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Membrane-bound mucin modular domains: from structure to function

Membrane-bound mucin modular domains: from structure to function

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Abstract

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4 Mucins belong to a heterogeneous family of large *O*-glycoproteins composed of a long
5 peptidic chain called apomucin on which are linked hundreds of oligosaccharidic chains.
6
7 Among mucins, membrane-bound mucins are modular proteins and have a structural
8 organization usually containing Pro/Thr/Ser-rich *O*-glycosylated domains (PTS), EGF-like
9 and SEA domains. *Via* these modular domains, **the membrane-bound** mucins participate in
10 cell signalling and cell interaction with their environment in normal and pathological
11 conditions. Moreover, the recent knowledge of these domains and their biological activities
12 led to the development of new therapeutic approaches involving mucins. In this review, we
13 show 3D structures of EGF and SEA domains. We also describe the functional features of the
14 evolutionary conserved domains of **membrane-bound** mucins and discuss consequences of
15 splice events.
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46 Keywords: mucin, evolution, structure-function, EGF-like, 3D structure
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1. Introduction

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2 Mucins belong to a heterogeneous family of large *O*-glycoproteins composed of a long
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4 peptidic chain called apomucin on which are linked hundreds of oligosaccharidic chains.
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6 Based on biochemical and molecular biology studies, mucins were separated into two
7
8 structurally distinct **categories**: the secreted and the membrane-bound mucins. Mucins have a
9
10 cell- and tissue-specific pattern of expression that is profoundly altered in epithelial cancers
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12 (loss of expression, over-expression, aberrant expression, neo-expression) suggesting their
13
14 implication in tumourigenesis [1, 2]. They play roles in cell signalling, cell proliferation,
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16 tumour progression or cell polarity, and mediate immune evasion. Mucins are considered as
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18 potent new therapeutic targets in mucosal biology, in malignant and inflammatory diseases of
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20 the epithelial tissues [3-6].
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26 **Secreted mucins**, gel-forming components of viscoelastic mucus gels protecting the epithelia,
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28 include **the 11p15 secretory mucins**: MUC2, MUC5AC, MUC5B, MUC6. **Next to that**
29
30 **family of genes, a fifth secretory mucin was described that is MUC19**. Their main
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32 function is to participate in mucus formation by forming a three-dimensional network *via*
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34 oligomerization domains in order to protect underlying epithelia against various injuries
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36 (inflammation, bacteria, virus, pollutants, pH, etc). MUC7 and MUC9 are smaller secreted
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38 mucins that do not oligomerize and do not form gels [3, 7].
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43 The membrane-bound mucins belong to an ever increasing **group** of type I membrane-
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45 anchored proteins. Based on their structure and localization at the cell surface they are thought
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47 to act in cell-cell and cell-matrix interactions and in cell signalling. The biological roles they
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49 play in cellular interactions and in cell signalling indicate that they are involved in regulating
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51 biological properties of epithelial cells [4, 5]. **Among the membrane-bound mucins, some**
52
53 **conform to the mucin definition (presence of a Pro/Thr/Ser (PTS) region in the peptidic**
54
55 **sequence) that are** MUC1, MUC3A/3B, MUC4, MUC12, MUC16, MUC17, MUC21 and
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1 MUC22 [8-11]. **Others were assigned the term MUC despite the absence of that PTS**
2 **domain that are MUC13, MUC15 and MUC20.** Because of their specific pattern of
3
4 expression during the different steps of carcinogenesis, membrane-bound mucins stay under
5
6 intense investigation as both potent new biomarkers and therapeutic targets in epithelial
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8 cancers [2-4].
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11 Analysis of the peptidic sequences of mucins allowed description of their modular
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13 organization. Membrane-bound mucins are modular proteins which share conserved domains
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15 such as epidermal growth factor-like (EGF) **or** Sea urchin sperm protein Enterokinase and
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17 Agrin (SEA) **domains** (Figure 1) [8, 9, 12]. **The PTS domain, which is a common feature**
18
19 **between mucins is the only domain not conserved at the genomic level although**
20
21 **similarities exist at the amino acid level.** The human MUC1, MUC3A/3B, MUC12,
22
23 MUC13, MUC16, and MUC17 mucins contain the SEA domains. Some domains are specific
24
25 to one mucin. For **example**, the MUC4 mucin contains NIDO, AMOP, and VWF-D domains
26
27 that are unique in the apomucin family. MUC22 (also called as Panbronchiolitis-related
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29 mucin-like protein 1, PBMUCL1) has recently been discovered in the disease-susceptibility
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31 locus for diffuse panbronchiolitis and contains both the typical PTS and a transmembrane
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33 (TM) domains [10]. Existence of other conserved domains in MUC22 remains to be
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35 demonstrated. **At this time, MUC15 is considered as a mucin-like since it lacks the**
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37 **characteristic tandem repeat (TR) region.**
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46 Understanding the structure and the function of membrane-bound mucin domains will bring
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48 new information about their biological roles in epithelium homeostasis as well as in
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50 pathological situations such as carcinogenesis or inflammatory processes in which membrane-
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52 bound mucin expression **and localization are** often altered. In this review, we will discuss the
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54 structural and functional features of the evolutionary conserved domains of **membrane-**
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56 **bound** mucins and their abilities to modulate the biological properties of epithelial cells.
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2. *Mucins and evolution*

Phylogenetic analyses have shown that the membrane-bound mucins are only found in mammals with the exception of MUC16 and MUC4 [13]. Mucin domains are shared among most of the MUC family members, suggesting common ancestors **or adoption of functional modules during evolution**. MUC1, MUC3, MUC12, MUC13 and MUC17 **share a peptidic sequence similar to** heparan sulfate proteoglycan of basement membrane (HSPG2) (Figure 2). *MUC3*, *MUC12* and *MUC17* genes are present contiguously on chromosome 7 (7q22) supporting the hypothesis of a process of duplication. *MUC16* evolved separately from agrin. MUC4 arose from two evolutionary events involving (i) NIDO and EGF-like domains from a common ancestor to nidogen and (ii) AMOP and VWF-D domains from a common ancestor to the Susd2 [13]. Beside its homology through the SEA domain, MUC1 has sequence similarities with other membrane-bound mucins and in fact evolved from repeated sequences of MUC5B secreted mucin [14]. Based on available sequence information, one can hypothesize that membrane-bound mucins evolved from several distinct ancestor genes and could be divided in different subgroups based on their conserved domains. Interestingly, mucins arising from a common ancestor gene are frequently clustered in a chromosome locus as illustrated on figure 2.

3. *The membrane-bound mucin prototype domains*

A prototype membrane-bound mucin is **characterized** by the presence of an extracellular *O*-glycosylated PTS domain, a TM domain and a cytoplasmic tail. Two EGF-like and one SEA domains are commonly found in most of membrane-bound mucins (Figure 1A).

3. 1 *The PTS domain*

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The hallmark of membrane-bound mucins is the large extracellular subunit that protrudes at the cell surface mainly composed by the PTS domain. The PTS domain is the substrate of important post-translational *O*-glycosylation modifications. This central domain is encoded by a large intronless genomic DNA sequence (>10 kb), typically characterized by a variable number of tandem repeat (VNTR) polymorphism [15]. PTS domain harbours extensive *O*-glycosylation (up to 80% of the total weight of the mature mucin) that forms chains of varying lengths, sequences, and compositions. The biosynthesis of mucin *O*-glycan chains is a step-by-step process occurring in the Golgi apparatus, involving specific glycosyl- and sulfo-transferases expressed in a tissue-specific manner [16, 17]. The *O*-glycans on PTS domain play major roles in the conformation and stability of the mucin and participate in defense of mucosae covering and protecting the underlying epithelium against various types of aggression (mechanical and chemical stress). In diseases such as cancer and inflammation, mucin *O*-glycosylation is altered, modifying the antigenic and adhesive properties of the glycan epitopes they bear [18]. Tumour-associated carbohydrate antigens (TAAs) are produced *via* incomplete synthesis of carbohydrate chains. TAA notably involve Tn and T antigens and their sialylated counterparts, sialyl-Tn and sialyl-T antigens. Cancer-associated *O*-glycans are often highly sialylated and less sulfated compared with *O*-glycans from normal mucins [19, 20].

Because of its extracellular localization **at the apical pole of the cell, the** MUC_{PTS} domain is mainly thought to act **as a sensor of the microenvironment. In the normal polarized epithelial cell, it may interact with immune cells, antibodies, viruses, bacteria to help maintain the epithelial homeostasis. In a depolarized cancer cell, the membrane-bound mucin is then expressed circumferentially and is going to then play different roles in cell-cell or cell-extracellular matrix interactions.** For example, **the** MUC1_{PTS} domain **is able** to interact with adhesion molecules such as the endothelial protein ICAM-1 and

1 consequently may assist **the** cancer cell during heterotopic adhesion and invasion into the
2 endothelium and reattachment at distant metastasis sites [21, 22].
3

4 Galectins belong to a family of carbohydrate binding proteins (β -galactoside-specific lectins).
5 Galectin-1 was shown to interact with MUC16. Galectin-3 interacts with MUC1, MUC4 and
6 MUC16. *O*-glycans on MUC4_{PTS} interact with galectin-3 at the cell surface and mediate
7 docking of tumour cells on endothelial cells [22, 23]. Galectin-3-MUC1 interaction alters
8 MUC1 cell surface polarization, enhances tumour cell homotypic aggregation, prevents
9 anoikis [24, 25], and regulates EGFR internalization, subcellular localization and ERK
10 signalling pathway activation [26].
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24 **3. 2 The epidermal growth factor (EGF)-like domain**

25 EGF-like domains are 30-40 residue-long and evolutionarily well-conserved. The EGF-like
26 domain contains six cysteine residues that form three different disulfide bonds within the
27 domain (C1–C3, C2–C4, and C5–C6). The first cysteine exhibits a mild conservation between
28 the different mucins whereas the five others are highly conserved suggesting that the resulting
29 tertiary structure leads to biological functions (Figure 3A/3B).
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39 The EGF-like domains of membrane-bound mucins are believed to direct the interactions with
40 different proteins. Mucin EGF-like domains are thought to act as ligands with membrane
41 receptor such as those of the ErbB family mostly based on the work conducted on
42 MUC4/rMuc4 biological roles. It has been speculated that EGF domains located in **the**
43 released extracellular domain of mucins resulting from cleavage could also act as growth
44 factors and serve as indicators of alteration of epithelial surfaces [5].
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53 Experimental evidence suggests that rat homologues of Muc4 and ErbB2 are regulators of
54 signalling related to growth, motility or differentiation of the cell *via* the EGF-like domains
55 [27]. Human MUC4 and ErbB2 were shown to physically interact *via* a peptidic region
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1 including the three EGF-like and intermediate domains of MUC4 [28]. Recombinant human
2 MUC3A TM-EGF1/EGF2 protein led to reduced apoptosis induced either by TNF- α or Fas
3 receptor stimulation. MUC17 EGF-like domains (also designated as Cys-rich domains, CRD)
4 were shown to have anti-apoptotic and pro-migratory activity *via* ERK phosphorylation but
5 did not stimulate cell proliferation [29, 30]. *In vivo* rectally administered MUC17 CRD, as
6 well as its mouse homologue, accelerated healing in an experimental mouse model of colitis
7 [29], suggesting a potential therapeutic use of EGF-like domains as treatment of mucosal
8 inflammatory diseases.
9

10 The deduced amino-acid (aa) sequences of the EGF-like domains of MUC4, MUC3A,
11 MUC12, MUC13, MUC16 and MUC17 (Table1) were used to predict their 3D structures
12 using the Phyre 2 server (Protein Homology/analogy Recognition Engine V2.0) [31]. The
13 predicted 3D structures of each EGF-like domain was compared with the resolved 3D
14 structure of one monomer of the human EGF available through RCSB (Research
15 Collaboratory for Structural Bioinformatics) protein data bank (pdb)
16 (<http://www.pdb.org/pdb/home/home.do>) (pdb code : 1j19) and with the other EGF domains
17 3D predicted structures using the molecular visualization system, PyMOL (The PyMOL
18 Molecular Graphics System), version 1.5.0.1 Schrödinger, LLC). PyMOL did not allow us to
19 predict the secondary structure of the third EGF domain of MUC4 (MUC4_{EGF3}) nor of the
20 EGF domain of MUC12. To determine whether the EGF-like domains of two membrane-
21 bound mucins were structurally homologous, PyMOL was used to measure the root-mean-
22 square-deviation (RMSD) of superimposed 3D structures. Human EGF resolved structure
23 consists notably of two anti-parallel β sheets. This topology is conserved in MUC_{EGF}
24 predicted structures as illustrated on Figure 4A. The RMSD illustrates the average distance
25 value between the backbone alpha carbons of two structures and consequently, the RMSD of
26 two aligned structures indicates their divergence from one another. If the RMSD value is
27

1 below 1.5Å, two 3D structures whose sequence alignment is over 30% can be considered as
2 almost homologous. The most significant result is then correlated with the highest number of
3 residues aligned. Regarding the mucin EGF domains, there is strong structural homology
4 between the 3D structure of the human EGF and the 3D predicted structure of MUC4_{EGF1},
5 MUC3A_{EGF} and MUC13_{EGF1} (Table 1). We also found high homology between MUC13_{EGF1}
6 and MUC3A_{EGF} and MUC12_{EGF}. The 3D predicted structure of MUC4_{EGF1} was shown **to be**
7 homologous to MUC16_{EGF}.
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19 **3. 3 The Sea urchin sperm protein Enterokinase and Agrin (SEA)**

20 The SEA module is a 120 aa domain found in many membrane-associated proteins at the cell
21 surface. SEA domains were initially described as extracellular domains associated with *O*-
22 glycosylation but more recently they have also been implicated in both cleavage events and
23 association of the subunits [32-34]. Mucins usually contain one SEA domain in their
24 extracellular region. However, MUC16 contains multiple SEA domains that were repeated up
25 to 56 times through duplication events [13]. Alignment analysis indicated a mild conservation
26 of the SEA domains of the different membrane-bound mucins (Figure 3C). We confirmed that
27 MUC3, MUC12 and MUC17, that are located in a cluster on the human 7q22 chromosome,
28 share the highest identity of SEA sequences (Figure 3D).
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43 The MUC1_{SEA} module is a self-cleaving domain [35]. Computer modeling of MUC1_{SEA}
44 domain initially suggested that it consists in three α -helixes and six β -strands forming an α/β
45 sandwich fold [33]. Macao *et al.* later determined that SEA consists **of** four-stranded
46 antiparallel β -sheets and four α -helixes occurring in the $\beta 1-\alpha 1-\alpha 2-\beta 2 \uparrow \beta 3-\alpha 3-\alpha 4-\beta 4$ order
47 and with the helixes packed against the concave surface of the β -sheet [35]. Moreover,
48 determination of the SEA domain structure from the murine Muc16 homologue using multi-
49 dimensional NMR spectroscopy allowed Maeda *et al.* [36] to propose the GSVVV motif as a
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1 proteolytic cleavage site located in the short loop connecting $\beta 2$ and $\beta 3$ sheets. Levitin et al.
2 proposed that this module likely functions as a site for proteolytic cleavage in all SEA
3 module-containing proteins [37]. Cleavage of membrane-bound mucins in the SEA domain is
4 not entirely sequence dependent, but may be related to structural features of the SEA domain.
5 Exposure of the cleavage site protruding outward from a compact structure enhances its
6 recognition and cleavage by cellular proteases [33]. In addition to its cleavage involvement,
7 MUC17 EGF1-linker-EGF2 recombinant protein induces anti-apoptotic activity that requires
8 an intact SEA module [29].

9 The deduced aa sequences of the SEA domains of MUC1, MUC3A, MUC12, MUC13,
10 MUC16 and MUC17 (Table 2) were used to predict their 3D structures using the Phyre 2
11 server. The 3D predicted structures of each SEA domain was compared with the 3D resolved
12 structure of the human mucin MUC1 available in the protein data bank (pdb code : 2ACM)
13 and with the 3D predicted structures of the other SEA domains using the molecular
14 visualization system, PyMOL. In the same manner and with the same criteria as for the 3D
15 prediction structures of the EGF domains, we looked at the 3D structural homology thanks to
16 the RMSD calculation. Bioinformatic analysis indicates that the 3D resolved structure of
17 MUC1_{SEA} is homologous with MUC17_{SEA}. MUC17_{SEA} is itself structurally close to
18 MUC3A_{SEA} and MUC12_{SEA}. Superimposition of the 3D structures showed that the α/β
19 sandwich fold of resolved MUC1_{SEA} is conserved in predicted structures of MUC3A_{SEA},
20 MUC12_{SEA}, MUC17_{SEA} (Figure 4B) [33].

21 **4. The unique domains of membrane-bound mucin MUC4**

22 Among membrane-bound mucins, MUC4 contains NIDO, AMOP, and VWF-D domains that
23 are unique in the apomucin family.
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4. 1. *The Nidogen-like (Nido) domain*

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2 NIDO nidogen-like domain is an extracellular domain of unknown function found in nidogen
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4 and other hypothetical proteins such as sushi nidogen and EGF-like domains 1 (SNED1),
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6 plexin domain containing-1/-2 (PLXDC-1/-2) and tectorin alpha (TECTA). Phylogenetic
7
8 analysis revealed the origin of the MUC4_{NIDO} domain from an ancestor common to the NIDO
9
10 protein [13]. Recent work demonstrated for the first time that MUC4_{NIDO} does not alter the
11
12 motility of pancreatic cancer cells but promotes invasion and extravasation. The MUC4_{NIDO}
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14 domain, which is similar to the G1-domain of fibulin-2 or extracellular matrix protein
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16 Nidogen/entactin, contributes to the interaction properties of MUC4. Senapati *et al.*
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18 hypothesized that MUC4_{NIDO} disrupts the interaction between NIDO/entactin and fibulin-2
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20 [38], a major component of murine liver blood vessels [39], in a competitive inhibition
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22 manner and thus may create a favorable environment in liver for the extravasation of
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24 metastatic pancreatic cancer cells. MUC4_{NIDO} domain is thus proposed as a mediator of
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26 protease-independent cell invasion in pancreatic cancer metastasis [38].
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4. 2 *The Adhesion-associated domain in MUC4 and Other Proteins (AMOP)*

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37 AMOP and vWF-D domains originated from a common ancestor to the Sushi-domain
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39 containing protein 2 (SUSD2) [13]. AMOP domain is uncommon in the human genome as it
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41 is described only in four proteins (Isthmin-1, -2, SUSD2 and MUC4) using Simple Modular
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43 Architecture Research Tool (SMART) database. The role of AMOP domain is unclear.
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45 Presence of AMOP in cell adhesion molecules could be indicative of a role for this domain in
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47 adhesion. Indeed, mouse Isthmin, an angiogenesis modulator, disrupts endothelial cell (EC)
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49 capillary network formation on Matrigel through its C-terminal AMOP domain [40]. From
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51 there, one could hypothesize such role for MUC4 in angiogenesis.
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4.3 The Von Willebrand Factor-D (vWF-D) domain

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2 The human gel-forming mucins contain vWF-D and C-terminal cysteine-knot domains that
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4 are responsible for their oligomerization [9, 41, 42]. Only MUC4 membrane-bound mucin
5
6 contains a vWF-D in its intracellular region [9]. VWF domains usually participate in forming
7
8 disulfide bonds. However, the cysteine residues, conserved in secreted mucins, are not
9
10 conserved in MUC4 suggesting a loss of function of this domain in membrane-bound mucins
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12 during evolution [43]. The crystal structures of three vWF domains are known. All three
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14 domains share identical three-dimensional fold with a α - β - α sandwiched model [44].
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5. The cytoplasmic tail

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24 Cytoplasmic tails (CT) of mucins are poorly conserved ruling out a potential common
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26 evolution. Size and aa sequence greatly vary among membrane-bound mucins leading to a
27
28 great variety of functions in cell signalling (Table 3). Indeed, MUC4 acts as a receptor partner
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30 at the membrane through its extracellular domain because of its short CT whereas MUC1 acts
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32 as an intracellular docking protein for signalling molecules. Among membrane-bound mucins,
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34 MUC1 has been under intense investigation.
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39 MUC1_{CT}, is 72 aa long, contains seven tyrosine residues, an Src Homology 2 (SH2)
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41 interaction domain, and is phosphorylated by several kinases [4, 22, 45, 46]. The MUC1_{CT}
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43 allows the direct interaction of MUC1 with a wide array of signalling pathways in tumour
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45 cells. MUC1_{CT} was shown to interact with Src family kinase including c-Src, Lyn and Lck
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47 and promotes phosphorylation of Tyr46 involved in protein-protein interaction. MUC1_{CT} is
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49 also targeted by kinases ζ chain associated protein kinase of 70 kDa (ZAP70), and the δ
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51 isoform of protein kinase C (PKC δ) [22, 47]. Interaction between MUC1_{CT} and proapoptotic
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53 kinase cAbl leads to phosphorylation of Tyr60 by its binding to the c-Abl SH2 domain [48].
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57 Binding of scaffolding protein Grb2 on MUC1_{CT} activates MAPK pathway, involved in
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1 proliferation of tumour cells [49]. MUC1_{CT} interacts with components of the IκB kinase
2 (IKK) complex enhancing IKKα-IKKβ interaction, promoting IKKβ phosphorylation, IKKα
3 degradation, NFκB-p65 targeting to the nucleus that leads to NF-κB p65 transcriptional
4 activity [50, 51]. MUC1_{CT} interacts with chaperone heat shock proteins (HSP) HSP70 and
5 HSP90 leading to mitochondrial translocation of MUC1_{CT} and therefore inhibiting apoptosis
6
7 [52]. MUC1_{CT} binds to Wnt signalling pathway components β-catenin and APC. MUC1_{CT}-β-
8 catenin interaction enhances the levels of nuclear β-catenin during disruption of cadherin-
9 mediated cell-cell adhesion and promotes expression of Wnt target genes [46, 53]. The
10 MUC1_{CT}-β-catenin binding is regulated by GSK3β and HSP90 in a competitive inhibition
11 manner [54]. MUC1_{CT} is phosphorylated by several cell surface receptors such as fibroblast
12 growth factor receptor 3 (FGFR3), platelet-derived growth factor receptor (PDGFR) and ErbB
13 receptor family altering interaction with proteins mentioned above. FGF1 induces Tyr46
14 YEKV phosphorylation whereas PDGF catalyses phosphorylation of Tyr35 and enhances
15 invasion *in vitro*, tumour growth and metastasis *in vivo* [55].

16 MUC1_{CT} also regulates its own nucleo-cytoplasmic transport by binding importin-β and
17 nucleoporin p62 (nup62) *via* a CQC motif and therefore alters transcriptional regulation of
18 numerous target genes [56]. In the cytoplasm of breast and lung cancer cells, MUC1_{CT} forms
19 dimers that are necessary for its nuclear localization *via* the CQC motif [56, 57]. In the
20 nucleus, MUC1_{CT} associates with transcription factors such as β-catenin/TCF4, p53,
21 CDKN1A (p21), nuclear factor-κB p65 or STATs [3]. The MUC1_{CT} domain also stabilizes
22 estrogen receptor-α (ERα) and is necessary for its nuclear localization [58].

23 MUC1_{CT} has been extensively characterised whereas other mucin CT are scarcely described.
24 MUC3_{CT} contains an YVAL aa motif which is similar to motifs recognized by the SH2
25 domain [59]. MUC13_{CT} contains a PKC phosphorylation site [60]. MUC16_{CT} contains
26 polybasic aa that are predicted to interact with ezrin/radixin/moesin (ERM) actin-binding
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1 proteins [61] and is proposed to cross-link MUC16 with actin-cytoskeleton. MUC16_{CT} is also
2 proposed to interact with JAK2 *via* its ERM domain and mediate breast cancer cell
3 proliferation [62]. PDZ-interacting domains are observed within MUC3_{CT}, MUC12_{CT} and
4 MUC17_{CT} but only phosphorylation sites of MUC17_{CT} exhibit a strong binding to PDZ
5 domain of PDZK1 [63]. It was also shown using *Pdzk1*^{-/-} mice that *Pdzk1* plays a specific role
6 in stabilizing mMuc3 (MUC17 mouse homologue) at the apical pole of polarized enterocytes.
7 MUC20_{CT} binds to a multifunctional docking site of hepatocyte growth factor (HGF) receptor
8 Met and therefore suppresses the Grb2-Ras pathway [64].

9 The tremendous variability of MUC_{CT} is thus associated with a wide array of signalling
10 pathways regulated by membrane-bound mucins and much is still to be discovered to fully
11 characterize their biological activities and effects on epithelial cell behavior.
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19 **6. Mucin splice variants**

20 **6. 1. MUC1**

21 Membrane-bound mucin isoforms or alternative splicing events have been reported for
22 MUC1, MUC3A, MUC4 and MUC17 that lead to new apomucin polypeptides possibly
23 lacking functional domains and presumably showing altered cellular/biological functions.
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25 MUC1/X is an alternate isoform of MUC1 in which the extracellular domain is comprised of
26 the SEA module in addition to thirty MUC1 N-terminal aa residues [37]. MUC1/Z and
27 MUC1/Y are two isoforms lacking the PTS domain. MUC1/Z isoform is proteolytically
28 cleaved after its synthesis and generates the two subunits MUC1 α and MUC1 β . On the
29 contrary, MUC1/Y contains a truncated SEA domain and therefore lacks the cleavage site,
30 resulting in a single uncleaved apomucin. MUC1/Y also contains the TM and cytoplasmic
31 domains. Interestingly, MUC1/Y isoform expression increases the tumorigenicity of DA3
32 mouse mammary epithelial cells [65]. An alternate isoform MUC1/ZD results from a reading
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frameshift caused by a splicing event that deletes the TR-PTS domain but contains the signal peptide and a subsequent stretch of 30 aa [66]. Variant MUC1/SEC lacks the TM and cytoplasmic sequences and has the potential to be directly secreted out of the cell. Interestingly, tumour cells expressing MUC1/SEC fail to develop tumours in immunocompetent mice. MUC1/SEC may inhibit tumour development and may support antitumour immune responses *via* the downregulation of Urokinase-Type Plasminogen Activator (uPA) and Signal Transducer and Activator of Transcription 1 (STAT1) [67]. Moreover, MUC1/SEC is capable of blocking expression of arginase 1 and production of reactive oxygen species (ROS) in myeloid-derived suppressor cells MDSCs that play a critical role in tumour-induced immunosuppression [68].

6.2. *MUC4*

Multiple splicing events have been described for *MUC4* [69]. Among them, MUC4/Y and MUC4/X lack the central TR/MUC4_{PTS} domain, the main feature of membrane-bound mucins [69]. Some MUC4 isoforms are also lacking TM domain and might be secreted. One could hypothesize that the EGF-like domains of MUC4 act as growth factors when released in the extracellular environment. Globally, more than twenty cDNA isoforms have been described for *MUC4* that are generated by several mechanisms (alternative use of cassette exons, exon skipping or use of cryptic splice donor/acceptor sites) [70]. However, till now no splice variants for Muc4 have been observed in mouse tissues [9]. So far, the existence at the protein level and the biological roles of the different MUC4 isoforms remain to be determined.

6.3. *Other mucin variants*

Transcription variants of *MUC3* encode truncated proteins, suggesting the possibility of expression of soluble forms [71]. Williams *et al.* confirmed the existence of two secreted

1 isoforms of MUC3 that lack the TM domain [72]. In a similar manner, an alternative MUC17
2 splice event occurs and lacks the second EGF domain, the TM domain, and the cytoplasmic
3 tail and leads to a secreted form MUC17/SEC [73].
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9 **7. Using MUC domains as therapeutic targets/tools**

10 Membrane-bound mucins display major biological activities in epithelium homeostasis and
11 pathologies and hold promise as biological tools for therapy in cancer or inflammatory
12 diseases [4].
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18 The extended knowledge of MUC1 domains and their biological significance led to the
19 development of peptide-based therapies that may have important clinical implications [1, 3, 4,
20 74]. As an example, MUC1 inhibitory peptide (MIP) was designed to block the intracellular
21 interactions between MUC1/ β -catenin and MUC1/EGFR. MIP was then fused to the protein
22 transduction domain, PTD4 (PMIP) in order to increase cellular uptake. Exogenous treatment
23 of PMIP led to a significant reduction in aggressiveness of metastatic breast cancer cells *in*
24 *vitro*, and inhibition of growth and recurrence of breast tumour in an *in vivo* transgenic model
25 [75]. Moreover, PMIP selectively inhibits lung adenocarcinoma proliferation, and inhibits ER
26 responses *via* the blocking of the MUC1-ER α interaction [76]. MUC1_{CT} dimerization may
27 also be targeted by reducing agents. Similarly, cell-penetrating peptide containing the
28 CQCRRKN sequence binds directly to the endogenous MUC1_{CT} and blocks its ability to
29 dimerize [57]. That blockage induces death of human breast cancer cells *in vitro* and in
30 xenograft tumour models [77].
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51 Since MUC1 displays ubiquitous expression in a wide variety of tumour types, numerous
52 studies are targeting MUC1. The MUC1 peptide core or glycopeptide has been used in
53 immunotherapy for immunization. The different strategies showed T cell- and antibody-
54 responses and then were challenged for tumour protection [78, 79]. Recently, immunization
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by a tripartite vaccine based on a synthetic 60-mer MUC1_{PTS} derived peptide covalently linked to a Toll-like receptor (TLR) agonist could elicit IgG antibodies that efficiently lysed MUC1-expressing cancer cells, stimulated cytotoxicity of T lymphocytes, and activated innate immune responses [80].

Trastuzumab (also called herceptin®), a monoclonal antibody, targets ErbB2-expressing tumours such as breast cancers and leads to inhibition of intracellular signalling and induction of an immune system-mediated antitumour response [81]. Human MUC4 and ErbB2 do physically interact *via* MUC4_{EGF3+1+2} region that contains the three EGF-like domains [28]. MUC4 overexpression decreases cancer cell sensitivity to trastuzumab, possibly by steric hindrance [82]. MUC4 is thought to impair access of trastuzumab to ErbB2 *via* its large PTS domain that protrudes outside the glycocalyx. More recently, we also showed an effect of MUC4 on gemcitabine sensitivity in pancreatic ductal adenocarcinoma [83] but via domains and partner still to identify.

MUC17_{EGF} domains display a strong potential in the treatment of mucosal inflammatory diseases as healing is accelerated in acetic acid and dextran sodium sulfate (DSS)-induced colitis in mice [29]. MUC1 also has a protective effect against DSS-induced colitis in mice. Since Muc1 is lacking EGF-like domains, this effect is most likely mediated by another mechanism. MUC1 could act through its ability to increase the mucus barrier but also by decreasing T cell recruitment to the afflicted site [84].

7. Conclusions

Membrane-bound mucins are characterized by a multi-domain organisation with a **PTS domain**, conserved EGF or SEA domains and unique domains such as AMOP, NIDO or vWF-D that could mediate specific functions for some membrane-bound mucins. Understanding the structure and the biological functions of each domain might help explain

the major roles of mucins in inflammatory and cancerous diseases of the epithelium. In the future, the better structural and functional characterization of these domains should give rise to new mucin-based therapeutic targets/tools.

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Figure legends

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5 **Figure 1: Membrane-bound mucin prototype.** (A) Membrane-bound mucins are modular
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7 proteins sharing conserved domains such as epidermal growth factor-like (EGF), Sea urchin
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9 sperm protein Enterokinase and Agrin (SEA) or Pro/Thr/Ser (PTS) domains or MUC4-
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11 specific domains (AMOP, Nido and vWF-D). (B) Alternative splicing events have been
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13 reported and lead to new secreted or membrane-bound apomucin polypeptides possibly
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15 lacking functional domains and presumably showing altered cellular/biological functions.
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21 **Figure 2: Evolution of the membrane-bound mucins.** Phylogenetic trees of membrane-
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23 bound mucins were built after aligning of amino acid sequences of various domains
24
25 conserved in mucin families. Three independent trees were deduced clustering (A) MUC1,
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27 MUC3, MUC12, MUC13, MUC17 evolving from SEA-containing protein HSGP2. MUC3_{EGF}
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29 domains display the highest identity with EGF. MUC1 cytoplasmic tail (CT) peptide
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31 sequence is related to MUC5B tandem repeat (TR) (B) MUC4 arises from two ancestor
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33 proteins common to Nidogen (Nido and EGF-like domains) and Susd2 (AMOP and vWF-D
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35 domains) (C) MUC16 arises from multiplication of SEA domain related to Agrin proteins.
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43 **Figure 3: EGF-like and SEA domains of membrane-bound mucins.** (A) Alignment of
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45 EGF domains of membrane-bound mucins and EGF. Peptidic sequences of the domains were
46
47 identified by Protein Knowledgebase (UniprotKB, <http://www.uniprot.org>) (B) Phylogenetic
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49 tree of identity of EGF domains. (C) Alignment of SEA domains of membrane-bound mucins.
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51 Peptidic sequences of the domains were identified by Protein Knowledgebase. * MUC16
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53 contains up to 56 SEA domains. For clarity purpose, we decided to use the SEA1 domain as a
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55 template for alignment analysis. (D) Phylogenetic tree of identity of SEA domains.
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2 **Figure 4: Three dimensional structure of EGF and SEA domains of membrane-bound**
3 **mucins.** (A) Ribbon diagram showing the superimposition of the 3D structure of the
4 EGF1 and EGF2 domains of MUC4 and the human EGF. (The PyMOL Molecular Graphics
5 System, Version 1.5.0.1 Schrödinger, LLC). (B) Ribbon diagram showing the
6 superimposition of the 3D structure of MUC1_{SEA} with the 3D predicted structure of
7 MUC3A_{SEA}, MUC12_{SEA} and MUC17_{SEA} (The PyMOL Molecular Graphics System, Version
8 1.5.0.1 Schrödinger, LLC).
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22 **Table 1. RMSD values of mucin EGF domains superimposition on alpha carbons.** The
23 3D structure predictions of the mucins EGF domains was carried out with the Phyre 2 server
24 (Protein Homology/analogy Recognition Engine V 2.0) by using the deduced amino-acids
25 sequences of each domain. The predicted 3D structure of each EGF domain was compared
26 with the resolved 3D structure of the human EGF (PDB code : 1j19) and with the other EGF
27 domains predicted structures using the molecular visualization system ,PyMOL (The PyMOL
28 Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC). To determine whether the
29 EGF domains were structurally homologous, PyMOL was used to measure the root-mean-
30 square-deviation (*RMSD): the average distance between the alpha carbons atoms (the
31 backbone atoms) of superimposed proteins. n.s:no 3D structure prediction. *RMSD : Root
32 Mean Square Deviation is the square root of the mean of the square of the distances between
33 the matched atoms. $RMSD = \sqrt{(\sum(d_{ii})^2)/N}$. d_{ii} is the distance between the i th atom
34 of structure 1 and the i th atom of structure 2 and N is the number of atoms matched in each
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Table 2: RMSD values of mucin SEA domains superimposition on alpha carbons. The

3D structure predictions of the mucin SEA domains was carried out with the Phyre 2 server (Protein Homology/analogy Recognition Engine V 2.0) by using the amino-acids sequence of each domain. The predicted 3D structure of each SEA domain was compared with the resolved 3D structure of the human SEA domain of MUC1 (PDB code : 2ACM) and with the others SEA domains predicted structures using the molecular visualization system ,PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC). To determine whether the SEA domains were structurally homologous, PyMOL was used to measure the root-mean-square-deviation (*RMSD).

Table 3: Sequence and functions of the cytoplasmic tails of membrane-bound mucins.

Table 1

Table 1. RMSD values of mucin EGF domains superimposition on alpha carbons

EGF	Human EGF	MUC4_EGF1	MUC4_EGF2	MUC4_EGF3	MUC3A_EG F	MUC17_EGF	MUC12_EGF	MUC13_EGF 1	MUC13_EGF 2	MUC13_EGF 3	MUC16_EG F
MUC4_EGF1	1.272 (25 atoms)		3.708 (21 atoms)	n.s	0.502 (15 atoms)	0.876 (13 atoms)	1.947 (14 atoms)	3.698 (12 atoms)	n.s	5.958 (30 atoms)	1.128 (27 atoms)
MUC4_EGF2	3.929 (24 atoms)	3.708 (21 atoms)		n.s	4.664 (24 atoms)	5.175 (35 atoms)	8.013 (16 atoms)	5.163 (26 atoms)	n.s	3.188 (14 atoms)	3.082 (30 atoms)
MUC4_EGF3	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	ns
MUC3A_EG F	0.502 (15 atoms)	2.177 (17 atoms)	4.664 (24 atoms)	n.s		4.776 (19 atoms)	7.122 (23 atoms)	0.148 (7 atoms)	n.s	8.884 (19 atoms)	2.559 (16 atoms)
MUC17_EGF	2.235 (22 atoms)	0.876 (13 atoms)	5.175 (35 atoms)	n.s	4.776 (19 atoms)		3.195 (23 atoms)	0.226 (4 atoms)	n.s	3.490 (18 atoms)	1.747 (21 atoms)
MUC12_EGF	2.093 (24 atoms)	1.947 (14 atoms)	8.013 (16 atoms)	n.s	7.122 (23 atoms)	3.195 (23 atoms)		1.455 (12 atoms)	n.s	1.812 (8 atoms)	2.643 (25 atoms)
MUC13_EGF 1	0.793 (22 atoms)	3.698 (12 atoms)	5.163 (26 atoms)	n.s	0.148 (7 atoms)	0.226 (4 atoms)	1.455 (12 atoms)		n.s	3.272 (27 atoms)	2.087 (24 atoms)
MUC13_EGF 2	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	ns
MUC13_EGF 3	1.178 (7 atoms)	5.958 (30 atoms)	3.188 (14 atoms)	n.s	8.884 (19 atoms)	3.490 (18 atoms)	1.812 (8 atoms)	3.272 (27 atoms)	n.s		2.867 (28 atoms)
MUC16_EGF	2.803 (41 atoms)	1.128 (27 atoms)	3.082 (30 atoms)	n.s	2.559 (16 atoms)	1.747 (21 atoms)	2.643 (25 atoms)	2.087 (24 atoms)	n.s	2.867 (28 atoms)	

Table 2. RMSD values of mucin SEA domains superimposition on alpha carbons

SEA	MUC1_SEA (2ACM)	MUC3A_SEA	MUC12_SEA	MUC13_SEA	MUC16-SEA	MUC17_SEA
MUC3A_SEA	3.812 (61 atoms)		4.615 (87 atoms)	11.740 (20 atoms)	1.759 (66 atoms)	0.004 (48 atoms)
MUC12_SEA	5.363 (66 atoms)	4.615 (87 atoms)		10.773 (45 atoms)	8.417 (82 atoms)	0.004 (56 atoms)
MUC13_SEA	11.740 (20 atoms)	13.996 (75 atoms)	10.773 (45 atoms)		3.361 (40 atoms)	6.417 (30 atoms)
MUC16_SEA	0.912 (44 atoms)	1.759 (66 atoms)	8.417 (82 atoms)	3.361 (40 atoms)		7.400 (48 atoms)
MUC17_SEA	1.204 (52 atoms)	0.004 (48 atoms)	0.004 (56 atoms)	6.417 (30 atoms)	7.400 (48 atoms)	

Table 3

apomucin	Cytoplasmic tail sequence	Protein binding-domains and phosphorylated tyrosines
MUC1	CQCRRKNYGQLDIFPARDTYHPMSEYPT YHTHGRYVPPSSTDRSPYEKVSAGNGGS SLSYTNPAVAATSANL	7 Tyr SH2 domain c-Src, Lyn, Lck, ZAP70, c-Abl, Grb2, IKK, HSP70, HSP90, b- catenin, GSK3, FGFR3
MUC3	AVRSGWWGGQRRGRSWDQDRKWFET WDEEVVGTFSNWFEDDGTDKDTNFH VALENDTTMKVHIKRPEMTSSSV	SH2 domain
MUC4	LRFWGCSGARFSYFLNSAEALP	1 Tyr
MUC12	SQRKRHREQYDVPQEWKKEGTPGIFQK TAIWEDQNLRESRFLENA YNNFRPTLE TVDSGTELHIQRPEMVA STV	2 Tyr
MUC13	VTARSNNKTKHIEEENLIDEDFQNLKLRS TGFTNLGAEGSVFPKVRITASRDSQMQN PYSRHSSMPRPDY	2 Tyr PKC
MUC15	GKRKTDSFSHRRLYDDRNEPVLRLDNAP EPYDVSVFGNSSYYNPTLND SAMP ESEEN ARDGIPMDDIPPLRTSV	4 Tyr
MUC16	VTTRRRKKEGEYNVQQQCPGY YQSHLD LEDLQ	3 Tyr moesin
MUC17	SIYSNFQPSLRHIDPETKIRIQRPQVM TTS F	1 Tyr PDZK1
MUC20	ENGGFLLRLSVASPEDLTDPRVAERLM QQLHRELHAHAPHFQVSLLRVRRG	c-Met
MUC21	RNSLSLRNTFNTAVYHHPHGLNHGLGPGP GGNHGAPHRPRWSPNWFWR RPVSSIAM EMSGRNSGP	1 Tyr
MUC22	SFCLRNLFPLRYCGIYYPHGSHSLGLD LNLGLGSGTFHSLGNALVHGGELEMGH GGTHGFGYGVGHGLSHIHGDGYGVNHG GHYGHGGGH	6 Tyr

Table 3: Sequence and functions of the cytoplasmic tails of membrane-bound mucins.
Tyrosines that can be phosphorylated are bold.

Figure 1
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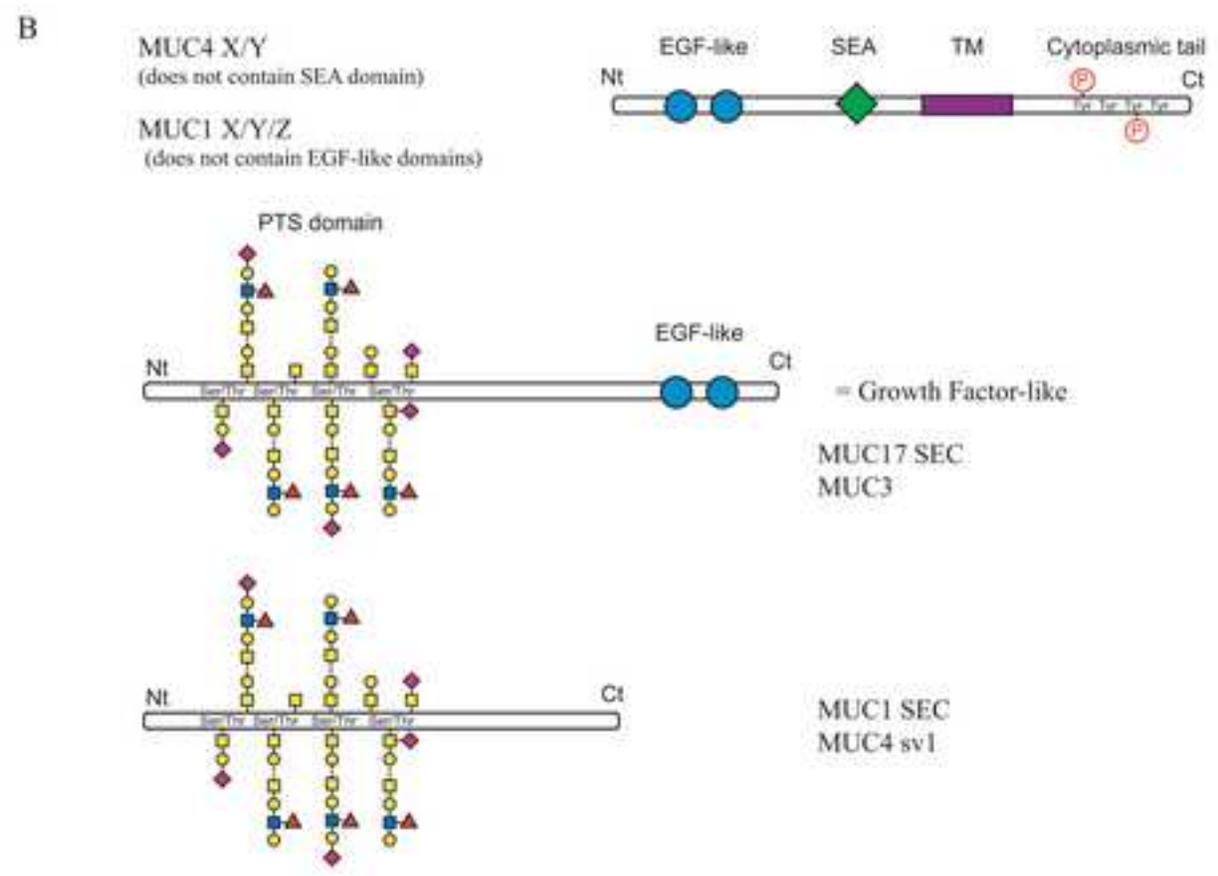
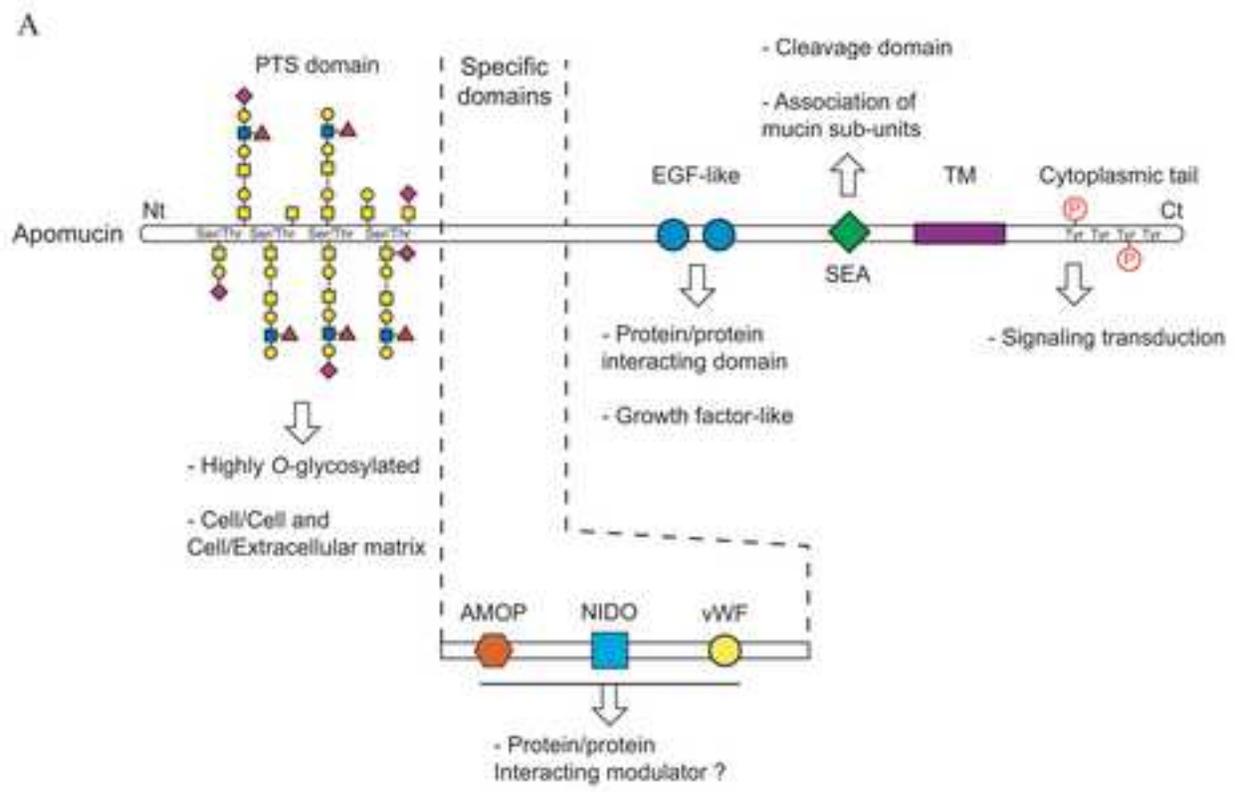


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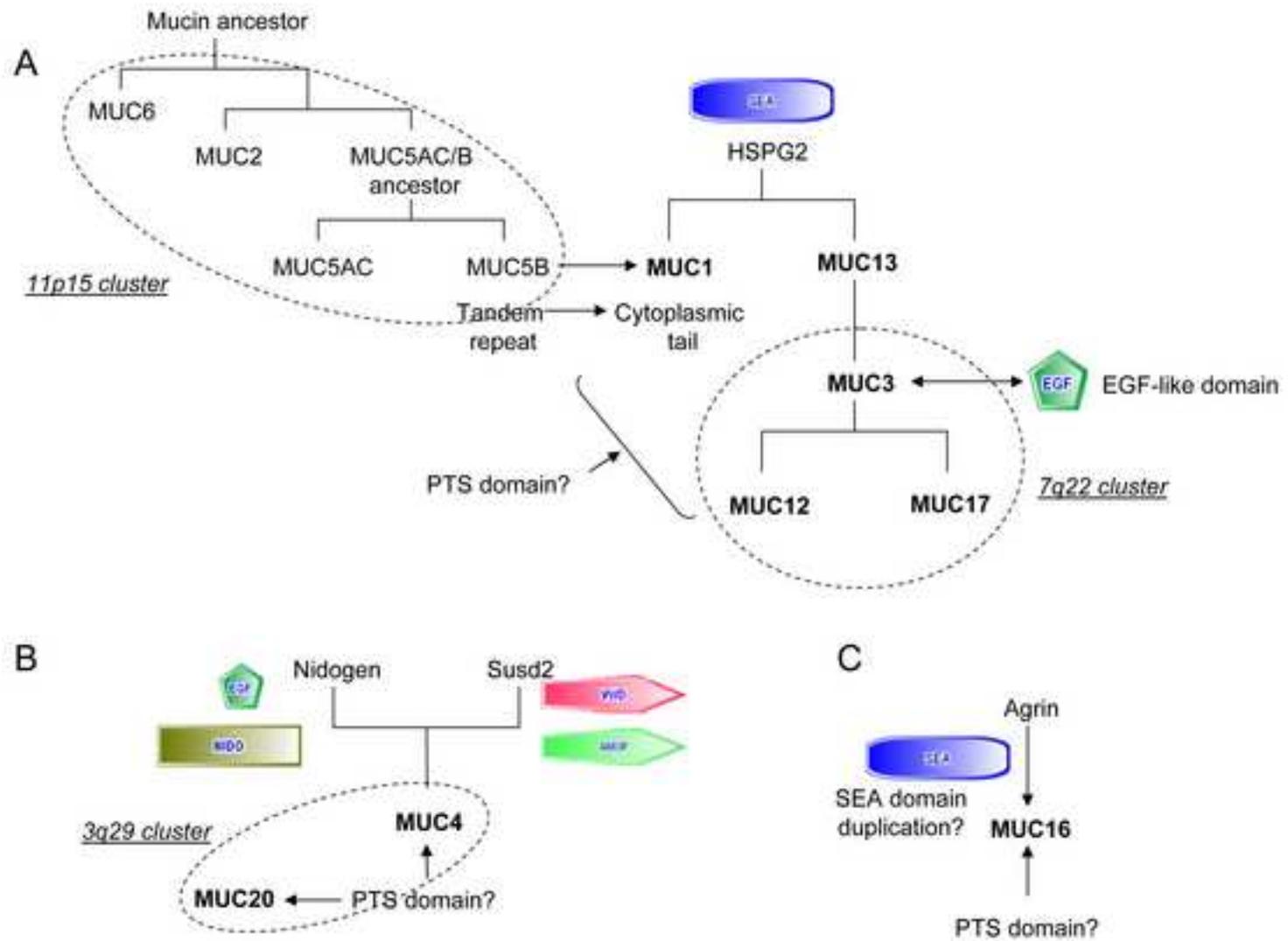


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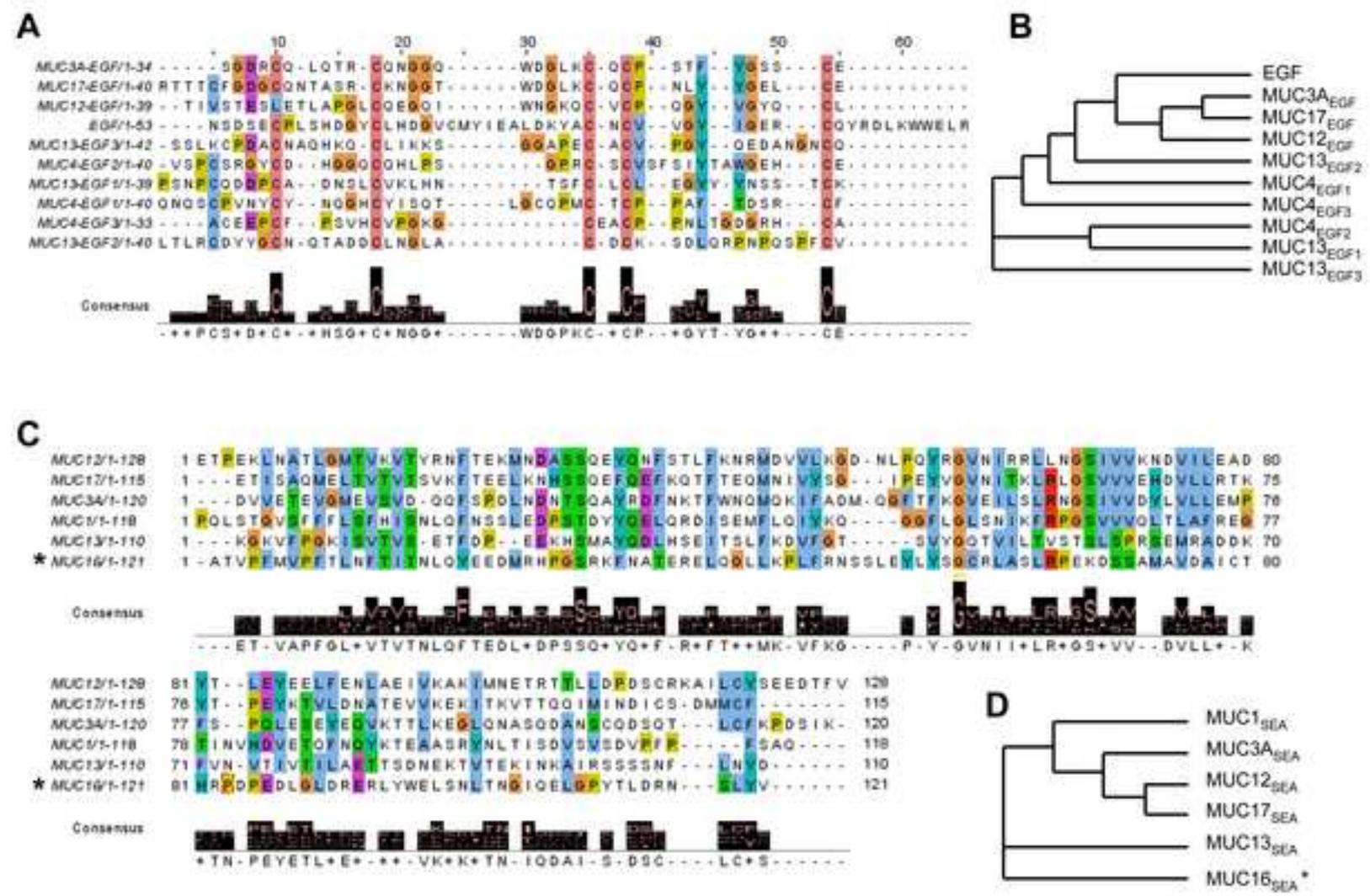
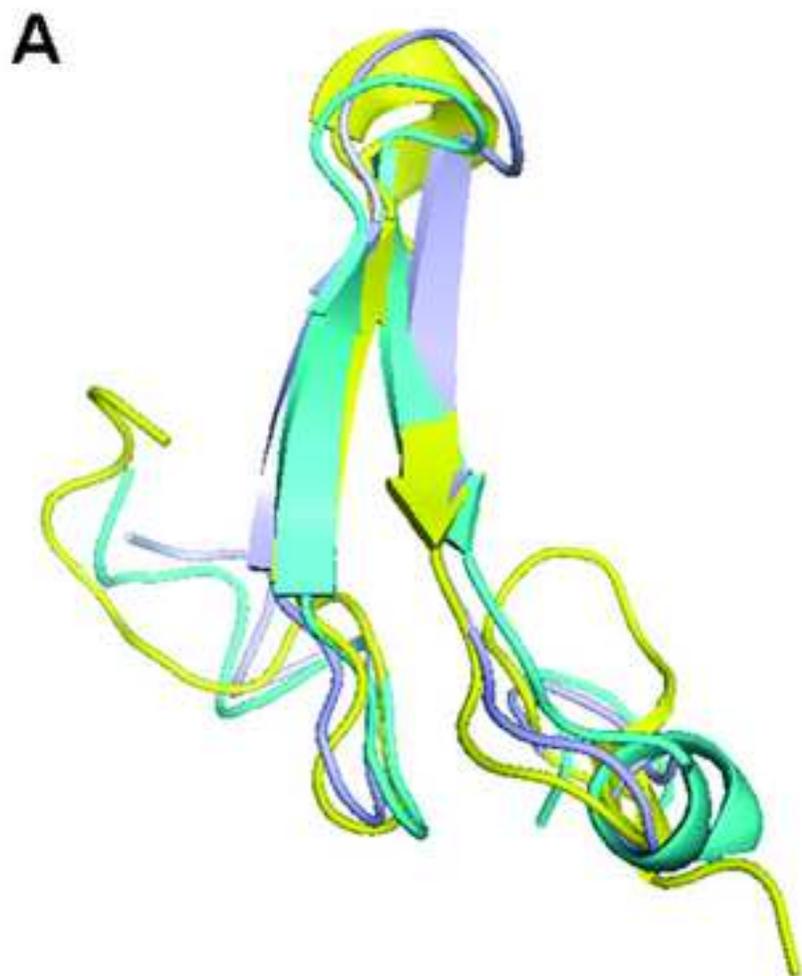
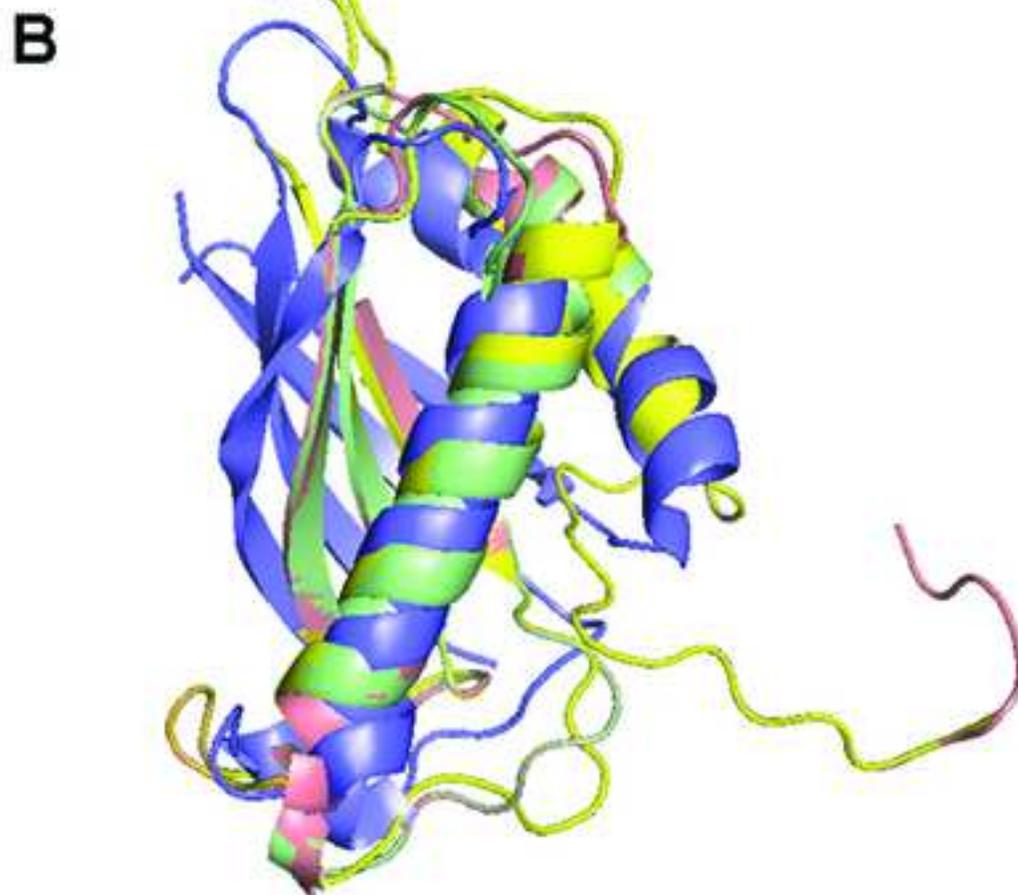


Figure 4
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	Predicted 3D structure of MUC4 _{EGF1}
	Predicted 3D structure of MUC4 _{EGF2}
	3D structure of human EGF



	Predicted 3D structure of MUC3A _{SEA}
	Predicted 3D structure of MUC12 _{SEA}
	Predicted 3D structure of MUC17 _{SEA}
	3D structure of MUC1 _{SEA}