Published in final edited form as:

J Proteome Res. 2016 February 5; 15(2): 339–359. doi:10.1021/acs.jproteome.5b00769.

Systems Proteomics View of the Endogenous Human Claudin Protein Family

Fei Liu^{*,†}, Michael Koval[‡], Shoba Ranganathan[†], Susan Fanayan[§], William S. Hancock^{§,||}, Emma K. Lundberg[⊥], Ronald C. Beavis[#], Lydie Lane[∇], Paula Duek[∇], Leon McQuade[◆], Neil L. Kelleher[○], and Mark S. Baker[§]

[†]Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW 2109, Australia [§]Department of Biomedical Sciences, Macquarie University, Sydney, NSW 2109, Australia [◆]Australian Proteome Analysis Facility, Macquarie University, Sydney, NSW 2109, Australia [‡]Department of Medicine, Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, and Department of Cell Biology, Emory University School of Medicine, 205 Whitehead Biomedical Research Building, 615 Michael Street, Atlanta, Georgia 30322, United States ^{||}Barnett Institute and Department of Chemistry and Chemical Biology, Northeastern University, Boston, Massachusetts 02115, United States [⊥]SciLifeLab, School of Biotechnology, Royal Institute of Technology (KTH), SE-171 21 Solna, Stockholm, Sweden [#]Department of Biochemistry and Medical Genetics, University of Manitoba, 744 Bannatyne Avenue, Winnipeg, Manitoba R3E 0W3, Canada [∇]SIB–Swiss Institute of Bioinformatics, CMU - Rue Michel-Servet 1, 1211 Geneva, Switzerland [○]Department of Chemistry, Department of Molecular Biosciences, and Proteomics Center of Excellence, Northwestern University, 2145 North Sheridan Road, Evanston, Illinois 60208, United States

Abstract

Claudins are the major transmembrane protein components of tight junctions in human endothelia and epithelia. Tissue-specific expression of claudin members suggests that this protein family is not only essential for sustaining the role of tight junctions in cell permeability control but also vital in organizing cell contact signaling by protein–protein interactions. How this protein family is collectively processed and regulated is key to understanding the role of junctional proteins in preserving cell identity and tissue integrity. The focus of this review is to first provide a brief overview of the functional context, on the basis of the extensive body of claudin biology research that has been thoroughly reviewed, for endogenous human claudin members and then ascertain existing and future proteomics techniques that may be applicable to systematically characterizing the chemical forms and interacting protein partners of this protein family in human. The ability to elucidate claudin-based signaling networks may provide new insight into cell development and differentiation programs that are crucial to tissue stability and manipulation.

*Corresponding Author, fei.liu@mq.edu.au. Tel: (61)-2-9850-8312. Fax: (61)-2-9850-8313..

The authors declare no competing financial interest.

Keywords

Membrane protein complexes; cell-contact signaling; systems proteomics; membrane proteomics; targeted proteomics; top-down proteomics; chemical proteomics

1. INTRODUCTION

Claudins are a family of transmembrane proteins for barrier and pore formation in metazoans, especially for vertebrates and tunicates.¹ Chordate claudins are the essential architectural proteins of tight junction strands in the apical junctional complex of epithelia and endothelia.^{2–9} These junctional strands, containing claudin protein complexes, act mainly as paracellular seals for large molecules and a semipermeable barrier to ions in tissues.^{5,6,10} Distinct claudin members are expressed in a tissue to confer tissue-specific permeability and barrier characteristics.^{7,11} Claudins also serve as protein scaffolds for assembling signaling complexes at cell junctions.^{3,12–15} Understanding how the claudin family of proteins are expressed, organized, and regulated in the cell with temporal and spatial resolution is critical to unraveling mechanisms that control paracellular barrier function, tissue integrity, and stability.

The seminal discovery of claudins by Furuse and Tsukita in 1998 came 25 years after the initial observation of cell–cell contact ultrastructures.^{16,17} Since this seminal work the claudin protein family has been the subject of intense investigations to understand their structure and function in physiological and pathological contexts. The number of claudin genes varies between species. For example, in the case of the puffer fish *Takifugu rubripes*, up to 56 claudin genes are found by genome sequence analysis,¹⁸ whereas in *Homo sapiens*, the claudin gene family has at least 23 and maybe more if all predicted claudin genes are found to be expressed as proteins.¹⁹ Because different claudins are differentially expressed with tissue specificity and temporal regulation, claudins are likely to confer several vital roles, particularly in control of paracellular permeability but also in cell differentiation, morphogenesis, and tissue maintenance.

Several reviews have appeared to account in detail advances in claudin physiology and biology of many organisms.^{1,4–10,14,18–21} These reviews represent a vast body of research in claudin physiology in humans and other organisms. The majority of insights into defining how claudins function have been obtained using epithelial cell lines where the ramifications of claudin expression are measured after manipulation by either cDNA overexpression or RNA silencing. Claudin-deficient²² and overexpression^{23,24} transgenic mouse models have also proven to be informative in identifying functions for specific claudins in regulating epithelial barrier function and other aspects of mammalian physiology relevant to human disease. These are valuable approaches. However, it is difficult to employ these models so that claudin expression is manipulated within the normal physiologic range of mRNA and protein expression. The claudin expression profile of many different tissues and cells, including normal and tumor cells, has been assessed at the mRNA and protein levels. While these approaches provide some insights into how the claudin proteome is organized in different tissues, they have two major limitations. First, claudin mRNA does not necessarily correlate with levels of protein expression,²⁵ since processes such as tight junction turnover are significantly regulated by protein–protein interactions²⁶ and post-translational modifications (PTMs) of claudins in both human and animal cell models.^{27–30} Second, direct measurements of claudin protein have been largely restricted to antibody-dependent approaches (e.g., immunoblot), which have significant utility but are difficult to use to measure the stoichiometry of claudin composition, which is a critical parameter needed to determine how differential claudin expression influences epithelial barrier function and other aspects of cell physiology.

The approach of systems proteomics, encompassing specialty technologies such as membrane proteomics,^{31,32} targeted proteomics,^{33–35} bottom-up/top-down proteomics,³⁶ structural proteomics,^{37,38} and chemical proteomics,^{39,40} has the potential to provide valuable adjuncts to other established methods currently used to define roles for claudins in cell and tissue function. However, the application of systems proteomics has lagged behind our understanding of functional roles of claudins in regulating human cells and tissues. There are several reasons for this. Claudins are highly hydrophobic proteins that are more difficult to isolate and analyze by mass spectrometry (MS) than hydrophilic proteins.⁴¹ Also, claudin function is highly dependent on cell localization that, in turn, is regulated by interactions with other proteins. Claudin–protein interactions range from weak, unstable interactions (e.g., claudin–claudin interactions^{14,42}) to interactions that effectively immobilize claudins onto the actin cytoskeleton.⁴³ These are classes of protein–protein interactions that are difficult to analyze and will require novel approaches to define.

In this review, human claudin genetics and biology are summarized first for a general outlook while also guiding the reader to other sources for further details on claudin cell and molecular physiology. Here, the discussion focuses on the chemical characteristics of human claudins with a particular emphasis placed on MS-centered systems proteomics approaches to studying human endogenous claudins. Some of the investigations on other organisms such as mice, rats, and canine cell/tissue models will be discussed only for occasional comparisons. Specialty proteomics technologies, such as membrane proteomics, structural proteomics, targeted proteomics, bottom-up/top-down proteomics, and chemical proteomics, are assessed for consideration of their further development and integration into investigating

human endogenous claudins. Because endogenous claudins are low in abundance with complex chemical modification and localization profiles, this will most certainly push technology development to new limits. Emerging MS approaches for elucidating membrane protein complexes in the relevant context of claudin biological function is also addressed here. Human claudin PTMs, revealed thus far by MS and/or immuno-detection, are discussed to highlight how claudin function may be regulated. Existing approaches for characterizing endogenous human claudins by MS in conjunction with chemical partition and enrichment techniques are also reviewed to underscore the challenges and also opportunities in unraveling the claudin protein networks holistically with improved temporal and spatial resolution. New chemical delineation of the native claudin protein system may lead to novel hypotheses into claudin biology and new cell assembly and tissue manipulation capabilities. The advanced technologies developed for claudin systems proteomics could also assist further biological investigations into junctional signaling networks involved in intercellular communication in metazoans.^{44–48}

2. GENERAL OVERVIEW OF THE GENETIC PROFILE, PROTEIN FAMILY, BIOLOGICAL ROLE, AND TISSUE EXPRESSION OF THE HUMAN CLAUDINS

As indicated earlier, recent reviews have already provided insightful accounts of claudin genetics and biology, along with their significant interactions with other important junction proteins. Information on human claudins from various databases, such as UniProt,⁴⁹ Ensembl,⁵⁰ and the Human Protein Atlas (HPA)⁵¹ is compiled in Table 1 to provide a general outlook of human claudins. Much of the recent claudin research is not yet captured in these databases, although they offer an overview of human claudins to help guide navigating the detailed reviews and current articles for in-depth analysis.

2.1. Genetic Analysis

Including transcript variants, 27 mammalian claudin genes have been reported, and at least 23 are found in humans (Table 1, column 1).^{20,52–54} The mammalian *CLDN13* gene is absent in humans but present in rodents, whereas *CLDN24*, 25, 26, and 27 are putative claudin genes.¹ The official gene names for claudins, attributed by the HUGO gene nomenclature committee,⁵⁵ are shown in Table 1 (columns 1 and 2) with other synonyms also in use (e.g., in GeneCards,⁵⁶ Table 1, column 3). The human claudin genes are spread across 13 chromosomes (1, 3, 4, 6, 7, 8, 11, 13, 16, 17, 21, 22, and X) with generally few or even no introns found for these genes (Table 1, column 4). Chromosomes 3 and 7 have the highest frequency of claudins (*CLDN1*, 11, 16, and 18 on chromosome 3; *CLDN3*, 4, 12, and 15 on chromosome 7). Some claudins also exhibit high pairwise sequence homology, such as *CLDN3* and 4, *CLDN6* and 9, *CLDN8* and 17, and *CLDN22* and 24. Coordinated gene expression is possible and has been observed for *CLDN3* and 4.⁵⁴ Mammalian claudins are classified into the classic (*CLDN1–10*, 14, 15, 17, and 19) and nonclassic (*CLDN11–13*, 16, 18, 20–24) groups on the basis of their phylogenetic relationship,⁵⁷ and it is also possible to split the claudins into eight groups based on their gene structures and phylogenetic distances.^{1,10}

2.2. Protein Family

From the protein structure and function perspective, the claudin protein family is included in the pfam00822 superfamily of proteins whose members are characterized by their four helical transmembrane regions.^{1,7,21} The pfam00822 superfamily also includes other subfamilies such as the PMP-22, EMP, MP20, and calcium channel CACNG proteins. Human claudins are in the range of 200–300 amino acids (20–35 kDa) and share the common transmembrane regions made of four helices (TM 1–4) with two extracellular loops (ECL1 and ECL2) and one intracellular loop (ICL).

The general molecular architecture of the claudin protein family, determined by an extensive body of genetic, biochemical, and biophysical experiments, is shown in Figure 1a. The four transmembrane regions (TM 1–4) are typically 24 amino acids in length, while the α -helix of TM3 extends beyond the membrane by about 10 amino acids.^{53,59} The ECL1 is generally around 50 amino acids with charged amino acids for paracellular pore formation. A Prosite signature sequence for the claudin family has been identified as [GN]-L-W-x(2)-C-x(7,9)-[STDENQH]-C in ECL1.^{1,60} A consensus sequence, W-X(17-22)-W-X(2)-C-X(8-10)-C is found in ECL1 with the first Trp flanking the end of TM 1.⁶¹ A signature motif, GLW, is found 2 amino acids away from the N-terminus of the conserved C-X(8-10)-C motif. Most claudins also have an Arg marking the end of the ECL1. Inside the cell, the claudin N-terminus (<10 amino acids), ICL (~12 amino acids), and C-terminus (25–55 amino acids) provide interaction sites with other proteins. Most notably, a PDZ⁶² domain binding motif YV at the C-terminus is completely conserved among classic claudins and also prevalent among the nonclassic ones.^{57,63} The YV motif is the main interaction site for binding other junction associated proteins such as ZO-1, -2, and -3, although other residues in proximity of the C-terminus also contribute to claudin's protein binding affinity.⁶³ The first mammalian claudin structure (mouse claudin-15) at 2.4 Å confirms this general claudin four-helix bundle that spans the full length of the lipid bilayer (Figure 1b).⁶⁴ Subsequently, a binary structure at 3.7 Å resolution of mouse claudin-19 complexed to the C-terminal region of *Clostridium perfringens* enterotoxin further validates the generality of the transmembrane, four-helix bundle scaffold and identifies the specific binding motifs in both the ECL1 and ECL2 loops to the bacterial toxin.⁶⁵

The claudin protein family expression level by immunoblotting is not necessarily correlated to its RNA level (Table 1, columns 5 and 6). Human claudins 24–27 have been tentatively assigned as members of the claudin family, and the evidence for these members remains tenuous even at the RNA level (Table 1, column 6). Many antibodies have been in use for claudin detection in both cells and tissues (Table 1, column 5), although polyclonal antibodies recognize multiple epitopes and antibody cross-reactivity is a significant concern in analyzing claudins. Currently, four human claudin proteins (claudin-8, -9, -20, and -22) are considered “missing”⁶⁶ at the MS level (as defined by HUPO, the Human Proteome Organization) with evidence for their presence only at the transcription level, although for human claudins 8 and 9, protein presence has been suggested by immunoblotting and likely requires further validation.^{67,68} In terms of good MS evidence, 11 of the human claudins, namely, claudins 1, 2, 3, 4, 5, 6, 7, 10, 11, 12, and 18, have been identified multiple times by more than one unique peptide with good log(*E*) scores (Table 2). Furthermore, four human

claudins (15, 16, 19, and 23) have been identified multiple times by just one unique peptide with good $\log(E)$ scores. In the case of claudin 9, only one nonunique peptide has been identified multiple times with good $\log(E)$ scores, which is also found in claudins 3, 4, and 6. Overall, of the 23 definitive human claudins (not including the disputed claudins 26 and 27), human claudins 8, 9, 14, 17, 20, 22, 24, and 25 do not have definitive MS-based protein evidence. Therefore, only about half of the human claudin protein family can be considered as identified with certainty by MS at the protein level, with the other half either missing or uncertain by MS. For human claudins (7, 10, 11, 18, and 19) with isoforms by alternative splicing, no credible MS evidence exists for their presence as protein isoforms. One caveat is that several of these may be enriched in tissues that have not been probed for claudin expression by MS. For instance, claudin-18 has been shown by immunologic techniques to be highly expressed at the protein level in stomach and lung.

3. GENERAL OVERVIEW OF THE TISSUE AND FUNCTIONAL PROFILES OF THE HUMAN CLAUDINS

Claudins are widely expressed, either as gene transcripts or expressed proteins, in diverse tissues.⁶⁹ The vast majority of claudin expression is found in barrier-forming epithelial and endothelial cells. Not all claudins are concurrently expressed by all tissues, and it is these differences in claudin expression that regulate cell function, most notably, paracellular barrier function. Reflecting a predominant role in regulating barrier function, claudins frequently are found concentrated at the tight junction; however, some claudins are also found at other intracellular locations, such as regions along the basolateral plasma membrane.^{1,14,70} Nuclear localization of claudins has also been observed.^{1,10,20,71–73} Roles for nonjunctional claudins are not well understood, although the literature correlating nuclear claudin localization with tumor metastasis suggests roles for these claudin pools in regulating cell growth, division, and migration.^{61,74–78}

The most prevalent roles for claudins are in barrier and pore formation, and the details of how individual claudins influence tight junctions have been extensively reviewed.^{21,57,61,79} The majority of claudins are primarily barrier forming, based largely on studies of cell line models where the effects of increasing and decreasing claudin expression on measures of barrier function were assessed, including transepithelial resistance and paracellular flux of low molecular weight tracers. By contrast, claudin-2, -10, -15, and -17 are primarily pore-forming and lead to increased paracellular permeability correlating with increased expression as a result of formation of paracellular ion and water channels. The high-resolution structure of claudin-15⁶⁴ has provided several insights into the structural basis for claudin paracellular ion selectivity. Homology modeling and structure determination of other claudins will likely provide a valuable approach to further define the permeability characteristics of other claudins. Claudins also interact with other junction membrane proteins, such as occludin, tricellulin, and junctional adhesion molecule-A (JAM-A), to stabilize and regulate their membrane retention for improved barrier formation.^{10,21,80}

Claudin mutations have been implicated in human diseases affecting ion homeostasis.^{61,74–77,81} One of the best characterized claudin-associated diseases is familial hypomagnesemia with hypercalciuria and nephrocalcinosis that is caused by mutations in

claudin-16 and -19.^{82–84} The majority of the mutant claudin-16 alleles (12 out of 16) show a missense mutation in the highly conserved amino acids in ECL1 or ECL2.^{83,85} Such mutations have been postulated to alter the tertiary structure of the extracellular domains in claudin complexes and thus change the cation specificity of claudin pores. Other mutations affect claudin-16/claudin-19 interactions, diminishing the transport and assembly of these claudins into tight junctions.⁸² Claudin mutations can be linked to diverse, tissue-specific pathologic outcomes, which underscores the need to understand the structural basis that regulates their function and protein–protein interactions.

4. POST-TRANSLATIONAL MODIFICATION OF CLAUDINS

PTMs greatly expand the claudin chemical diversity found in cells and in turn further diversify claudin function.⁸⁶ Potential biological processes that may be differentially regulated through claudin PTM include protein interaction partnerships, trafficking, subcellular localization, oligomer (hetero- or homo-) assembly, and finally net claudin homeostasis.¹³ Here, we discuss several classes of claudin PTMs: phosphorylation, palmitoylation, ubiquitination, and glycosylation (Figure 2). Proteolysis is another way by which claudins can be subject to PTM. It is not well established whether any proteolytic claudin fragments have biological activity or whether this largely serves to decrease the total claudin protein pool.

4.1. Phosphorylation

Currently, phosphorylation on Ser/Thr/Tyr residues is the most abundantly observed claudin PTM. Theoretical predictions of potential phosphorylation sites indicate that up to 10 phosphorylation sites are possible by a variety of protein kinases (e.g., PKA/C, MAPK, WNK, Src, and Eph).⁸⁶ Most of these predicted phosphorylation sites are located in the C-terminal tail. One important feature of claudin phosphorylation is that the Tyr residue found in the highly conserved PDZ binding motif at the end of the C-terminus can be a phosphorylation site by the Eph receptor tyrosine protein kinase, thus providing a means to regulate its binding activity. For example, in the case of claudin-4 in HT29 colon carcinoma cells, EphA2 activation leads to claudin-4 tyrosine phosphorylation, and this specific Tyr phosphorylation lowers its association with ZO-1 and leads to less claudin-4 integration into cell junctions and higher paracellular permeability in MDCK cell models.⁸⁷ This result indicates that EphA2 Tyr phosphorylation near the claudin C-terminus may alter the function of the tight junction and promote a regime change in signaling at the tight junction. Tyr phosphorylation for claudin-5, detected by a general phosphor-Tyr antibody, has also been linked to increased paracellular permeability in cultured human brain capillary endothelial cells.⁸⁸ Similarly, Ser/Thr phosphorylation of claudins is known to influence the barrier strength of cell junctions. Phosphorylation of T192 of claudin-3 by PKA has been found in ovarian cancer cells as a mechanism for disruption of normal junctional function.⁸⁹ In other instances, Ser/Thr phosphorylation of claudin-7 can upregulate the caspase pathway to increase the chemosensitivity of lung cancer cells toward cisplatin or increase paracellular permeability to chloride anions in kidney cells.^{90,91} Large-scale phosphoproteomics investigations (from PhosphoSitePlus⁹² with specific journal article references and the Global Proteome Machine⁹³) have identified multiple Tyr/Ser/Thr sites of claudin-1–12,

-15, -16, -18, and -23 in human T cells, embryonic stem cells, and cancer cells (summarized in Table 3).^{90,91,94–113} However, the specific roles of these claudin PTMs remain largely unknown without functional validation from biological experiments.

The effects of phosphorylation are claudin-specific, in regard to the specific residue modified, and vary even among highly homologous claudins. For example, S208 phosphorylation of claudin-2 is required for its membrane retention and reduced trafficking to lysosomes in MDCKII cells; however, this phosphorylation does not alter its binding to ZO-1 or ZO-2.³⁰ In the case of claudin-5, PKA phosphorylation at T207 in endothelial cells, while conducive to its translocation into the tight junction membrane, leads to increased paracellular permeability.^{114,115} In addition to barrier function, claudin phosphorylation is also known to control selective ion permeability. For example, reduction of claudin-16 phosphorylation at S217 results in impaired Mg^{2+} permeability in renal MDCK cells.¹¹⁶ As an additional confounding variable, tyrosines are subject to nitrosylation under conditions of oxidative stress.¹¹⁷ Evidence for tyrosine nitration of claudins has been found only in rats at this point, where renal claudin-2 nitration was detected in early diabetic rats.¹¹⁸

4.2. Palmitoylation

Similar to phosphorylation, palmitoylation can significantly alter claudin localization, protein interactions, trafficking, and stability.⁸⁶ TM2 and TM4 of claudins are each flanked by a cysteine residue that has a palmitoylation motif. Some claudins have additional cysteine residues potentially available for palmitoylation in the ICL and C-terminal tail. For example, in MDCK cells, two cysteine residues in the ICL and two more in the C-terminal tail of claudin-14 must be palmitoylated as a requirement for efficient plasma membrane localization and tight junction assembly.¹¹⁹ However, in transfected HEK cells, palmitoylation of claudin-7 is associated with partitioning into glycosphingolipid membrane microdomains and inhibits integration into tight junctions, suggesting a model where palmitoylation controls the relative amounts of claudin-7 in the basolateral vs tight junction pools.¹²⁰ Protein S-acylation has been profiled on the proteome scale,^{121–123} and given the difficulties in measuring palmitoylation by standard biochemical techniques, this is an area that would be ideally suited for state-of-the-art proteomics analysis.

4.3. Ubiquitination and SUMOylation

Evidence for human claudin ubiquitination and SUMOylation is scarce but more frequently observed in model cell lines of other organisms. Polyubiquitination of claudin-5 is observed in claudin-5 transfected HeLa cells at K199 to target the protein for proteasomal degradation.¹²⁴ This results in claudin-5 being removed from the plasma membrane and loss of the tight junction strands, indicating that with this particular PTM claudin stability can be compromised by a potential mechanism of increased turnover. Claudin ubiquitination has been more consistently observed in canine, mouse, and rat cell models according to PhosphositePlus. For example, polyubiquitination of claudin-1 in MDCK cells appears to be dependent on LNX1p80 overexpression, but the sites of ubiquitination are not specified and also do not involve Lys48, a site that is typically ubiquitinated for proteasome-dependent degradation.¹²⁵ SUMOylation of claudin-2 at K218 is also reported in MDCK cells in which claudin-2 is removed from the lower lateral membrane but not the tight junction.¹²⁶ It is

noteworthy that not all claudins contain ubiquitination sites, although the classic claudins are thought to be the primary candidates for ubiquitination, and this is supported by Lys ubiquitination sites identified by large-scale proteomics (Table 3, claudin-7 and -12). The current understanding of claudin ubiquitination/SUMOylation on claudin localization, function, or stability is based primarily on studies of non-human claudins. It is likely that direct evidence for ubiquitination/SUMOylation of human endogenous claudins will be forthcoming.

4.4. Glycosylation

Experimental evidence for claudin glycosylation is again scarce, perhaps due to the low abundance level of these proteins that requires specific enrichment prior to such analyses. This lack of data also suggests that claudin glycosylation is a rare event. Putative N- and O-glycosylation sites on some claudins have been identified.^{127,128} Some of these predicted O-glycosylation sites overlap with predicted phosphorylation sites. These overlaps are known as the Yin Yang sites, where the two modifications can reciprocally occur on the same Ser/Thr residue or occupy adjacent residues. For example, in the case of human claudin-1, eight potential O-GlcNAc modifications sites were predicted to be located at T190, T191, S24, S56, S185, S192, S205, and S206. Of these eight potential glycosylation sites, four (T191, S192, S205, and S206) were also predicted as Yin Yang sites. However, there is no compelling evidence suggesting a role for this PTM overlap in claudin function. In addition, N-glycosylation sites have been predicted to exist in the ECL1 of claudin-1 and -12.¹²⁹ Furthermore, claudin-26 homologue TMEM114 is also predicted to be N-glycosylated at position N54 and N88.^{129,130}

In this review, we have undertaken prediction of N-glycosylation sites in all claudin proteins using the NetNGlyc 1.0 Server,¹³¹ where a threshold >0.5 was applied for this analysis. This server predicts N-glycosylation sites in human proteins using artificial neural networks that examine the sequence context of Asn-Xaa-Ser/Thr motifs. The predicted N-glycosylation sites tend to be much less abundant compared to those for O-glycosylation. While potential glycosylation sites were observed for claudins-1, -7, -10, -12, -15, -18, -22, -23, and -24 (all with jury values between 7/9 and 9/9, except for claudin-7 with a jury value of 5/9), most of the predicted N-glycosylation sites are in the helical regions and therefore not likely to be accessible to glycosylation machinery, except for claudin-12 at 47 NLTV, a segment in the ECL1 region. Claudin-12 expressed by transfected HeLa cells is N-glycosylated, as determined by a complex banding pattern observed by immunoblot that compresses into a single band when treated with Peptide-N-Glycosidase F (Koval, unpublished results), indicating that this is a functional glycosylation site. Claudin-12 transfected COS-7 kidney fibroblasts show a similar banding pattern, also suggesting that it is glycosylated.¹³² However, immunoblots of claudin-12 expressed by endothelial cells and in human brain homogenates show predominantly a single band,¹³³ which supports the notion that in an endogenous setting claudin-12 glycosylation is less frequent and instead likely to be a regulated process. Further work is needed to determine the extent of claudin glycosylation in a physiological setting, although most data in the literature suggests that N-glycosylation of claudins in situ is an exceptionally rare event.

Another program called NetOglyc,¹³⁴ which produces neural network predictions of mucin type GalNAc O-glycosylation sites in mammalian proteins, was also used to analyze claudins. Only sites with scores >0.5 are predicted as glycosylated and marked as positive for O-glycosylation. Using this approach, all members of the claudins, except 4, 7, 9, and 24, were predicted to be O-glycosylated (Table 4).

Interestingly, all of the predicted O-glycosylation sites are in close proximity to known or predicted phosphorylation sites of claudins in the C-terminal region.^{72,89,135} This close proximity of the two PTM types may suggest potential crosstalk between O-GlcNAc modification and phosphorylation at these positions. The YinOYang server¹³¹ can calculate the O-glycosylation potential for all Ser, Thr, and Tyr residues in a protein sequence and crosscheck these sites against NetPhos 2.0¹³⁶ predictions for potential phosphorylation sites to determine potential Yin Yang sites with a high possibility for both modifications. Currently, the experimental evidence for mammalian claudin glycosylation exists only in mice on S241 of claudin-16.¹³⁷

It should be noted that the above predictions are purely sequence-based and do not incorporate the structural information on the individual claudin proteins. Given the importance of glycosylation in protein sorting and endocytosis, a more in-depth investigation of potential claudin glycosylation in human is warranted to help understand whether these modifications play roles in regulating claudin localization and recycling.

4.5. Proteolysis

Claudin proteolysis is known to alter the barrier function of intestinal epithelial cells in human and animals.^{138,139} Intestinal barrier integrity is often compromised in autoimmune diseases such as celiac or inflammatory bowel disease, in which gut/intestinal permeability is higher than normal.¹⁴⁰ Such barrier disruption is frequently mediated by proinflammatory cytokines such as IFN- γ , partly through downstream selective cleavage of some claudins. In particular, in T84 (intestinal human epithelial) cells, claudin-2 is specifically cleaved, by yet-to-be identified proteases, in either the ICL or ECL2 after IFN- γ incubation, whereas claudin-1, -3, and -4 in the same cells remain intact.¹⁴¹ In addition, the basal expression level of all claudins is reduced by IFN- γ treatment, and a nonspecific Ser protease inhibitor (AEBSF) is able to significantly rescue this claudin-2 expression loss and cleavage. SwissProt bioinformatics analysis has identified a Ser protease cleavage site in ECL2 of claudin-2 but not in claudin-1, -3, and -4. The transmembrane claudin-2 cleavage is restricted to the Triton X-100 soluble membrane fractions but not in the Triton X-100 insoluble cytoskeletal fraction that contains uncleaved claudin-2. This may suggest that claudin proteolysis is dependent on claudin localization and thus spatially limited, perhaps due to limited access of proteolytic enzymes to the tight junction-associated claudin pool. Nonetheless, most evidence to date suggests that the net effect of proteolysis is to decrease overall junction-associated claudin protein content, perhaps by inhibiting incorporation of newly synthesized claudin into tight junctions. Claudin proteolysis may also contribute to post-translational control of claudin turnover. For instance, claudin-2 and -4 have half-lives of 12 and 4 h, respectively, which is determined by the C-terminal cytoplasmic tail, as demonstrated using chimeric claudin constructs in MDCK cells.¹⁴² Claudin-5 turnover is

cell-dependent, ranging from 70 min in HUVEC¹²⁴ to over 3 h for bovine retinal endothelial cells.¹⁴³

5. MASS SPECTROMETRY OF ENDOGENOUS CLAUDINS

5.1. Mass Spectrometry of Claudins and Their Partners from Cells or Tissues

More recently, MS analysis, often coupled with affinity enrichment, has seen wider use in characterization of endogenous claudins at the protein level given the advantage of the technique in identifying low-abundance membrane proteins. An early demonstration of a claudin-targeted enrichment strategy prior to MS employed a GST-fusion affinity column made from *Clostridium perfringens* enterotoxin (CPE) that binds to the ECL2 of claudin-3, -4, and -7.^{144,145} In these reports, affinity-enriched claudins from cultured rat cholangiocytes were trypsin digested and subjected first to liquid chromatography (LC) and then MS to identify the enriched claudins. Co-elution of some non-CPE binding claudins was also observed by immunoblotting and MS. Relative MS protein quantification using SILAC (stable isotope labeling by amino acids in cell culture) also identified potential plasma membrane-associated claudin-binding proteins as well as claudin-binding proteins in the cytosol, nucleus, and mitochondria. Endogenous human claudin-5 in brain endothelial cells has been enriched by cell fractionation for mass spectrometry.¹⁴⁶ Claudin-5 partners were also identified by coimmunoprecipitation and LC-MS. It was demonstrated with additional siRNA experiments that G-protein subunit *αi2* was a new partner of claudin-5 and required for proper claudin-5 assembly in the tight junction.

Recently, a claudin interactome for *Drosophila* has been reported.¹⁴⁷ Specific antibodies were generated against the claudin Megatrachea in the fly embryos. Subsequent immunoprecipitation, followed by MS analysis, identified 142 protein partners of the claudin Megatrachea, including 10 bona fide members of the septate junctional complex, the invertebrate equivalent to the apical junctional complex. Further validation by RNA interference uncovered new protein partners for Megatrachea interaction including members of the clathrin-mediated vesicle proteins. This is analogous to human claudin-4 internalization by clathrin-mediated endocytosis that depends on a sorting sequence on the ICL and also suggest that there are significant parallels between invertebrate and vertebrate claudin-based protein complexes and regulation.¹⁴⁸ A very recent proteomics investigation on proteins proximal to claudin-4 and occludin in MDCK II cells also significantly expanded the inventory of proteins at the tight junction, particularly in signaling and endocytic trafficking proteins.¹⁴⁹ While a comprehensive analysis of the endogenous human claudins and their interactomes in various cell types remains absent, these studies serve as important foundations to further proteome-wide investigations for human endogenous claudins.

5.2. Membrane Proteomics of Endogenous Claudins

Because endogenous claudins are low-abundance proteins in vertebrate epithelial cells, it is difficult to profile their expression comprehensively without protein-enrichment techniques.¹⁵⁰ Nonetheless, it is apparent that there are tissue specificities to the expression of claudin members and claudin PTMs and that signaling proteins have substrate preferences

for some claudin members over others.¹² Claudin antibodies are widely available and used for specific immuno-detection; however, these need to be carefully monitored and tested for cross-reactivity and nonspecificity.¹⁵¹ Antibodies enable relative claudin expression levels to be monitored by standard immunoblotting methods as well as immunofluorescence imaging. While these approaches are powerful for investigating individual claudins, they are limited in that they do not enable direct calculation of relative claudin stoichiometry and are difficult to employ to measure PTMs.

The accurate and systematic expression and PTM profiling of claudins with spatial and temporal resolution are currently inaccessible without rigorous methods that can isolate, enrich, and distinguish endogenous claudins. Bottom-up membrane proteomics methods have been applied specifically to enrich membrane junction proteins from intestinal human epithelial cells (T84) or mouse liver tissues.^{152,153} A junction-targeting antibody, as validated by immunofluorescence staining, was used to enrich cell junction proteins from T84 that were then immunoblotted to verify the presence of tight junction proteins, silver-stained to verify protein size, and digested for analysis by mass spectrometry.¹⁵² More than 900 proteins were identified, half of which were synaptic or signaling proteins. However, the coverage of known junctional proteins was low. Nevertheless, this study provided a starting point for more in-depth and adequate analysis of membrane junction proteins such as claudins. A subsequent proteomics investigation utilized membrane protein-enrichment protocols involving cell fractionation followed by repeated guanidine treatment and NP-40 extraction. This optimized analysis was able to identify claudin-1 and -3 by MS from the bile canaliculi fraction of mouse livers.¹⁵³ More success in claudin protein identification has been reported in rat renal inner medullar collecting duct cells, in which claudin-3, -4, -7, -8, and -10 were found and listed on the IMCD Proteome Database.^{154,155}

The low abundance of the plasma membrane proteome, concomitant with the inadequate proteomic coverage of the full complement of membrane proteins, has been recognized for some time and explains the challenges in proteomic investigations on claudins or other junctional proteins. Hydrophobic proteins are difficult to capture into matrices to enable separation and subsequent identification, which results in hydrophilic proteins being vastly over-represented in proteomic analyses. Methods for the capture and enrichment of hydrophobic membrane proteins addressing this imbalance include isolation in sodium bicarbonate,¹⁵⁶ aqueous two-phase partitioning using poly(ethylene glycol) and dextran,^{157,158} or nonionic detergents and sucrose gradients for highly insoluble membrane proteins.¹⁵⁹ Recently, a membrane proteome analysis of human embryonic stem cells (hESCs) identified three claudins: claudin-3, -6, and -7.¹⁶⁰ Claudin-6 was the most abundant of the three, which is consistent with genetic analysis showing claudin-6 as the highest expressed claudin member in hESCs.⁶⁹ Although claudin-2, -4, and -12 genes were also reported to be expressed in many hESCs, their protein products were not identified in this analysis. Compared to a prior hESC proteomics study that did not identify any claudin,¹⁶¹ methods with membrane protein enrichment may help with enhancing claudin and tight junction membrane protein coverage.

5.3. Top-Down Proteomics of Claudins

Top-down MS has been in decades of development for characterizing intact proteins.^{162–166} Recently, top-down proteomics has emerged as a powerful approach for characterizing proteins in their native and intact form (Figure 3).^{167–171} Unlike bottom-up proteomics, in which proteins are broken into peptide fragments for identification, top-down proteomics reveals the precise complete proteoform(s) of a protein without loss of the chemical information embedded in the structure of the protein or proteoform.¹⁷⁰ While bottom-up proteomics will remain a powerful approach for obtaining a comprehensive claudin overview, the high level of homology among claudin family members requires further validation for reliable protein identification and characterization. Top-down proteomics in this regard holds the definite promise of capturing the exact chemical details of claudins, which are well below 50 kDa in size, with high fidelity and thus will be able to uncover authentic biological activities encoded by these claudin protein characteristics. In addition, the coverage bias in bottom-up proteomics toward more abundant proteins is better mitigated in the top-down approach. This is particularly relevant to endogenous claudins because, generally, low-abundance membrane proteins with a high level of homology can be prone to misidentification and irreproducible quantification in proteomics.^{172,173}

Advances in top-down proteomics methodology, in particular with improved protein ion excitation and resolution in mass spectrometry for large protein ion fragmentation, have already enabled large-scale, robust protein identification in human cells.^{174–176} For example, HeLa S3 cells have been fractionated by multiple methods in sequence including solution isoelectric focusing, gel-eluted liquid fraction entrapment electrophoresis, and nanocapillary liquid chromatography.¹⁷⁴ The resulting fractions were then analyzed by MS in which whole protein molecules could be ionized and fragmented in tandem for protein identification. In total, over 3000 protein products from 1043 genes were found, displaying a full range of protein diversification by PTMs, RNA splicing, and proteolysis. Even proteins as large as over 100 kDa and up to 11 transmembrane helices were mapped. More importantly, unknown isoforms of endogenous proteins related to senescence were uncovered, thus demonstrating the discovery power of top-down proteomics in unravelling complex biology. This approach has also been expanded to other cell lines such as H1299 with improved proteome coverage of over 5000 proteoforms, including previously unknown lipid-anchored and hyper-phosphorylated proteins. In these HeLa S3 cells, multiple claudin proteoforms have been identified, including claudin-2, -7, -15, and -17. The top-down proteomics approach, combined with specific claudin-enrichment protocols, will likely further increase claudin coverage with reliable PTM information in many other cell types. In addition to a combination of bottom-up and top-down proteomics techniques to ensure sensitive detection and extensive coverage of low-abundance membrane proteins in their native states with accurate PTM information, the middle-down approach, in which limited digestion is used in conjunction with top-down techniques,^{177–180} is also amenable to extracting chemical information in proteomics. This approach has been in use for understanding histone and ribosome PTMs and may very well be suited for the claudin system.

5.4. Targeted Proteomics of Claudins

In targeted proteomics, the mass analysis is focused on one protein or a small set of proteins to reduce the mass bias and interference inherent in complex biological samples for improving sensitivity and quantification. As such, targeted proteomics of the claudin system represents a more quantitative option for claudin stoichiometry and PTM characterization with enhanced accuracy and precision. One earlier example of such approach was demonstrated in quantifying low-abundance proteins, such as growth hormones, in very complex and highly dynamic human plasma samples.¹⁸¹ Since then, target proteomics has become widely adopted in both top-down and bottom-up proteomics for biomarker validation,¹⁸² human plasma proteome analysis,^{183–185} multiplex protein activity assays,¹⁸⁶ PTM profiling,^{187,188} and mass-linked immuno-selective assays.¹⁸⁹ Great progress has also been made in improving the quantification, throughput, and bioinformatics analysis aspects essential for targeted proteomics to scale up and accommodate broader biological applications.^{33–35,190–194} Recently, targeted proteomics has enabled quantitation of claudin-5 expression in the plasma membrane fraction of a human hCMEC/D3 cell line (0.879 fmol of claudin-5/ μ g protein) as well as in human brain microvessels (3.39 fmol of claudin-5/ μ g protein).^{195–197} Similar levels of claudin-5 expression (6–8 fmol/ μ g protein) are also reported from investigations with mouse brain capillaries.⁴¹ For the claudin system that has many homologous members, these advances in targeted proteomics will continue to facilitate a more reliable analysis of low-abundance claudin expression and PTM profiles with temporal and spatial resolution.

6. CHEMICAL CAPTURE AND ENRICHMENT OF ENDOGENOUS CLAUDINS FOR CHEMICAL PROTEOMICS

6.1. Claudin Extraction and Subcellular Fractionation

Existing subcellular or cell compartment fractionation protocols provide spatial segregation and consequentially also enrichment of proteins.^{198,199} However, cytoplasmic aggregates are often observed and can render fractionation inefficient. Because every cultured cell line has different cytoplasmic and cytoskeletal organizations, optimal conditions for an ideal homogenate before fractionation can be highly variant and difficult to obtain. Nonetheless, cellular fractionation has been used regularly provided that some quality control measures such as marker enzymes or morphological analysis are also employed. Subcellular localization of claudins has been investigated in monolayers of intestinal Caco-2 cells, where knockdown of endogenous protein kinase C θ activity decreased membrane- and cytoskeletal-associated claudin-1 and -4 and increased the cytosolic pool of these claudins, as detected by fractionation and immunoblotting.²⁰⁰ In Caco-2 cells, depletion of cholesterol by methyl- β -cyclodextrin leads to displacement of claudin-3, -4, and -7 from the cholesterol-rich membrane domains along with other TJ proteins such as JAM-A and occludin. However, claudin-1 is not affected by this depletion.²⁰¹ Overall, claudin extraction methods have been investigated, albeit more consistently in animal cell models such as MDCK cells.¹⁵⁰ For example, in MDCK II cells, the extractability of claudins is not affected by cholesterol levels; however, different detergents, such as Triton X-100 or CHAPS, partition different claudins into different sizes of aggregates.²⁰² Also in MDCK cells, sodium caprate

is shown to increase solubilities of claudin-4 and -5 in Triton X-100 but not claudin-1, -2, and -3.²⁰³ As it is widely recognized that the ability of a detergent to extract membrane proteins is cell-type-dependent and different detergents differ in their ability to solubilize different membrane proteins,^{150,204} extraction or fractionation of claudins or claudin proteoforms from various cell types and cellular compartments will remain extremely challenging and likely require cell-type-specific optimization of isolation protocols prior to mass spectrometry.

6.2. Covalent Tagging of Endogenous Claudins

Another related issue is claudin enrichment, which can be partially achieved by subcellular fractionation. However, multispan membrane proteins such as claudins are, in general, more resistant to extraction and identification. While methods on the basis of noncovalent binding are helpful,²⁰⁵ approaches for covalent labeling of endogenous claudins are needed for a reproducible capture and enrichment protocol with spatial resolution even at very low expression levels. The approach of chemical proteomics with protein tagging, in which proteins are modified by small molecule probes carrying an affinity anchor, fluorescent reporter, and other functional groups for post-capture analysis, may be suited for system-wide profiling of protein or enzyme activities.^{206–210} Covalent labeling of claudins in the plasma membrane is frequently achieved by using amine- or thiol-reactive small molecule probes.^{211–215}

The thiol-based labeling strategy is used more widely for labeling cysteines in claudins from both human and other organisms.^{216,217} Reactive epitopes such as non-cell-permeable biotinylated NHS esters were used to label HEK293 cells transfected with a mutant claudin-5 expression vector in order to quantify the plasma membrane localization level of the claudin-5 mutant.²¹² Cysteine mutants of claudin-2 were generated in MDCK I cells for thiol-labeling in order to determine the protein surface accessibility of claudin-2 in cells.²¹³ A thiol cross-linker was also successfully utilized to identify, by MS after covalent labeling, the tetraspanin interacting partners in A431 and A549 cells.²¹¹ Amine-reactive labels have also shown the ability to label claudins in cells or animals.^{218,219} New-born mice with or without claudin-1 were subcutaneously injected with an amine-reactive biotinylation solution in order to assess the extent of barrier function loss in the mice epidermis lacking claudin-1.²¹⁸ Amine-reactive biotinylation agents likewise showed labeling of tight junction claudins in the mouse Eph4 cells junctions for permeability visualization upon treatment with fluorescent molecule-labeled avidin.²¹⁹ These precedents suggest that cell surface claudins can be accessible to covalent chemical tagging.

Claudins have also been found in other cellular compartments such as endosomes that likely reflect intracellular compartments active in tight junction turnover.^{69,71} Specifically, surface claudins in MDCK II cells have been differentiated from internal claudins by covalent surface biotinylation labeling of the cell surface. This differential labeling in these cells was able to demonstrate in MDCK cells that the endocytic recycling of claudin-1 and -2 was affected in the presence of an endocytosis inhibitor, resulting in the intracellular accumulation of these claudins, whereas other claudins were not affected.²¹⁵ Chemical tagging of claudins with spatial resolution may be desirable for tracing claudin dynamics in

the cell but requires further development of new chemical tags that are specific for endogenous claudins. Such improved chemical tools, in conjunction with subcellular fractionation and high-fidelity methods from advanced proteomics technology, will help to chemically characterize claudin protein forms as potentially the combinatorial protein codes that coordinate cell contact signaling and regulate claudin turnover to control tissue identity and integrity.⁷⁵

7. TOWARD MOLECULAR IDENTIFICATION OF CLAUDIN-MEDIATED CELL-CONTACT SIGNALING PLATFORMS

Multicellular organisms rely on coordinated cellular processes to maintain tissue stability and coordinate cell physiology. Cell–cell contact sites represent a critical structure that enables intercellular communication by organizing the formation of large multiprotein complexes (Figure 4). For example, intercellular junctions act as sensors for cell contact that regulate signaling by forming protein complexes that activate kinases and also regulate gene expression by coordinating the transit of transcription factors between the plasma membrane and nucleus.^{220–222,15,44,223} Relevant to claudins, the relatively unstructured intracellular C-terminal region contains several putative and identified binding motifs that promote protein–protein interactions, most prominently the PDZ binding motif.¹ Loss of the C-terminal tail leads to intracellular retention of claudins in the ER and proteasome degradation, possibly by improper protein folding or lack of binding to trafficking partners.^{224–226} These serial truncation experiments, however, demonstrate that the PDZ binding motif is not required for proper claudin trafficking to the plasma membrane and reveal the critical role of the juxtamembrane region of about 20 amino acids in localization control of claudins.

Many excellent reviews and articles have already emphasized that specific claudins, localized to various subcellular locations, interact with many other proteins to form specific scaffolds for signaling complexes in different cell types of various organisms.^{8,14,46,47,86,227,228} Some recent examples in human cells are highlighted here, while many other related examples are also known in other mammals such as rats and mice,^{132,229–231} along with recent proteomic analyses of MDCK-II cells that significantly expand the list of known and new signaling proteins assembled in and around the tight junction as discussed earlier.¹⁴⁹

HEK293 cells are commonly used as nonpolarized, tight junction-free cell models for cell–contact investigations after transfection with claudins.²³² In transfected HEK293 cells, classic claudins such as claudin-1, -3, and -5, are found mainly at the cell–cell contact points and provide multilateral interaction with other tight junction proteins such as occludin, tricellulin, and MarvelD3 in order to secure the TJ strand network for stable barrier function.^{80,233–235} The dynamic aspect of claudin localization in the cell membrane is also demonstrated for claudin-2 and -4 in SKCO 15 or Caco-2 BBE (brush border expressing) cells in response to environmental changes such as cytokine exposure.²³⁶ This is consistent with the existing notion that claudins can mediate junction remodeling and recruit other TJ proteins.

Claudin expression and dynamics are also known to be regulated by epithelial cell adhesion molecule (EpCAM) in epithelial cells.²³⁷ Complexes between claudin-7 and EpCAM have been demonstrated by coimmunoprecipitation experiments and shown to recruit proteins into tetraspanin-enriched membrane microdomains.²³⁸ In HEK293 cells expressing EpCAM, the proliferative activities of EpCAM require formation of a complex with claudin-7 in order to disrupt EpCAM oligomerization and activate mitogenic signaling. Mice injected with HEK–EpCAM–claudin-7 cells developed tumors, whereas HEK cells without the EpCAM–claudin-7 complex did not induce tumors in the animal.²³⁹ In a transgenic mouse model expressing human claudin-1, Notch signaling, a critical regulator of intestinal epithelial cell differentiation and lineage determination, is upregulated by claudin-1 over-expression with concurrent upregulation of MMP-9 activity and p-ERK signaling.²⁴⁰ In MDA-MB-231 cells, claudin-5 overexpression increases cell motility and coimmunoprecipitates with N-WASP and ROCK1, suggesting a possible role of claudin-5 in metastasis.²⁴¹ In HEK293 cells, claudin-2 expression is regulated by components in Wnt signaling, whereby expression of both LEF-1 and β -catenin enhanced the promoter activity of claudin-2.²⁴² In human colonic cancer cell line SW480, claudin-1 is also identified as a downstream target of Wnt/ β -catenin signaling, and increased claudin-1 expression is observed in all 16 primary colorectal cancers investigated with significant claudin-1 localization detected in cell–cell boundaries and the cytoplasm.²⁴³

The nonjunctional localization of some claudins has also been linked to disease progression in various epithelial cancers, which has been extensively reviewed.^{74–77,89,244–246} For example, in craniopharyngioma, claudin-1 expression is reduced in invasive tumors compared to that in noninvasive ones, and increased cytoplasmic claudin-1 localization is detected by immunostaining in tumor cells that border brain tissues and dura.²⁴⁷ In invasive breast cancer, claudin-1 is frequently downregulated, but it is upregulated in some aggressive subtypes of basal-like breast cancer.²⁴⁸ Higher claudin-1 protein expression is observed in tumors from older patients, accompanied by more frequent cytoplasmic claudin-1 localization.²⁴⁹ In melanoma tissue samples, both cytoplasmic and nuclear claudin-1 expression is frequently observed, and nuclear claudin-1 expression is drastically reduced in lymph node metastases.²⁵⁰ In vitro models demonstrate a positive link between higher MMP-2 activity and higher cytoplasmic claudin-1 expression level, suggesting a role for claudin-1 in melanoma progression. This is not restricted to claudin-1, since, in proliferating human lung adenocarcinoma A549 cells or tissues, claudin-2 is found in the nucleus as part of a complex containing ZO-1, ZONAB, and cyclin D1.⁷³ Compared to the wild type, a S208A mutant of claudin-2 exhibits a higher extent of nuclear localization in its dephosphorylated form, suggesting that nuclear claudin-2 may serve to retain ZONAB and cyclin D1 in the nucleus to enhance cell proliferation. Such a proliferation-promoting effect is also suggested for claudin-1 in osteoblasts.²⁵¹ Likewise, both claudin-7 transcript and protein expression levels are frequently elevated in epithelial ovarian tumors but not in normal ovarian tissues, and in many ovarian cancer cell lines both cytoplasmic and cell membrane claudin-7 expression are detected by immunostaining.²⁵² However, the mechanisms and molecular details behind the change of claudin localization remain unclear, particularly when localized to the cytosol or nucleus.

The diverse localization and binding partners identified using standard biochemical techniques suggest that regulation of claudin function is complex. The acquisition of the exact chemical characteristics of the specific claudin protein forms, from different cell locations by MS, in addition to their levels of expression, may help to elucidate the details of the signaling context specific to cell/tissue types or disease states. Initial attempts at identifying protein partners of claudins are listed in Table 5. Those interactions were retrieved from SwissProt and IntAct databases. Reflecting the most prominent function of claudins, namely, formation of epithelial barriers via tight junctions, claudin–claudin and claudin–scaffold protein interactions are well-represented in Table 5. This list will certainly be expanded and refined as more claudin-specific protein–protein interactions are characterized from an expanded range of host tissues.

The range of protein partnership for claudins can be illustrated by a protein interaction network diagram using human claudin-1 as an example (Figure 5). Direct protein interactions with human claudin-1 were obtained by careful mining of the literature. Extended human protein interactions between protein partners of claudin-1 and other proteins were retrieved from UniProtKB/SwissProt and from IntAct (only interactions found by more than one experiment). The domain-specific annotations of these protein interactions with claudin-1 are listed in Table 6. As not all the interactions described in the literature are yet imported into UniProtKB/SwissProt and IntAct this interaction network may be more complex.

While the human claudin-1 diagram does not include its non-human protein partners, pathogenic proteins have been found to interact with human claudin-1. For example, human claudin-1 along with occludin and the tetraspanin receptor CD81 mediates the initial step of hepatitis C virus (HCV) infection.²⁶⁸ During this process, CD81 directly interacts with the envelope glycoproteins HCVE1 and HCVE2.²⁶⁹ The direct physical interaction between claudin-1 and the HCVE1/2 glycoproteins has not been definitively established but inferred by the ability of the particles expressing the HCV glycoproteins to bind claudin-1-expressing cells.^{270,271} A direct interaction between claudin-1 and the premembrane (prM) protein from the dengue virus has been suggested to be important for dengue virus entry.²⁷² Interestingly, some of the protein partners of human claudin-1 were also shown to interact with viral proteins: MPDZ with the adenovirus type 9 E4 protein,²⁷³ LNX1 with the endogenous retrovirus K protein Np9,²⁷⁴ and SRC with the hepatitis E virus ORF3 protein.²⁷⁵ Human claudin-1 is also involved in cell infection by nonviral pathogens. A recent study suggests that the intestinal epithelial damage caused by the protozoan *Entamoeba histolytica* is initiated by the interaction of the virulence complex EhCPADH112 (formed by CP112/EhCP112 and ADH112/EhADH112) with claudin-1, occludin, TJP1/ZO-1, and TJP2/ZO-2, followed by their degradation.²⁷⁶

In addition to standard biophysical techniques, MS is a particularly useful technique for acquiring complementary information on stoichiometry and topology of protein complexes.^{277–283} Although lower in resolution as compared with techniques with atomic resolution, MS-based information is crucial to understanding dynamic protein interactions and networks that govern biological function.²⁸⁰ Integrated approaches that combine PTM analysis by MS-based proteomics, protein crossing-linking, protein complexes

characterization by MS, and structural modeling may offer new structural understanding of endogenous protein partnerships or interactomes.^{278,279,282} Examples of complex multicomponent systems that have been successfully analyzed by MS-coupled quantitative proteomics include large protein assemblies such as the human initiation factor (eIF)3 complex and the yeast RNA exosome. Recent advances in ionization and dissociation methods, including surface-induced collision and ion-mobility MS procedures, also greatly improve the prospect of MS in characterizing intact protein complexes in their native states.^{277,281} These new technological capacities, in conjunction with enrichment techniques, may begin to help shed light on the supramolecular structures of claudin protein complexes that define the signaling context at and beyond cell junctions.

8. CONCLUSIONS

The predominant role for claudins in regulating epithelial and endothelial barrier function necessitates that they need to be able to interact with a multiplicity of proteins in diverse ways, including trans (intercellular) interactions with extracellular domains of proteins across intercellular junctions, cis (in the plane of the plasma membrane) interactions with trans-membrane proteins, and binding to scaffold proteins that regulate their organization and stability.²⁸⁴ Thus, claudins have two roles in regulating tight junction permeability: a direct role by forming different classes of paracellular channels and a more indirect role by participating in the formation of the large multiprotein complexes associated with claudin-containing tight junction strands. Although considerable progress has been made to understand how claudin diversity affects paracellular channel selectivity, precisely defining claudin binding partners at a quantitative level is still at an early stage of research. The ensemble of proteins linked to claudins in tight junctions affects paracellular flux through control of strand morphology, localization, and stability. Understanding how protein cofactors mediate intercellular interactions, cross-linking of claudins with the actin cytoskeleton, and integration with signal transduction pathways will provide new insights into how tissue barrier function is regulated in normal and diseased organs, as well as potential therapeutic targets for the manipulation of paracellular flux.

The regulatory role conferred by the claudin family of proteins in tight junctions is complemented by roles in morphogenesis, tissue maintenance, growth control, and cell migration. All of these processes require constant protein processing and modification. Individual claudin members have been investigated biochemically or in cell models/tissues for their expression and localization profile, and some claudin members have been characterized by MS in addition to antibody-based detection. Many claudin PTMs, such as C-terminal phosphorylation and palmitoylation, have been identified. There are no doubt many more claudin phosphorylation sites remaining to be identified, and future studies are needed to provide added evidence in support of PTMs such as ubiquitination, SUMOylation, palmitoylation, and glycosylation.

More importantly, it remains an enormous challenge to acquire a direct and accurate profile of claudins, PTMs, and interacting partners when considering that claudins are low-abundance, membrane-bound, and heavily modified transmembrane proteins. While the function of tight junction-associated claudins is largely to regulate the paracellular barrier,

nonjunctional claudins in the lateral plasma membrane, cytosol, or nucleus likely have distinct roles in cell regulation. Complementary to antibody-based detection that will continue to be a major method of investigation, advanced chemical capture, enrichment, and analysis tools, tailored for high-precision MS-based proteomic profiling in a multipronged and integrated manner, could provide unbiased approaches to identify claudin PTM and interacting proteins. Defining claudin PTMs and interacting proteins in a quantitative and stoichiometric manner has the potential to determine whether the spectrum of claudin expression not only controls paracellular flux but also integrates the flow of information by acting in effect as a cell “identity code” that is recognized across intercellular junctions. Understanding this context in which claudins affect cells could enable a nuanced approach to therapeutic design by either modulating claudin barrier function (e.g., for the purposes of drug delivery) or affecting other nonbarrier cell functions regulated by claudins, such as cell proliferation and migration. By defining the different molecular contexts of claudin expression, systems proteomics is a valuable approach that will make it possible to consider specifically targeting junctional vs nonjunctional roles for claudins.

ACKNOWLEDGMENTS

We thank the Australian Research Council (LIEF150100161 to F.L. and S.R.), the National Institute of Health (R01-HL116958 to M.K.), the National Health and Medical Research Council of Australia (NHMRC APP1010303 to M.S.B.), and the New South Wales Cancer Council (RG10-04 and RG08-16 to M.S.B.) for financial support.

REFERENCES

1. Gunzel D, Yu AS. Claudins and the modulation of tight junction permeability. *Physiol. Rev.* 2013; 93:525–569. [PubMed: 23589827]
2. Schneeberger EE, Lynch RD. The tight junction: a multifunctional complex. *Am. J. Physiol Cell Physiol.* 2004; 286:C1213–C1228. [PubMed: 15151915]
3. Tsukita S, Furuse M, Itoh M. Structural and signalling molecules come together at tight junctions. *Curr. Opin. Cell Biol.* 1999; 11:628–633. [PubMed: 10508648]
4. Furuse M. Molecular Basis of the Core Structure of Tight Junctions. *Cold Spring Harbor Perspect. Biol.* 2010; 2:a002907–a002907.
5. Tsukita S, Furuse M. The structure and function of claudins, cell adhesion molecules at tight junctions. *Ann. N. Y. Acad. Sci.* 2000; 915:129–135. [PubMed: 11193568]
6. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell Biol.* 2001; 2:285–293. [PubMed: 11283726]
7. Van Itallie CM, Anderson JM. Claudins and Epithelial Paracellular Transport. *Annu. Rev. Physiol.* 2006; 68:403–429. [PubMed: 16460278]
8. Koval M. Claudins—key pieces in the tight junction puzzle. *Cell Commun. Adhes.* 2006; 13:127–138. [PubMed: 16798613]
9. Van Itallie CM, Anderson JM. Architecture of tight junctions and principles of molecular composition. *Semin. Cell Dev. Biol.* 2014; 36:157–165. [PubMed: 25171873]
10. Gunzel D, Fromm M. Claudins and other tight junction proteins. *Comprehensive Physiology.* 2012; 2:1819–1852. [PubMed: 23723025]
11. Van Itallie CM, Anderson JM. The role of claudins in determining paracellular charge selectivity. *Proc. Am. Thorac. Soc.* 2004; 1:38–41. [PubMed: 16113410]
12. Matter K, Balda MS. Signalling to and from tight junctions. *Nat. Rev. Mol. Cell Biol.* 2003; 4:225–236. [PubMed: 12612641]
13. Findley MK, Koval M. Regulation and roles for claudin-family tight junction proteins. *IUBMB Life.* 2009; 61:431–437. [PubMed: 19319969]

14. Van Itallie CM, Anderson JM. Claudin interactions in and out of the tight junction. *Tissue Barriers*. 2013; 1:e25247. [PubMed: 24665401]
15. Zihni C, Balda MS, Matter K. Signalling at tight junctions during epithelial differentiation and microbial pathogenesis. *J. Cell Sci*. 2014; 127:3401–3413. [PubMed: 25125573]
16. Farquhar MG, Palade GE. Junctional complexes in various epithelia. *J. Cell Biol*. 1963; 17:375–412. [PubMed: 13944428]
17. Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J. Cell Biol*. 1998; 141:1539–1550. [PubMed: 9647647]
18. Loh YH, Christoffels A, Brenner S, Hunziker W, Venkatesh B. Extensive expansion of the claudin gene family in the teleost fish, *Fugu rubripes*. *Genome Res*. 2004; 14:1248–1257. [PubMed: 15197168]
19. Lal-Nag M, Morin PJ. The claudins. *Genome Biol*. 2009; 10:235. [PubMed: 19706201]
20. Angelow S, Ahlstrom R, Yu ASL. Biology of claudins. *AJP: Renal Physiology*. 2008; 295:F867–F876. [PubMed: 18480174]
21. Anderson JM, Van Itallie CM. Physiology and Function of the Tight Junction. *Cold Spring Harbor Perspect. Biol*. 2009; 1:a002584–a002584.
22. Tamura A, Tsukita S. Paracellular barrier and channel functions of TJ claudins in organizing biological systems: advances in the field of barrierology revealed in knockout mice. *Semin. Cell Dev. Biol*. 2014; 36:177–185. [PubMed: 25305579]
23. Troy TC, Turksen K. The targeted overexpression of a Claudin mutant in the epidermis of transgenic mice elicits striking epidermal and hair follicle abnormalities. *Mol. Biotechnol*. 2007; 36:166–174. [PubMed: 17914196]
24. Pope JL, et al. Claudin-1 overexpression in intestinal epithelial cells enhances susceptibility to adenomatous polyposis coli-mediated colon tumorigenesis. *Mol. Cancer*. 2014; 13:167. [PubMed: 24997475]
25. Daugherty BL, et al. Developmental regulation of claudin localization by fetal alveolar epithelial cells. *American journal of physiology. Lung cellular and molecular physiology*. 2004; 287:L1266–1273. [PubMed: 15347569]
26. Shen L, Weber CR, Turner JR. The tight junction protein complex undergoes rapid and continuous molecular remodeling at steady state. *J. Cell Biol*. 2008; 181:683–695. [PubMed: 18474622]
27. Camire RB, et al. Biphasic modulation of paracellular claudin-5 expression in mouse brain endothelial cells is mediated through the phosphoinositide-3-kinase/AKT pathway. *J. Pharmacol. Exp. Ther*. 2014; 351:654–662. [PubMed: 25281324]
28. Garcia-Hernandez V, et al. EGF regulates claudin-2 and -4 expression through Src and STAT3 in MDCK cells. *J. Cell. Physiol*. 2015; 230:105–115. [PubMed: 24909426]
29. Twiss F, et al. HGF signaling regulates Claudin-3 dynamics through its C-terminal tyrosine residues. *Tissue Barriers*. 2013; 1:e27425. [PubMed: 24665413]
30. Van Itallie CM, et al. Phosphorylation of claudin-2 on serine 208 promotes membrane retention and reduces trafficking to lysosomes. *J. Cell Sci*. 2012; 125:4902–4912. [PubMed: 22825868]
31. Wu CC, Yates JR 3rd. The application of mass spectrometry to membrane proteomics. *Nat. Biotechnol*. 2003; 21:262–267. [PubMed: 12610573]
32. Tan S, Tan HT, Chung MC. Membrane proteins and membrane proteomics. *Proteomics*. 2008; 8:3924–3932. [PubMed: 18763712]
33. Deutsch EW, Lam H, Aebersold R. PeptideAtlas: a resource for target selection for emerging targeted proteomics workflows. *EMBO Rep*. 2008; 9:429–434. [PubMed: 18451766]
34. Surinova S, et al. Automated selected reaction monitoring data analysis workflow for large-scale targeted proteomic studies. *Nat. Protoc*. 2013; 8:1602–1619. [PubMed: 23887179]
35. Toprak UH, et al. Conserved peptide fragmentation as a benchmarking tool for mass spectrometers and a discriminating feature for targeted proteomics. *Mol. Cell. Proteomics*. 2014; 13:2056. [PubMed: 24623587]

36. Valaskovic GA, Kelleher NL. Miniaturized formats for efficient mass spectrometry-based proteomics and therapeutic development. *Curr. Top. Med. Chem.* 2002; 2:1–12. [PubMed: 11899062]
37. Sali A, Glaeser R, Earnest T, Baumeister W. From words to literature in structural proteomics. *Nature.* 2003; 422:216–225. [PubMed: 12634795]
38. Heck AJ, Van Den Heuvel RH. Investigation of intact protein complexes by mass spectrometry. *Mass Spectrom. Rev.* 2004; 23:368–389. [PubMed: 15264235]
39. Greenbaum D, et al. Chemical approaches for functionally probing the proteome. *Mol. Cell. Proteomics.* 2002; 1:60–68. [PubMed: 12096141]
40. Adam GC, Sorensen EJ, Cravatt BF. Chemical strategies for functional proteomics. *Mol. Cell. Proteomics.* 2002; 1:781–790. [PubMed: 12438561]
41. Uchida Y, et al. A study protocol for quantitative targeted absolute proteomics (QTAP) by LC-MS/MS: application for interstrain differences in protein expression levels of transporters, receptors, claudin-5, and marker proteins at the blood-brain barrier in ddY, FVB, and C57BL/6J mice. *Fluids Barriers CNS.* 2013; 10:21. [PubMed: 23758935]
42. Koval M. Differential pathways of claudin oligomerization and integration into tight junctions. *Tissue Barriers.* 2013; 1:e24518. [PubMed: 24665398]
43. Ivanov AI, Parkos CA, Nusrat A. Cytoskeletal regulation of epithelial barrier function during inflammation. *Am. J. Pathol.* 2010; 177:512–524. [PubMed: 20581053]
44. McCrean PD, Gu D, Balda MS. Junctional music that the nucleus hears: cell-cell contact signaling and the modulation of gene activity. *Cold Spring Harbor Perspect. Biol.* 2009; 1:a002923.
45. Matter K, Balda MS. SnapShot: Epithelial tight junctions. *Cell.* 2014; 157:992–992.e1. [PubMed: 24813618]
46. Gonzalez-Mariscal L, Tapia R, Chamorro D. Crosstalk of tight junction components with signaling pathways. *Biochim. Biophys. Acta, Biomembr.* 2008; 1778:729–756.
47. Balda MS, Matter K. Tight junctions and the regulation of gene expression. *Biochim. Biophys. Acta, Biomembr.* 2009; 1788:761–767.
48. Capaldo CT, Nusrat A. Claudin switching: Physiological plasticity of the Tight Junction. *Semin. Cell Dev. Biol.* 2015; 42:22–29. [PubMed: 25957515]
49. UniProt Consortium. UniProt: a hub for protein information. *Nucleic Acids Res.* 2015; 43:D204–212. [PubMed: 25348405]
50. Cunningham F, et al. Ensembl 2015. *Nucleic Acids Res.* 2015; 43:D662–669. [PubMed: 25352552]
51. Uhlen M, et al. Towards a knowledge-based Human Protein Atlas. *Nat. Biotechnol.* 2010; 28:1248–1250. [PubMed: 21139605]
52. Morita K, Furuse M, Fujimoto K, Tsukita S. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. *Proc. Natl. Acad. Sci. U. S. A.* 1999; 96:511–516. [PubMed: 9892664]
53. Mineta K, et al. Predicted expansion of the claudin multigene family. *FEBS Lett.* 2011; 585:606–612. [PubMed: 21276448]
54. Baltzegar DA, Reading BJ, Brune ES, Borski RJ. Phylogenetic revision of the claudin gene family. *Marine Genomics.* 2013; 11:17–26. [PubMed: 23726886]
55. Gray KA, Yates B, Seal RL, Wright MW, Bruford EA. Genenames.org: the HGNC resources in 2015. *Nucleic Acids Res.* 2015; 43:D1079–1085. [PubMed: 25361968]
56. Safran M, et al. GeneCards Version 3: the human gene integrator. *Database.* 2010; 2010:baq020. [PubMed: 20689021]
57. Krause G, et al. Structure and function of claudins. *Biochim. Biophys. Acta, Biomembr.* 2008; 1778:631–645.
58. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat. Methods.* 2008; 5:621–628. [PubMed: 18516045]
59. Kollmar R, Nakamura SK, Kappler JA, Hudspeth AJ. Expression and phylogeny of claudins in vertebrate primordia. *Proc. Natl. Acad. Sci. U. S. A.* 2001; 98:10196–10201. [PubMed: 11517306]

60. Sigrist CJA, et al. PROSITE, a protein domain database for functional characterization and annotation. *Nucleic Acids Res.* 2010; 38:D161–D166. [PubMed: 19858104]
61. Turksen K. Barriers built on claudins. *J. Cell Sci.* 2004; 117:2435–2447. [PubMed: 15159449]
62. Harris BZ, Lim WA. Mechanism and role of PDZ domains in signaling complex assembly. *J. Cell Sci.* 2001; 114:3219–3231. [PubMed: 11591811]
63. Nomme J, et al. Structural Basis of a Key Factor Regulating the Affinity between the Zonula Occludens First PDZ. Domain and Claudins. *J. Biol. Chem.* 2015; 290:16595–16606. [PubMed: 26023235]
64. Suzuki H, et al. Crystal structure of a claudin provides insight into the architecture of tight junctions. *Science.* 2014; 344:304–307. [PubMed: 24744376]
65. Saitoh Y, et al. Tight junctions. Structural insight into tight junction disassembly by *Clostridium perfringens* enterotoxin. *Science.* 2015; 347:775–778. [PubMed: 25678664]
66. Horvatovich P, et al. Quest for Missing Proteins: Update 2015 on Chromosome-Centric Human Proteome Project. *Journal of proteome research.* 2015; 14:3415. [PubMed: 26076068]
67. Uhlen M, Oksvold P, Algenas C, Hamsten C, Fagerberg L, Klevebring D, Lundberg E, Odeberg J, Ponten F, Kondo T, Sivertsson A. Antibody-based protein profiling of the human chromosome 21. *Mol. Cell. Proteomics.* 2012; 11:M111.013458.
68. Zheng A, Yuan F, Li Y, Zhu F, Hou P, Li J, Song X, Ding M, Deng H. Claudin-6 and claudin-9 function as additional coreceptors for hepatitis C virus. *J. Virol.* 2007; 81:12465–12471. [PubMed: 17804490]
69. Hewitt KJ, Agarwal R, Morin PJ. The claudin gene family: expression in normal and neoplastic tissues. *BMC Cancer.* 2006; 6:186. [PubMed: 16836752]
70. Yoshida Y, Ban Y, Kinoshita S. Tight junction transmembrane protein claudin subtype expression and distribution in human corneal and conjunctival epithelium. *Invest. Ophthalmol. Visual Sci.* 2009; 50:2103–2108. [PubMed: 19117933]
71. Dhawan P, Singh AB, Deane NG, No Y, Shiou S-R, Schmidt C, Neff J, Washington MK, Beauchamp RD. Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. *J. Clin. Invest.* 2005; 115:1765–1776. [PubMed: 15965503]
72. French AD, et al. PKC and PKA phosphorylation affect the subcellular localization of claudin-1 in melanoma cells. *Int. J. Med. Sci.* 2009; 6:93–101. [PubMed: 19305641]
73. Ikari A, Watanabe R, Sato T, Taga S, Shimobaba S, Yamaguchi M, Yamazaki Y, Endo S, Matsunaga T, Sugatani J. Nuclear distribution of claudin-2 increases cell proliferation in human lung adenocarcinoma cells. *Biochim. Biophys. Acta, Mol. Cell Res.* 1843; 1843:2079–2088.
74. Morin PJ. Claudin proteins in human cancer: promising new targets for diagnosis and therapy. *Cancer Res.* 2005; 65:9603–9606. [PubMed: 16266975]
75. Gupta IR, Ryan AK. Claudins: unlocking the code to tight junction function during embryogenesis and in disease. *Clin. Genet.* 2010; 77:314–325. [PubMed: 20447145]
76. Kwon MJ. Emerging roles of claudins in human cancer. *Int. J. Mol. Sci.* 2013; 14:18148–18180. [PubMed: 24009024]
77. Singh AB, Sharma A, Dhawan P. Claudin family of proteins and cancer: an overview. *J. Oncol.* 2010; 2010:541957. [PubMed: 20671913]
78. Singh AB, Dhawan P. Claudins and cancer: Fall of the soldiers entrusted to protect the gate and keep the barrier intact. *Semin. Cell Dev. Biol.* 2015; 42:58. [PubMed: 26025580]
79. Anderson JM, Van Itallie CM, Fanning AS. Setting up a selective barrier at the apical junction complex. *Curr. Opin. Cell Biol.* 2004; 16:140–145. [PubMed: 15196556]
80. Cording J, et al. In tight junctions, claudins regulate the interactions between occludin, tricellulin and marvelD3, which, inversely, modulate claudin oligomerization. *J. Cell Sci.* 2013; 126:554–564. [PubMed: 23203797]
81. Krug SM, Schulzke JD, Fromm M. Tight junction, selective permeability, and related diseases. *Semin. Cell Dev. Biol.* 2014; 36:166–176. [PubMed: 25220018]
82. Hou J, Rajagopal M, Yu AS. Claudins and the kidney. *Annu. Rev. Physiol.* 2013; 75:479–501. [PubMed: 23140368]

83. Konrad M, Hou J, Weber S, Dotsch J, Kari JA, Seeman T, Kuwertz-Broking E, Peco-Antic A, Tasic V, Dittrich K, Alshaya HO, von Vigier RO, Gallati S, Goodenough DA, Schaller A. CLDN16 genotype predicts renal decline in familial hypomagnesemia with hypercalciuria and nephrocalcinosis. *J. Am. Soc. Nephrol.* 2008; 19:171–181. [PubMed: 18003771]
84. Konrad M, et al. Mutations in the tight-junction gene claudin 19 (CLDN19) are associated with renal magnesium wasting, renal failure, and severe ocular involvement. *Am. J. Hum. Genet.* 2006; 79:949–957. [PubMed: 17033971]
85. Simon DB, et al. Paracellin-1, a renal tight junction protein required for paracellular Mg²⁺ resorption. *Science.* 1999; 285:103–106. [PubMed: 10390358]
86. González-Mariscal L, Garay E, Quirós M. Regulation of Claudins by Posttranslational Modifications and Cell-Signaling Cascades. *Curr. Top. Membr.* 2010; 65:113–150.
87. Tanaka M, Kamata R, Sakai R. EphA2 Phosphorylates the Cytoplasmic Tail of Claudin-4 and Mediates Paracellular Permeability. *J. Biol. Chem.* 2005; 280:42375–42382. [PubMed: 16236711]
88. Shen W, et al. Tyrosine phosphorylation of VE-cadherin and claudin-5 is associated with TGF-beta1-induced permeability of centrally derived vascular endothelium. *Eur. J. Cell Biol.* 2011; 90:323–332. [PubMed: 21168935]
89. D'Souza T, Agarwal R, Morin PJ. Phosphorylation of claudin-3 at threonine 192 by cAMP-dependent protein kinase regulates tight junction barrier function in ovarian cancer cells. *J. Biol. Chem.* 2005; 280:26233–26240. [PubMed: 15905176]
90. Hoggard J, et al. Claudin-7 increases chemosensitivity to cisplatin through the upregulation of caspase pathway in human NCI-H522 lung cancer cells. *Cancer Sci.* 2013; 104:611–618. [PubMed: 23433123]
91. Tatum R, et al. WNK4 phosphorylates ser(206) of claudin-7 and promotes paracellular Cl(–) permeability. *FEBS Lett.* 2007; 581:3887–3891. [PubMed: 17651736]
92. Hornbeck PV, et al. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res.* 2015; 43:D512–520. [PubMed: 25514926]
93. Fenyo D, Eriksson J, Beavis R. Mass spectrometric protein identification using the global proteome machine. *Methods Mol. Biol. (N. Y., NY, U. S.).* 2010; 673:189–202.
94. Mayya V, et al. Quantitative phosphoproteomic analysis of T cell receptor signaling reveals system-wide modulation of protein-protein interactions. *Sci. Signaling.* 2009; 2:ra46.
95. Rigbolt KT, et al. System-wide temporal characterization of the proteome and phosphoproteome of human embryonic stem cell differentiation. *Sci. Signaling.* 2011; 4:rs3.
96. Bai Y, Li J, Fang B, Edwards A, Zhang G, Bui M, Eschrich S, Altioik S, Koomen J, Haura EB. Phosphoproteomics identifies driver tyrosine kinases in sarcoma cell lines and tumors. *Cancer Res.* 2012; 72:2501–2511. [PubMed: 22461510]
97. Shiromizu T, Adachi J, Watanabe S, Murakami T, Kuga T, Muraoka S, Tomonaga T. Identification of missing proteins in the neXtProt database and unregistered phosphopeptides in the PhosphoSitePlus database as part of the Chromosome-centric Human Proteome Project. *J. Proteome Res.* 2013; 12:2414–2421. [PubMed: 23312004]
98. Kim J-Y, Welsh EA, Oguz U, Fang B, Bai Y, Kinose F, Bronk C, Remsing Rix LL, Beg AA, Rix U, Eschrich SA, Koomen JM, Haura EB. Dissection of TBK1 signaling via phosphoproteomics in lung cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* 2013; 110:12414–12419. [PubMed: 23836654]
99. Phanstiel DH, Brumbaugh J, Wenger CD, Tian S, Probasco MD, Bailey DJ, Swaney DL, Tervo MA, Bolin JM, Ruotti V, Stewart R, Thomson JA, Coon JJ. Proteomic and phosphoproteomic comparison of human ES and iPS cells. *Nat. Methods.* 2011; 8:821–827. [PubMed: 21983960]
100. Gu T-L, Deng X, Huang F, Tucker M, Crosby K, Rimkunas V, Wang Y, Deng G, Zhu L, Tan Z, et al. Survey of tyrosine kinase signaling reveals ROS kinase fusions in human cholangiocarcinoma. *PLoS One.* 2011; 6:e15640. [PubMed: 21253578]
101. Ding VMY, Boersema PJ, Foong LY, Preisinger C, Koh G, Natarajan S, Lee D-Y, Boekhorst J, Snel B, Lemeer S, Heck AJR, Choo A. Tyrosine phosphorylation profiling in FGF-2 stimulated human embryonic stem cells. *PLoS One.* 2011; 6:e17538. [PubMed: 21437283]
102. Moritz A, Li Y, Guo A, Villen J, Wang Y, MacNeill J, Kornhauser J, Sprott K, Zhou J, Possemato A, et al. Akt-RSK-S6 kinase signaling networks activated by oncogenic receptor tyrosine kinases. *Sci. Signaling.* 2010; 3:ra64.

103. Han G, Ye M, Liu H, Song C, Sun D, Wu Y, Jiang X, Chen R, Wang C, Wang L, Zou H. Phosphoproteome analysis of human liver tissue by long-gradient nanoflow LC coupled with multiple stage MS analysis. *Electrophoresis*. 2010; 31:1080–1089. [PubMed: 20166139]
104. Van Hoof D, Munoz J, Braam SR, Pinkse MWH, Linding R, Heck AJR, Mummery CL, Krijgsveld J. Phosphorylation dynamics during early differentiation of human embryonic stem cells. *Cell Stem Cell*. 2009; 5:214–226. [PubMed: 19664995]
105. Guo A, Villen J, Kornhauser J, Lee KA, Stokes MP, Rikova K, Possemato A, Nardone J, Innocenti G, Wetzel R, et al. Signaling networks assembled by oncogenic EGFR and c-Met. *Proc. Natl. Acad. Sci. U. S. A.* 2008; 105:692–697. [PubMed: 18180459]
106. Rikova K, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell*. 2007; 131:1190–1203. [PubMed: 18083107]
107. Herskowitz JH, et al. Phosphoproteomic analysis reveals site-specific changes in GFAP and NDRG2 phosphorylation in frontotemporal lobar degeneration. *J. Proteome Res.* 2010; 9:6368–6379. [PubMed: 20886841]
108. Kim W, et al. Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol. Cell*. 2011; 44:325–340. [PubMed: 21906983]
109. Aono S, Hirai Y. Phosphorylation of claudin-4 is required for tight junction formation in a human keratinocyte cell line. *Exp. Cell Res.* 2008; 314:3326–3339. [PubMed: 18786529]
110. Imami K, Sugiyama N, Kyono Y, Tomita M, Ishihama Y. Automated phosphoproteome analysis for cultured cancer cells by two-dimensional nanoLC-MS using a calcined titania/C18 biphasic column. *Anal. Sci.* 2008; 24:161–166. [PubMed: 18187866]
111. Depontieu FR, et al. Identification of tumor-associated, MHC class II-restricted phosphopeptides as targets for immunotherapy. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106:12073–12078. [PubMed: 19581576]
112. Ikari A, et al. Tight junctional localization of claudin-16 is regulated by syntaxin 8 in renal tubular epithelial cells. *J. Biol. Chem.* 2014; 289:13112–13123. [PubMed: 24659781]
113. Kettenbach AN, et al. Quantitative phosphoproteomics identifies substrates and functional modules of Aurora and Polo-like kinase activities in mitotic cells. *Sci. Signaling*. 2011; 4:rs5.
114. Ishizaki T, Chiba H, Kojima T, Fujibe M, Soma T, Miyajima H, Nagasawa K, Wada I, Sawada N. Cyclic AMP induces phosphorylation of claudin-5 immunoprecipitates and expression of claudin-5 gene in blood–brain-barrier endothelial cells via protein kinase A-dependent and -independent pathways. *Exp. Cell Res.* 2003; 290:275–288. [PubMed: 14567987]
115. Soma T, et al. Thr207 of claudin-5 is involved in size-selective loosening of the endothelial barrier by cyclic AMP. *Exp. Cell Res.* 2004; 300:202–212. [PubMed: 15383327]
116. Ikari A, Matsumoto S, Harada H, Takagi K, Hayashi H, Suzuki Y, Degawa M, Miwa M. Phosphorylation of paracellin-1 at Ser217 by protein kinase A is essential for localization in tight junctions. *J. Cell Sci.* 2006; 119:1781–1789. [PubMed: 16608877]
117. Overgaard CE, Daugherty BL, Mitchell LA, Koval M. Claudins: control of barrier function and regulation in response to oxidant stress. *Antioxid. Redox Signaling*. 2011; 15:1179–1193.
118. Molina-Jijon E, Rodriguez-Munoz R, Namorado M. d. C. Pedraza-Chaverri J, Reyes JL. Oxidative stress induces claudin-2 nitration in experimental type 1 diabetic nephropathy. *Free Radical Biol. Med.* 2014; 72:162–175. [PubMed: 24726862]
119. Van Itallie CM, Gambling TM, Carson JL, Anderson JM. Palmitoylation of claudins is required for efficient tight-junction localization. *J. Cell Sci.* 2005; 118:1427–1436. [PubMed: 15769849]
120. Heiler S, Mu W, Zoller M, Thuma F. The importance of claudin-7 palmitoylation on membrane subdomain localization and metastasis-promoting activities. *Cell Commun. Signaling*. 2015; 13:29.
121. Yang W, Di Vizio D, Kirchner M, Steen H, Freeman MR. Proteome scale characterization of human S-acylated proteins in lipid raft-enriched and non-raft membranes. *Mol. Cell. Proteomics*. 2010; 9:54–70. [PubMed: 19801377]
122. Dowal L, Yang W, Freeman MR, Steen H, Flaumenhaft R. Proteomic analysis of palmitoylated platelet proteins. *Blood*. 2011; 118:e62–73. [PubMed: 21813449]
123. Martin BR, Wang C, Adibekian A, Tully SE, Cravatt BF. Global profiling of dynamic protein palmitoylation. *Nat. Methods*. 2011; 9:84–89. [PubMed: 22056678]

124. Mandel I, et al. The ubiquitin-proteasome pathway regulates claudin 5 degradation. *J. Cell. Biochem.* 2012; 113:2415–2423. [PubMed: 22389112]
125. Takahashi S, et al. The E3 ubiquitin ligase LNX1p80 promotes the removal of claudins from tight junctions in MDCK cells. *J. Cell Sci.* 2009; 122:985–994. [PubMed: 19295125]
126. Van Itallie CM, Mitic LL, Anderson JM. SUMOylation of claudin-2. *Ann. N. Y. Acad. Sci.* 2012; 1258:60–64. [PubMed: 22731716]
127. Ahmad W, Shabbiri K, Ijaz B, Asad S, Sarwar MT, Gull S, Kausar H, Fouzia K, Shahid I, Hassan S. Claudin-1 required for HCV virus entry has high potential for phosphorylation and O-glycosylation. *Virology*. 2011; 8:229. [PubMed: 21569618]
128. Butt AM, et al. Role of post translational modifications and novel crosstalk between phosphorylation and O-beta-GlcNAc modifications in human claudin-1, -3 and -4. *Mol. Biol. Rep.* 2012; 39:1359–1369. [PubMed: 21617949]
129. Price MG, Davis CF, Deng F, Burgess DL. The alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptor trafficking regulator “stargazin” is related to the claudin family of proteins by its ability to mediate cell-cell adhesion. *J. Biol. Chem.* 2005; 280:19711–19720. [PubMed: 15760900]
130. Maher GJ, et al. The cataract-associated protein TMEM114, and TMEM235, are glycosylated transmembrane proteins that are distinct from claudin family members. *FEBS Lett.* 2011; 585:2187–2192. [PubMed: 21689651]
131. Gupta R, Brunak S. Prediction of glycosylation across the human proteome and the correlation to protein function. *Pac Symp. Biocomput.* 2002;310–322. [PubMed: 11928486]
132. Fujita H, et al. Differential expression and subcellular localization of claudin-7, -8, -12, -13, and -15 along the mouse intestine. *J. Histochem. Cytochem.* 2006; 54:933–944. [PubMed: 16651389]
133. Schrade A, et al. Expression and localization of claudins-3 and -12 in transformed human brain endothelium. *Fluids Barriers CNS.* 2012; 9:6. [PubMed: 22373538]
134. Steentoft C, et al. Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. *EMBO J.* 2013; 32:1478–1488. [PubMed: 23584533]
135. Awan FM, et al. In-silico analysis of claudin-5 reveals novel putative sites for post-translational modifications: Insights into potential molecular determinants of blood-brain barrier breach during HIV-1 infiltration. *Infect., Genet. Evol.* 2014; 27:355–365. [PubMed: 25120100]
136. Blom N, Gammeltoft S, Brunak S. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J. Mol. Biol.* 1999; 294:1351–1362. [PubMed: 10600390]
137. Trinidad JC, et al. Global identification and characterization of both O-GlcNAcylation and phosphorylation at the murine synapse. *Mol. Cell. Proteomics.* 2012; 11:215–229. [PubMed: 22645316]
138. Buzza MS, et al. Membrane-anchored serine protease matriptase regulates epithelial barrier formation and permeability in the intestine. *Proc. Natl. Acad. Sci. U. S. A.* 2010; 107:4200–4205. [PubMed: 20142489]
139. Groschwitz KR, et al. Mast cells regulate homeostatic intestinal epithelial migration and barrier function by a chymase/Mcpt4-dependent mechanism. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106:22381–22386. [PubMed: 20018751]
140. Zeissig S, et al. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut.* 2007; 56:61–72. [PubMed: 16822808]
141. Willemsen LEM, Hoetjes JP, Van Deventer SJH, Van Tol EAF. Abrogation of IFN-gamma mediated epithelial barrier disruption by serine protease inhibition. *Clin. Exp. Immunol.* 2005; 142:275–284. [PubMed: 16232214]
142. Van Itallie CM, Colegio OR, Anderson JM. The cytoplasmic tails of claudins can influence tight junction barrier properties through effects on protein stability. *J. Membr. Biol.* 2004; 199:29–38. [PubMed: 15366421]
143. Murakami T, Felinski EA, Antonetti DA. Occludin phosphorylation and ubiquitination regulate tight junction trafficking and vascular endothelial growth factor-induced permeability. *J. Biol. Chem.* 2009; 284:21036–21046. [PubMed: 19478092]

144. Ebihara C, Kondoh M, Hasuike N, Harada M, Mizuguchi H, Horiguchi Y, Fujii M, Watanabe Y. Preparation of a claudin-targeting molecule using a C-terminal fragment of *Clostridium perfringens* enterotoxin. *J. Pharmacol. Exp. Ther.* 2005; 316:255–260. [PubMed: 16183701]
145. Lohrberg D, et al. A strategy for enrichment of claudins based on their affinity to *Clostridium perfringens* enterotoxin. *BMC Mol. Biol.* 2009; 10:61. [PubMed: 19545418]
146. Luissint A-C, et al. Guanine nucleotide-binding protein Gai2: a new partner of claudin-5 that regulates tight junction integrity in human brain endothelial cells. *J. Cereb. Blood Flow Metab.* 2012; 32:860–873. [PubMed: 22333621]
147. Jaspers MHJ, et al. The Claudin Megatrachea Protein Complex. *J. Biol. Chem.* 2012; 287:36756–36765. [PubMed: 22930751]
148. Ivanov AI, Nusrat A, Parkos CA. Endocytosis of Epithelial Apical Junctional Proteins by a Clathrin-mediated Pathway into a Unique Storage Compartment. *Mol. Biol. Cell.* 2003; 15:176–188. [PubMed: 14528017]
149. Fredriksson K, et al. Proteomic analysis of proteins surrounding occludin and claudin-4 reveals their proximity to signaling and trafficking networks. *PLoS One.* 2015; 10:e0117074. [PubMed: 25789658]
150. Mitic LL, Unger VM, Anderson JM. Expression, solubilization, and biochemical characterization of the tight junction transmembrane protein claudin-4. *Protein Sci.* 2003; 12:218–227. [PubMed: 12538885]
151. Tsukita S, Yamazaki Y, Katsuno T, Tamura A, Tsukita S. Tight junction-based epithelial microenvironment and cell proliferation. *Oncogene.* 2008; 27:6930–6938. [PubMed: 19029935]
152. Tang VW. Proteomic and bioinformatic analysis of epithelial tight junction reveals an unexpected cluster of synaptic molecules. *Biol. Direct.* 2006; 1:37. [PubMed: 17156438]
153. Yamazaki Y, Okawa K, Yano T, Tsukita S, Tsukita S. Optimized proteomic analysis on gels of cell-cell adhering junctional membrane proteins. *Biochemistry.* 2008; 47:5378–5386. [PubMed: 18416558]
154. Pisitkun T, et al. High-throughput identification of IMCD proteins using LC-MS/MS. *Physiol. Genomics.* 2006; 25:263–276. [PubMed: 16449382]
155. Hoffert JD, Wang G, Pisitkun T, Shen RF, Knepper MA. An automated platform for analysis of phosphoproteomic datasets: application to kidney collecting duct phosphoproteins. *J. Proteome Res.* 2007; 6:3501–3508. [PubMed: 17683130]
156. Fujiki Y, Hubbard AL, Fowler S, Lazarow PB. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 1982; 93:97–102. [PubMed: 7068762]
157. Fischer F, Wolters D, Rogner M, Poetsch A. Toward the complete membrane proteome: high coverage of integral membrane proteins through transmembrane peptide detection. *Mol. Cell. Proteomics.* 2005; 5:444–453. [PubMed: 16291997]
158. Franzel B, Fischer F, Trotschel C, Poetsch A, Wolters D. The two-phase partitioning system—a powerful technique to purify integral membrane proteins of *Corynebacterium glutamicum* for quantitative shotgun analysis. *Proteomics.* 2009; 9:2263–2272. [PubMed: 19322788]
159. Mathias RA, et al. Triton X-114 phase separation in the isolation and purification of mouse liver microsomal membrane proteins. *Methods.* 2011; 54:396–406. [PubMed: 21272644]
160. McQuade LR, Schmidt U, Pascovici D, Stojanov T, Baker MS. Improved membrane proteomics coverage of human embryonic stem cells by peptide IPG-IEF. *J. Proteome Res.* 2009; 8:5642–5649. [PubMed: 19899800]
161. Schulz TC, et al. A large-scale proteomic analysis of human embryonic stem cells. *BMC Genomics.* 2007; 8:478. [PubMed: 18162134]
162. Han X, Jin M, Breuker K, McLafferty FW. Extending top-down mass spectrometry to proteins with masses greater than 200 kDa. *Science.* 2006; 314:109–112. [PubMed: 17023655]
163. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray ionization for mass spectrometry of large biomolecules. *Science.* 1989; 246:64–71. [PubMed: 2675315]
164. Loo JA, Edmonds CG, Smith RD. Primary sequence information from intact proteins by electrospray ionization tandem mass spectrometry. *Science.* 1990; 248:201–204. [PubMed: 2326633]

165. Demirev PA, Ramirez J, Fenselau C. Tandem mass spectrometry of intact proteins for characterization of biomarkers from *Bacillus cereus* T spores. *Anal. Chem.* 2001; 73:5725–5731. [PubMed: 11774913]
166. Bogdanov B, Smith RD. Proteomics by FTICR mass spectrometry: top down and bottom up. *Mass Spectrom. Rev.* 2005; 24:168–200. [PubMed: 15389855]
167. Kelleher NL. Peer Reviewed: Top-Down Proteomics. *Anal. Chem.* 2004; 76:196A–203A.
168. Mann M, Kelleher NL. Precision proteomics: The case for high resolution and high mass accuracy. *Proc. Natl. Acad. Sci. U. S. A.* 2008; 105:18132–18138. [PubMed: 18818311]
169. Savaryn JP, Catherman AD, Thomas PM, Abecassis MM, Kelleher NL. The emergence of top-down proteomics in clinical research. *Genome Med.* 2013; 5:53. [PubMed: 23806018]
170. Smith LM, et al. Proteoform: a single term describing protein complexity. *Nat. Methods.* 2013; 10:186–187. [PubMed: 23443629]
171. Yates JR, Kelleher NL. Top Down Proteomics. *Anal. Chem.* 2013; 85:6151–6151. [PubMed: 23705843]
172. Nesvizhskii AI, Aebersold R. Interpretation of shotgun proteomic data: the protein inference problem. *Mol. Cell. Proteomics.* 2005; 4:1419–1440. [PubMed: 16009968]
173. Nesvizhskii AI, Vitek O, Aebersold R. Analysis and validation of proteomic data generated by tandem mass spectrometry. *Nat. Methods.* 2007; 4:787–797. [PubMed: 17901868]
174. Tran JC, et al. Mapping intact protein isoforms in discovery mode using top-down proteomics. *Nature.* 2011; 480:254–258. [PubMed: 22037311]
175. Catherman AD, et al. Large-scale Top-down Proteomics of the Human Proteome: Membrane Proteins, Mitochondria, and Senescence. *Mol. Cell. Proteomics.* 2013; 12:3465–3473. [PubMed: 24023390]
176. Kelleher NL. A cell-based approach to the human proteome project. *J. Am. Soc. Mass Spectrom.* 2012; 23:1617–1624. [PubMed: 22976808]
177. Moradian A, Kalli A, Sweredoski MJ, Hess S. The top-down, middle-down, and bottom-up mass spectrometry approaches for characterization of histone variants and their post-translational modifications. *Proteomics.* 2014; 14:489–497. [PubMed: 24339419]
178. Wu C, Tran JC, Zamborg L, Durbin KR, Li M, Ahlf DR, Early BP, Thomas PM, Sweedler JV, Kelleher NL. A protease for 'middle-down' proteomics. *Nat. Methods.* 2012; 9:822–824. [PubMed: 22706673]
179. Cannon J, et al. High-throughput middle-down analysis using an orbitrap. *J. Proteome Res.* 2010; 9:3886–3890. [PubMed: 20557100]
180. Liu X, et al. De Novo Protein Sequencing by Combining Top-Down and Bottom-Up Tandem Mass Spectra. *J. Proteome Res.* 2014; 13:3241. [PubMed: 24874765]
181. Wu SL, et al. Targeted proteomics of low-level proteins in human plasma by LC/MSn: using human growth hormone as a model system. *J. Proteome Res.* 2002; 1:459–465. [PubMed: 12645918]
182. Immler D, Greven S, Reinemer P. Targeted proteomics in biomarker validation: detection and quantification of proteins using a multi-dimensional peptide separation strategy. *Proteomics.* 2006; 6:2947–2958. [PubMed: 16619308]
183. Whiteaker JR, et al. A targeted proteomics-based pipeline for verification of biomarkers in plasma. *Nat. Biotechnol.* 2011; 29:625–634. [PubMed: 21685906]
184. Omenn GS, Baker MS, Aebersold R. Recent Workshops of the HUPO Human Plasma Proteome Project (HPPP): a bridge with the HUPO CardioVascular Initiative and the emergence of SRM targeted proteomics. *Proteomics.* 2011; 11:3439–3443. [PubMed: 21850651]
185. Anderson NL. The Human Plasma Proteome: History, Character, and Diagnostic Prospects. *Mol. Cell. Proteomics.* 2002; 1:845–867. [PubMed: 12488461]
186. Everley RA, Kunz RC, McAllister FE, Gygi SP. Increasing throughput in targeted proteomics assays: 54-plex quantitation in a single mass spectrometry run. *Anal. Chem.* 2013; 85:5340–5346. [PubMed: 23662842]
187. Peng Y, et al. Top-down targeted proteomics for deep sequencing of tropomyosin isoforms. *J. Proteome Res.* 2013; 12:187–198. [PubMed: 23256820]

188. Li M, et al. Oncogene-induced cellular senescence elicits an anti-Warburg effect. *Proteomics*. 2013; 13:2585–2596. [PubMed: 23798001]
189. Madian AG, Rochelle NS, Regnier FE. Mass-linked immuno-selective assays in targeted proteomics. *Anal. Chem*. 2013; 85:737–748. [PubMed: 22950521]
190. Qeli E, Omasits U, Goetze S, Stekhoven DJ, Frey JE, Basler K, Wollscheid B, Brunner E, Ahrens CH. Improved prediction of peptide detectability for targeted proteomics using a rank-based algorithm and organism-specific data. *J. Proteomics*. 2014; 108:269–283. [PubMed: 24878426]
191. Reker D, Malmstrom L. Bioinformatic challenges in targeted proteomics. *J. Proteome Res*. 2012; 11:4393–4402. [PubMed: 22866949]
192. Maiolica A, Junger MA, Ezkurdia I, Aebersold R. Targeted proteome investigation via selected reaction monitoring mass spectrometry. *J. Proteomics*. 2012; 75:3495–3513. [PubMed: 22579752]
193. Qiu X, Zhang H, Lai Y. Quantitative targeted proteomics for membrane transporter proteins: method and application. *AAPS J*. 2014; 16:714–726. [PubMed: 24830943]
194. Gillet LC, Navarro P, Tate S, Rost H, Selevsek N, Reiter L, Bonner R, Aebersold R. Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol. Cell. Proteomics*. 2012; 11:O111.016717. [PubMed: 22261725]
195. Uchida Y, et al. Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. *J. Neurochem*. 2011; 117:333–345. [PubMed: 21291474]
196. Shawahna R, et al. Transcriptomic and quantitative proteomic analysis of transporters and drug metabolizing enzymes in freshly isolated human brain microvessels. *Mol. Pharmaceutics*. 2011; 8:1332–1341.
197. Ohtsuki S, et al. Quantitative targeted absolute proteomic analysis of transporters, receptors and junction proteins for validation of human cerebral microvascular endothelial cell line hCMEC/D3 as a human blood-brain barrier model. *Mol. Pharmaceutics*. 2013; 10:289–296.
198. Pasquali C, Fialka I, Huber LA. Subcellular fractionation, electromigration analysis and mapping of organelles. *J. Chromatogr., Biomed. Appl*. 1999; 722:89–102.
199. Huber LA, Pfaller K, Vietor I. Organelle Proteomics: Implications for Subcellular Fractionation in Proteomics. *Circ. Res*. 2003; 92:962–968. [PubMed: 12750306]
200. Banan A, Zhang LJ, Shaikh M, Fields JZ, Choudhary S, Forsyth CB, Farhadi A, Keshavarzian A. Theta Isoform of Protein Kinase C Alters Barrier Function in Intestinal Epithelium through Modulation of Distinct Claudin Isotypes: A Novel Mechanism for Regulation of Permeability. *J. Pharmacol. Exp. Ther*. 2005; 313:962–982. [PubMed: 15900076]
201. Lambert D, O'Neill CA, Padfield PJ. Methyl-beta-cyclodextrin increases permeability of Caco-2 cell monolayers by displacing specific claudins from cholesterol rich domains associated with tight junctions. *Cell. Physiol. Biochem*. 2007; 20:495–506. [PubMed: 17762176]
202. Lynch RD, et al. Cholesterol depletion alters detergent-specific solubility profiles of selected tight junction proteins and the phosphorylation of occludin. *Exp. Cell Res*. 2007; 313:2597–2610. [PubMed: 17574235]
203. Sugibayashi K, Onuki Y, Takayama K. Displacement of tight junction proteins from detergent-resistant membrane domains by treatment with sodium caprate. *Eur. J. Pharm. Sci*. 2009; 36:246–253. [PubMed: 19013238]
204. Schuck S, Honsho M, Ekroos K, Shevchenko A, Simons K. Resistance of cell membranes to different detergents. *Proc. Natl. Acad. Sci. U. S. A*. 2003; 100:5795–5800. [PubMed: 12721375]
205. Nagase S, Doyama R, Yagi K, Kondoh M. Recent advances in claudin-targeting technology. *Biol. Pharm. Bull*. 2013; 36:708–714. [PubMed: 23649330]
206. Speers AE, Cravatt BF. A tandem orthogonal proteolysis strategy for high-content chemical proteomics. *J. Am. Chem. Soc*. 2005; 127:10018–10019. [PubMed: 16011363]
207. Sadaghiani AM, Verhelst SH, Bogyo M. Tagging and detection strategies for activity-based proteomics. *Curr. Opin. Chem. Biol*. 2007; 11:20–28. [PubMed: 17174138]
208. Heal WP, Dang TH, Tate EW. Activity-based probes: discovering new biology and new drug targets. *Chem. Soc. Rev*. 2011; 40:246–257. [PubMed: 20886146]

209. Jing C, Cornish VW. Chemical tags for labeling proteins inside living cells. *Acc. Chem. Res.* 2011; 44:784–792. [PubMed: 21879706]
210. Hayashi T, Hamachi I. Traceless affinity labeling of endogenous proteins for functional analysis in living cells. *Acc. Chem. Res.* 2012; 45:1460–1469. [PubMed: 22680975]
211. Kovalenko OV, Yang XH, Hemler ME. A novel cysteine cross-linking method reveals a direct association between claudin-1 and tetraspanin CD9. *Mol. Cell. Proteomics.* 2007; 6:1855–1867. [PubMed: 17644758]
212. Piontek J, et al. Formation of tight junction: determinants of homophilic interaction between classic claudins. *FASEB J.* 2007; 22:146–158. [PubMed: 17761522]
213. Angelow S, Yu AS. L. Structure-Function Studies of Claudin Extracellular Domains by Cysteine-scanning Mutagenesis. *J. Biol. Chem.* 2009; 284:29205–29217. [PubMed: 19690347]
214. Cukierman L, Meertens L, Bertaux C, Kajumo F, Dragic T. Residues in a Highly Conserved Claudin-1 Motif Are Required for Hepatitis C Virus Entry and Mediate the Formation of Cell-Cell Contacts. *J. Virol.* 2009; 83:5477–5484. [PubMed: 19297469]
215. Dukes JD, Whitley P, Chalmers AD. The PIKfyve Inhibitor YM201636 Blocks the Continuous Recycling of the Tight Junction Proteins Claudin-1 and Claudin-2 in MDCK cells. *PLoS One.* 2012; 7:e28659. [PubMed: 22396724]
216. Giron P, Dayon L, Sanchez JC. Cysteine tagging for MS-based proteomics. *Mass Spectrom. Rev.* 2011; 30:366–395. [PubMed: 21500242]
217. Dabrowski S, et al. Redox-sensitive structure and function of the first extracellular loop of the cell-cell contact protein claudin-1: lessons from molecular structure to animals. *Antioxid. Redox Signaling.* 2015; 22:1–14.
218. Furuse M, et al. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J. Cell Biol.* 2002; 156:1099–1111. [PubMed: 11889141]
219. Umeda K, et al. ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. *Cell.* 2006; 126:741–754. [PubMed: 16923393]
220. Fagotto F, Gumbiner BM. Cell contact-dependent signaling. *Dev. Biol.* 1996; 180:445–454. [PubMed: 8954717]
221. Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell.* 1996; 84:345–357. [PubMed: 8608588]
222. Brucher BL, Jamall IS. Cell-Cell Communication in the Tumor Microenvironment, Carcinogenesis, and Anticancer Treatment. *Cell. Physiol. Biochem.* 2014; 34:213–243. [PubMed: 25034869]
223. Guillemot L, Paschoud S, Pulimeno P, Foglia A, Citi S. The cytoplasmic plaque of tight junctions: A scaffolding and signalling center. *Biochim. Biophys. Acta, Biomembr.* 2008; 1778:601–613.
224. Arabzadeh A, Troy TC, Turksen K. Role of the Cldn6 Cytoplasmic Tail Domain in Membrane Targeting and Epidermal Differentiation In Vivo. *Mol. Cell. Biol.* 2006; 26:5876–5887. [PubMed: 16847338]
225. Muller D, Kausalya PJ, Meij IC, Hunziker W. Familial hypomagnesemia with hypercalciuria and nephrocalcinosis: blocking endocytosis restores surface expression of a novel Claudin-16 mutant that lacks the entire C-terminal cytosolic tail. *Hum. Mol. Genet.* 2006; 15:1049–1058. [PubMed: 16501001]
226. Ruffer C, Gerke V. The C-terminal cytoplasmic tail of claudins 1 and 5 but not its PDZ-binding motif is required for apical localization at epithelial and endothelial tight junctions. *Eur. J. Cell Biol.* 2004; 83:135–144. [PubMed: 15260435]
227. Nagasawa K, et al. Possible involvement of gap junctions in the barrier function of tight junctions of brain and lung endothelial cells. *J. Cell. Physiol.* 2006; 208:123–132. [PubMed: 16547974]
228. Kojima T, et al. Cx32 but not Cx26 is associated with tight junctions in primary cultures of rat hepatocytes. *Exp. Cell Res.* 2001; 263:193–201. [PubMed: 11161718]
229. Blackman B, Russell T, Nordeen SK, Medina D, Neville MC. Claudin 7 expression and localization in the normal murine mammary gland and murine mammary tumors. *Breast cancer research: BCR.* 2005; 7:R248–255. [PubMed: 15743505]

230. Liebner S, Corada M, Bangsow T, Babbage J, Taddei A, Czupalla CJ, Reis M, Felici A, Wolburg H, Fruttiger M, et al. Wnt/beta-catenin signaling controls development of the blood-brain barrier. *J. Cell Biol.* 2008; 183:409–417. [PubMed: 18955553]
231. Beeman N, Webb PG, Baumgartner HK. Occludin is required for apoptosis when claudin-claudin interactions are disrupted. *Cell Death Dis.* 2012; 3:e273. [PubMed: 22361748]
232. Inai T. The coculture method to examine interactions between claudin isoforms in tight junction-free HEK293 cells and tight junction-bearing MDCK II cells. *Methods Mol. Biol. (N. Y., NY, U. S.).* 2011; 762:101–114.
233. Milatz S, et al. Probing the cis-arrangement of prototype tight junction proteins claudin-1 and claudin-3. *Biochem. J.* 2015; 468:449. [PubMed: 25849148]
234. Rossa J, et al. Claudin-3 and claudin-5 protein folding and assembly into the tight junction are controlled by non-conserved residues in the transmembrane 3 (TM3) and extracellular loop 2 (ECL2) segments. *J. Biol. Chem.* 2014; 289:7641–7653. [PubMed: 24478310]
235. Rossa J, et al. Molecular and structural transmembrane determinants critical for embedding claudin-5 into tight junctions reveal a distinct four-helix bundle arrangement. *Biochem. J.* 2014; 464:49–60. [PubMed: 25174580]
236. Capaldo CT, et al. Proinflammatory cytokine-induced tight junction remodeling through dynamic self-assembly of claudins. *Mol. Biol. Cell.* 2014; 25:2710–2719. [PubMed: 25031428]
237. Wu CJ, Mannan P, Lu M, Udey MC. Epithelial cell adhesion molecule (EpCAM) regulates claudin dynamics and tight junctions. *J. Biol. Chem.* 2013; 288:12253–12268. [PubMed: 23486470]
238. Kuhn S, et al. A complex of EpCAM, claudin-7, CD44 variant isoforms, and tetraspanins promotes colorectal cancer progression. *Mol. Cancer Res.* 2007; 5:553–567. [PubMed: 17579117]
239. Nubel T, Preobraschenski J, Tuncay H, Weiss T, Kuhn S, Ladwein M, Langbein L, Zoller M. Claudin-7 Regulates EpCAM-Mediated Functions in Tumor Progression. *Mol. Cancer Res.* 2009; 7:285–299. [PubMed: 19276185]
240. Pope JL, et al. Claudin-1 regulates intestinal epithelial homeostasis through the modulation of Notch-signalling. *Gut.* 2014; 63:622–634. [PubMed: 23766441]
241. Escudero-Esparza A, Jiang WG, Martin TA. Claudin-5 is involved in breast cancer cell motility through the N-WASP and ROCK signalling pathways. *J. Exp. Clin. Cancer Res.* 2012; 31:43. [PubMed: 22559840]
242. Mankertz J, et al. Functional crosstalk between Wnt signaling and Cdx-related transcriptional activation in the regulation of the claudin-2 promoter activity. *Biochem. Biophys. Res. Commun.* 2004; 314:1001–1007. [PubMed: 14751232]
243. Miwa N, et al. Involvement of claudin-1 in the beta-catenin/Tcf signaling pathway and its frequent upregulation in human colorectal cancers. *Oncol. Res.* 2001; 12:469–476. [PubMed: 11939410]
244. Oku N, Sasabe E, Ueta E, Yamamoto T, Osaki T. Tight junction protein claudin-1 enhances the invasive activity of oral squamous cell carcinoma cells by promoting cleavage of laminin-5 gamma2 chain via matrix metalloproteinase (MMP)-2 and membrane-type MMP-1. *Cancer Res.* 2006; 66:5251–5257. [PubMed: 16707450]
245. Tokes AM, et al. Claudin-1, -3 and -4 proteins and mRNA expression in benign and malignant breast lesions: a research study. *Breast cancer research: BCR.* 2005; 7:R296–305. [PubMed: 15743508]
246. Swisshelm K, Macek R, Kubbies M. Role of claudins in tumorigenesis. *Adv. Drug Delivery Rev.* 2005; 57:919–928.
247. Stache C, et al. Tight junction protein claudin-1 is differentially expressed in craniopharyngioma subtypes and indicates invasive tumor growth. *Neuro-oncology.* 2014; 16:256–264. [PubMed: 24305709]
248. Blanchard AA, et al. Claudins 1, 3, and 4 protein expression in ER negative breast cancer correlates with markers of the basal phenotype. *Virchows Arch.* 2009; 454:647–656. [PubMed: 19387682]

249. Blanchard AA, et al. Claudin 1 expression in basal-like breast cancer is related to patient age. *BMC Cancer*. 2013; 13:268. [PubMed: 23721519]
250. Leotlela PD, et al. Claudin-1 overexpression in melanoma is regulated by PKC and contributes to melanoma cell motility. *Oncogene*. 2007; 26:3846–3856. [PubMed: 17160014]
251. Alshbool FZ, Mohan S. Differential expression of claudin family members during osteoblast and osteoclast differentiation: Cldn-1 is a novel positive regulator of osteoblastogenesis. *PLoS One*. 2014; 9:e114357. [PubMed: 25479235]
252. Dahiya N, Becker KG, Wood WH, Zhang Y, Morin PJ. Claudin-7 Is Frequently Overexpressed in Ovarian Cancer and Promotes Invasion. *PLoS One*. 2011; 6:e22119. [PubMed: 21789222]
253. Shannon P, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003; 13:2498–2504. [PubMed: 14597658]
254. Itoh M, et al. Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. *J. Cell Biol*. 1999; 147:1351–1363. [PubMed: 10601346]
255. Hamazaki Y, Itoh M, Sasaki H, Furuse M, Tsukita S. Multi-PDZ domain protein 1 (MUPP1) is concentrated at tight junctions through its possible interaction with claudin-1 and junctional adhesion molecule. *J. Biol. Chem*. 2002; 277:455–461. [PubMed: 11689568]
256. Roh MH, Liu CJ, Laurinec S, Margolis B. The carboxyl terminus of zona occludens-3 binds and recruits a mammalian homologue of discs lost to tight junctions. *J. Biol. Chem*. 2002; 277:27501–27509. [PubMed: 12021270]
257. Nakatsukasa M, et al. Tumor-associated calcium signal transducer 2 is required for the proper subcellular localization of claudin 1 and 7: implications in the pathogenesis of gelatinous drop-like corneal dystrophy. *Am. J. Pathol*. 2010; 177:1344–1355. [PubMed: 20651236]
258. Singh AB, Sharma A, Dhawan P. Claudin-1 expression confers resistance to anoikis in colon cancer cells in a Src-dependent manner. *Carcinogenesis*. 2012; 33:2538–2547. [PubMed: 22941059]
259. DiTommaso T, et al. Keratin 76 is required for tight junction function and maintenance of the skin barrier. *PLoS Genet*. 2014; 10:e1004706. [PubMed: 25340345]
260. Guo Z, et al. Proteomics strategy to identify substrates of LNX, a PDZ domain-containing E3 ubiquitin ligase. *J. Proteome Res*. 2012; 11:4847–4862. [PubMed: 22889411]
261. Harris HJ, Farquhar MJ, Mee CJ, Davis C, Reynolds GM, Jennings A, Hu K, Yuan F, Deng H, Hubscher SG, et al. CD81 and claudin 1 coreceptor association: role in hepatitis C virus entry. *J. Virol*. 2008; 82:5007–5020. [PubMed: 18337570]
262. Raleigh DR, et al. Occludin S408 phosphorylation regulates tight junction protein interactions and barrier function. *J. Cell Biol*. 2011; 193:565–582. [PubMed: 21536752]
263. Cording J, et al. Redox Regulation of Cell Contacts by Tricellulin and Occludin: Redox-Sensitive Cysteine Sites in Tricellulin Regulate Both Tri- and Bicellular Junctions in Tissue Barriers as Shown in Hypoxia and Ischemia. *Antioxid. Redox Signaling*. 2015; 23:1035–1049.
264. Furuse M, Sasaki H, Tsukita S. Manner of interaction of heterogeneous claudin species within and between tight junction strands. *J. Cell Biol*. 1999; 147:891–903. [PubMed: 10562289]
265. Lim TS, Vedula SR, Kausalya PJ, Hunziker W, Lim CT. Single-molecular-level study of claudin-1-mediated adhesion. *Langmuir*. 2008; 24:490–495. [PubMed: 18095722]
266. Miyamori H, et al. Claudin promotes activation of pro-matrix metalloproteinase-2 mediated by membrane-type matrix metalloproteinases. *J. Biol. Chem*. 2001; 276:28204–28211. [PubMed: 11382769]
267. Tanaka M, Kamata R, Sakai R. Phosphorylation of ephrin-B1 via the interaction with claudin following cell-cell contact formation. *EMBO J*. 2005; 24:3700–3711. [PubMed: 16211011]
268. Douam F, Lavillette D, Cosset FL. The mechanism of HCV entry into host cells. *Progress in molecular biology and translational science*. 2015; 129:63–107. [PubMed: 25595801]
269. Cocquerel L, Kuo CC, Dubuisson J, Levy S. CD81-dependent binding of hepatitis C virus E1E2 heterodimers. *J. Virol*. 2003; 77:10677–10683. [PubMed: 12970454]
270. Douam F, et al. Critical interaction between E1 and E2 glycoproteins determines binding and fusion properties of hepatitis C virus during cell entry. *Hepatology (Hoboken, NJ, U. S.)*. 2014; 59:776–788.

271. Hopcraft SE, Evans MJ. Selection of a hepatitis C virus with altered entry factor requirements reveals a genetic interaction between the E1 glycoprotein and claudins. *Hepatology* (Hoboken, NJ, U. S.). 2015; 62:1059–1069.
272. Gao F, et al. Novel binding between pre-membrane protein and claudin-1 is required for efficient dengue virus entry. *Biochem. Biophys. Res. Commun.* 2010; 391:952–957. [PubMed: 19962368]
273. Lee SS, Glaunsinger B, Mantovani F, Banks L, Javier RT. Multi-PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins. *J. Virol.* 2000; 74:9680–9693. [PubMed: 11000240]
274. Armbruester V, Sauter M, Roemer K, Best B, Hahn S, Nty A, Schmid A, Philipp S, Mueller A, Mueller-Lantzsch N. Np9 protein of human endogenous retrovirus K interacts with ligand of numb protein X. *J. Virol.* 2004; 78:10310–10319. [PubMed: 15367597]
275. Korkaya H, et al. The ORF3 protein of hepatitis E virus binds to Src homology 3 domains and activates MAPK. *J. Biol. Chem.* 2001; 276:42389–42400. [PubMed: 11518702]
276. Betanzos A, et al. The EhCPADH112 complex of *Entamoeba histolytica* interacts with tight junction proteins occludin and claudin-1 to produce epithelial damage. *PLoS One.* 2013; 8:e65100. [PubMed: 23762290]
277. Zhou M, Wysocki VH. Surface induced dissociation: dissecting noncovalent protein complexes in the gas phase. *Acc. Chem. Res.* 2014; 47:1010–1018. [PubMed: 24524650]
278. Stengel F, Aebersold R, Robinson CV. Joining forces: integrating proteomics and cross-linking with the mass spectrometry of intact complexes. *Mol. Cell. Proteomics.* 2012; 11:R111.014027. [PubMed: 22180098]
279. Lasker K, et al. Integrative structure modeling of macromolecular assemblies from proteomics data. *Mol. Cell. Proteomics.* 2010; 9:1689–1702. [PubMed: 20507923]
280. Benesch JL, Ruotolo BT. Mass spectrometry: come of age for structural and dynamical biology. *Curr. Opin. Struct. Biol.* 2011; 21:641–649. [PubMed: 21880480]
281. Barrera NP, Robinson CV. Advances in the mass spectrometry of membrane proteins: from individual proteins to intact complexes. *Annu. Rev. Biochem.* 2011; 80:247–271. [PubMed: 21548785]
282. Zhou M, Robinson CV. When proteomics meets structural biology. *Trends Biochem. Sci.* 2010; 35:522–529. [PubMed: 20627589]
283. Heck AJ. Native mass spectrometry: a bridge between interactomics and structural biology. *Nat. Methods.* 2008; 5:927–933. [PubMed: 18974734]
284. Krause G, Protze J, Piontek J. Assembly and function of claudins: Structure-function relationships based on homology models and crystal structures. *Semin. Cell Dev. Biol.* 2015; 42:3. [PubMed: 25957516]

Figure 1.

(a) General scheme of the claudin protein structure; (b) secondary structural motifs found in the ECL loops of the X-ray structure of mouse claudin-15.⁶⁴

Figure 2.

Known or hypothetical PTMs of claudins and the regions in which they are predicted to occur. Predicted PTMs include phosphorylation (red oval), palmitoylation (yellow line), ubiquitination (blue circle), proteolysis (green marks), and glycosylation (gray box).

Figure 3.

Top-down and bottom-up proteomics: advantages (in bold) and disadvantages (in gray).

Figure 4.

Simplified scheme of the epithelial apical junctional complex, showing a subset of protein constituents, including tetraspan transmembrane proteins (claudins, occludin), single-pass transmembrane proteins (JAM-A, cadherin), cytosolic scaffold proteins (ZO-1, ZO-2, catenins), signaling proteins (RhoA, MLCK), and the actin cytoskeleton. The Crumbs/PALS/PATJ polarity complex is also shown, which defines the apical/basolateral axis and is directly associated with tight junctions via scaffold protein interactions.

Figure 5.

Diagram of the protein interaction network of claudin-1. The diagram was made using Cytoscape.²⁵³ In orange are the intrinsic membrane proteins with at least one transmembrane domain. In yellow are the peripheral membrane proteins. These two groups contain plasma membrane proteins and endomembrane system proteins (endosome, endoplasmic reticulum, Golgi apparatus, and cytoplasmic vesicles). Membrane proteins from mitochondria or nucleus are not included in these two groups. In green are cytosolic, nuclear, and mitochondrial proteins. In blue are secreted proteins (with a signal peptide and no transmembrane domains). The pink border indicates proteins found in tight junctions. Red edges connect CLDN1 (diamond) with its direct interacting partners: TJP1/ZO-1, TJP2/ZO-2, TJP3/ZO-3, MPDZ, INADL, TACSTD2, SRC, KRT76, LNX1, CD81, CD9, OCLN, MARVELD2, MARVELD3, CLDN3, CLDN7, MMP14, MMP2, and EFNB1 (rectangles).

Interactions with non-human proteins are not included in the network but briefly described in the text.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Overview of the Human Claudin Family Members from Databases

gene	protein IDs (UniProt/Ensembl) ^b	synonyms (GeneCards)	chromosome localization ^e	tissue frequency with HPA evidence ^f	tissue-specific RNA FPKM ⁱ (max in nonspecific tissue)
CLDN1	O95832/163347	SEMP1, ILVASC	3q28	very common	– (skin: 224.1)
CLDN2	P57739/165376	SP82	Xq22	common ^g	– (kidney: 114.5)
CLDN3	O15551/165215	CPETR2	7q11	very common	– (colon: 94.8)
CLDN4	O14493/189143	CPER, CPETR1, WBSCR8	7q11	common ^h	– (colon: 160.9)
CLDN5	O00501/184113	AWAL, TMVCF	22q11	very common	adipose tissue: 110.8; lung: 32.1 (spleen: 10.4)
CLDN6	P56747/184697	UNQ757, PRO1488, Skullin	16p13	very common	– (placenta: 1.3)
CLDN7	O95471/181885	CEPTL2, CPETRL2	17p13	very common	– (colon: 297.3)
CLDN8	P56748/156284	UNQ779, PRO1573	21q22	very common	– (kidney: 63.6)
CLDN9	O95484/213937		16p13	very common	– (pancreas: 2.0)
CLDN10	P78369/134873	CPETRL3, OSP-L	13q32	less common	– (salivary gland: 150.7)
CLDN11	O75508/013297	OSP, OTM	3q26	less common	– (testis: 167.0)
CLDN12	P56749/157224		7q21	very common	– (liver: 31.9)
CLDN14	O95500/159261	UNQ777, PRO1571, DFNB29	21q22	NA	kidney: 5.3; liver: 8.4 (appendix: 0.5)
CLDN15	P56746/106404		7q22	very common	duodenum: 184.0; small intestine: 140.1 (spleen: 12.6)
CLDN16	Q9Y5I7/113946	PCLN1, PCLN-1, HOMG3	3q28	rare	kidney: 39.0 (thyroid gland: 6.8)
CLDN17	P56750/156282	UNQ758 PRO1489	21q22	NA	esophagus: 6.4 (testis: 0.3)
CLDN18	P56856/066405	UNQ778 PRO1572, SFTA5, SFTPJ	3q22	rare	lung: 246.6; stomach: 556.1 (heart muscle: 7.3)
CLDN19	Q8N6F1/164007	HOMG5	1p34	NA	kidney: 21.9; placenta: 11.3 (adipose tissue: 0.2)
CLDN20	P56880/171217		6q25	NA	– (uterus: 0.3)
CLDN21 ^a		see CLDN24			
CLDN22	Q8N7P3/177300		4q35	NA	– (adrenal gland: 0.1)
CLDN23	Q96B33/253958	2310014B08RikCLDNL, hCG1646163	8p23	NA	– (stomach: 17.6)
CLDN24	A6NM45/185758	also named CLDN21	4q35	NA	– (kidney: 0.7)
CLDN25	C9JDP6/228607		11q23	rare	– (adipose tissue: 0.0)
CLDN26	B3SHH9 ^c	TMEM114 ^d	16p13	NA	– (testis: 0.4)

gene	protein IDs (UniProt/Ensembl ^b)	synonyms (GeneCards)	chromosome localization ^e	tissue frequency with HPA evidence ^f	tissue-specific RNA FPKM ⁱ (max in nonspecific tissue)
CLDN27	A6NFC5 ^c	TMEM235 ^d	17q25	NA	– (testis: 0.1)

^aMerged with CLDN24; see <http://www.ncbi.nlm.nih.gov/gene/53843>.

^bEnsemble code prefix: ENSG000000.

^cNot identified as claudin but as TMEM (transmembrane) proteins.

^dTracked from GeneCards synonyms.

^eCLDN13 is absent in human. CLDN 6 and 9, as well as CLDN 8 and 17, may be considered as paralogs.⁵⁴

^fHPA evidence was calculated based on the manual curation of western blot, tissue profiling, and subcellular location using a limited number of claudin antibodies that have been submitted to HPA and validated by HPA. Many other claudin antibodies are available but not included in the HPA list. Very common = high or medium levels in at least 20 tissues; common = high or medium levels in at least 10 tissues; less common = high or medium levels in more than 3 but less than 10 tissues; rare = high or medium levels in only 1–3 tissues; NA = no antibodies.

^gIn nucleus but not nucleoli, cell junctions by immunofluorescence analysis.

^hIn plasma membrane by immunofluorescence analysis.

ⁱFPKM value = number of fragments per kilobase gene model and million reads. The FPKM threshold value for detection is >1.⁵⁸ FPKM measurements reflect measured tissues represented in the database and are not necessarily representative of a complete mRNA expression profile.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 2

Peptide Evidence for Claudin Proteins by MS Analysis^a

protein	no. of observations	best log (E)	evidence code ^b	sequences with good evidence (EC = 4) ^b (amino acid residue numbers in parentheses) ^c
CLDN1	323	-111	4	VFDLLNLSTLQATR (66-81); CLEDEEVQK (107-115); CLEDEEVQKMR (107-117); IVQEFYDPMTPVNAR (144-158); FYDPMTPVNARYE (148-160); KITTSYTPRPYPK (189-201); TTSYTPRPYPK (190-201); PYPKPAPSSGKDYV (198-211); PYPKPAPSSGK (198-208)
CLDN2 ^f	100	-31	4	DFYSPLVPDSMK (146-157); SNYYDAYQAQPLATR (192-206); SEENSYSLTGGYV (219-230)
CLDN3	1695	-78	4	VYDLSLLALPQDLQAAAR (65-80) ^d ; DFYNPVVPEAQK (145-156); DFYNPVVPEAQKR (145-157); VVYSAPRSTGPGASLTGYDR (196-216); STGPGASLTGYDRKDYV (203-220); STGPGASLTGYDRK (203-217); STGPGASLTGYDR (203-216); STGPGASLTGYDRKD (203-218)
CLDN4	861	-51	4	VYDLSLLALPQDLQAAAR (66-81) ^d ; CTNCLDESAK (104-114); CTNCLDESAKAK (104-116); NIIQDFYNPLVASGQK (142-157)
CLDN5	169	-85	4	GLWMSCVVQSTGHMQCK (134-150); VYDSVLALSTEVQAAAR (151-166); EFYDPSVPVSQK (231-242); RPTATGDYDKK (290-300)
CLDN6	997	-59	4	VYDLSLLALPQDLQAAAR (66-81) ^d ; DFYNPLVAEAAQK (146-157); DFYNPLVAEAAQKR (146-158); YSTSAPAIRSGPSEYPTKNYV (200-220); YSTSAPAIRSGPSEYPTK (200-217); GPSEYPTK (210-217)
CLDN7	372	-69	4	SSYAGDNIITAQAMYK (33-48); AGDNIITAQAMYK (36-48); GLWMDCVTQSTGMMSCK (49-65) ^e ; KMYDSVLALSAAQATR (65-81); MYDSVLALSAAQATR (66-81); SVLALSAAQATRALMV (69-85); VSLVLGLAMFVATMG (86-101); QIVTDFYNPLIPTNIK (143-158)
CLDN8	18	-7	2	
CLDN9	703	-28	4	VYDLSLLALPQDLQAAAR (66-81) ^d
CLDN10	102	-24	4	ACVTDSTGVSNCK (52-64); ITTEFFDPLFVEQK (142-155); YTYNGATSVMSR (192-204); YHGGEDFK (207-214)
CLDN11	336	-362	4	GLWADCVMATGLYHCK (51-66); GLWADCVMATGLYH (51-64); CKPLVDILILPGYVQACR (65-82); PLVDILILPGYVQACR (67-82); ILILPGYVQACR (71-82); ILPGYVQACR (73-82); MGQEPGVAK (108-116); FYYTAGSSSPH (190-201); FYYTAGSSSPHAK (190-203) ^e ; FYYTAGSSSPH (190-202); YTTAGSSSPHAK (191-203); YTAGSSSPHAK (192-203)
CLDN12	259	-17	4	SSVPNIK (115-121); SRLSAIEIDIPVVSHTT (228-244); SRLSAIEIDIPVVSH (228-242); LSAIEIDIPVVSHTT (230-244)
CLDN14	14	-4	2	
CLDN15 ^g	11	-8	4	RPYQAPVSVMPVATSDQEGDSSFGK (197-221)
CLDN16 ^g	34	-15	4	TCDEYDSILAEHPLK (130-144)
CLDN17	5	-2	2	
CLDN18	82	-87	4	ESSGFTECR (56-64); GYFTLLGLPAMLOAVR (65-80); AVSYHASGHVAVYK (208-221); SVAYKPGGFK (217-226); TEDEVQSYPSKHDYV (247-261); TEDEVQSYPSK (247-257)

protein	no. of observations	best log (E)	evidence code ^b	sequences with good evidence (EC = 4) ^b (amino acid residue numbers in parentheses) ^c
CLDN19 ^h	18	-11	4	LYDSLLALDGHQISAR (66–81)
CLDN20	2	not found	1	
CLDN21	0			see CLDN24
CLDN22	10	-5	2	
CLDN23 ^h	91	-15	4	RSSVSTIQVEWPEPDLAPAIK (202–222) ^e
CLDN24	54	-9	2	
CLDN25	2	-3	2	

^aFor each claudin, peptides with good MS evidence are listed (data compiled from www.hcggpm.org and www.nextiprot.org).

^bEvidence code: 4 = good evidence; 3 = moderate evidence; 2 = weak evidence; 1 = no evidence.

^cAll sequences are at least 8 amino acid residues in length.

^dThe peptide is common to claudins 3, 4, 6, and 9.

^eSingle nucleotide polymorphism-associated peptide variants found for this peptide.

^fClaudin 2 is considered missing in neXtProt.

^gIn neXtProt, only peptides by single reaction monitoring were available for claudins 15 and 16.

^hIn neXtProt, additional peptides have been identified for the claudin although not identified as level 4 evidence by GPM.

Table 3Human Claudin PTM Sites from PhosphoSitePlus or the Global Proteome Machine (GPM) Databases^a

claudin	PTM sites
1	T195-p; Y199-p; S205-p ; S206-p; Y210-p
2	S192-p; Y194-p; Y195-p; Y198-p; S208-p; S219-p
3	Y198-p; S199-p; S203-p; T204-p; S209-p; T212-p; Y214-p; Y219-p
4	Y193-p; S194-p; Y197-p; Y208-p
5	Y148-p; Y152-p; Y154-p ; S155-p; T207-p; Y212-p; Y217-p
6	Y152-p ; S201-p; T202-p; S203-p; S208-p; S212-p; Y214-p; Y219-p
7	K203-ub; S204-p; S206-p; S207-p; K208-ub; Y210-p
8 ^b	Y67-p; S69-p; S74-p; S215-p
9	Y200-p
10	S94-p; T198-p; S199-p; S202-p
11	Y191-p; Y192-p; T193-p ; S196-p; S197-p; S198-p
12	T111-p; S115-p; S116-p; K121-ub; Y211-p; S216-p; Y220-p; S228-p; S231-p
15	T84-p; S111-p; S211-p; S217-p; S218-p
16	S217-p; S278-p
18	Y206-p; S210-p; Y211-p; S214-p; S217-p ; Y220-p; T229-p ; Y241-p; T247-p; S253-p; Y254-p; S256-p; Y260-p
23	S203-p; S204-p; S206-p ; S207-p; Y223-p; Y224-p

^a p = phosphorylation; ub = ubiquitination.¹⁰⁸ Sites in bold are from GPM without a specific article reference from PhosphoSitePlus.^b Phosphorylated claudin-8 peptides were reported.⁹⁴

Table 4Identification of Potential O-Glycosylation Sites in Human Claudins Using NetOGlyc 4.0^{134,a}

gene/protein	O-glycosylation in the C-terminal tail
CLDN1	Y (T195)
CLDN2	Y (T204, S206, S207)
CLDN3	Y (S196, S200, T201, S206)
CLDN4	N
CLDN5	Y (S201, T207)
CLDN6	Y (S194, S201, T202, S203, S208, S212)
CLDN7	N
CLDN8	Y (S201, T204, T205, S208, S215)
CLDN9	N
CLDN10	Y (T198, S199, S202, T215, T216)
CLDN11	Y (S196)
CLDN12	Y (S212)
CLDN14	Y (T201, T202, T203, T204, T207, S224)
CLDN15	Y (S204, S211)
CLDN16	Y (S287, S289)
CLDN17	Y (T207, T213, T214)
CLDN18	Y (S210, S228)
CLDN19	Y (S195, S204)
CLDN20	Y (T198, S203)
CLDN22	Y (T204)
CLDN23	Y (S197, S203, S204, S206)
CLDN24	N
CLDN25	Y (S207)

^a A threshold of 0.5 was applied for this analysis.

Table 5Protein–Protein Interactions with Claudins^a

gene/protein	protein/protein interaction partners (UniProtKB/SwissProt manual annotation)	binary interactions (IntAct annotation ^g)
CLDN1 ^b	CLDN3 (but not CLDN2); TJP-ZO ^f ; MPDZ; INADL; HCV E1/E2 ^d	BRD4; DRG1; TACSTD2
CLDN2 ^b	CLDN3 (but not CLDN1); TJP-ZO ^f	KRTAP4-12; KRT31; NOTCH2NL; TJP1
CLDN3 ^b	CLDN1 and CLDN2; TJP-ZO ^f	
CLDN4	TJP-ZO ^f ; EPHA2	TACSTD2
CLDN5	TJP-ZO ^f ; MPDZ	
CLDN6	TJP-ZO ^f	
CLDN7	TJP-ZO ^f ; EPCAM ^c	HGD; RHOXF2; SYNE4; TACSTD2
CLDN8	TJP-ZO ^f	CCDC155; SYNE4
CLDN11	TSPAN3	
CLDN12		ECHS1; SEC13; STRN4
CLDN15	CLDN15 (linear homooligomers)	GEM
CLDN16		CLDN19; Cldn14 ^e
CLDN19		CLDN16; SRPK2; Cldn14 ^e

^a Compiled from www.swissprot.org and <http://www.ebi.ac.uk/intact/>.

^b Can form homo- and heteropolymers with other claudins.

^c Interaction requires claudin phosphorylation.

^d Direct interaction with HCV proteins has not been experimentally validated (see text).

^e Experiment done with mouse proteins.

^f TJP1/ZO-1; TJP2/ZO-2; TJP3/ZO-3; these protein partners are common to claudins 1–8.

^g In bold when found by two independent experiments.

Table 6
Domain-Specific Annotations of Protein Interactions with Claudin-1

claudin-1 domains	protein/protein interaction partners
cytosolic tail	TJP1/ZO-1 ²⁵⁴
	TJP2/ZO-2 ²⁵⁴
	TJP3/ZO-3 ²⁵⁴
	MPDZ/MUPP1 ²⁵⁵
	INADL/PATJ ²⁵⁶
	TACSTD2 ²⁵⁷
	SRC ²⁵⁸
	KRT76 ²⁵⁹
	LNX1 ^{125,260}
cis (transmembrane)	CD81 ²⁶¹
	CD9 ²¹¹
	OCL ²⁶²
	MARVELD2 ²⁶³
	MARVELD3 ⁸⁰
	CLDN3 ^{233,264}
	CLDN1 ²⁶⁵
trans junction/extracellular	CLDN7 ²³⁷
	CLDN1 ²⁶⁵
	CLDN3 ²⁶⁴
	MMP14 ²⁶⁶
	MMP2 ²⁶⁶
	EFNB1 ²⁶⁷