. For instance, LAT1’s role in tryptophan uptake has direct implications in the immune response (Hayashi and Anzai, 2022) and regulation of the aryl hydrocarbon receptor transcription factor (D’Amato et al., 2015), which uses a tryptophan metabolite (kynurenine) to reprogram the transcriptome. Another example is that ASC2 transports the amino acid glutamine, a critical metabolite for the generation of essential molecules (e.g., glucose) and amino acids. The depletion of glutamine uptake and biosynthesis attenuates the growth of ovarian cancer cells (Furusawa et al., 2018). Thus, there is a significant need to further understand the regulatory function of the claudin-4/ASC2 interaction.

Claudin-4 and survival. High expression of claudin-4 is associated with reduced survival in ovarian cancer patients (Human Protein Atlas). Breed and associates (Breed et al., 2019) found that high expression in OVCAR3 variants reduced the apoptotic response to paclitaxel and that the molecule interacted with tubulin. The response could be restored by treatment of the cells with a claudin-4 targeting peptide. A major question is how claudin-4 produces these effects. We have recently shown that loss of claudin-4 expression in ovarian cancer cells decreases DNA repair capacity and increases the sensitivity to PARP inhibitors (Yamamoto et al., 2019, 2022). Consistently, human tumors with low claudin-4 expression correlate with both higher mutational burden and genomic instability. The connection between these findings and the localization of claudin-4 is currently not clear.

Other Claudins in cancer cells. Claudin-4 is not the only claudin in ovarian cancer cells (Li, 2021; Rangel et al., 2003). CLDN3 is expressed at a higher average level in ovarian tumors in the TCGA than either CLDN4 or CLDN1, while CLDN6 and CLDN7 are also expressed at significant levels. Claudin-3 was localized throughout the cell in many ovarian cancers although the precise membranes in which it was distributed were not determined (Corsini et al., 2018). An important question is whether Claudins-3, 6, 7 are localized in membrane or membrane adjacent compartments in cancer cells. What are the biological actions of all these Claudins in epithelial cancer cells?

Phosphorylated Claudin-4. Phosphorylated claudin-4 is relatively understudied. In OVCAR3 cells, claudin-4 phosphorylated on Y208 was localized to cytoplasmic puncta that also contained non-phosphorylated focal adhesion proteins, raising the question of whether phosphorylated claudin-4 is involved in the formation of focal adhesion complexes. Tanaka and colleagues found that claudin-4 was phosphorylated on Y208 by EPHA2, a process that attenuated its interaction with ZO-1 in the tight junctions of MDCK cells (Tanaka et al., 2005). While there is no evidence that phosphorylated claudin-4 interacts with tight junction proteins such as ZO-1 (also known as TJP1) in ovarian cancer cells, it is clear that it is tightly regulated and confined to cytoplasmic puncta in these cells. Phosphorylated claudin-4 appeared to be present at about the same level in cells where CLDN4 is knocked down (shCLDN4 cells) as in control cells (shCTRL; Figure 2B) where total non-phosphorylated claudin-4 is significantly diminished. Whether this finding is the result of a saturation of the phosphorylation mechanism in the control cells, or some other mechanism, remains to be determined.

Summary and limitations of this study. The overall aim of this study was to determine the localization of claudin-4 in ovarian cancer cells. In the course of these experiments we found that claudin-4 is localized to most membranes of the cell, that native and phosphorylated claudin-4 did not colocalize, that about 20% of claudin-4 was localized to the Golgi in both normal and claudin-4 knockdown cells, and that claudin-4 continued its Golgi localization in cells where the Golgi is dispersed including dividing cells where it colocalized with both the Golgi marker GM130 and with the plasma membrane receptor Notch2.

One limitation of this study is that neither other types of ovarian cancer cells nor actual ovarian tumors were studied. There do not seem to be studies of the localization of Claudins in other types of epithelial cancers; it seems possible that Claudins other than claudin-4 may be localized in membrane areas that are not tight junctions. This finding would have important implications for therapeutic agents. For example, claudin-4 has been shown to reduce the response of ovarian cancer cells to paclitaxel (Breed et al., 2019); the mechanism of this action is unknown.

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4.1. Methods

Cell lines and cell culture. Human derived OVCAR3 and OVCAR8 cells were obtained from the laboratory of Monique Spillman at the University of Colorado and cultured in RPMI-1640 medium (Gibco, Thermo Fisher Scientific) plus 10% heat inactivated fetal bovine serum and penicillin/streptomycin at 37 °C and 5% CO2. Cell lines were authenticated by short tandem repeat profiling at the University of Arizona Genetics Core.

shRNA knockdown. OVCAR3 cells were plated 3.2 × 10^4 in a 96-well plate and incubated at 37 °C for 24 h. When cells reached 70% confluence, 10 μL of claudin-4 short hairpin RNA (shCLDN4; TRC#: TRCN0000116627, TRCN0000116628, or TRCN0000116631) or control (shCTRL; SHC001, pLK0.1-puro Empty Vector) lentiviral suspension was added to the cells, which were incubated overnight at 37 °C. Fresh medium was added to remove lentivirus and cells were allowed to recover for 24 h before being treated with 0.5 mg/mL puromycin for selection and expansion of transduced cells. Western blot analysis confirmed the decrease in claudin-4 expression.

Data from The Cancer Genome Atlas (TCGA, 2011). The TCGA contains expression values for 20532 mRNAs from 209 ovarian cancer patients. RNA-seq data was downloaded from cbioportal and compiled. The patient values were averaged, and the standard deviation and standard errors of the mean determined.

Bio ID. Constructs - pTR2E hyg myc bion ligase CLDN4 plasmid was a gift from Melvin Anderson at NIH. The myc bion ligase CLDN4 was excised using Bam HI and Sal I and ligated into a pBABE vector. A control plasmid with a stop codon before CLDN4 was also constructed using site directed mutagenesis. Cells were transduced separately with these plasmids.

Purification of Biotinylated Proteins. Method adapted from (Van Itallie et al., 2013). 50μM bion (Sigma B4639) was added to nine 150mm dishes of 80% confluent cells and incubated 16 h. Cells were washed three times with PBS, scraped into PBS, pelleted, lysed in 5 ml RIPA (1% Triton X-100, 0.5% deoxycholate, 0.2% SDS, 50mM Tris pH 7.5, 150mM NaCl and protease inhibitors), sonicated, incubated on ice for 10 min, sonicated again, and centrifuged for 20 min at 12,000 x g. The supernatant was transferred to microfuge tubes with 500μl prewashed Dyna-beads MyOne Streptavidin C1 and incubated at 40 °C for 4 h in an end-over-end mixer. The beads were then washed twice with 2% SDS,
once with 0.1% deoxycholate, 1% Triton X-100, 500mM NaCl, 1mM EDTA, 50mM Hepes pH 7.3, once with 250mM LiCl, 1mM EDTA, 0.5% deoxycholate, 0.5% Triton X-100, 10mm Tris pH 8.0, and twice with 50mM Tris pH 7.5, 50mM NaCl. Proteins were eluted from the beads and mass spectrometry carried out by Monika Dzieciatkowska, PhD, in the University of Colorado School of Medicine Biological Mass Spectrometry Proteomics Core Facility.

The experiment was repeated four times, twice with OVCAR3 cells and twice with OVCAR8 cells. The resulting numerical values for levels of biotinylated proteins were analyzed by the difference between cells receiving the gene for claudin with biotin ligase and claudin alone. Only proteins where the difference between the biotinylated and control was greater than 10 and the log2 ratio was greater than 1 were selected. The final list and summary data are given in Supplementary Table 1. In most cases, all four experiments met the criteria for selection. In a few cases, only three experiments met the criteria, but these proteins were retained in the list. The final list contained 52 proteins. Sixteen of these proteins had been identified previously in a BioID experiment in MDCK cells examining claudin-4 (asterisks in Supplementary Table 1) (Fredriksson et al., 2015).

**Immunohistochemistry.** Cells grown on Type I collagen (Sigma) coated glass slides were fixed with 10% phosphate buffered formalin (Sigma) at room temperature for 15 min. Slides were washed twice with phosphate buffered saline (PBS), permeabilized with 0.5% Triton X-100 (Sigma) for 5 min at room temperature, washed twice with PBS, blocked with 2% bovine serum albumin (Sigma) for 1 h at room temperature, and then treated with primary antibodies (Supplemental Table 3) overnight at 4°C. Slides were washed with PBS five times before application of secondary antibodies (dilution 1:100) to the same species as the primary antibodies and conjugated to a fluorescent probe (Jackson Immuno Research) as well as 5 μg/ml 4',6-diamidina-2-phenylindole (DAPI, Sigma) for 45 min at room temperature following five washes with PBS. OPDA (20 mg/ml, o-phenylenediamine dihydrochloride (Sigma) in 1M Tris, (pH 8.5 and 10% glycerol) was applied to slides for preservation of fluorescence and slides were coverslip mounted. Imaging was performed in the Advanced Light Microscopy Core part of the Neuro Technology Center at University of Colorado Anschutz Medical Campus supported in part by Rocky Mountain Neurological Disorders Core Grant Number P30 NS048154 and by Diabetes Research Center Grant Number P30 DK116073.

**Quantitation of immunohistochemistry.** Quantitation of the area occupied by a particular-colored stain was carried out using Slidebook software (Intelligent Imaging Innovations Inc., Denver, CO).

**Statistical analysis.** Quantitative data are presented as mean +/- SEM. An unpaired Student t-test was used for statistical comparison between control and treatment groups. A P-value of <0.05 was considered significant.

**Declarations**

**Author contribution statement**

Margaret C. Neville: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Patricia G. Webb: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Heidi K. Baumgartner: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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**Data availability statement**

Data included in article/supp. material/referenced in article.

**Declaration of interest's statement**

The authors declare no conflict of interest.

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**Additional information**

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