Biochemical and functional studies on mouse ficolin-B, a novel pattern recognition molecule of the innate immune system

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Preetham Elumalai aus, Indian

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Board of examiners:

Chairman: Prof. Dr. Oliver Reiser
First Examiner: Prof. Dr. Jörg Heilmann
Second Examiner: Prof. Dr. Daniela Männel
Third Examiner: Prof. Dr. Armin Buschauer

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt und keine anderen als die hier angegebenen Quellen und Hilfsmittel verwendet habe.

……..

Preetham Elumalai

To my daughter Prarthana who came to this

world at the same time as this thesis.

"The scientist is not a person who gives the right answers,

he's one who asks the right questions"

~Claude Lévi-Strauss (1908 – 2009)

Abstract

Ficolins are members of the collectin family of proteins which in human and mice are able to recognize pathogen associated molecular patterns (PAMPs) on microbial surfaces. Ficolins trigger the activation of the innate immune system by initiating the complement cascade in serum upon binding to their specific PAMPs. Our group recently published the first observation on the cellular localization of mouse ficolin-B. In contrast to the human ortholog M-ficolin which is secreted, ficolin-B was only detected intracellularly in peritoneal macrophages (Runza *et al*., 2006). Investigations on the expression profile in our laboratory indicated that ficolin-B expression is down-regulated upon maturation of myeloid cells such as macrophages, granulocytes, and bone marrow-derived dendritic cells suggesting a critical role of ficolin-B during early stages of cell activation upon pathogen encounter. In contrast to others who have shown that ficolin-B does not associate to serine proteases and, therefore, is unable to activate the complement system (Endo et al., 2005) unpublished findings by our group show complement activation by ficolin-B. However, the biological relevance of these findings and the function of mouse ficolin-B upon bacterial challenge remains to be elucidated.

An established method for the recombinant production of ficolin-B in an eukaryotic (insect) expression (DS2 cells) system exists in our lab. This method is, however, expensive and time consuming. The first goal of this work was to establish an alternative expression system in *E*.*coli* to produce recombinant ficolin-B without tag. The biological activity of the *E*.*coli*-expressed ficolin-B was to be compared to the activity of the DS2-expressed ficolin-B. The protein should then be used to immunize rats to generate monoclonal antibodies.

In the second part of the project functional characterization of ficolin-B through mutational analysis should be tested. Ficolin-B muteins are expected to define the differences in fine specificity as shown by *Xenopus*, mouse, and human ficolins and, as such, bring evidence for adaptive changes during evolution. Ficolin-B has a conserved collagen binding site (Girija *et al.*, 2007) that has been linked to important functions such as lectin pathway activation and collaboration with the blood coagulation system by interacting with serine proteases like MASPs (Endo *et al.,* 2010). Weak adjacent sites of the MASP binding domain may enhance or decrease affinity for binding, but little is known about the biological role of this affinity modulation. The aim was to alter the biological activity of ficolin-B by introduction of a single amino acid mutation in the collagen like domain. Protein biochemical and chromatographic studies were performed to compare the ficolin-B wild type and mutant forms.

Zusammenfassung

Ficoline sind Mitglieder der Collectin-Proteinfamilie, die in Mensch und Maus Pathogen-assoziierte molekulare Muster (PAMP) auf Oberflächen erkennen. Ficoline starten das angeborene Immunsystem, indem sie im Serum nach Bindung an spezifische PAMP die Complement-Kaskade aktivieren. Unsere Arbeitsgruppe zeigte als erste, dass Maus-Ficolin-B intrazellulär lokalisiert ist. Im Gegensatz zum humanen Ortholog, M-Ficolin, wurde Ficolin-B nur intrazellulär in Peritonealmakrophagen nachgewiesen (Runza et al., 2006). Untersuchungen zum Expressionsprofil aus unserem Labor zeigten, dass Ficolin-B während der Reifung myeloider Zellen, wie Makrophagen, Granulozyten und vom Knochenmark abgeleiteten Dendritischen Zellen, herunterreguliert wird, was für eine wichtige Funktion von Ficolin-B in den frühen Stadien der Zellaktivierung nach Pathogen-Kontakt spricht. Ebenfalls im Gegensatz zu Befunden anderer Gruppen, die zeigten, dass Ficolin-B nicht mit Serinproteasen assoziiert und daher unfähig ist das Complementsystem zu aktivieren (Endo et al. 2005), finden wir eine Aktivierung des Complementsystems durch Ficolin-B (unveröffentlicht). Die biologische Bedeutung dieser Befunde und die Funktion von Maus-Ficolin-B nach bakterieller Belastung müssen jedoch noch geklärt werden.

Eine Methode zur Produktion von rekombinantem Ficolin-B in Insekten(DS2)-Zellen ist in unserem Labor etabliert. Die Methode ist jedoch teuer und langwierig. Das erste Ziel dieser Arbeit war es, ein alternatives Expressionsystem in *E. coli* zu etablieren, um rekombinantes Ficolin-B ohne *Tag* zu produzieren. Die biologische Aktivität des *E. coli*-exprimierten Ficolin-B sollte mit der Aktivität von DS2-Zellen-exprimiertem Ficolin-B verglichen werden. Das Protein selbst sollte zur Immunisierung von Ratten zur Herstellung monoklonaler Antikörper dienen.

Im zweiten Teil des Projektes sollte Ficolin-B funktionell durch Mutations-Analyse charakterisiert werden. Ficolin-B-Muteine könnten Spezifitätsunterschiede erklären, wie schon für adaptive Veränderungen während der Evolution durch Vergleich von *Xenopus*-, Maus- und Mensch-Ficolin-B-Ortholog geschehen. Ficolin-B hat eine konservierte Collagen-Bindungsstelle (Girija et al., 2007), die mit den wichtigen Funktionen wie Lektinweg-Aktivierung und Interaktion mit dem Gerinungssystem durch Interaktion mit Serinproteasen wie MASP verknüpft ist (Endo et al., 2010). Schwache benachbarte Bindungsstellen der Bindungsstelle können die Affinität erhöhen oder erniedrigen, wobei wenig über die biologische Rolle dieser Affinitätsmodulation bekannt ist. Durch Mutation einer einzigen Aminosäure in der Collagen-Bindungsstelle von Ficolin-B sollte die biologische Aktivität verändert werden. Biochemische und chromatographische Untersuchungen sollten durchgeführt werden, um Wild-Typ- und Mutein-Ficolin-B zu vergleichen.

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1. Introduction

1.1. The immune system

The immune system is a complex network of cells, tissues, and organs that work together to defend the body against any “foreign” invaders. These are primarily microbes—tiny organisms such as bacteria, parasites, and fungi that can cause infections. The human body provides an ideal environment for many microbes. It is the immune system’s job to attack and wipe out nearly all of them.

This is done in two ways: The adaptive or acquired immunity, consisting of B and T cells, uses a vast set of antibodies and T cell receptors with a high specificity against any foreign components. The highly effective acquired immunity is responsible for elimination of infection in a late phase and for the establishment of immunological memory. By contrast, the innate immune system constitutes the first-line of host defence in the early phase of infection. It recognizes only a limited number of conserved structures of invading microorganisms through a limited number of germline encoded receptors and it seems to be independent of immunologic memory (Akira *et al.,* 2006). Furthermore, innate immunity provides co-stimulatory molecules and cytokines to direct the adaptive immune response.

1.1.1. Overview of the innate immune system

The immune system consists of a diverse but interconnected group of defence mechanisms that prevent an individual from infection and disease (Jeanne Kelly, NIH publication No. 07-5423, sept. 2007). Many of these constituents are present from birth or act without the need for prior exposure to foreign material. These include physical barriers like the skin and mucous membranes, production of antimicrobial compounds and cellular killing processes. Collectively these mechanisms are defined as innate immunity and share certain characteristics that differentiate them from acquired immunity, the other main branch of the immune system.

“Innate immunity is evolutionary ancient” (Agrawal, 1998). The innate immune response, phylogenetically conserved and present in almost all multicellular organisms, is the first line of host defence and is responsible for immediately recognizing and countering microbial invasion (Hoffmann *et al*., 1999). This arm of the immune system is comprised mainly of complement proteins, phagocytic cells such as macrophages and neutrophils, which can ingest and kill the invading pathogens. These cells can also instruct the adaptive immune response about the nature of the pathogenic challenge through cytokine and chemokine

production, expression of costimulatory molecules, and presentation of microbial antigens to lymphocytes leading to the development of a highly specific adapative immune response.

Mutations in innate immunity genes are usually lethal. In comparison, acquired immunity is slower to develop but is required for the removal of pathogens that have evolved to evade the innate response (Schatz *et al.,* 1992). Through somatic rearrangement of antigen receptor genes, the adaptive immune system is capable of recognizing an unlimited variety of proteins and carbohydrates. However, without underlying innate immunity, these processes are ineffective.

The main distinction between these is the receptor types used to recognize pathogens (Ruslan Medzhitov, 2007). Innate immune recognition is mediated by pattern recognition receptors (PRRs), which are germline encoded, and each receptor has broad specificities for conserved and invariant features of microorganisms (Janeway, 1989). By contrast, adaptive immune recognition is mediated by antigen receptors: the genes encoding these receptors are assembled from gene segments in the germ line, and somatic recombination of these segments enables the generation of a vast diverse collection of receptors with random but narrow specificities (Schatz, 1992).

1.1.2. Recognition of microorganisms by the innate immune system

Whenever a microorganism is able to cross the epithelium, myeloid cells, located in the submucosal tissues, are the first cells to encounter the pathogens, and they are soon strengthend by the recruitment of large numbers of neutrophils to the site of infection (Janeway, Jr. and Medzhitov, 2002). Macrophages and neutrophils, both phagocytes, recognize microorganisms by means of their cell-surface receptors that can discriminate between self and non-self. These receptors include the mannose receptor (only on macrophages), scavenger receptors, which bind negatively charged ligands such as lipoteichoic acids (LTA, cell-wall components of Gram-positive bacteria), and CD14, a receptor for lipopolysaccharides (LPS, cell-wall component of Gram-negative bacteria) (Feizi, 2000). Upon ligation of many of these receptors pathogen binding will result in phagocytosis. Phagocytosis is an active process in which the bound pathogen is first surrounded by the phagocyte membrane and then internalized in a membrane-bound vesicle known as the phagosome, where the microbe is killed by acidification of the vesicle. In addition, macrophages and neutrophils also possess vacuoles called lysosomes that contain enzymes, proteins, and peptides that can mediate intracellular killing of the bacteria. The phagosome fuses with one or more lysosomes to generate a phagolysosome in which the lysosomal contents are released to destroy the pathogen. During phagocytosis, macrophages and neutrophils produce toxic products like nitric oxide (NO), the superoxide

anion (O_2-), and hydrogen peroxide (H_2O_2) that helps kill microorganisms (Janeway, Jr. *et al.,* 2005). The interaction between pathogens and tissue macrophages leads to release of cytokines, chemokines (chemotactic cytokines), and other mediators that attracts neutrophils and other cells to the site of infection. Furthermore, it stimulates the expression of co-stimulatory molecules (i.e. B7.1/CD80 and B7.2/CD86) on macrophages and on dendritic cells to intiate an adaptive immune response (Janeway, Jr. and Medzhitov, 2002).

1.1.3. Toll-like receptors: discriminating 'self' from 'non-self'

Toll-like receptors (TLR) are membrane-bound proteins that activate a leukocyte when bound to its ligands. (Akira *et al.,* 2006) TLRs widely recognize viral nucleic acids and several bacterial products, including lipopolysaccharide and lipoteichoic acids. Currently there are 10 known human TLRs (Lichtman, 2003). Although they may share a similar function in immunity, the TLRs vary in cellular expression, ligand specificity, cellular location, and adapter molecules (Ishii, 2008). Toll-like receptor ligands are primarily pathogen associated molecular patterns (PAMPs). These molecules are structures that are evolutionary conserved in many different pathogens. For example, TLR4 recognizes lipopolysaccharide (LPS) a common surface molecule of Gram-negative bacteria, fungal mannans, and viral envelope proteins (Fig 1.1). TLR3 recognises viral double-stranded RNA, TLR4 LPS, TLR5 flagellin and TLR9 bacterial CpG DNA. With the exception of TLR3, TLRs induce nuclear factor (NF)-kB-dependent expression of proinflammatory and immunoregulatory genes via a common signaling adaptor, the myeloid differentiation primary-response protein 88 (MyD88). However, TLR3, as well as TLR4, upon endocytosis into endosomes, can signal through the Toll/IL-1R(TIR)-domain-containing adapter inducing IFN-β (TRIF) pathway, that triggers activation of the interferon regulatory factor-3 (IRF-3) and type I interferon production (O'Neil, 2008). When bound, the ligand receptor complex recruits signaling proteins and initiates signalling cascades (e.g., NF-κB or MAPK) which leads to the transcription and expression of a wide range of inducible proteins such as inflammatory cytokines (TNF), chemokines (IL-8), or costimulatory molecules (CD80) which coordinates local and systemic inflammatory responses (Blander, 2008). Furthermore, IL-1β, together with IL-6, activates hepatocytes to produce acute phase proteins, including collectins and pentraxins. These proteins, in turn, activate complement and opsonize pathogens for phagocytosis by macrophages and neutrophils. In this way, TLRs indirectly elicit an antimicrobial response.

In addition to TLRs, there are several other classes of pattern recognition receptors. The family of nucleotide-binding oligomerization domain (NOD)-like receptors, unlike TLRs, is cytoplasmic and is capable of sensing PAMPs created by viruses (Iwasaki, 2009) or intracellular bacteria.

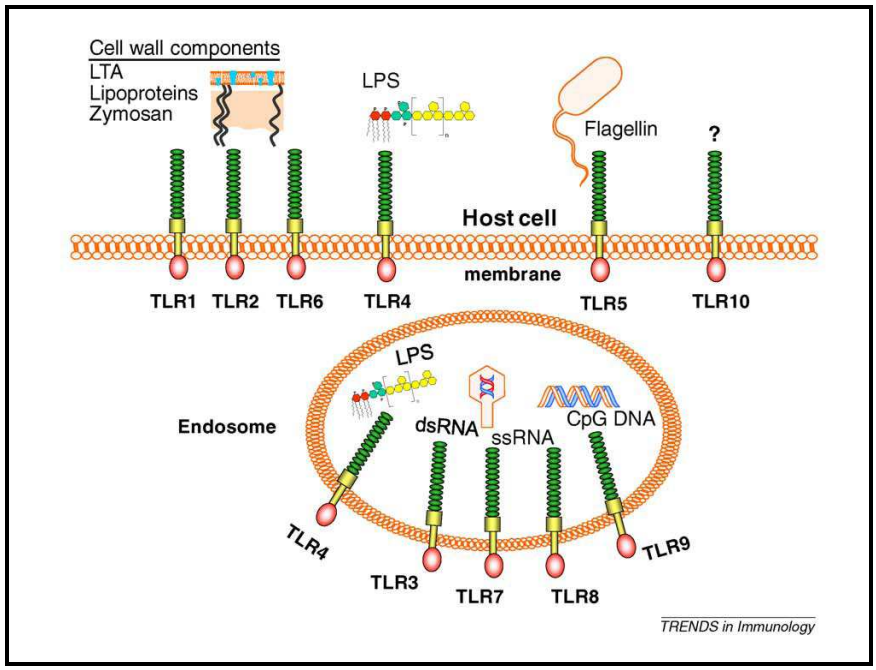


Figure 1.1 | Major forms of TLRs and their specific ligands.(Lambris, 2010)

1.2. The complement system

The complement system is the central component of the innate immune system comprising of at least 35 proteins which collaborate in an intricate manner in the elimination of microorganisms and in the removal of apoptotic cells, but also serves as a natural adjuvant, enhancing and directing the adaptive immune response (Walport, 2001).

There are three major pathways of complement activation, namely, the classical pathway which is activated by certain isotypes of antibodies bound to antigens; the alternative pathway which is activated on microbial cell surface in the absence of antibody and the lectin pathway which is activated by plasma lectins that bind to carbohydrate residues on microbes. MBL and ficolins are the complement-activating PRM which act via the lectin pathway.

In the following sections, the activation, regulation, and biological effects of the complement system will be described.

1.2.1. Complement intiation and amplification

The **classical pathway (CP)** starts when C1q binds to the pathogen surface. The binding of this molecule to a microorganism can occur in three different ways. First, it can bind directly to bacterial surface components such as certain proteins of the cell wall and polyanionic surface structures (i.e. LTA on Gram-positive bacteria). Second, C1q can bind to the C-reactive protein, which is a human plasma protein with specificity for phosphocholine residues in bacterial polysaccharides (i.e. the pneumococcal C polysaccharide). Third, as a

linker between the innate and the adaptive immune systems, C1q binds to the Fc domains of antibody:antigen complexes (Arlaud *et al*., 2002). C1q is part of the C1 complex which has a single C1q molecule bound to two molecules of zymogens C1r and C1s (Janeway CA Jr, *et al*.,2001). C1q is a calcium-dependent sugar-binding protein, which has six globular heads, linked together by a collagen-like tail, which surround the (C1r:C1s)2 complex. Binding of more than one of the C1q heads to a pathogen surface causes a conformational change in the (C1r:C1s)2 complex, which leads to activation of an autocatalytic enzymatic activity in C1r; the active form of C1r then cleaves its associated C1s to generate an active serine protease. Activated C1r then activates C1s, which in turn cleaves C4 and C2 to generate C3 convertase C4b2a. This consequently leads to the activation of the central component of complement C3. The generated C3b opsonizes pathogens to facilitate their elimination by phagocytosis, and the generated convertases C4b2a and C3bBb activate C5 on pathogens, inducing the lytic pathway via the late components of complement, C5 to C9.

In the **lectin pathway (LP),** MBL and ficolins serve as recognition molecules against pathogens which are molecules similar to C1q. While activated MASP-2, which is one of three human MASPs (MASP-1, MASP-2 and MASP-3) and a main effector of the lectin pathway, activates C4 and C2 to generate C3 convertase C4b2a, like C1s in the classical pathway (Matsushita *et al*., 2000b; Rossi *et al.,* 2001) (Fig. 1.2). It was reported that MASP-1 activates MASP-2 (Takahashi *et al*., 2008). MASP-1 also activates factor D, which is an early component of the alternative pathway, suggesting that MASP-1 is an initiator of the alternative pathway (Takahashi *et al*., 2010). MASPs, C1r and C1s are each members of the MASP/C1r/C1s family, a subfamily of the serine protease superfamily (Endo *et al.,* 1998, 2003). Thus the lectin pathway initiates complement activation in a way similar to the classical pathway, forming a C3 convertase from C2b bound to C4b (Janeway, Jr. *et al.,* 2005).

The third pathway, **alternative pathway (AP)** of the complement system is independent of the presence of antibodies and activated by different molecules and pathogens including gram negative bacteria and cell wall of gram positive bacteria (Pangburn and Muller-Eberhard 1984). The spontaneous hydrolysis of C3 which is also known as the “tickover” leads to the formation of C3i. The C3i acts as a binding site for Factor B which is cleaved by Factor D to form Ba (small fragment of Factor B). The initial convertase, C3(H2O)Bb, is highly unstable and is able to cleave C3 to C3a and C3b. If present, C3b binds to an activating surface. Together with Factor B and Factor D, the complex C3bBb is produced and is stabilized by binding of Properdin to form the C3 convertase (C3iBb). This creates an amplification loop to generate more C3 convertase. C3b can bind to the C3 convertase to produce C5 convertase which can cleave C5 to C5a and C5b. C5b can bind to C6-9 to

produce the membrane-attack complex (MAC) leading to the lysis of target cell. The MAC is intiated by enzymatic cleavage of C5 and then leads to the sequential binding of C6, C7, C8 and C9. Several C9 molecules bind to the C5b678 complex, producing the pore channel, MAC. Once MAC forms into the cell or pathogen membrane, this will lead to cell lysis due to the formation of ion-permeable pores or channel or leaky patches (Muller-Eberhard 1986; Morgan 1999).

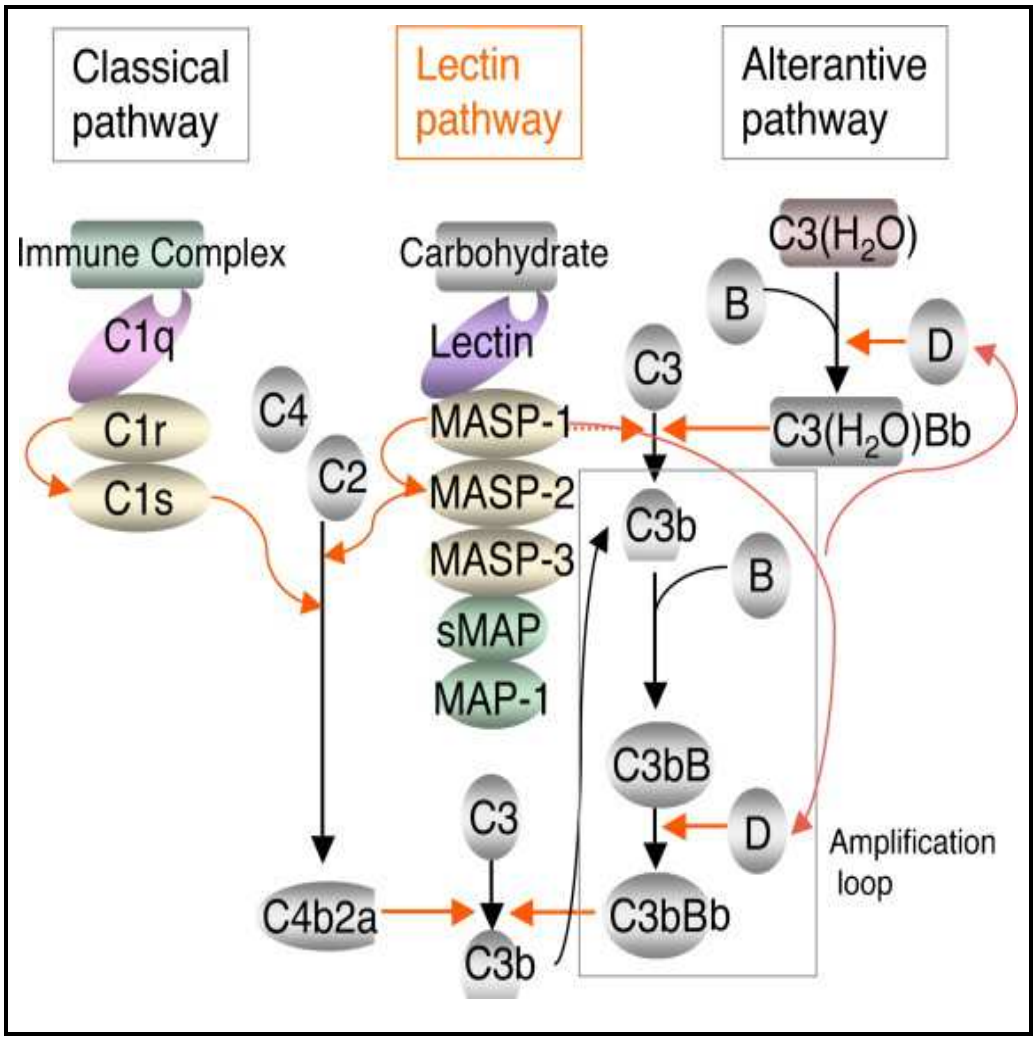


Fig. 1.2 | A schematic representation of the three pathways of complement activation: The classical, alternative and lectin pathways. The classical pathway is initiated by the binding of the C1 complex to antibodies that are bound to antigens on the surface of bacteria. The lectin pathway is initiated by the binding of either mannose-binding lectin (MBL) or ficolin — associated with MBL-associated serine protease 1 (MASP1), MASP2, MASP3 and small MBL-associated protein (sMAP) — to an array of carbohydrate groups on the surface of a bacterial cell. The alternative pathway is initiated by the low-grade activation of C3 by hydrolysed C3 ($C3(H_2O)$) and activated factor B (Bb). Red arrows depict the activation reactions (Endo, 2011).

1.2.2. Effector functions of complement

When PAMPs are detected on invading microorganisms, one or several complement initiation pathways that aim to eliminate microbial intruders are triggered. The complement cascade leads to the formation of the MAC on the pathogen surface which finally leads to the eventual destruction of the pathogen (Parker and Sodetz, 2002; Janeway, Jr*. et al.,* 2005). In addition to the MAC formation, all along the cascade the complement generates large numbers of activated proteins that either opsonize the pathogen or act as chemotactic molecules. Acute-phase proteins increases markedly at the early stage of infection and key components of this response is the secreted PRRs: collectins, ficolins and pentraxins. They activate the complement system by opsonising microbial cells for phagocytosis. Collectins and ficolins activate the lectin pathway of the complement system (Endo *et al*., 2006; Holmskov *et al.,* 2003), whereas, pentraxins activate the classical pathway (Bottazi *et al*., 2006).

C3a and especially C5a are strong chemoattractants that guide neutrophils, monocytes and macrophages towards sites of complement activation and promote phagocytosis through interaction with opsonins (Ricklin *et al*., 2010). There are various complement receptors (CRs) that act as opsonins. Among these receptors, CR1 (CD35), is expressed on macrophages and polymorphonuclear lymphocytes (PMNs), and can bind to C3b, C4b and iC3b (Whaley and Schwaeble, 1997) to promote neutrophil-mediated phagocytosis and regulatory degradation of its ligands by factor I (fI) (Atkinson *et al.,* 2001). CR2 (CD21) is found on B cells as part of a co-receptor for B-cell activation and differentiation and also binds to the inactive forms of C3b, C3dg that remain attached to the pathogen surface (Whaley and Schwaeble, 1997). CR3 (CD11b:CD18), and CR4 (CD11c:CD18) are integrin receptors that binds iC3b fragment and contributes to phagocytosis. CR3 also regulates cytokine responses, leukocyte trafficking and synapse formation.

C3a, C4a, and C5a are small complement fragments that act on specific receptors to produce local inflammatory responses (Janeway CA Jr, Travers P, Walport M, *et al.* 2001). When produced in large amounts or injected systemically, they induce a generalized circulatory collapse, producing a shocklike syndrome similar to that seen in a systemic allergic reaction involving IgE antibodies. Such a reaction is termed „anaphylactic shock" and these small complement fragments are therefore often referred to as anaphylatoxins. Of the three, C5a is the most stable and has the highest specific biological activity. All three induce smooth muscle contraction and increase vascular permeability. C3a and C5a are constantly released and trigger proinflammatory signaling through their corresponding G-protein–coupled receptors, C3a receptor (C3aR) and C5a receptor (C5aR; also called CD88). A third,

G-protein–independent anaphylatoxin receptor, C5L2 (GPR77), has more recently been discovered. However, its exact roles are not yet fully determined (Ward, 2009).

In summary, the changes induced mainly by C5a and C3a recruit antibodies, complement components, and phagocytic cells to the site of infection leading to defence aganist microbial intruders.

1.2.3. Complement regulation

Soluble and cell-bound complement regulators play a major role in complement regulation and help to control complement attack (Zipfel, 2009; Fig. 1.3). C1 esterase inhibitor (C1-INH), a secreted glycoprotein of the serpin family that inhibits several proteases of the classical and lectin pathways, In addition to that, two other lectin pathway modulators have been identified: sMAP and MAP-1 are nonproteolytic splice products of the MASP2 and MASP1/3 genes, respectively, that apparently compete with MASPs for binding to MBL and ficolins. The C2 receptor inhibitor also binds to C2 and inhibits its activation by C1s (Skerka, 2009). In the alternative pathway, activation in solution is mainly controlled by the abundant factor H (fH) and its truncated homolog, factor H-like protein 1 (FHL-1). fH mainly acts on C3 convertases in the alternative pathway, either competitively removing Bb from the C3bBb complex (decay acceleration) or serving as a cofactor for the factor I (fI)-mediated degradation of C3b. Another fluid-phase regulator, C4b-binding protein (C4BP), has similar effects on classical pathway and lectin pathway convertases. Most importantly, fH, FHL-1 and C4BP also support complement regulation on human cells by engaging host-specific surface patterns (such as sialic acid or glycosaminoglycans), thereby contributing to self-recognition and prevention of self-attack. On the other hand, during complement activation, some C5b67 complexes gets deposited on nearby cells which leads to cell lysis. To prevent this, cell-based regulators like Clusterin bind to C5b67 which prevent insertion of the complex to the membrane (Tschopp and French 1994). Protectin or CD59 inhibits insertion and unfolding of C9 into membranes. Vitronectin inhibits the membrane-damaging effect of TCC.

Most human cells also expose convertase regulators (membrane regulators) that act as decay accelerators, such as CR1 or decay-accelerating factor (DAF, also called CD55), or as cofactors for fI, such as CR1 and membrane cofactor protein (MCP or CD46) (Jalali, 2010). Only a few C5-specific regulators have been described so far; whereas fH-related protein 1 directly binds C5 and inhibits C5 convertase activity, CRIg regulates the C3b-containing C3 and C5 convertases, although the physiological implications of this mechanism are unknown. Finally, carboxypeptidase-N quickly converts anaphylatoxins to their desarginated forms;C3adesArg and C5adesArg and they can trigger important functions like hematopoietic

stem-progenitor cell (HSPC) mobilization (Ratajczak, 2006) or lipid metabolism (Mclaren, 2008).

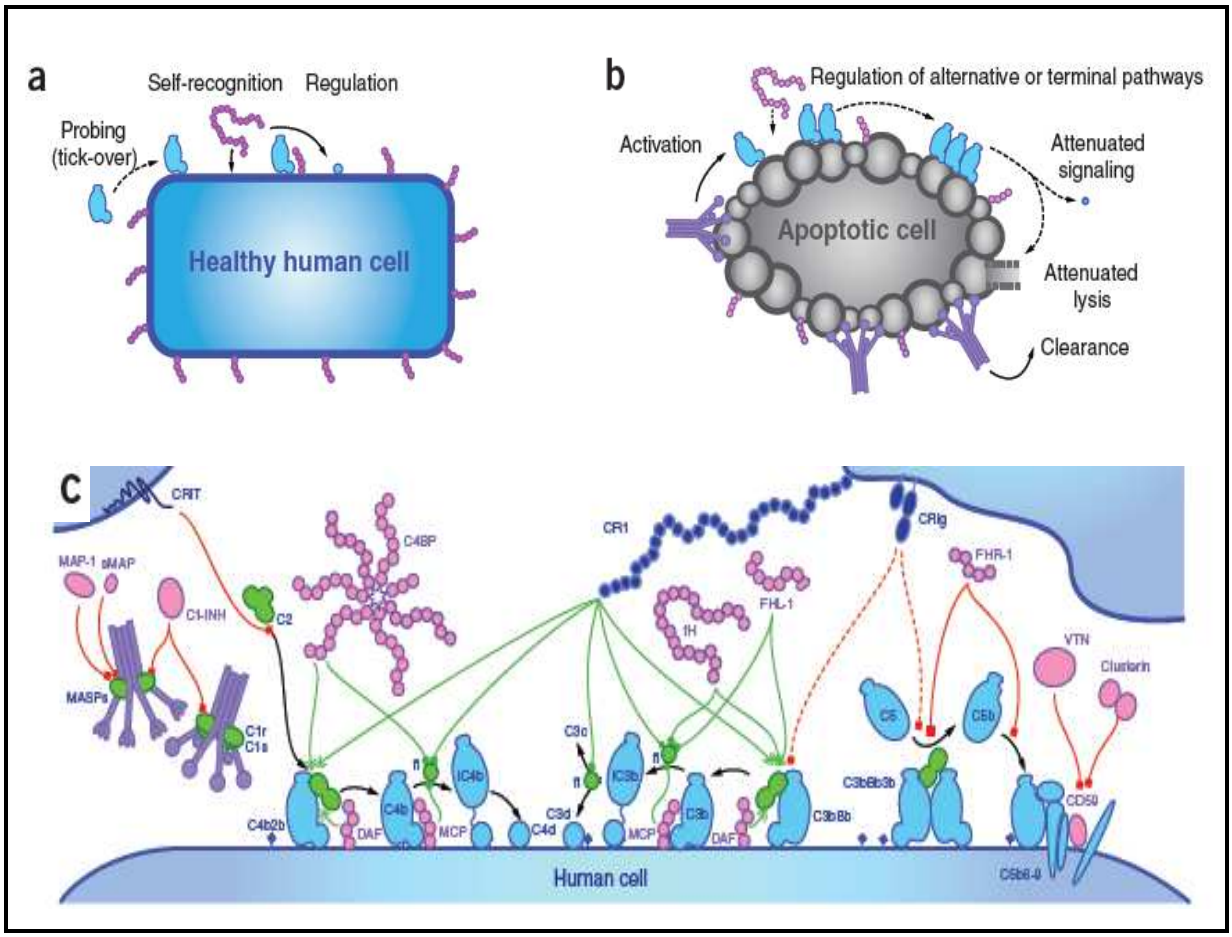


Figure 1.3 | Immune regulation functions of complement. (Lambris, 2010)

1.2.4. Complement in health and diseases

Besides the more 'classical' roles of complement in the elimination of microbial intruders and clearance of apoptotic debris, complement has important roles in cell homeostasis and disease. In Alzheimer's disease, both C1q abd C3 recognize accumulating amyloid fibrils and induce activation of complement. In sepsis (Fig.1.4a), microorganisms poison the blood and cause excessive complement activation with release of C5a that contributes to severe effects like immune depletion to severe inflammation and disseminated coagulation, all of which may result in tissue damage, multi-organ failure and death (Ward, 2004). Complement has a role in synaptogenesis (Fig. 1.4b), where it eliminates weak or immature synapses. "An unknown signal derived from immature astrocytes promotes recognition by C1q, which leads to opsonisation with C3b and iC3b and facilitates complement receptor (CR)-mediated phagocytosis by activated microglia" (Schafer *et al.,* 2010). Although the role of the C5L2 receptor in the immune response is unclear, it seems to play a vital role in lipid metabolism (Fig. 1.4c). Adipocytes secrete C3, fB and fD, and this expression can be promoted by stimuli

such as insulin or lipids, leading to a higher turnover of the alternative pathway and generation of C3a, which is transformed into the C3adesArg fragment (ASP). This fragment can induce lipid clearance, glucose uptake and triglyceride (TG) synthesis in adipocytes through C5L2 signaling. Complement is likely to have a dual role in cancer (Fig. 1.4d): It contributes to protection through direct activation of complement or as part of the complement-dependent cytotoxicity of tumor-directed therapeutic antibodies (Lambris et al., 2009). However, many tumors escape complement attack by expressing and secreting complement inhibitors that largely prevent amplification, TCC formation or complement-mediated phagocytosis. The generation of C5a in the tumor microenvironment can attract myeloid-derived suppressor cells (MDSC) and induce the generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) through the C5a receptor (C5aR), which impairs the tumor-directed effect of T cells (Markiewski *et al.,* 2008).

The deficiency of classical pathway C proteins will lead to SLE. Patients with C-deficiencies are at particular risk of developing serious infections with encapsulated organisms such as *S.pneumoniae* and *Neisseria meningitides.*

The deficiency of lectin pathway results from low concentrations of MBL and has been associated with an increased risk, severity and autoimmune disorders (Sorensen *et al.,* 2005) and increased susceptibility to fungal, protozoal and viral infections (Hajela *et al.,* 2008). MBL deficiency has also been associated with infections of the respiratory tract (Eisen, 2010) and with increased disease severity in cystic fibrosis (Fidler *et al*., 2009). MBL deficiency is one of the most common human immunodeficiencies and arises primarily from three single point mutations in exon 1 of the MBL-2 gene. This mutations result in a failure to assemble fully functional multimeric protein (Turnet, 2003). To date, clinical studies have shown that MBL deficiency predispose to infectious diseases such as pneumococcal infection (Eisen, 2010).

The deficiency of the alternate pathway is due to the components which include fB, factor D, properdin and C3. Meningococcal infections have been seen in multiple individuals Neisseria meningitides as the most common pathogen encountered. Factor I deficiency has been associated with glomerulonephritis with reduced levels of CR1 expression (Ponce *et al.,* 2001). General deficiencies of C5, C6, C7, or C8 lead to increased suspetibility to meningococcal infections. C9 individuals have some haemolytic and bactericidal activity, but they are still predisposed to meningococcal infections (Nagata *et al.,* 1989).

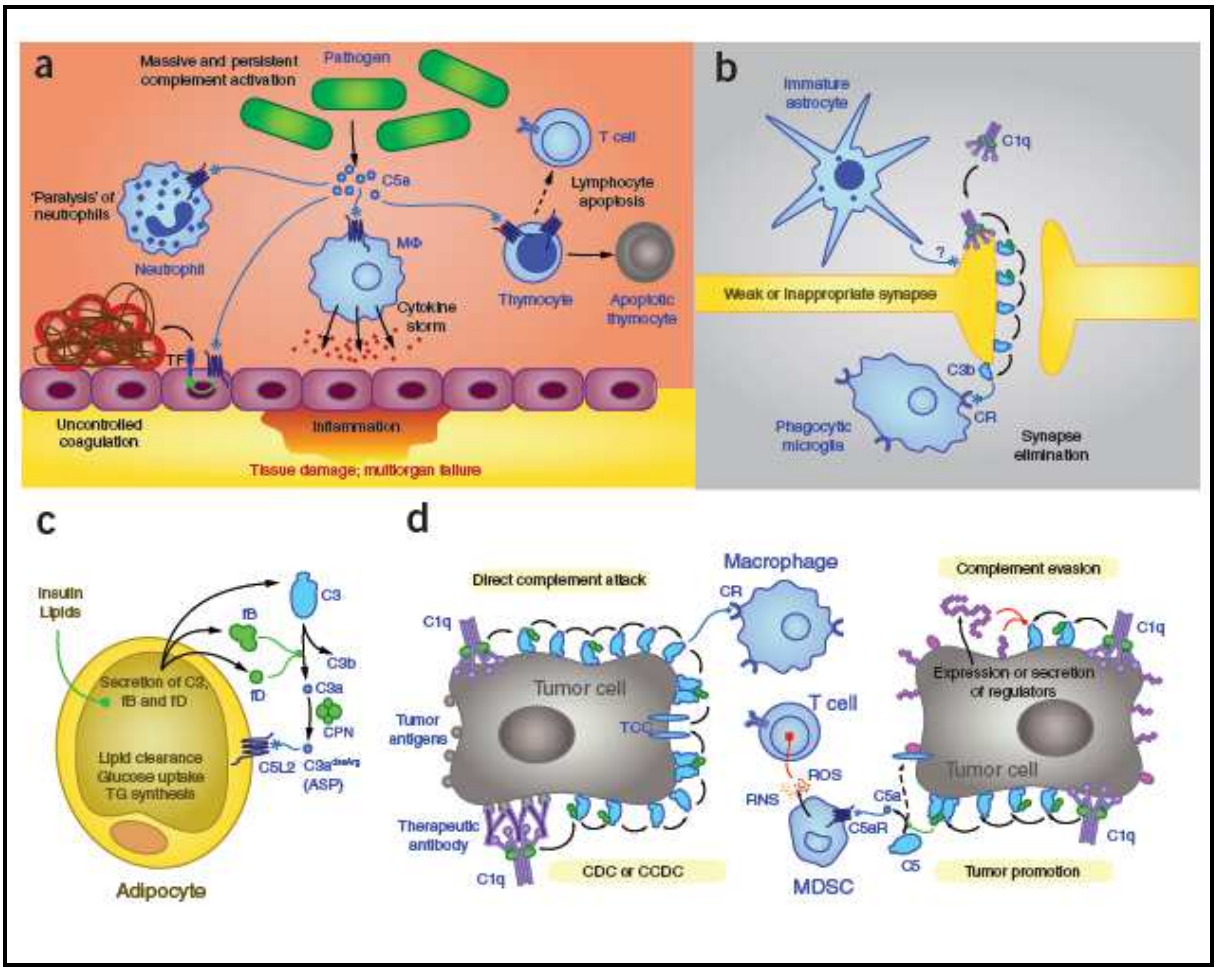


Figure 1.4 | Emerging roles of complement in health and disease. Role of complement (a) in sepsis b) in synaptogenesis (c) in lipid metabolism (d) a dual role in cancer (Lambris, 2010).

To date no ficolin deficiencies have been reported. However studies on L-ficolin polymorphisms have been reported (Hummelshoj *et al*., 2005; Herpers et al., 2005), and it shows that these genetic variations are associated with both the serum levels and the N-acetyl-D-glucosamine (GlcNAc) binding activity. It was reported that low L-ficolin is associated to prematurity, low birth weight and infection in neonates (Swierzko *et al.,* 2008), allergic rhinitis (Cedzynski *et al*., 2009), and severe streptococcal infection (Messias-Reason *et al.*, 2009). Low H-ficolin deficiency is associated with recurrent infection (Munthe-fog *et al.,* 2009), fever and neutropenia (Schlapbach *et al*., 2010).

1.3. Lectins – "The Pattern recognition molecule"

Lectins are carbohydrate-binding proteins that play an important role in innate immunity by recognizing a wide range of pathogens and by aggregating and opsonising them. This recognition is mediated by a set of pattern-recognition receptors (PRR) that recognize conserved pathogen-associated molecular patterns (PAMPs) shared by broad classes of microorganisms, thereby successfully discriminating pathogens from self. PAMPs are essential for the survival of certain microbial groups, and are, therefore, highly conserved among the different classes of pathogens (Medzhitov and Janeway, Jr., 2000b). Lectins form a class of PRRs that bind specifically to the unique carbohydrate moieties on microbes (Lu *et al.,* 2002). Among the lectins known, MBL and ficolins are the typical pattern recognition proteins.

1.3.1. Mannose binding lectin (MBL)

MBL consists of large oligomers assembled from identical polypeptide chains (Drickamer and Taylor, 1993). Three polypeptide chains assemble into structural subunits, which associate into higher oligomeric forms. Each subunit is composed of an N-terminal cysteine-rich domain followed by a collagenous domain, an α-helical coiled coil or neck region and three C-terminal C-type carbohydrate-recognition domains (CRDs) which is the ligand binding domain (Fig.1.5). The N-terminal cysteine rich region is stablized by hydrophobic interaction and interchain disulphide bonds. In serum, MBL consists of oligomers of the trimeric subunit, ranging from dimers to hexamers. MBL of higher order oligomers (e.g. tetramers to hexamers) are necessary for stable binding of MBL to pathogens which leads to effective forms of the protein functions, for examples, the glycan interaction and complement activation on microbial surfaces (Kawasaki *et al.,* 1995).

MBLs recognise foreign cells via multivalent interactions with the carbohydrate epitopes commonly found on pathogens. Each CRD has a single binding site for monosaccharides such as mannose, fucose or N-acetylglucosamine, which occur only rarely at the terminal positions of mammalian oligosaccharides on glycoproteins and glycolipids but are present in high-density arrays on many bacterial, fungal and parasitic cells (Drickamer, 1993).

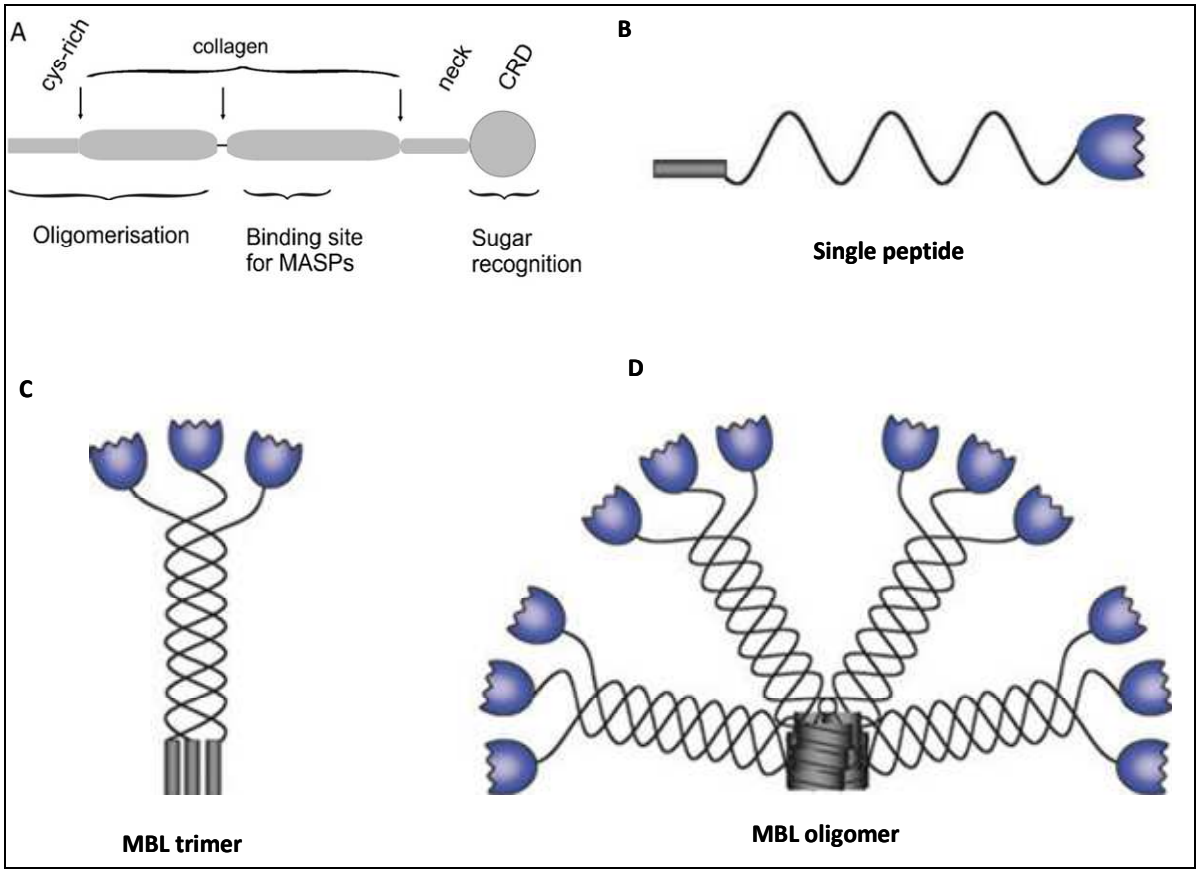


Fig. 1.5 | Domain organisation of MBL (A) Structural organisation of MBL. Each subunit has N-terminal cys-rich region, middle collagen-like domain and c-terminal darbohydrate recognition domain. The MASPs binding site resides in the collagen domain. Arrows show potentially flexible regions (Wallis, 2007) (B) Single polypeptide chain (C) the basic structure subunit of MBL formed by three polypeptide chains (D) MBL oligomers formed by multiple subunits (Stuart *et al*., 2009).

There are two human MBL genes but MBL-1 is a pseudogene and only MBL-2 encodes a protein product. In rodents two MBL genes are expressed, namely MBL-A and MBL-C. It has been shown that MBL is an acute phase protein that increases in serum levels following infection and trauma (Thiel *et al.,* 1992). Liver is the main site for MBL biosynthesis, but its expression is also seen in other tissues of mouse (Uemura *et al.*, 2002; Wagner *et al*., 2003). The average median concentration of MBL in plasma is around 1.5μg/ml and this concentration varies to a large degree in every individuals due to polymorphisms in the promoter region and in exon 1 of the gene (reviewed in Turner *et al*., 2000, Garred *et al.,* 2006). MBL has been shown to bind to a wide range of micro-organisms (Neth *et al*., 2000) and further interaction with MASPs leads to the activation of the lectin pathway of the complement system.

1.3.2. Ficolins

Ficolins are a group of oligomeric lectins which are able to recognize pathogen-associated molecular patterns (PAMP) on microbial surfaces. Upon binding to their specific PAMP, ficolins may trigger activation of the complement system via the lectin pathway. Ficolins were first identified as transforming growth factor-β1 (TGF-β1)-binding proteins on pig uterus membranes (Ichijo *et al*., 1991). Their primary structure revealed that they are mainly composed of fibrinogen- and collagen-like domains and, this unique feature gave them their name ficolins (Ichijo *et al*., 1993). It was demonstrated that all ficolins (except mouse ficolin-B) can associate with MASPs and small MBL-associated protein (sMAP), suggesting they play a role in the complement activation through the lectin pathway (Matsushita *et al.*, 2000a, 2002; Liu *et al*., 2005a).

Table 1 - Characteristics of ficolins

Species	Ficolins	mRNA expression	Protein identified	Binding substance	Function
Human	L-ficolin	liver	serum/plasma	GlcNAc (acetyl group); β-(1→3)-D-glucan; *N*-acetylneuraminic acid; lipoteichoic acid; C-reactive protein; fibrinogen, fibrin; DNA; elastin;corticosteroid	complement activation; opsonin
	H-ficolin	liver, lung, glioma cell	serum/plasma, bile duct, bronchus, alveolus	GlcNAc, GalNAc, fucose; lipopolysaccharide; polysaccharide preparations	complement activation; opsonin
	M-ficolin	lung, monocyte, spleen	plasma, monocyte, neutrophil, alveolar epithelial cell	GlcNAc, GalNAc; sialic acid	complement activation; phagocytic receptor
Mouse	Ficolin A	liver, spleen	serum/plasma	GlcNAc, GalNAc; elastin	complement activation
	Ficolin B	bone marrow, spleen	macrophage	GlcNAc, GalNAc; sialic acid	opsonin
Pig	Ficolin α	liver, lung, bone marrow	serum/plasma, uterus membranes	GlcNAc; lipopolysaccharide, lipoteichoic acid, elastin; TGF-β1	antiviral activity
	Ficolin β	neutrophil, bone marrow	neutrophil, uterus membranes	TGF-β1	ND
Hedgehog	Erinacin	ND	muscle	metalloprotease	antihemorrhagic activity
Xenopus	XeFCN1	liver, spleen, heart	serum	GlcNAc, GalNAc	ND
	XeFCN2	lung, spleen, leukocyte	NI	ND	ND
	XeFCN3	ND	NI	ND	ND
	XeFCN4	lung, spleen	NI	ND	ND
Ascidian	p40	hepatopancreas	hemolymph plasma	GlcNAc, GalNAc	ND
	p50	hepatopancreas	hemolymph plasma	GlcNAc	ND

ND = Not determined; NI = not identified.

(Matsushita, 2010)

There are two types of ficolin present in each species, a serum type (or plasma type) and a non-serum type (or non-plasma type) (Endo *et al.,* 2007). The former is expressed mainly in the liver and is present in the circulation as a serum lectin, including human L-ficolin and H-ficolin, M-ficolin (Garred *et al*., 2008), mouse ficolin-A and porcine ficolin - α. While the latter is expressed mainly in non-liver tissues, such as peripheral leukocytes and bone marrow, including human M-ficolin, mouse ficolin-B and porcine ficolin-β. To date, the ficolin homologues (Table 1) have been isolated from human (Endo *et al*., 1996, Lu *et al*., 1998) and non-human vertebrate species such as mice (Fujimori *et al.,* 1998; Ohashi and Erickson, 1998), hedgehog (Omori-Satoh *et al*., 2000) and *Xenopus* (Kakinuma *et al.,* 2003), and also from an invertebrate species, ascidian (*Halocynthia roretzi*)(Kenjo *et al.,* 2001).

1.3.2.1. Characteristics of human and mouse ficolins

1.3.2.1.1. Structure, expression, and location of human ficolins

There are three types of human ficolins known, L, H, and M. From a structural point of view, ficolins are assembled from basal homotrimeric subunits comprising a collagen-like triple helix and a globular recognition domain composed of 3 fibrinogen-like domains (fig.1.6). Two cysteines at the N-terminal end of the polypeptide chains form interchain disulfide bonds that mediate assembly into higher oligomeric structures. Like MBL, ficolins are able to trigger the complement cascade through activation of the serine protease MASP-2 (Fujita *et al.,* 2001). The MASP interaction site within the collagen-like regions of human MBL and ficolins has been recently mapped and shown to have a conserved lysine residue (Girija *et al.,* 2007). The fibrinogen-like domain is globular, and the overall structure of L-ficolin resembles a “bouquet”. The proposed structure is a tetramer consisting of 4 triple helices formed by 12 subunits (Garred *et al.,* 2007). There is one potential N-linked glycosylation site in the fibrinogen domain and five potential O-linked sites in the collagen-like domain (Wallis *et al.,* 2011).

H-Ficolin was first identified as a serum antigen recognized by an autoantibody present in patients with systemic lupus erythematosus matosus (Inaba *et al*., 1978). H-ficolin is an oligomer of 34-kDa subunits (Yae *et al.,* 1991). The H-ficolin gene (FCN3) is located on chromosome 1p35.3 and consists of 8 exons with similar organization as the L-ficolin gene. H-ficolin mRNA is expressed in the liver and lung. In the liver, H-ficolin is produced by bile duct epithelial cells and hepatocytes and is secreted into bile and serum (Akaiwa *et al.*, 1999). In the lungs, H-ficolin is produced by both ciliated bronchial epithelial cells and type II alveolar epithelial cells and is secreted into the bronchus and alveolus. H-ficolin mRNA expression was found in human glioma cell line T98G (Kuraya *et al.,* 2003).

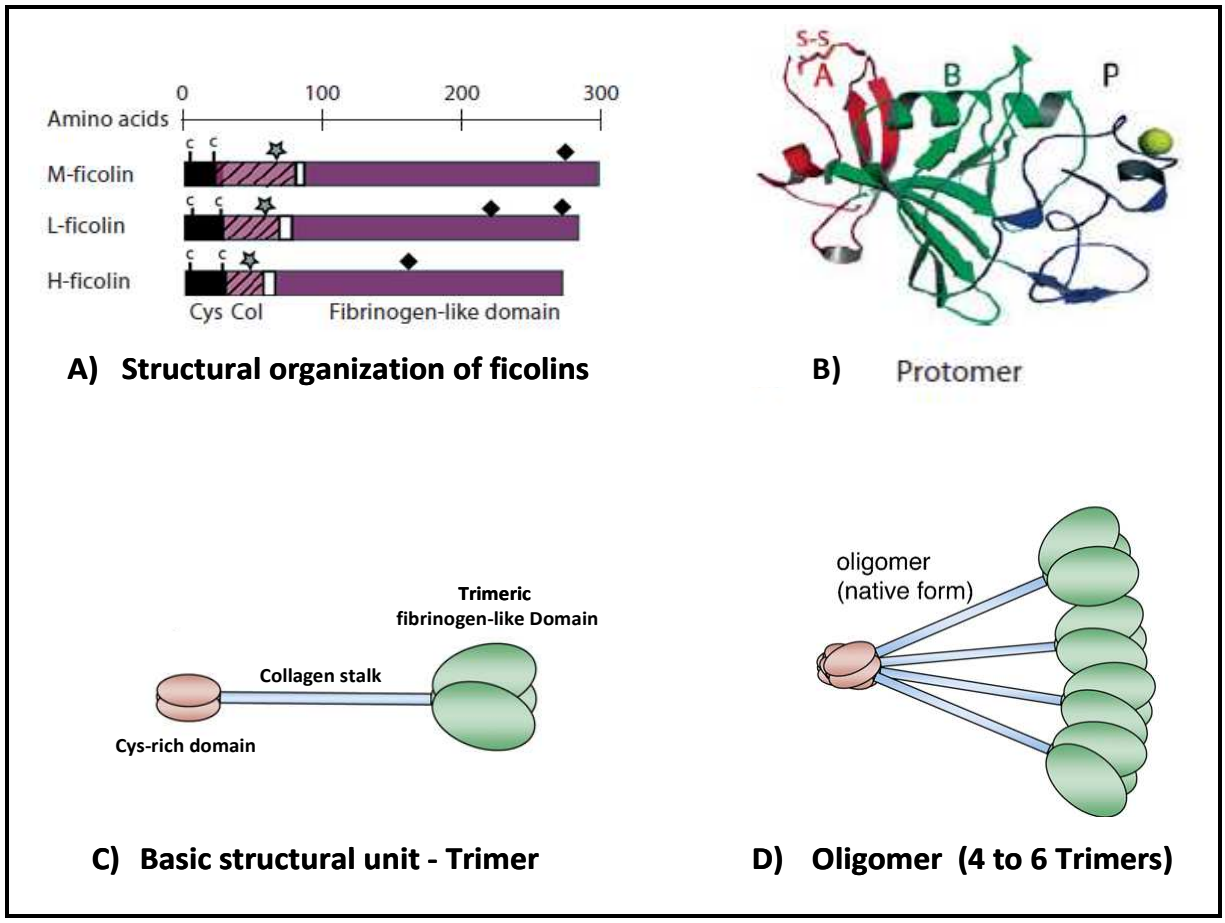


Fig. 1.6 | Structural organization of ficolins and their multivalent recognition function. **A)** Each mature polypeptide chain comprises a cysteine-rich N-terminal sequence (black), a collagen-like sequence (hatched), a linker segment (white) and a fibrinogen-like sequence (purple). The N-linked oligosaccharides (♦), the binding site for the MASPs (∗) and the 2 N-terminal cysteine residues are indicated. **B)** X-ray structure of the fibrinogen-like protomer. Domains A, B and P are colored red, green and blue respectively. The N-terminal disulfide bond (S-S) is shown in red. A Ca 2+ ion is represented by a yellow sphere. (Garlatti et al., 2010). **C)** Monomeric subunits assemble to form the trimer via the collagen-like domain.**D)** Four to six trimers assemble the multimer (Oligomer), potentially by crosslinking via disulfide bridges in the N-terminal region (Endo *et al.,* 2011).

The mRNA of M-ficolin is expressed in monocytes, lungs, and the spleen (Lu *et al*., 1996). The M-ficolin gene (FCN1) is located on chromosome 9q34. M-ficolin has an extra exon encoding an additional segment of 4 Gly-Xaa-Yaa repeats. M-ficolin has been reported to be expressed on the surface of peripheral blood monocytes and pro-monocytic U937 cells (Teh *et al.,* 2000). M-ficolin is a secretory protein and it has been found to be located in secretory granules in the cytoplasm of peripheral neutrophils and monocytes and in type II alveolar epithelial cells in the lungs (Liu *et al.,* 2005). Recent report has shown that M-ficolin is present in human plasma at very low levels (Garred *et al.,* 2008).

L-Ficolin is an oligomeric protein consisting of 35-kDa subunits (Mizuochi *et al.,* 1998). Two cysteine residues in the N-terminal domain are thought to mediate multimerization by disulfide bonding. The L-ficolin gene (FCN2) is located on chromosome 9q34 and contains 8 exons (Fujita *et al.,* 1996). The mRNA of L-ficolin is mainly expressed in the liver and its protein product is secreted into serum.

1.3.2.1.2. Expression and location of mouse ficolins

Mice have 2 types of ficolins, termed ficolin-A and ficolin-B. The ficolin-A gene was first isolated from a mouse liver library (Fujimori *et al.,* 1998). Both ficolin-A and ficolin-B genes are located on chromosome 2A3. Ficolin-A is the plasma protein with a molecular mass of 37 kDa, highly expressed in liver and spleen (Fujimori *et al.,* 1998). The ficolin-A protein is present as a tetramer with 12 subunits (Ohashi *et al.,* 1998). Ficolin-A mRNA is expressed during ontogenesis as early as on embryonic day (E) 12.5, displaying an increase in abundance during development, peaking around birth, and slightly declining in the adult stages (Liu *et al.,* 2005a). In addition, *In situ* hybridization studies indicated that ficolin-A mRNA was mainly localized in the linings of the hepatic sinusoids in the liver, and in the red pulp of the spleen. These observations suggest that ficolin-A mRNA is expressed by cells of the monocyte-macrophage lineage (Liu *et al.,* 2005a).

Ficolin-B was first characterized by Ohashi and Erickson in 1998 with strong mRNA expression in bone marrow and weak expression in spleen. The expression of ficolin-B in the liver increases with development, peaks two or three days before birth, and thereafter rapidly declines to an undetectable level. Thus, the expression of ficolin-B switches from the embryonic liver to postnatal bone marrow and spleen. The different spatial–temporal expression patterns suggest that ficolin-A and ficolin-B might play distinct roles during the prenatal and postnatal stages (Liu *et al.,* 2005). Our group (Runza *et al.,* 2006) recently published the first observation on ficolin-B expression at the protein level. Ficolin-B was found to be expressed by peritoneal macrophages of C57Bl/6 mice by immunocytochemistry, and surprisingly a positive staining was detected only after permeabilization of the cells indicating an intracellular expression and not a cell-association of this protein. Recently, it has been demonstrated that mouse ficolin-A and ficolin-B show lectin activities for GlcNAc and GalNAc, and like human M-ficolin, ficolin-B additionally recognizes sialic acid residues inthe sugar chain (Endo *et al.,* 2005). The binding-specificity of M-ficolin and ficolin-B to sialic acid residue suggests a unique role for non-serum type ficolin.

Sequence alignments reveal that (Lys^{55}) which is a conserved residue in the collagen domain of MBL is also conserved in ficolins and plays a critical role by forming the key contacts with the MASP-2 binding (Lacroix et al., 2008) and the adjacent residues mainly methionine or alanine might help to stabilize the interaction (Girija *et al*., 2007). Although mouse ficolin-B possesses a similar binding motif, it is unusual compared to other ficolins because it contains an acidic residue adjacent to the key conserved lysine: Hyp-Gly-Lys-**Glu**-Gly-Pro. This adjacent glutamic acid residue is likely to disrupt MASP binding and activation, explaining the lack of complement activation by mouse ficolin-B (Girija *et al.,* 2011).

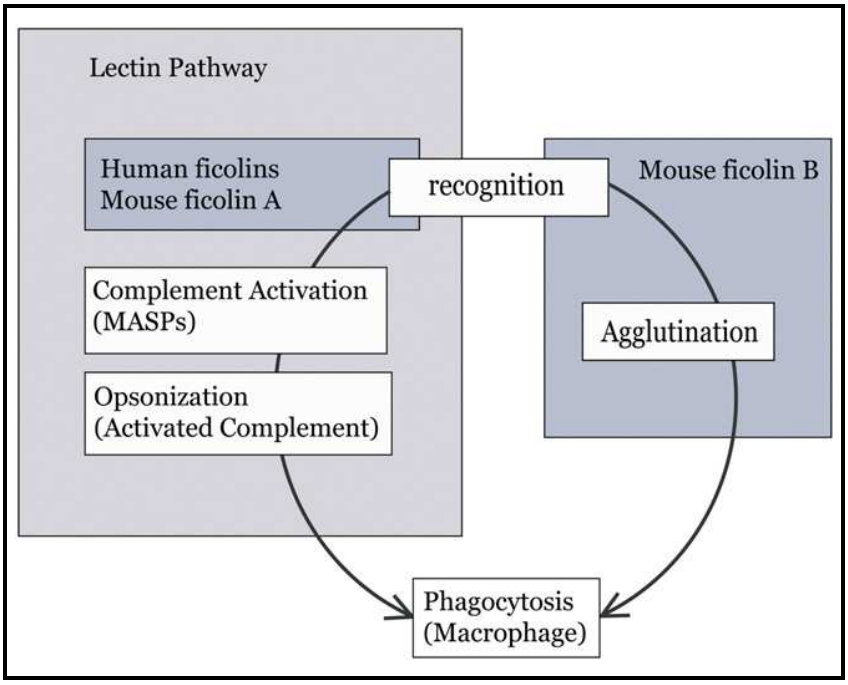


Fig. 1.7 | Two routes of ficolin function: The human ficolins and mouse ficolin-A execute their functions through the lectin pathway, while mouse ficolin-B does through its strong agglutination activity. The existence of the latter route is supported by observation that ficolin-B aggregates bacteria stronger than ficolin-A leading to enhanced phagocytosis of the aggregated bacteria by mouse peritoneal macrophages (Endo *et al.,* 2007).

Interestingly, the recombinant mouse ficolin-B, but not ficolin-A, had a strong activity to aggregate *Staphyloccus aureus* which leads to phagocytosis of the bacteria by mouse peritoneal macrophages without involvement of serum components (Endo *et al.,* 2007). This result suggests that ficolin has two distinct ways to eliminate pathogens: one is a classical route through the lectin pathway executed by human ficolins and mouse ficolin-A, the other is a primitive opsonophagocytosis executed by mouse ficolin-B (Fig. 1.7) (Endo *et al.,* 2007).

1.3.2.2. Functions of ficolins

1.3.2.2.1. Carbohydrate and pathogen recognition of ficolins

The fibrinogen-like domains of ficolin are responsible for carbohydrate and pathogen recognition. X-ray crystallography studies on ficolins demonstrated the molecular basis of GlcNAc-recognition in the fibrinogen-like domains (Garlatti *et al*., 2007a, 2007b; Tanio *et al.,* 2007). In L-ficolin, four ligand binding sites S1 to S4 were identified (Fig 1.8) (Garlatti *et al.,* 2007a). The Ca2+ is bound to the loop region located adjacent to the ligand-binding site. However, it remains controversial as to whether this recognition requires Ca2+ or not (Ohashi and Erickson, 1997). (Matsushita *et al.,* 1996; Endo et al., 2006) reported that the binding of L-ficolin to GlcNAc requires Ca2+. The four ligand-binding sites provide a continuous recognition surface, which can detect various acetylated and neutral carbohydrates on microbes. The binding sites can be abolished by EDTA and sodium acetate (Lacroix *et al.,* 2009). The S1 binding site gets disrupted and exhibits increased flexibility at acidic pH (Tanio *et al.,* 2006).

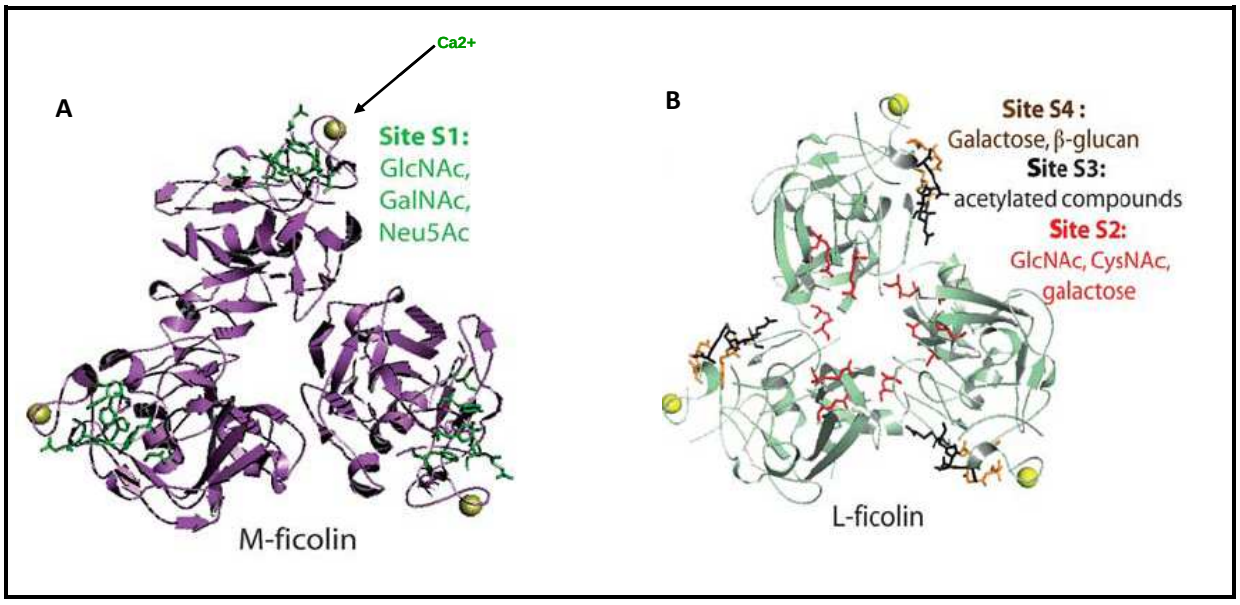


Fig. 1.8 | The ligand binding sites in M- and L-ficolins. A) An outer binding site (S1) showing the binding regions for GlcNAc, GalNAc and Neu5Ac, on each protomer of the trimeric fibrinogen-like domain of M-ficolin. B) Three binding sites S2, S3, and S4 were observed in the trimeric fibrinogen-like domain of L-ficolin, which binds to a wide variety of acetylated ligands and some neutral sugars (galactose, β-D-glucan) (Garalatti., 2010).

L-ficolin binds to GlcNAc-BSA and complex-type oligosaccharide chains with GlcNAc linked to the trimannosyl core (Matsushita *et al.,* 1996). L-ficolin can bind specifically to lipoteichoic acid (LTA), a cell wall constituent of Gram-positive bacteria (Lynch *et al.,* 2004). It also binds to peptidoglycan, a component of the bacterial cell wall, and 1, 3-β-D-glucan, a component of

yeast and fungal cell walls (Ma *et al.,* 2004). L-ficolin binds to Gram-positive bacteria such as serotype III group B *streptococci, Strept. pneumoniae* 11F and *S.* aureus, and to Gram-negative bacteria such as *E*. *coli* and *S*. *typhimurium,* TV119 (Matsushita *et al.,* 1996; Krarup *et al.,* 2003; Aoyagi *et al.,* 2005). L-ficolin was shown to enhance the uptake of *S*. *typhimurium* strains by neutrophils, suggesting that it serves as an opsonin for phagocytosis (Matsushita *et al.,* 1996). It was also demonstrated that L-ficolin recognizes DNA, which enables the binding of L-ficolin to late apoptotic and necrotic cells (Jensens *et al.,* 2007).

The recombinant M-ficolin binds to GlcNAc-BSA, N-acetylgalactosamine (GalNAc)-BSA and sialyl N-acetyllactosamine- BSA (Liu *et al.,* 2005a). Specific binding to sialic acid was confirmed by the binding of M-ficolin to the glycoprotein fetuin, and not to its asialo derivative (Liu *et al.,* 2005). M-ficolin has been found on the surface of monocytes and U937 cells (Teh *et al.,* 2000). M-ficolin binds to *S*. aureus and *S*. *typhimurium* LT2 (Liu *et al.,* 2005a).

H-ficolin also binds to GlcNAc and GalNAc, but not to mannose and lactose (Sugimoto *et al.,* 1998). H-ficolin-induced agglutination of human erythrocytes, coated with lipopolysaccharides (LPS) derived from *S*. *typhimurium*, *S*. *minnesota* and *E*. *coli* (O111), was inhibited by GlcNAc, GalNAc and fucose (Misao *et al.,* 2002). H-ficolin binds neither *S*.*aureus* nor *S*. *pneumoniae,* but can bind to *Aerococcus viridans*, and inhibits its growth (Tsujimura *et al.,* 2002).

The mouse ficolin-A and ficolin-B also bind to GlcNAc and GalNAc, and ficolin-B, like M-ficolin, additionally recognizes sialic acid residues (Endo *et al.,* 2005). Thus, all ficolins in common can recognize GlcNAc. All these observations show that, like MBL, ficolins play an important role in the clearance of both apoptotic cells and microorganisms.

1.3.2.3. Ficolin complex formation with MASPs and sMAP

Like MBL, all ficolins activate the lectin pathway in association with the key enzymes, MASPs (Matsushita *et al.,* 2000a, 2000b, 2002; Liu *et al.,* 2005a). The MASP and sMAP binding is mediated through the collagen region of ficolin (Lacroix *et al.,* 2009). Upon binding of ficolin to ligands the associated MASP exhibit proteolytic activities and activates complement components. As shown in (Fig 1.9), the carbohydrate residue on the microbe is recognized and sensitizes the fibrinogen domain of ficolin. This leads to the activation of serine protease MASP2 and triggers complement components of lectin pathway. MASP-2 is the only MBL-associated protease that efficiently can activate the complement cascade (Thiel *et al.,* 1997).

It was previously reported that unlike ficolin-A, mouse ficolin-B failed to form complexes with MASP-2 and sMAP (Endo *et al.,* 2005). However, “it was recently reported that unlike mouse ficolin-B, rat ficolin-B associated to and activated MASP-2 (Girija *et al.,* 2011). This result

might suggest that like the other mammalian ficolins, murine ficolin B also plays a role in the complex with MASPs in the lectin pathway" (Endo *et al.,* 2011).

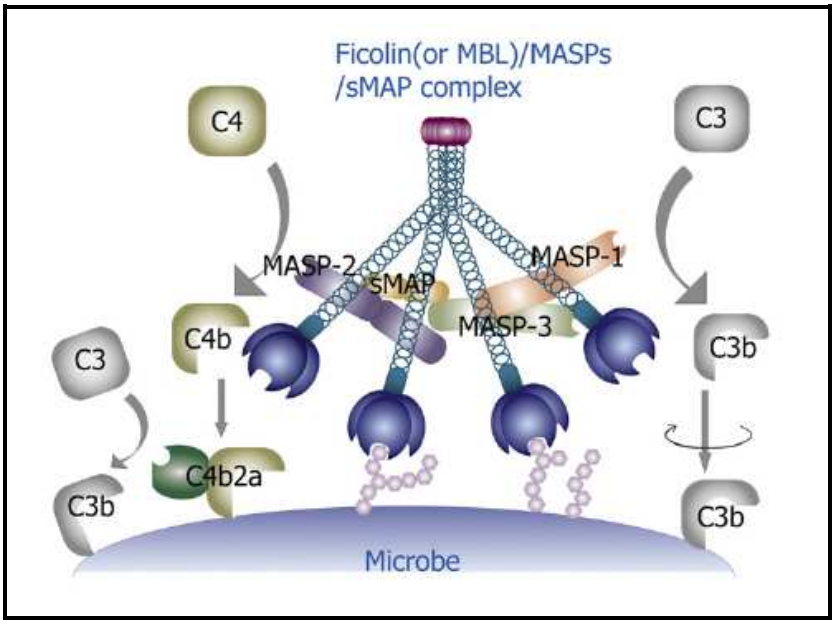


Fig. 1.9 | Ficolin-MASPs/sMAP complex. The sugar residue on the microbe is recognized by the fibrinogen domain of ficolin and triggers the lectin pathway. The complexed MASPs are converted to the active forms and activates the lectin pathway of the complement system (Endo., 2007).

1.3.2.4. Collaboration of ficolin with other defense systems

Ficolins and MBL are higher oligomers composed of monomeric subunits and therefore have multivalent ligand-binding sites. Fibrinogen (Fbg) is a dimer of 3 subunits, formulated as (Aα)2(Bβ)2α2, and fibrin (Fbn) is a polymer consisting of (α2, β2 , γ2)n . The tetrapeptide GlyProArgPro, a known inhibitor of blood coagulation, mimics the N-terminal sequences of fibrin α- and β-chains. Recently, Endo *et al.,* (2010) confirmed that ficolin-A binds to the Aα- and Bβ-chains of fibrinogen and the α- and β-chains of fibrin, but not to the γ-chain, and that MBL preferentially binds to the fibrin α- and β-chains. Based on these observations, he suggested the hypothesis that ficolins can interact with fibrinogen and/or fibrin and the lectin pathway might collaborate with the coagulation system in the first-line host defence against pathogens. Ficolin or MBL bind to sugar residues on microbes at the ligand binding site and also bind to fibrinogen or fibrin with their remaining binding sites (Fig.1.10). Thrombin and MASP2 activate factor XIII to XIIIa and this leads to form a cross-link between fibrin and pathogen and within fibrin. This binding results in a complex network on the pathogen and activates MASP2 for further intiation of the downstream cascade of the lectin pathway.

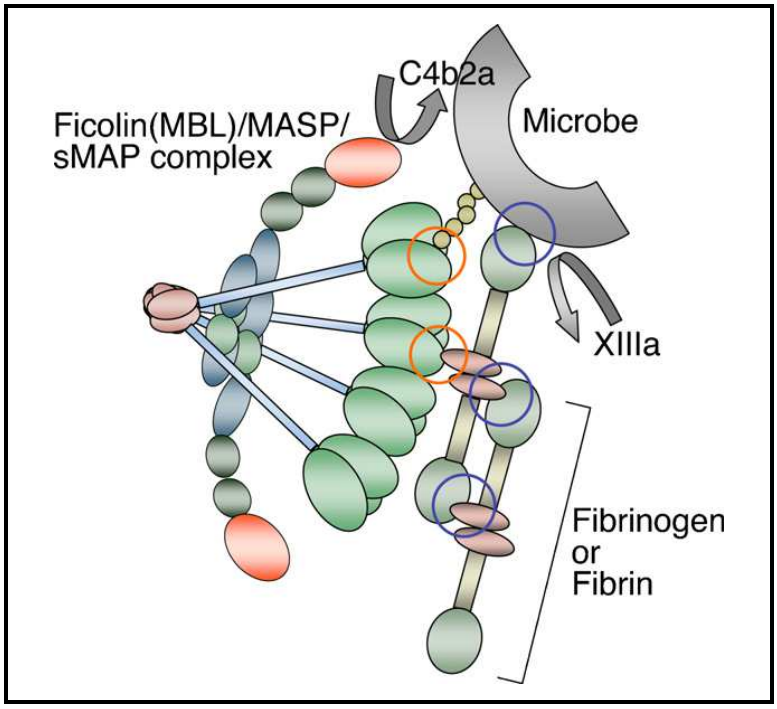


Fig. 1.10 | A schematic model showing the collaboration of the lectin pathway with blood coagulation. (Endo.,*et al* 2011).

It has also been shown that human C-reactive protein (CRP) can bind to *S.enterica* and L-ficolin. This interaction between CRP and L-ficolin stabilizes the binding of CRP to the bacteria and increases complement activation (Ng *et al.,* 2007; Zhang *et al.,* 2009). This observation shows that the recognition molecule–pathogen complex is stabilized by another plasma protein and, in turn, this stabilization boosts the lectin pathway. Thus, it is suggested that the lectin pathway 'cross talks' and collaborates with other defense systems, such as blood coagulation and acute phase inflammation, to eliminate pathogens with high efficiency.

1.3.3. Mannose binding lectin associated serine proteases (MASPs)

MASPs are serine proteases that are activated when complexed with MBL. They cleave complement C4 and C2 to form C4b2a, the classical pathway C3 convertase (Matsushita and Fujita, 1992). There are three known serine proteases, namely MBL-associated serine proteases (MASP-1, -2 and -3). A small non-enzymatic protein, MAp19 (or sMAP), is an alternatively spliced product which shares its origin with MASP-2 and another non-catalytic protein, MAp44 (or MAP1), which shares its origin with MASP-1 and MASP-3 (Garred *et al.*, 2010). MASPs are homodimers which are composed of a CUB domain at the N-terminal end followed by an epidermal growth factor (EGF) domain, a second CUB domain, two complement control protein (CCP) domains, and a serine protease domain (Fig. 1.11) (Wallis, 2002). Binding is mediated through a portion of the collagenous domain of MBL and

the N-terminal domains of the MASP. All three N-terminal MASP domains (CUB1–EGF–CUB2) are necessary and sufficient to reproduce the binding properties of the full length proteins. The CUB1–EGF domains bind to MBL in a Ca2+ manner.

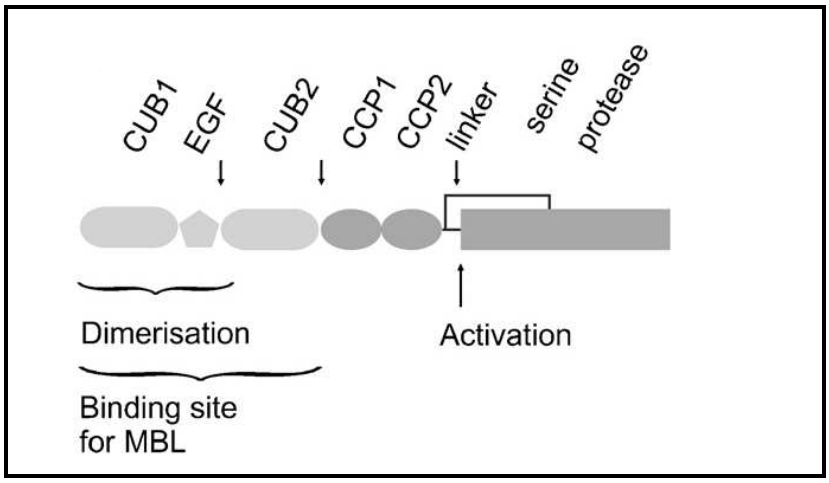


Fig. 1.11 | Domain organisation of MASPs (Wallis, 2002)

MASPs normally circulate as zymogens. However, when MBL/MASP or ficolin/MASP complexes bind to target epitopes on pathogens, MASPs-1 and -2 activate through autolysis at a single site within the short linker region between the CCP-2 module and serine protease domain. The active protease domain remains attached to the N-terminal fragment through a single disulphide bond. MASP-3 is also activated through cleavage of the linker region.

However, the zymogen cannot auto-activate, it might be probably activated through an unidentified serum protease (Zundel *et al*., 2004). MASP-2 is the only MBL-associated protease that efficiently can activate the complement cascade (Thiel *et al.,* 1997). MASP-1 activates factor D, which is an early component of the alternative pathway, MASP-1 most likely enhances complement activation initiated by MASP-2 (Rossi *et al.,* 2001). MASP-3 shows no proteolytic activities and therefore is not directly involved in the initiation of the lectin pathway of complement (Zundel *et al.,* 2004). On contrast, MASP-3 has been shown to down-regulate H-ficolin mediated complement activation (Skjoedt *et al.,* 2009). MAp19 is an alternative splice product of the MASP-2 gene (Stover *et al.,* 1999a; Stover *et al.,* 1999b). It is found in association with MBL or in complex with MASP-1 in serum, but its physiological role has not been determined yet (Thiel *et al.,* 2000). Another inhibitor Map44 was recently characterized as competing with MASP-2 for binding to ficolin and MBL resulting in inhibition of complement activation (Skjoedt *et al.,* 2009).

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Item	Manufacturer
Agarose, electrophoresis grade	Invitrogen
Acetic acid	AppliChem
Acetone	Merck
Acrylamide/Bisacrylamide	Bio-Rad
Ampicillin, >98%	Sigma-Aldrich
APS, ammonium persulfate	Biorad
Bromphenol Blue	Sigma-Aldrich
BSA, bovine serum albumin, fraction V	Biomol
Calcium chloride	Sigma-Aldrich
Chloramphenicol	Sigma-Aldrich
Chelating Sepharose Fast Flow	GE Healthcare
CNBr-activated Sepharose 4B	GE Healthcare
Coomassie Brilliant Blue R250	Fluka
Deoxynucleotides, PCR grade	Roche Applied Science
DMEM medium	Invitrogen
DMSO, dimethyl sulfoxide	Sigma-Aldrich
Dried milk powder	Nestlé
Dithiothreitol	NEB
EDTA	Sigma-Aldrich
Eppendorf combitips	Eppendorf
Eppendorf tubes	Eppendorf

Ethanol, abs.	AppliChem
Ethidium bromide	Sigma-Aldrich
FCS, fetal calf serum	PAN Biotech
Gentamycin	PAA Laboratories
Glutamine	Biochrom AG
Glutathione (Oxdised)	Sigma-Aldrich
Glutathione (Reduced)	Sigma-Aldrich
Glutathione Sepharose® 4B	Amersham
Glycerol, ultrapure	Invitrogen
Guanidine hydrochloride	Sigma-Aldrich
H_2O (deionized)	Milli Q UF Plus system
HEPES	Sigma-Aldrich
Hygromycin-B	Invitrogen
IPTG, isopropyl-β-D-thiogalactoside	Biomol
Kanamycin	Invitrogen
LB Broth Base	Sigma-Aldrich
Lysozyme	Sigma-Aldrich
ß-mercaptoethanol for cell culture	Invitrogen
ß-mercaptoethanol for molecular biology	Sigma-Aldrich
Methanol, technical grade	Merck
Magnesium chloride	Sigma-Aldrich
Ni-NTA (Nitrilotriacetic acid) Agarose	QIAGEN
Nowa Solution A+B (ECL)	MoBiTec
Nuclease free water	Promega
PBS buffer (10X Dulbecco's) powder	Applichem
Polyacrylamide, Rotiphorese® Gel30 (37,5:1)	Carl Roth

Penicillin/Streptomycin	Gibco
Phenol/chloroform, for DNA purification	Carl Roth
PMSF	Sigma-Aldrich
2-Propanol	Merck
RNAse A, DNAse free	Roche Applied Science
RPMI-1640	Sigma-Aldrich
SDS ultra pure	Sigma-Aldrich
Sodium acetate	Sigma-Aldrich
Sodium chloride	Sigma-Aldrich
Sodium hydroxide	Sigma-Aldrich
SDS-PAGE Molecular weight standard, broad range	Biorad
TEMED, Tetramethylethylenediamin	Biorad
Triton X-100	GE Healthcare
Tris Base	Sigma-Aldrich
Tryptone	Difco
TSR (Template suppression reagent)	Applied Biosystems
Tween 20	Fluka
Urea	Molecular Probes
Whatman round filter	Whatman
X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside)	Biomol
Yeast extract	Difco
β-Mercaptoethanol	Sigma-Aldrich

Aspergillus fumigatus from Prof. Dr. Frank Ebel, Max-von-Pettenkofer-Institut, München.

Unless otherwise noted all the buffer-substances and chemicals were purchased from Sigma-Aldrich®, Fluka® or Applichem®. DNA-purification-Kits were purchased from QIAgen® and ProOmega.

2.1.2. Buffers and Solutions

2.1.2.1. Acrylamide Gel electrophoresis (Protein SDS-PAGE)

Separating Gel

	6.5%	**7.5%**	**10%**	**12%**
H2O dest	20.2 ml	17.1 ml	14.5 ml	12.3 ml
3M Tris-HCl pH 9.0	3.8 ml	3.8 ml	3.8 ml	3.8 ml
Acrylamid Roti 30	6.8 ml	7.9 ml	10.5 ml	12.7 ml
20% SDS	0.15 ml	0.15 ml	0.15 ml	0.5 ml
TEMED	0.03 ml	0.03 ml	0.03 ml	0.03 ml
10% APS	0.15 ml	0.15 ml	0.15 ml	0.15 ml

Stacking Gel

H2O dest	7.7 ml
1M Tris-HCl pH 6.8	1.25 ml
Acrylamid Roti 30	1.66 ml
20% SDS	0.05 ml
TEMED	0.015 ml
10% APS	0.1 ml

Blocking for Western Blot	5% Milk powder in TBS-T
Blot buffer (Western blot)	190mM Glycine
	25 mM Tris base
	20% (v/v) Methanol
Coomassie-Staining solution	10% Acetic acid
	40% Ethanol
	0,2% Coomassie Brilliant Blue R250

Coomassie-Destaining solution	10% Acetic acid
	40% Ethanol
Blot stripping buffer	62.5mM Tris
	2%SDS
	0.04% 2-Mercaptoethanol
	0.005 % Bromphenolblue
DNA Loading Dye Solution (6x)	0.25% Bromphenolblue
	0.25% Xylencyanol
	30% Glycerol in H2O
Electrophoresis buffer (10x)	25 mM Tris Base
	190 mM Glycine
	0.1% SDS at pH 8.6
Gel filtration buffer	20mM Tris, pH 8.0
	50 mM KCl
Lysis buffer (Protein)	10 mg/ml Lysozyme
	60 mg/ml Na-Desoxycholate
	1 M Mgcl2
	6 mg/ml DNaseI
PBS	137 mM NaCl
	6.5 mM Na2HPO4 x H2O
	1.5 mM KH2PO4
	2.7 mM KCl
PBS-T	0.05% Tween 20 in PBS-buffer
Ponceau S	0.2% Ponceau S
	3% Trichloroacetic acid

SDS-loading buffer (1x)	60mM Tris-HCL, pH 6.8
	10% Glycerin
	3% SDS
	5% β-Mercaptoethanol
	0.005% Bromophenol blue
TAE-buffer	40 mM Tris-Acetate
	1 mM EDTA
TBS-buffer	8 g NaCl
	0.2 g KCl
	3 g Tris Base
TBS-T	0.05 % Tween 20 in TBS-buffer
Tris-buffer for SDS-PAGE	1,5 M Tris-HCl (pH 8.8)
	0.5 M Tris-HCl (pH 6.8)
10x TBE buffer (DNA electrophoresis)	890 mM Tris
	89 mM Boric acid
	20 mM EDTA

IMPACT Kit buffers

Lysis buffer:	20 mM Tris-HCl, pH 8.5
	500 mM NaCl
Column buffer	20 mM Tris-HCl, pH 8.5
	500 mM NaCl
Cleavage buffer	20 mM Tris-HCl
	500 mM NaCl
	50 mM DTT
Stripping Solution	0.3 M NaOH

2.1.3. Analytical Kits

BCA protein assay Kit	Pierce, Rockford, IL, USA
Dc Protein Assay	Biorad
Gel Extraction Kit QIAEX II	QIAGEN
HiFi PCR Master Kit	Roche
IMPACT™ Kit	NEB
pGEM – T –Easy vector system	Promega
Plasmid Purification Maxi Kit	QIAGEN
Plasmid Purification Mini Kit	QIAGEN
Prime-It® II Random Primer Labelling Kit	Stratagene
Reverse Transcription Kit	Promega
RNA Isolation Kit	Macherey Nagel
RNeasy Mini Kit	QIAGEN
RNase-free DNase Set	QIAGEN
TOPO TA Cloning® Kit	Invitrogen
Wizard® *SV gel* and PCR clean-Up system	Promega
Wizard® *Plus* Miniprep DNA Purification System	Promega

2.1.4. Standards

Standards	**Manufacturer**
1 kb ladder for DNA	NEB
Kaleidoscope prestained standards	Bio-Rad
Precision Plus Protein dual color standards	Bio-Rad
Pre-stained Molecular Weight Standard Mixture for protein	Fermentas

2.1.5. Enzymes

Enzyme	Manufacturer
Calf Intestinal Phosphatase (CIP)	NEB
DNase I	Sigma
Endonucleases	NEB and MBI-Fermentas
Lysozyme	Sigma-Aldrich
Klenow fragment	Roche
M-MLV Reverse Transcriptase	Promega
Pfu DNA-Polymerase	Promega
Pfx DNA-Polymerase	Invitrogen
RNase A	Roche
T4 DNA ligase	NEB
Taq DNA-Polymerase	peQLab

2.1.6. Antibodies

2.1.6.1. Primary antibodies

Antibody/ Antigen	Species	Application	Working Dilution	Manufacturer
Chicken anti-ficolin - B (polyclonal)	Chicken polyclonal	WB	1:2000	GmBH Davids Biotech
Anti chitin binding domain(monoclonal)	Rabbit	WB	1.2000	NEB
Rabbit anti-ficolin-B (polyclonal)	ELISA / WB / IP	WB	1:2000	Lab source
Rat anti-ficolin-B (monoclonal) IA4	ELISA	WB	1:500	Lab source
Anti-GST / anti-GST	goat monoclonal	WB	1:10000	Amersham,

2.1.6.2. Secondary antibodies

Antibody/Antigen	Species	Application	Working Dilution	Manufacturer
Anti-goat horseradish-peroxidase linked	sheep polyclonal	WB	1:5000	Amersham, NA933
Anti Chicken-IgY	Hen	WB	1.3000	GmBH Davids Biotech
Goat anti-rabbit-IgG-HRP	WB	WB	1:1000	Sigma
Rat anti-ficolin-B (monoclonal)	ELISA	WB	1:500	Lab source

2.1.7. Expression vectors

Plasmid	Annotation	Marker	Supplier
pTXB1	vector for high–level expression of C–terminally intein tagged proteins in bacteria	Ampicillin	NEB
pGEX-4T-1	vector for high–level expression of C–terminally GST–tagged proteins in bacteria	Ampicillin	Pharmacia
pGEX-4T-1-N -TEV	vector for high–level expression of C–terminally GST–tagged proteins in bacteria with TEV site for cleavage	Ampicillin	MPI, Dortmund
pCR®2.1-TOPO	Vector with single 3'-thymidine overhangs for direct cloning of *Taq*-amplified PCR products	Ampicillin	Invitrogen
pMT/BiP/V5-His A	Vector includes the *Drosophila* metallothionein (MT) promoter for high-Level, copper-inducible expression.	Ampicillin	Invitrogen
pProEXHTb	Bacterial expression vector, genes cloned into the MCS will be expressed as fusion proteins with an N-terminally 6xHis tag	Ampicillin	Invitrogen
pET28	vector for high–level expression of N –terminal His tagged proteins in bacteria	Kanamycin	Novagen

2.1.8. Organisms and cell lines

2.1.8.1. Bacterial strains

Strains	Derivative	Genotype	Antibiotic resistance
E.coli Dh5α	K12	F–,ø80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deo*R, *recA*1, *endA*1, *hsdR*17(rK–, mK+), *phoA*, *supE*44, λ–, *thi*-1, *gyrA*96, *relA*1	Ampicilin
BL21 (DE3) "Rosetta"	BL21	F-*ompT hsdSB*(rB-mB-) *gal dcm* pRARE2 (Camr)	Chloramphenicol (34 µg/ml)
BL21 (DE3) pLysS	B 834	F- ompT hsdSB(rB –mB -) gal dcm (DE3)	Chloramphenicol (34 µg/ml)
TG1	K12	supE hsdΔ5 thi Δ (lac±proAB) F´[traD36 proAB+lacIq lacZΔM15]	None
OneShot®TOP10F'	MC1061	*mcr*A□(*mrr-hsd*RMS-*mcr*BC) 80lacZM15 *lac*X74*rec*A1*ara*D139(*ara-leu*)7697*gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Ampicilin
XL-1 blue	K12	*rec*A1*end*A1 *gyrA96 thi-1 hsdR17 supE44 relA1 lac* {F´ *proAB lac*Iq*ZΔM15* Tn*10* (Tetr)}	Tetracycline

2.1.8.2. Cultured cell lines

Cell line	Medium and antibiotic resistance	Annotation
Drosophila Schneider 2 (S2) cell line	Insect media (Insect X-press, Cambrex), kanamycin	Derived from a primary culture of late stage *Drosophila melanogaster* embryos

2.1.9. Oligonucleotides

All primers used for cloning, sequencing or screening were purchased from Metabion at a concentration of 100 pmol/µl, and stored at -20℃ in aliquots of 10 pmol/ µl until use.

The primers used in this work are listed below:

Primers	Usage	5' → 3' Sequence
Ficolin B Intein Nde 5´	Forward Primer	5´GGT GGT CAT ATG TGC CCA GAA CTG AAA GTC

Ficolin B Intein Sap 3´	Reverse primer	5´GGT GGT TCG TCT TCC GCA GAT GAG CCG CAC CTT CAT
FicolinB-BamHI-5´	Forward Primer	5´ CC GGA TCC ATG GCC CTG GGA TCT GCT TGC
FicolinB-XhoI stop-3´	Reverse primer	5´ CC CTC GAG CAG AGA ACA AGC ACC TTT TGA AA
FicolinB- XhoI- 5´	Forward Primer	5´ CC CTC GAG AGA GGA GAG AGT GGC C
FicolinB-XhoI stop-3´	Reverse primer	5´ CC CTC GAG TTA GGG CCC AGA GTC TCC
E72A_For	Qucik-change-mutagenesis-primer sense E72A mutation	5´ CTT CCT GGA ATT CCT GGA AAA GCA GGA AAA GCA GGA CCA ACT GGA CCC AAA GGA
E72A_Rev	Qucik-change-mutagenesis-primer Antisense E72A mutation	5´ TCC TTT GGG TCC AGT TGG TCC TGC TTT TCC AGG AAT TCC AGG AAG

2.1.10. Equipment

Apparatus	**Manufacturer**	**Apparatus**	**Manufacturer**
Agarose electrophoresis gel chamber	Stratagene	Neubauer counting chamber	Brand
Autoclave	Webeco	Nitrocellulose transfer, 0.45µM	Whatman
Blotting chamber	Hoefer	Petri dishes	Hartenstein
Blotting papers Grade 3 MM Chr	Whatman	Pipettes	Eppendorf, Abimed
Centrifuges	Eppendorf, Sorvall, Heraeus	Photometer	Hitachi
Computer	Dell	Precision balance	Sartorius
Counting chamber	Neubauer (improved)	Shaker	Bellco Biotechonology

Coulmns - Mini	NEB	Dialysis Cassettes	Thermo-Scientific
Digital chemical balance	Sartorius	Sterile bench	Heraeus
Freezer	Liebherr, Nunc	Thermocycler	Eppendorf, Perkin Elmer
Ice maker	Scotsman	UV crosslinker	Stratagene
Incubator	Heraeus, Memmert	X-ray film	Fujifilm

2.1.11. Software and databases

Product name	Supplier
Adobe Photoshop CS	Adobe Systems Inc. USA
Gene runner	Hastings software
Gel filtration analysis	Unicorn evaluation software
Literature	http://www.ncbi.nlm.nih.gov/pubmed/
Microsoft Office	Microsoft, USA
Sequence analysis	http://www.uniprot.org/
Statistical analysis	http://www.graphpad.com/prism/prism.htm

2.2. Methods

2.2.1. Molecular Biological Methods

2.2.1.1. Amplification of DNA by polymerase chain reaction

2.2.1.1.1. PCR

The polymerase chain reaction (PCR) is a technique for oligonucleotide primer directed enzymatic amplification of a specific DNA sequence of interest. The PCR product is obtained from the DNA template *in vitro* applying a heat-stable DNA polymerase. An automated thermal cycler puts the reaction through 30 or more cycles of denaturation of dsDNA, annealing of primers and polymerization. Denaturation separates the complementary strands and was carried out at 92 ℃. Annealing temperature was calculated based on the primer sequence (Tm). Primers are single-stranded oligonucleotides complementary to the flanking areas of the targeted segment which serve as a starting point for DNA replication. During polymerization the enzyme catalyzes the synthesis of a new strand DNA. The optimal temperature varies according to the polymerase species. PCR products were visualized on an agarose gel.

Taq PCR components	(µl)
Taq Pol buffer 10x	5
$MgCl_2$ (25 mM)	3
Plasmid DNA (~200 ng/µl)	1
5'-Primer (10 pmol/µl)	2
3'-Primer (10 pmol/µl)	2
dNTPs (10 mM)	1
Taq (3 U/µl)	0.4
$_{MP}H_2O$	ad 50

Pfu PCR components	(µl)
Pfu Pol buffer 10x	10
Plasmid DNA (~200 ng/µl)	1
5'-Primer (10 pmol/µl)	2
3'-Primer (10 pmol/µl)	2
dNTPs (25 mM)	1
Pfu Pol (3 U/µl)	1
$_{MP}H_2O$	ad 100

Pfx PCR components	(µl)
Pfx Pol buffer 10x	5
Plasmid DNA (~200 ng/µl)	1
5'-Primer (10 pmol/µl)	3
3'-Primer (10 pmol/µl)	3
Pfx Pol (2.5 U/µl)	1
$_{MP}H_2O$	ad 50

Standard 1-step PCR Program

#	Step	Temperature	Time	Cycle (s)
	hot start			
1	Denaturating	92°C	2 min	1
2	Denaturating	92°C	0.25 min	
	Annealing	T_{mR}	0.25 min	30
	Elongation	72°C	1 min*/kb	
3	Completion	72°C	10 min	1
4	Storage	15°C	hold	

Standard 2-step PCR Program

#	Step	Temperature	Time	Cycle(s)
	hot start			
1	Denaturation	92°C	2 min	1
2	Denaturation	92°C	0.25 min	
	Annealing	T_{mr}	0.25 min	2
	Elongation	72°C	1 min*/kb	
3	Denaturation	92°C	0.25 min	
	Annealing	T_{mR}	0.25 min	30
	Elongation	72°C	1 min*/kb	
4	Completion	72°C	10 min	1
5	Storage	15°C	hold	

*1 min per 1000 bp of DNA sequence of interest was calculated for the duration of the elongation step based on the average processivity of the specific polymerase.

Taq Pol → 1 min/kb DNA sequence

Pfu Pol → 2 min/kb DNA sequence

Pfx Pol → 1 min/kb DNA sequence, operates at 68℃

The basic melting temperature Tm of the primers was calculated with the formula:

Tm= 2 x (*a*A +*b*T) + 4 x (*c*G +*d*G) [℃], where *a*, *b*, *c*, and *d* are the numbers of bases A, T, G and C in the primer sequence, respectively.

Tmr= Tm – 5 (or 4) [℃], without taking into consi deration the restriction endonuclease recognition site sequence (additional to the gene specific sequence)

TmR= Tm – 5 (or 4) [℃], calculating the entire pr imer, including the introduced endonuclease recognition site.

2.2.1.1.2. Reverse transcription PCR (RT-PCR)

The RT-PCR method is used to translate RNA to its DNA complement (complementary DNA, or cDNA) by using the enzyme reverse transcriptase. The cDNA is then amplified using a traditional PCR with a DNA polymerase. Three different types of primers could be used for the RT-step: random primers, oligo (dT) primers or gene specific primers. There are two ways to perform a RT-PCR, first the RT-step could be performed in the same reaction tube like the following PCR-step (one-step RT-PCR). On the other hand, the PCR-step could be performed in another reaction tube (two-step RT-PCR).

In this work, the RT reaction was carried out with the Reverse Transcription Kit in a 20 µl final volume under the following conditions:

	Final concentration
$MgCl_2$	**5 mM**
dNTPs	**1 mM each**
Oligo $(dT)_{15}$ primer	**0.5 µg**
RT Buffer	**1x**
AMV reverse transcriptase	**15 U/µl**
RNasin	**1 U/µl**
RNA template	**1 µg**

The reaction was incubated at 42° for 15 minutes (first strand cDNA synthesis) followed by 5 minutes duration at 95℃ and cooling down to 4℃ for 5 minutes. The first strand of cDNA synthesized during this step was used as the template for the coupled standard PCR reaction.

2.2.1.1.3. Colony PCR

Colony PCR is a rapid method for screening for positive bacteria clones on a plate subsequent to DNA cloning. Gene specific primers of the desired sequence were used for a Taq-PCR reaction mix. The reaction mix was aliquoted to 10 PCR tubes on ice. Single isolated bacteria colonies from a LB-plate were picked with an autoclaved toothpick into the PCR-tubes containing the mix. The tested colonies were transferred to a fresh LB-plate for further usage. Cell lysis was achieved by an additional initialization heating step at 95℃ for 3 min in the thermocycler (*Eppendorf*) prior to the standard PCR-program. PCR-products were analyzed by agarose gel electrophoresis. Cultures for positives clones were prepared for plasmid DNA isolation.

2.2.1.2. DNA Cloning

DNA cloning is a technique for DNA fragments reproduction. A vector is required to carry the DNA fragment of interest into a host cell. All cloning vectors used in the course of this work were plasmids. Plasmids are small circular molecules of double stranded DNA derived from natural plasmids that occur in bacterial cells. A DNA fragment can be inserted into a plasmid if both, the circular plasmid and the source of DNA, have recognition sites for the same restriction endonuclease. The plasmid and the foreign DNA are cut by a restriction endonuclease producing intermediates with sticky and complementary ends. After ligation the new plasmid can be introduced into bacterial cells and produce many copies of the inserted DNA.

2.2.1.2.1. Generating a DNA insert

DNA inserts were generated by PCR and products were separated on an agarose gel and purified. Subsequential incubation with restriction endonucleases produced blunt ends or single-stranded overhangs (cohesive ends also called sticky ends) complementary to those in the vector. Primers were designed to provide a restriction site at each end of the PCR product. In other cases the PCR products contained the cleavage site. Accurate digestion was verified by agarose gel electrophoresis, followed by purification of the digested products.

Preparative restriction double digestion	(µl)	U
PCR cDNA product (~500 ng)	20	
10x reaction buffer	3	
restriction endonuclease I	variable	10-20
restriction endonuclease II	variable	10-20
$_{MP}H_2O$	ad 30	

The temperature of incubation, the choice of reaction buffer as well as the amount of units applied were dependent on the restriction endonuclease's requirements. Duration of incubation was in most cases minimum 2 h. In case double digestion was not suitable, the cDNA was subjected to cleavage by one enzyme at a time, followed by gel electrophoresis and extraction.

2.2.1.2.2. Generating recombinant plasmid DNA

Plasmid DNA was cleaved by corresponding restriction endonucleases which produced cohesive ends complementary to the insert DNA. After verification of a successful plasmid linearization and vector fragment integrity by gel electrophoresis, the vector was purified using the Promega Gel Extract Kit according to manufacturer's instructions.

Preparative restriction double digestion	(µl)	U
plasmid DNA (~1µg)	variable	
buffer 10x	3	
restriction endonuclease I	variable	5-10
restriction endonuclease II	variable	5-10
$_{MP}H_2O$	ad 30	

The temperature of incubation and the choice of reaction buffer, as well as the amount of units applied were dependent on the restriction endonuclease's requirements. Duration of the incubation was 0.5-1 h. In case a double digestion was not suitable DNA was subjected to cleavage by one enzyme at a time, followed by a gel electrophoresis and extraction.

2.2.1.2.3. Noncohesive ends cloning

If not having compatible restriction sites for a cloning procedure at hand, a blunt (noncohesive end) cloning was performed. Also, provided a restriction endonuclease generated noncohesive ends upon restriction of an insert, a blunt end cloning was carried out. The insert and vector terminii were both blunted by using *E. coli* DNA polymerase I large fragment (Klenow fragment, *Roche*) according to the manufacturer's instructions. Klenow has 5'-3' polymerase and 3'-5' exonuclease, but not 5'-3' exonuclease activities. Next, the vector was dephosphorylated by using the calf intestinal phosphatase (CIP) to decrease the background of non-recombinants that arise from self-ligation. CIP was also employed according to the manufacturer's instructions. Gel purification of the vector was performed to reduce the frequency of aberrant clones and were recovered from the gel by using the NucleoTrap gel extraction kit (Pro Omega).

2.2.1.2.4. Site-directed Mutagenesis

Specific deletions were introduced into cloned DNA using completely overlapping sense and antisense primers containing the mutation of interest. Mutagenic primers lacking the DNA sequences designated for deletion were employed for PCR amplification using cDNA for full-length wild-type ficolin-B as template and the Pfx DNA Polymerase (*Invitrogen*), according to the manufacturer's conditions. Primers were extended for 10 min at 68℃ during which the desired mutations were incorporated and subsequently amplified. The PCR products obtained were two different linear DNA fragments, the parental template and the mutated DNA. Parental template DNA was subjected to degradation by DpnI digestion (30 U/50 µl PCR sample, for 1 h at 37℃). Subsequently, DpnI en donuclease was heat inactivated for 15 min at 65℃. Mutant DNA was circularized by blunt e nd ligation, achieved by transforming competent *E. coli* DH5α bacterial cells with DpnI-treated PCR sample. The sequences of mutant was confirmed by double-stranded DNA sequencing (Geneart, Regensburg).

2.2.1.2.5. Ligation

Ligation is the process of joining linear DNA fragments together with a covalent bond. A phosphodiester bond between the 3'-hydroxyl termini and the 5'-phosphate is catalyzed by the T4 DNA ligase. The enzyme originates from the T4 bacteriophage and ligates DNA fragments having blunt and sticky ends annealed together. The optimal incubation

temperature for T4 DNA ligase is 16℃. Following ge neration and purification of insert and vector DNA fragments, the fragments were eluted together in 1x T4 ligase buffer and supplemented with T4 DNA ligase (*NEB*). To maximize the yield, a ligation reaction was set in an estimated vector:insert molar ratio of 3:1. Following over night incubation at 16℃ or 2hours at room temperature, the total reaction was used for transformation of competent cells.

Ligation	(µl)	U
DNA fragments	19	
T4 DNA ligase	1	400 U/µl
final volume	20	

2.2.1.2.6. Transformation and culturing of *E.coli* DH5α bacterial cells

Transformation is the process of uptake of exogenous plasmid DNA into a bacterium. Bacteria that are capable of being transformed are called competent. Competent cells are prepared by treatment with rubidium chloride (RbCl). Cells are treated with a hypotonic solution containing RbCl. It increases the ability of a prokaryotic cell to incorporate plasmid DNA allowing them to be genetically transformed. The addition of rubidium chloride to a cell suspension promotes the binding of plasmid DNA to the cell surface, which can then pass into the cell. Transformation of bacterial cells is achieved by an uptake of plasmid DNA. The procedure involves heat shocking and ice shocking alternately. 20 µl of a ligation reaction were added to 200 µl competent bacterial cells (*E. coli* DH5α) previously thawed on ice, gently mixed and incubated on ice for further 40 min. Cells were heat shocked for 90 s at 42℃ and chilled on ice for 2 min. 1 ml LB was adde d and the cells were incubated under shaking for 30-60 min at 37℃. Cells were collected by centrifugation at 3500 rpm for 3 min at RT. Supernatant was discarded and the cell pellet was dissolved in 150 µl LB. Next, 50 µl and 100 µl were plated out on LB agar plates containing the appropriate antibiotic as selection marker and plates were incubated overnight at 37℃. Colonies of transformants were inoculated for further investigation or plates were stored for several weeks at 4℃. For bacterial culture and subsequent plasmid purification, designated transformed *E. coli* DH5α clones were shaken over night in LB broth supplemented with either 100 µg/ml ampicillin or 100 µg/ml kanamycin at 37℃. Later cells were pelleted a nd plasmid DNA was isolated employing the Plasmid Maxi Kit (*Qiagen*) according to manufacturer's protocol.

2.2.1.3. Separation of nucleic acids by Agarose gel electrophoresis

Agarose gel electrophoresis is a technique used to separate DNA fragments based on their size. DNA is negatively charged in solution and is forced to migrate through an agarose gel to the positive pole by an electric field. Migration rate is inversely proportional to the logarithm of the number of base pairs. Longer molecules move more slowly due to their entrapment in the gel matrix. Gel agarose concentration is chosen to be appropriate for the size of DNA fragments. Concentration of 0.7%-agarose gels shows good resolution of large DNA molecules (up to 12 kb), whereas 2%-agarose gels are suitable for smaller molecules (down to 0.2 kb). DNA can be visualized by illumination by UV light (Bachofer GmBH) based on the incorporation of fluorescent dye (ethidium bromide) into the DNA helix. If the DNA was to be used for further cloning procedures only brief exposure to UV light was performed to minimize the risk of DNA damage. The agarose (% (w/v) = gel concentration) was dissolved by boiling in 0.5x TBE buffer (microwave). After cooling to 60℃ ethidium bromide was added to a final concentration of 5 µg/ml. DNA-loading buffer was added to the DNA samples. Gels were run usually at 100 volts for 30-60 min. The agarose gel areas containing the DNA fragments of interest were excised and DNA was recovered from the gel by employing Quiagen or Promega Gel Extraction Kit according to manufacturer's protocol.

2.2.1.4. Isolation of nucleic acids from aqueous solutions

2.2.1.4.1. Alcohol precipitation

Ethanol precipitation is a rapid technique to concentrate DNA. A DNA precipitate is allowed to form in ethanol in the presence of moderate concentration of monovalent cations (0.1 volumes of 3M Sodium acetate). Initial precipitation was done with 2 to 2.5 volumes of ice cold absolute ethanol for 20 min at low temperature (-20℃). DNA was recovered by centrifugation at 15000 g for 20 min at RT. Next, the precipitate was redissolved in ice cold 70% ethanol, followed by a brief centrifugation eliminating the residual salt. After drying the pellet was resuspended in an appropriate buffer or $_{MP}H_2O$. Isopropanol can be used instead of ethanol. One volume isopropanol was added to the aqueous solution. Precipitation was achieved by mixing and incubation for 10 min at RT. DNA precipitate was collected by centrifugation at 15000 g for 10 min at RT. Isopropanol and salt were removed by subsequent wash with ice cold 70% ethanol.

Isolation of plasmid DNA from bacterial cells was carried out by using *Qiagen* Plasmid Maxi Kit and *Qiagen* Plasmid Giga Kit following the manufacturer's instructions.

2.2.1.5. Isolation and purification of plasmid DNA

2.2.1.5.1. Mini-scale isolation of plasmid DNA ("Minipreps")

Mini-scale isolation of plasmid DNA was performed using the Wizard Plus SV Minipreps DNA Purification System (Promega). This kit combines two techniques: alkaline lysis and silica resin-based DNA purification. Cells are partially lysed using an alkaline solution of the detergent SDS. This allows small plasmid DNA molecules to escape from the cell, while genomic DNA remains within the cells. When a concentrated potassium acetate solution is added to the cell lysate, cell debris (containing high molecular weight genomic DNA) is precipitated, while plasmids and soluble proteins remain in solution. If cell membranes are dissolved completely, sheared genomic DNA may be released, contaminating the plasmid preparation. To avoid this, the lysis step is carried out for a limited time – just enough for the solution to clear. The adhesion of DNA to a silica matrix is based on the observation that nucleic acids adhere to silica in high-salt conditions, but not in low-salt conditions. DNA will bind to silica in the lysis solution, and will be eluted from the matrix by TE buffer (see section "Buffers").

Colonies were picked and cultured in LB medium containing 100 µg/ml ampicillin at 37℃ overnight under vigorous shaking. The experimental procedure was carried out according to the manufacturer's instructions. Briefly, 3 ml of overnight bacterial culture were centrifuged for 5 min and the pellet resuspended in 250 µl of Resuspension Solution. After lysis with one volume of Cell Lysis Solution the reaction was stopped by adding 350 µl of Neutralization Solution and pelleted at top speed for 10 min at RT. The clear lysate was then decanted into a Spin Column containing the silica-resin, and washed extensively before the DNA was eluted with 100 µl of TE buffer. Plasmid identity was checked by restriction digestion and the DNA stored at 4℃.

2.2.1.5.2. Scaled-up preparation of plasmid DNA ("Maxipreps")

Maxiprep isolation of DNA was performed to obtain high yields of purified plasmid DNA for further transformations and transfections. Positive clones were chosen and plasmids were isolated using the Plasmid Maxi Kit (QIAGEN). This protocol is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. While RNA, proteins, dyes and low-molecular-weight impurities are removed by a medium-salt wash, plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation.

Briefly, 200 ml LB medium containing 100 μg/ml ampicillin was inoculated with the starter culture in a 1/1000 ratio, and incubated overnight at 37℃ under vigorous shaking. After spinning down at 6500x*g* for 15 minutes, cells were resuspended extensively in Buffer P1 containing RNase A, sequentially lysed in Buffer P2 for 5 min and neutralized in chilled Buffer P3. The lysate was then centrifuged (20,000x*g,* 30 min) to pellet the cell debris and the clear supernatant was decanted into a QIAGEN-tip 500 containing the anion-exchange resin. The resin was washed with medium-salt- and DNA eluted with high-salt-containing buffers. DNA precipitation was performed by adding 0,7 volumes of isopropanol and further centrifugation (14,500x*g* for 30 min). Before resuspending the DNA in water, the pellet was washed with 70% ethanol. All centrifugation steps were carried out at 4℃. Plasmid identity was checked by restriction digestion and the DNA stored at 4℃.

2.2.1.6. Isolation of total RNA

To investigate the expression pattern of ficolin B in mouse tissue, total RNA was isolated from different organs using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Briefly, after excision samples (up to 30 mg, depending on the organ) were immediately placed in RNA*later* solution which permeates the tissue to stabilize and protect cellular RNA *in situ*. Stabilized tissue samples were then disrupted in buffer containing highly denaturing guanidine isothiocyanate (to inactivate RNases) by a rotor-stator homogenizer (Ultra-Turrax T25, Ika® Labortechnik), which causes disruption by a combination of turbulence and mechanical shearing. Disrupted tissue was then homogenized to reduce viscosity of the cell lysate by shearing the high-molecular-weight genomic DNA and cellular components to create a homogeneous lysate. Homogenization was performed using the QIAshredder Kit (QIAGEN). Ethanol was then added to provide appropriate binding conditions and the lysate applied to a column where the total RNA binds to the membrane and contaminants are washed away. Eventually, on-column DNase digestion was carried out with the RNase-Free DNase Set (QIAGEN) and high-quality RNA was finally eluted with DEPC-water.

2.2.1.7. Gel DNA recovery

Following gel electrophoresis analysis DNA fragments of interest were excised and recovered from the gel by using the Promega Gel Extract Kit following the manufacturer's instructions.

2.2.1.8. Quantification of nucleic acids by spectrophotometry

ssDNA, dsDNA and RNA absorb light in ultraviolet range, most strongly in 254-260 nm range. The measurement at 260 nm GeneQuantII photometer (Pharmacia Biotech) allows a calculation of the dsDNA concentration. An OD260 of 1 corresponds to approximately 50 µg/ml of dsDNA. The ratio between measurements at 260 nm and 280 nm provides an estimate of the purity of the samples. Proteins absorb light at 280 nm.

Formulas:

C (double stranded DNA) = OD260 x factor of dilution x 50 [µg/ml]

C (RNA and oligos) = OD260 x factor of dilution x 40 [µg/ml]

1 µl DNA (or RNA) sample was dissolved in $_{MP}H_2O$ (1:100) and photometrically examined (*Hitachi*). Nucleic acid concentration was calculated employing the formulas (Lambert-Beer's law) described above.

2.2.1.9. Sequencing

The integrity of plasmid constructs was checked by sequencing before proceeding to further applications. DNA plasmids were sequenced by Geneart, Regensburg. The obtained sequence data were compared with the published sequence in the database (PubMed, Swisprot, uniprot). Clones without any mutations were used for further work.

2.2.2. Protein biochemical Methods

2.2.2.1. Electrophoretic separation of proteins (SDS-PAGE)

Polyacrylamide gel electrophoresis (PAGE) is applied to separate protein mixtures based on their migration in solution in response to an electric field. The sample is run in a support matrix which separates the molecules by size and provides a record of the electrophoretic run. Proteins are amphoteric compounds, therefore their net charge is determined by the pH of the solution. The net charge is independent of its size, i.e. the charge is carried per unit mass. Under denaturing conditions the anionic detergent sodium dodecyl sulfate (SDS) disrupts the secondary and tertiary structure and binds to proteins quite specifically in a mass ratio of 1.4:1. Therefore SDS confers a negative charge to the polypeptides in proportion to their length (equal charge per unit length). 2-mercaptoethanol reduces disulphide bridges in proteins. During denaturing SDS-PAGE separation is achieved by polypeptide migration determined not by its intrinsic electrical charge, but by its molecular weight.

Throughout the course of this work, protein samples were subjected to SDS-PAGE using a discontinuous buffer system providing a good resolution. In a discontinuous system a non-restrictive large pore gel, called stacking gel, is layered on top of a separating gel, called a resolving gel. Each gel was made with a different buffer along with the tank buffer being different from the gel buffers. Prior to loading, protein samples were added 1x SDS-loading buffer and boiled for 5 min at 100℃. Protein sizes were analyzed using the pre-stained Protein Ladder (*Fermentas, Bio-Rad*) as molecular mass marker. Electrophoresis was performed at 36 mA at RT until the BPB (bromphenol blue) dye reached the bottom of the gel.

2.2.2.2. Electroblotting and immunodetection

Proteins were transferred efficiently from a polyacrylamide gel to a PVDF membrane by electroblotting (Western blot) using a Biorad blotting tank. The transfer was carried out for 1.5 to 2 h at 150 mA at RT in western transfer buffer. Protein transfer was verified by staining the nitrocellulose membrane with Ponceau S solution (*Sigma*). For immunodetection the PVDF membrane was blocked over night in blocking buffer at 4℃. After blocking the membrane was incubated in blocking buffer containing the corresponding primary antibody for 3 h at RT. Subsequently, the membrane was washed 3 times with PBST for 10 min at RT with shaking. Next, the membrane was incubated in PBST: blocking buffer 3:1 (v/v) containing the secondary HRP-linked antibody for 45 min at RT followed by a second washing as described above. Finally, the exceed liquid was removed and the antibody coupled peroxidase was detected with an ECL reagent kit (enhanced chemoluminescence, *Amersham*). Signals were detected by autoradiography using an ECL-hyperfilm (*Amersham*).

2.2.2.3. Ponceau S staining

To control the efficient transfer of the proteins to the PVDF membrane, the membrane was incubated in Ponceau solution for 1 min and briefly washed with TBST-buffer. The positions of the standard marker proteins were labeled with a pencil. Afterwards, the membrane was destained in TBST buffer for several times until the red color was completely removed. For the preparation of the staining solution 2 g Ponceau S (Sigma-Aldrich) was dissolved in 100 ml 3 % trichchloracetic acid.

2.2.2.4. Dot blot

Dot blots were performed either to assess the ability of poly- and monoclonal antibodies in detecting non-reduced ficolin-B or to evaluate the binding affinity of ficolin-B to different organic compounds. In each case ficolin-B (in different concentrations) or the supernatant (1 to 5µl) were spotted on a nitrocellulose membrane at the corresponding concentration and let

air-dry for 30 minutes before blocking with 1% low fat dry milk powder in TBS/0,05%Tween. The following detection steps were identical to a Western Blot.

2.2.2.5. Western Blot stripping

Membrane stripping is the removal of antibodies (including primary and secondary) from a western blot membrane, to allow the further incubation of the membrane with other primary and secondary antibodies. The membrane was incubated with prewarmed 50 ml Stripping buffer (-mercaptoethanol was freshly added) at 60 °C for 15 minutes. That incubation step was repeated 3 times. After the third incubation, the membrane was washed with PBST four times for 10 minutes at room temperature.

2.2.2.6. BCA assay

Protein concentrations were determined using the Pierce Endogen BCA (Thermo Scientific Inc., Rockford, USA) assay according to the manufacturer's protocol (microplate procedure). Preparation of the BCA™ working reagent:

Prepare working reagent by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B. (e.g. 5 mL of Reagent A with 100 µL of Reagent B)

Microplate procedure protocol:

1. Pipette 10 µL of each standard or unknown sample replicate into a microplate well (working range = 20 – 2000 µg/mL).
2. Add 200 µL of the working reagent to each well and mix the plate thoroughly on a plate shaker for 30 seconds.
3. Cover Plate and incubate at 37℃ for 30 minutes. Cool plate at RT.
4. Measure the absorbance at 562 nm.

2.2.2.7. Bradford assay

Normalization of total protein amounts was performed using Bio-rad Bradford reagent. 5µl of protein lysates was added to 795 µl of milli Q water. 200µl of reagent was added and reading was taken after 5 min at λ_{595} using a Bio-Rad spectrophotometer 3000.

2.2.2.8. ELISA

Enzyme-linked immunosorbent assay (ELISA), is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. Nunc Maxisorb plates were coated with AcBSA (10µg/ml), BSA (10µg/ml), or 100ul of chitin beads (NEB) in coating buffer (5mM Ca^{2+}/TBST) for overnight at 4℃ in 100 µl/well volume. Blocking of unspecific sites was performed with 5% milk powder in TBS (200ul) for 2 hours at RT and were subsequently washed thrice in TBST. In order to evaluate whether the binding was Ca^{2+} dependent, two buffer conditions were used. Ficolin-B is added to the plates in increasing concentrations (0.6, 1.25, 2.5, 5 µg/ml) and in different buffers (5mM Ca^{2+}/TBST (or) 50mM EDTA/TBST) as indicated and kept for overnight at 4℃. The same blocking buffer was used to dilute the reagents of the following steps. Primary antibody (IA4- anti ficolin-B monoclonal antibody) was added and incubated at RT shaking for 2 hours. After washing, HRP or Pox conjugated goat anti-rat antibody was used in a 1:3000 dilution for 2 hours at RT. Plates are washed and developed for 10-15min with OPD substrate containing H_2O_2. Addition of 50 µl/well sulphuric acid terminated the enzymatic reaction. The absorbance at 450 nm was measured using a microplate biokinetics reader.

2.2.2.9. Recombinant protein expression in prokaryotes

The optimization of protein is carried out with various factors like temperature, IPTG concentration and time of induction. Induction of protein expression at 12–15℃ can often help the folding and the solubility of the fusion protein and increase the cleavage efficiency. The optimal incubation temperature and time for induction will vary depending on the target protein.

Briefly, *E. coli* strain BL21DE3pLys was transformed with the recombinant protein expression vector pTXB1, coding for C-terminally Intein-fused proteins. The bacterial culture was grown in LB broth supplemented with 100 g/ml ampicillin and 34 g/ml chloramphenicol. The BL21DE3pLys strain contains a pRARE plasmid (camr) coding for tRNAs of the rarely used codons Arg, Ile, Gly, Leu and Pro, enabling high protein expression yields (Novy *et al*., 2001). The recombinant protein transcription driven by a lacZ promotor was then induced with isopropyl-β-D-thiogalactopyranoside (IPTG). Initially, the recombinant host was grown over night to saturation in LB with shaking (180 rpm) at 37℃. Next, 1 litre of LB broth were inoculated with the primary culture (10:1) and incubated with shaking (180 rpm) at 37℃ to A600 of 0.6. Recombinant expression was then induced by 1 mM IPTG (to 0.1 mM) and cells were cultured for 4 hours with shaking (180 rpm) at 30℃. Finally, cells were pelleted

(*Beckmann* GSA, 10000 g, 10 min, 4℃), subsequently washed once with PBS and if required stored at -20℃.

To optimize expression, varying induction temperature and time was tested at (37℃ for 2–4 hours, 30℃ for 4–6 hours, 22–25℃ for 6–16 hours and 12–15℃ overnight using 0.4 mM IPTG). One sample with no IPTG should be incubated as a control for uninduced cells. Varying IPTG concentrations (up to 1 mM) can also be tested. Lowering the IPTG concentration (0.01–0.1 mM) may also reduce the fusion protein expression in inclusion bodies. For low temperature induction (e.g.12–15℃) the culture can be incubated at 37℃ until the OD_{600} reaches 0.6–0.7 and further induction carried at low temperature. Fusion protein expression can be examined by SDS-PAGE, followed by Coomassie staining or western blot analysis. If the fusion protein is not detected by Coomassie staining, a Western blot with the anti-Chitin Binding Domain Serum may be performed as the intein fusion protein contains a Chitin binding domain (CBD) site.

A frozen stock of *E.coli* BL21 (DE3) pLysS carrying the desired vector was used for inoculation of 5 mL of LB medium supplemented with the respective selective antibiotic. Cells were grown at 37℃ in a shaking incubator at 180 rpm for 14 to 16 hours. 2 mL of this culture were used for inoculation of 100 mL of fresh LB medium containing the selective antibiotic and this culture was then grown at 30℃ to an OD_{600} of 0.6 to 0.8. Before induction of protein expression, a 2 mL aliquot of the suspension was collected and centrifuged at 14000x g for 2 minutes in a tabletop centrifuge. The supernatant was discarded and the pellet was resuspended in 100 µL of 1x protein sample buffer and stored on ice at -20℃ for overnight. Protein expression was induced by the addition of IPTG (final concentration of 0.1 mM) and the incubation temperature was reduced to 20℃ (another sample at 30℃). Thereafter, several samples (2 mL) were taken at indicated timepoints and centrifuged for 2 minutes at 14000x g a tabletop centrifuge. Supernatants were discarded and pellets were stored on ice at -20℃ for overnight.

The 2 mL overnight stored pellets were lysed with cell resuspension buffer pH 7,6 and further treated with PMSF, DTE and ß-Mercaptoethanol. The samples are vortexed and placed in ice for 15 minutes. This was again resuspended in Lysozyme, Sodium deoxycholate, Magnesium chloride and DNaseI with each time repeated vortexing and placement of the sample on ice for 15 minutes. Finally the lysed and resuspended sample was centrifuged at 4℃, 14000rpm for 30 minutes to get it separated into pellet and supernatant.

2.2.3. Chromatography Methods

2.2.3.1. Intein based affinity chromatography

The Ficolin-B was overexpressed and purified by using the intein–CBDsystem according to the basic protocol provided by New England Biolabs. pTXB1 (NEB) contains a mini-intein from the *Mycobacterium xenopi gyrA* gene (Mxe GyrA intein; 198 amino acid residues) is used as tag for proteins for expression and purification applications. The target protein is fused at its C-terminus to a self-cleavable intein tag (~28 kDa) that contains the chitin binding domain (CBD, 6 kDa) allowing for affinity purification of the fusion precursor on a chitin column.

2.2.3.1.1. Protein purification from the soluble fraction

For purification, the cells from 1 liter culture after a 4 hours incubation at 30℃ in the presence of 1 mM IPTG were harvested. The pellet (about 8 g wet wt cells) was resuspended in 20ml of lysis bufferA (Section 2.1.2.1). All subsequent steps were performed at 4℃. The cells were disrupted by sonication, and the insoluble debris was removed by ultracentrifugation for 1 h at 16,000*g*. After lysis, cell debris was removed by ultracentrifugation for 1 h at 16,000*g*. The cleared lysate was applied directly onto a chitin column (New England Biolabs) preequilibrated with 10 vol of buffer A. After loading, the column was washed with 10 bed vol of buffer A. The column buffer was exchanged with cleavage buffer (Section 2.1.2.1) by quickly washing the column with 3 bed vol. The flow was then stopped and the column was left at 4℃ for 20 h. The ficolin-B elution was carried out by continuing the column flow with buffer A. Chromatography eluted samples were concentrated by ultrafiltration using Amicon Ultra (Mwco: 10K) ultrafiltration-devices (Millipore). The ficolin-B content of the fraction was analyzed by SDS– PAGE, and protein concentrations were determined by using the Bradford dye binding assay (Bio-Rad Labora- tories) and bovine serum albumin as the standard.

2.2.3.1.2. Protein purification from inclusion bodies

Protein pellet was resuspended in cell lysis buffer (Section 2.1.2.1) and centrifuged at 16,000*g* for 20 min at 10 ℃. The cells were disrupted by s onication and spinned down cell debris containing the inclusion bodies at 16,000 g at 4℃ for 30 minutes. The supernatant was discarded and the pellet was resuspend in 100 ml breaking Buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl and 7M Guanidine-HCL). The solution was stirred for 1 hour at 4℃ and remaining cell debris were spin down at 16,000 g and 4℃ for 30 minutes. The supernatant was loaded into dialysis bag and dialyzed against renaturation Buffer A, B, C, D and 2 times

E according to IMPACT kit manual. Each step is against 1 L of a renaturation buffer and should take at least 3 hours at 4℃. During dialysi s the buffer should be continuously stirred. Finally Centrifuge the dialyzed solution containing the renatured protein at 16,000 g and 4℃ for 30 minutes to remove any remaining impurities or incorrectly folded protein which is again aggregated. After sample centrifugation, the standard protocol for chitin chromatography (New England Biolabs, Beverly, MA, USA) and cleavage reaction was used to elute the protein product and further analyzed both the eluate and chitin beads for cleavage efficiency and protein solubility.

Chromatography eluted samples were concentrated by ultrafiltration using Amicon Ultra (Mwco: 10K) ultrafiltration-devices (Millipore). Stripping of intein-CBD from chitin column was carried out using 20 mM Tris–HCl buffer, pH 8, containing 500 mM NaCl and 1% SDS solution.

2.2.3.2. Gel filtration

Gel filtration analysis is a method used for the analysis of the apparent size of proteins or protein complexes under native conditions. For purified recombinant proteins, gel filtration provides evidence about the formation of di-, tri, or oligomers if the elution of the protein of interest is compared to that of marker molecules of known molecular mass.

Molecular weight determinations by gel filtration are carried out by comparing average elution volume parameter, such as Kav of the protein of interest, with values obtained for several known calibration standards. A calibration curve was prepared by measuring the elution volumes of several standard proteins, calculating their corresponding Kav values and plotting their Kav values versus the logarithm of their molecular weight. The apparent molecular weight of an unknown protein can be determined from the calibration curve once its Kav value is calculated from the measured elution volume. The Kav was calculated using the formulae given below.

$$K_{av} = \frac{\text{Elution volume - Void volume}}{\text{Total volume - Void volume}}$$

The calibration curve was plotted from the calculated Kav to logarithm of the molecular mass for the various standards (Fig.1). To calculate the molecular mass of the unknown protein from Kav a formulae was deduced from the calibration curve using the computer program.

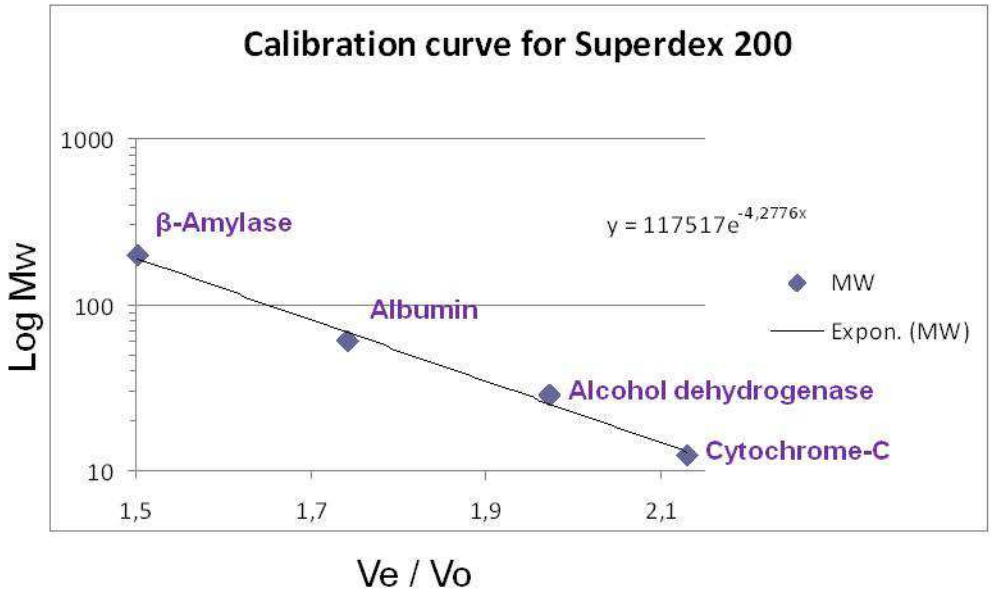


Fig. 2.1 Plot of the Elution volume Ve/Vo versus log MW in gel filtration.

Gel filtration chromatography was conducted on a computer-controlled ÄKTA^{TM} purifier high performance liquid chromatography equipped with Superdex 200 column (10 mm x 30 cm; GE Healthcare) equilibrated in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl at a flow rate of 0.5 ml/min, at room temperature. The injection volume of the proteins was 100µl with concentrations ranging from 0.226mg/ml for the "Old ficolin-B" and 0.408mg/ml for the "fresh ficolin-B". Eluted proteins were collected in 1 mL fractions and analysed by Western blot to verify the identity of the proteins in the distinct peak fractions.

Molecular weight calibration of the analytical gel filtration column was done using the Sigma gel filtration molecular mass standard kit 12,000–200,000 Da (cytochrome *c*, 12.4 kDa; carbonic anhydrase, 29 kDa; bovine serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa; b-amylase, 200 kDa). Data analysis and processing were done with the Unicorn evaluation software (GE Healthcare) and Sigma Plot 9.0 (Systat Software®). Values in brackets are the Stoke's radii of protein standards. The Stoke's radii of ficolin-A oligomers were determined from a graph of $[-\log(K_{av})]^{1/2}$ against the Stokes radius of protein standards, where K_{av} is the partition coefficient, V_e is the elution volume of the protein, and V_0 and V_t are the void and total volumes of the gel filtration column, respectively.

2.2.4. Cell Biological Methods

2.2.4.1. Cell culture techniques- Drosophila Schneider-2 (S2) cells

Transfection of S2 cells

S2 cells were stably transfected with the “pMT-pCoHygro-ficolin-B” (fusion vector) wild type and mutant constructs in order to express the ficolin -B proteins, respectively. The fusion vector allows us for single transfection and leads to have more positive clones.

Stable transfection is optimal for long-term storage, high protein expression, and large-scale production of the desired protein. In addition, the Drosophila Expression System (DES®, Invitrogen) offers a convenient non-lytic system that uses simple plasmids for stable expression of heterologous proteins in S2 cells. Protein expression using the DES occurs in healthy, logarithmically growing cells, so high yields of high-quality protein can be produced (Johansen et al. 1989), (Deml et al. 1999). *Drosophila melanogaster* transfected cell lines generally contain multicopy inserts of the foreign gene that form arrays of more than 500-1000 copies in a head to tail fashion.

In order to generate a stable recombinant cell line, the plasmid (pMT-pCoHygro) containing the sequence of interest was transfected. The plasmid has a resistance-bearing pCoHygro vector that constitutively expresses the hygromycin resistance gene (HPH) (Gritz and Davies 1983) for selection of transfectants with the antibiotic hygromycin B. When added to cultured S2 cells, hygromycin B acts as an aminocyclitol to inhibit protein synthesis by disrupting translocation and promoting mistranslation.

In this work, transfection was performed with the DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate). DOTAP is a liposomal transfection reagent for the delivery of negatively charged biomolecules such as DNA, RNA, oligonucleotides, and protein into eukaryotic cells. It can be used for the highly efficient transfection of DNA into eukaryotic cells for transient or stable gene expression. Due to these features, it assembles DNA into compact structures, thus optimizing its entry into the cell. DOTAP-DNA complexes possess a net positive charge, which allows them to bind to negatively charged receptors (e.g. sialylated glycoproteins) on the surface of eukaryotic cells. Once inside, the reagent buffers the lysosome after it has fused with the endosomes, leading to pH inhibition of lysosomal nucleases and stability of DOTAP-DNA complexes.

Cells were seeded onto a 6cm plate 24 hrs prior to transfection such that they were 50-70% confluent on the day of transfection. For each transfection in a 6cm plate, 2µg of total plasmid DNA was suspended in HBS and was added to 14µl of DOTAP (Roche) and DNA-liposomal complexes were allowed to form by incubating for 10 min. Meanwhile, the medium

from the plates was replaced with DMEM without FBS. DNA-Liposomal complexes formed were added to the cells and incubated at standard growth conditions. The medium was replaced with normal growth medium containing FBS 4 hrs later. Cells were cultivated for 3 days under normal conditions and then the selection reagent was incorporated to the normal growing medium (300 μg hygromycin B per ml of medium) which was replaced every 4-6 days. Selection took place over 4 weeks and was monitored by comparison to the control flask containing cells transfected in the absence of the pCoHygro vector. Cells transfected with the empty pMT-pCoHygro vector served as a negative control.

2.2.4.2. Induction of protein expression

Stable transfectants were induced to express the protein by the addition of 500 μM $CuSO_4$ (final concentration) to the culture medium. To check for protein expression, supernatant aliquots were taken 2-3 days after induction and analysed by Western Blot. For high yield production of the recombinant protein supernatant was collected 10 days after induction and subjected to metal-affinity chromatography.

2.2.4.3. Protein purification

Ten days after induction, the conditioned medium was collected and cleared by centrifugation at 3000x*g* for 10 minutes at 4℃. Binding to the resin was per formed batchwise (1 ml resin/L medium, enough to bind approximately 5 mg His-tagged protein) overnight at 4℃ under rotation. The resin-Cu^{2+}-protein slurry was then poured into a column and attached to the BioRad Econo System device (BioRad) to facilitate the forthcoming steps. Washing was performed sequentially at a rate of 0.5 ml/min with PBS until baseline UV absorbance monitored at 280 nm and then again with 10 mM imidazole in 0.5 M/PBS to remove non-specifically bound proteins. Competitive elution of the desired protein was carried out with 250 mM imidazole in 50 mM Tris pH 8.0. Elution fractions were collected in 0.5 ml aliquots and analysed by SDS-PAGE (see section II.2.4.2). Finally, the column was stripped with 20 mM EDTA in PBS to remove any metal bound to the resin and re-equilibrated with 50 vol of deionized water.

Positive elution fractions were pooled, dialysed overnight against PBS at 4℃ and stored in aliquots at -20℃. Freezing/thawing cycles were alw ays avoided. Protein concentration of the samples was assessed by BCA assay (see section 2.2.2.6)

3. Results

The use of bacteria in order to produce recombinant protein has grown in the recent years. Using recombinant DNA and inserting it into a plasmid of rapidly reproducing bacteria enables the manufacture of recombinant protein. These recombinant proteins can be a variety of different types, such as antibodies, antigens, hormones, and enzymes.

Therefore, the first aim of this work was the synthesis of tag-free recombinant mouse ficolin – B in an prokaryotic expression system, the genes of which have been sequenced (Fujimori *et al.,* 1998;Ohashi and Erickson, 1998) and scanned in search of conserved domains and potential posttranslational modification sites. However, very little is known about the properties of the proteins they encode. The whole process towards the generation of the recombinant ficolin-B consisted of (i) the cloning of the ficolin genes into an prokaryotic expression vector for (ii) protein production in prokaryotes and (iii) further purification by affinity chromatography.

3.1. Cloning, expression, and purification of the intein-ficolin-B fusion protein

In this work, mouse ficolin-B was expressed and purified from *E. coli* cultures taking advantage of the intein-mediated approach. The ficolin- B cDNA contains a 942-base pair (bp) open reading frame (ORF) encoding 314 amino acids. Figure 3.1 and 3.2 show the full length cDNA of ficolin-B.

The aim of the present work was to demonstrate that the fusion of ficolin-B to intein could lead to an efficient purification strategy in a single chromatographic step. For this purpose, ficolin-B was fused to the C-terminus of the intein gene in *pTXB1 vector* (Impact Kit), and the expression of intein-ficolin-B chimeric protein (Morassutti *et al.,* 2005, Pezza *et al.,* 2004) was induced in *E. coli* cultures. Ficolin-B cDNA was cloned to the C-terminus of intein into the pTXB1 vector.

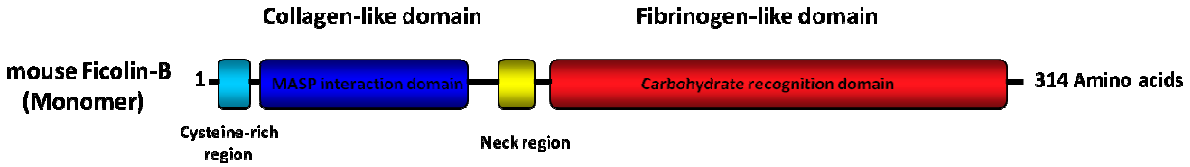
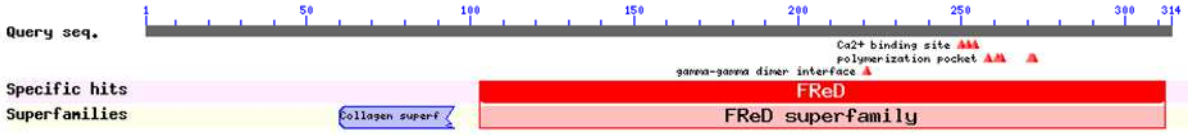


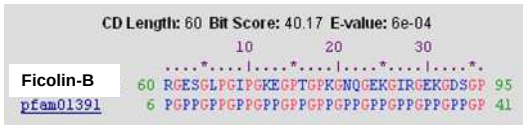
Fig 3.1. Domain architecture of Ficolin-B protein: All known ficolin proteins share a common structure with an N-terminal Cysteine rich region followed by Collagen-like domain and a small neck region in the center. The C-terminus comprises of a Fibrinogen-like domain which is responsible for carbohydrate recognition.

A)

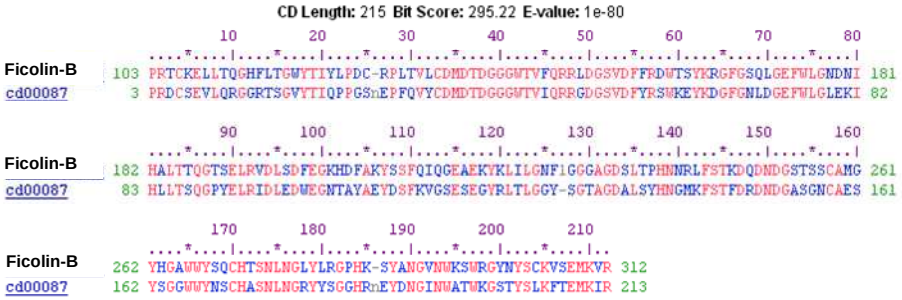
Ficolin-B protein full length



Collagen-like domain:



Fibrinogen-like domain:



B)

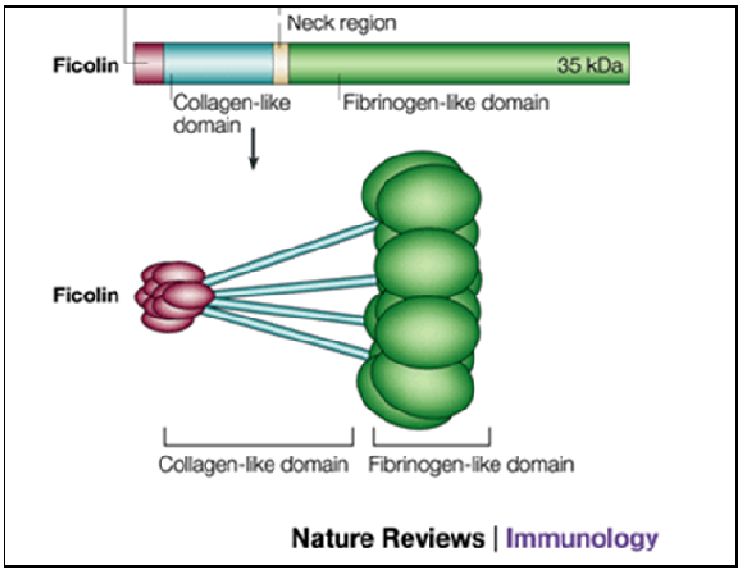


Fig. 3.2: Ficolin-B nucleotide and deduced amino acid sequences. A) Sequence alignment of ficolin-B mapping the collagen and fibrinogen domain. B) Ficolins are oligomers of structural subunits, each of which is composed of three identical 35-kDa polypeptides, respectively. Each subunit contains: an amino-terminal, cysteine-rich region; a collagen-like domain and a carboxy-terminal fibrinogen like-domain. (Fujita *et al.,* 2002).

The IMPACT (Intein Mediated Purification with an Affinity Chitin-binding Tag) system is a novel protein purification system which utilizes the inducible self-cleavage activity of protein splicing elements (termed inteins) to separate the target protein from the affinity tag. It distinguishes itself from all other purification systems by its ability to purify, a native recombinant protein without the use of a protease in a single chromatographic step (Chong *et al.,* 1997, Evans *et al.* 1998*)*. Each intein tag contains a chitin binding domain (CBD) for the affinity purification of the fusion protein on a chitin resin. Induction of on-column cleavage, using thiol reagents such as dithiothreitol (DTT), releases the target protein from the intein tag (Figure 3.3).The vectors included in this kit allow for the fusion of the target protein at its C-terminus (pTXB1) or at its N-terminus (pTYB21) to the intein tag. The intein-CBD chimeric protein can bind to the chitin beads and on-column intein-mediated cleavage of ficolin-B is induced by DTT addition (Manual Impact kit E6901).

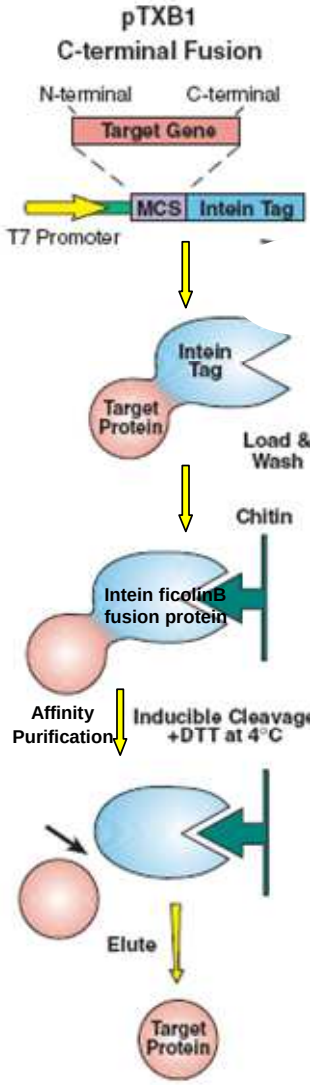


Fig. 3.3: Schematic illustration of the IMPACT System.

3.1.1. Cloning of a ficolin-B expression construct in pTXB1 impact kit vector

In order to clone the ficolin-B sequence into the pTXB1 prokaryotic expression vector, primer pairs (numbered from 1 and 2 in section 2.1.9) were designed to amplify the cDNA of ficolin-B using cDNA derived from HoxB-8 cells (cDNA from HoxB-8 - neutrophil progenitor cells). These PCR product were first cloned into the pGEMT vector, which is an activated plasmid ready to accept PCR products as inserts. The primers also contained endonuclease restriction sites at the 5' ends to further digest the pGEMT-ficolin-B vector with NdeI/SapI inorder to subclone the ficolin-B cDNA into the pTXB1 vector (Fig 3.4), which has been digested with the same enzymes.

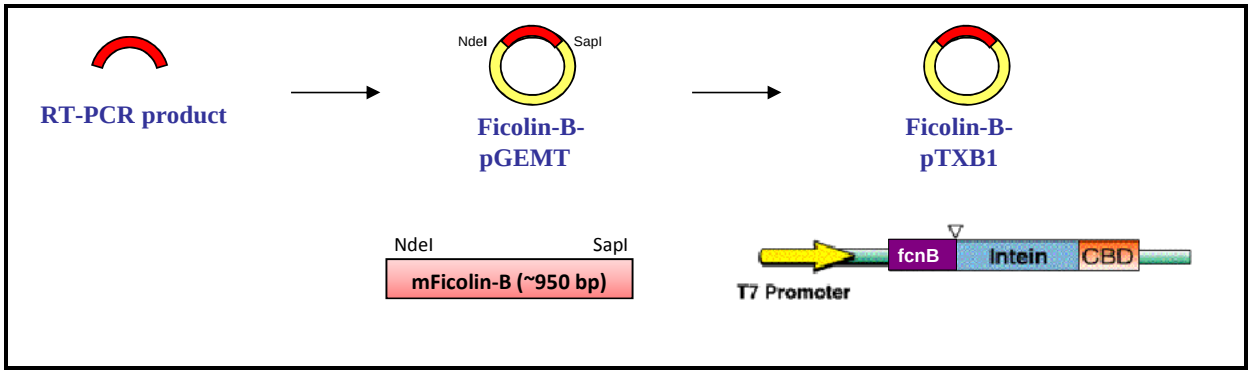


Fig. 3.4: Diagram describing the construction of the ficolin-B pTXB1 plasmid, pTXB1 containing T7 promoter-driven system to achieve high levels of expression and tight transcriptional control in E. coli. The arrow head indicates the self-cleavage site of the intein which allows the release of the target protein (Ficolin-B) from the chitin-bound C-terminal intein tag.

Briefly, based on the known mouse ficolin-B gene sequence (Gen-Bank Accession No. M 070497) the specific primers for PCR amplification were designed to amplify the cDNA fragment containing the ATG of the endogenous. Appropriate restriction sites, absent in the target gene, are incorporated in the forward and reverse primers when a target gene is generated by PCR. Cloning into the NdeI and the SapI sites results in the fusion of the intein to the C-terminus of the ficolin-B protein, without any extra amino acids on the protein after cleavage of the intein tag. PCR was done as described in the materials and methods (Section 2.2.1.1.1). The amplification products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (EtBr). A specific 1Kb PCR product was obtained (Figure 3.5). The amplification product (500 ng), digested with NdeI and SapI, was isolated from an agarose gel bands using Gel-Out kit (Promega).

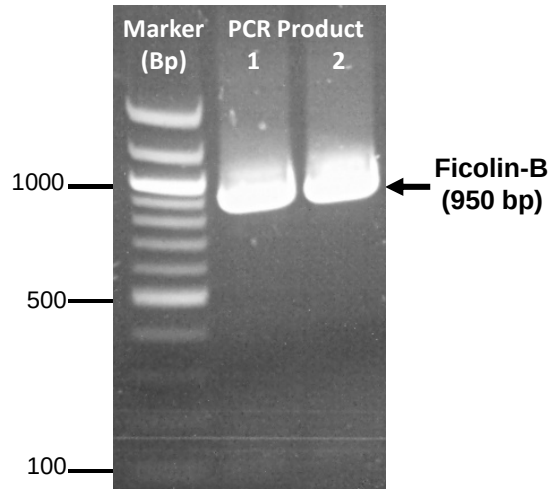


Fig. 3.5: The PCR products of the target genes: DNA samples were separated on a 1.5% (w/v) agarose gel containing EtBr Lane M: NEB (1Kb) DNA high range marker ready to use and lane 2 PCR product. Lane M, DNA marker and lane 1,2 PCR product of mficolin-B.

Prior to ligation into the pTXB1 vector, the vector was digested with restriction enzymes (NdeI/SapI) and was dephosphorylated by Calf intestinal alkaline phosphatase (CIAP) treatment to prevent religation. After ligation of *E.coli* XL1blue cells were transformed. Transformed *E.coli* colonies were screened by colony-PCR with the primers mentioned above. PCR products were analysed by DNA gel electrophoresis. Figure 3.6 shows that PCR of colonies 1 to 33, amplified bands with a size of 950bp that correlated with the calculated size of ficolin-B cDNA. Lane (NC-negative control) showed no bands at the expected size indicating that these clones did not carry the vector containing the correct insert.

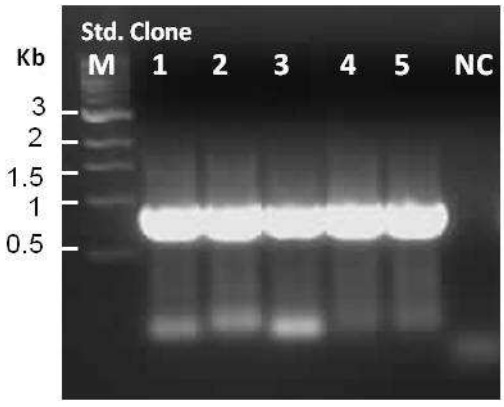


Fig. 3.6: Colony PCR to quickly amplify and screen positive clones : Colonies containing a 950bp fragment cloned into the pTXB1 vector were grown on ampicillin plates at either single-copy or high-copy number were picked for PCR Screen. Colony PCR was performed using primers homologous to the ends of the cloning vector. Lane M, DNA size marker; The top panel shows clones 1 to 5. The resulting PCR fragments from the positive clones are ~950kb, as expected. NC, *E.coli* XL-1 Blue (negative control).

To check whether those clones giving positive results in the PCR screening indeed carried the insert of interest, a plasmid preparation of an overnight *E.coli* culture of every each individual colony was made. The plasmid DNA was digested with the restriction enzymes NdeI and SapI and loaded on a 1% (w/v) agarose gel containing EtBr. Figure 3.7 shows the restriction digestion of clone 4. The gel was run with the undigested plasmid DNA (Lane1), with single digestions (Lane 2,3) and double digestions (Lane4). The double digested (NdeI/SapI) clone 4 contained a DNA insert in the size of 950 bp matching the expected size for ficolin-B.

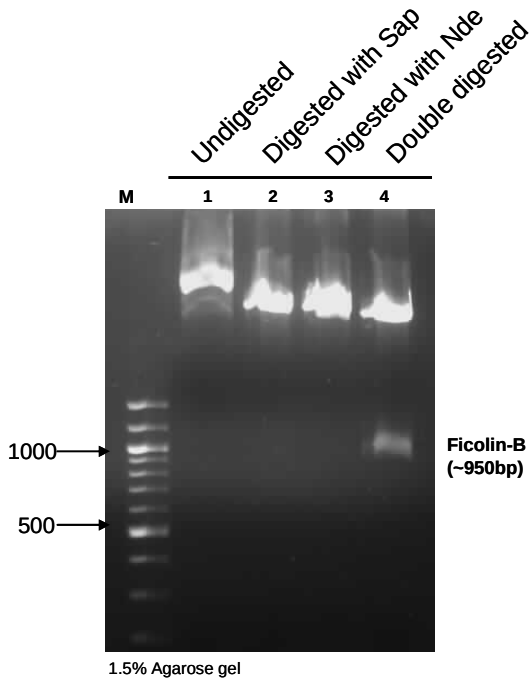


Fig. 3.7: mficolin-B in pTXB1 vector SapI/NdeI control digest: Plasmid DNA (Lanes 1 to 4) was digested with restriction enzymes SapI/NdeI and checked on a 1.5% (w/v) agarose gel containing EtBr. Lane M: NEB 100 bp DNA ladder marker; Lane1, Undigested control; Lane2, the digestion of pTXB1-ficolin-B recombinant with SapI; Lane3, the digestion of pTXB1-ficolin-B recombinant with NdeI; the digestion of pTXB1-ficolin-B recombinant with SapI/NdeI.

Isolated plasmid DNA samples of the positive clone (clone 4) was verified by sequencing (Geneart Regensburg, Germany) using standard T7 primers. As the standard primers were not sufficient to obtain full-length sequences, internal primers were designed. Obtained sequences were matched with the mficolin-B sequence (NCBI Accession No.: M070497) by BLAST analysis (data not shown).

3.1.2. Expression and purification of intein-ficolin-B fusion protein

3.1.2.1. Expression of the Intein-ficolin-B fusion protein

In order to determine the expression of intein ficolin-B fusion protein, the vector containing the ficolin-B DNA was transformed into *E.coli* BL21 (DE3) pLysS cells and protein expression of ficolin-B over a time period of 4 hours was analysed. Expression was induced with two different concentrations of IPTG (0.1 and 0.4 mM) and protein expression over time was

detected by SDS-PAGE (Fig 3.8) and staining with Coomassie brilliant blue (Materials and Methods - section 2.3.1).

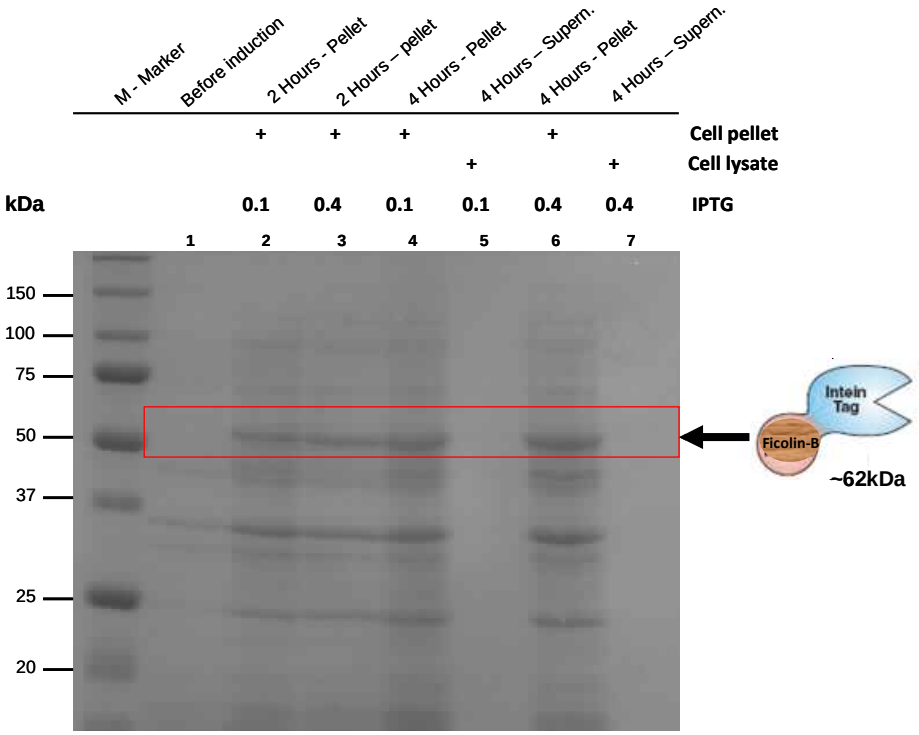


Fig. 3.8: Test expression of pTXB1-Ficolin-B recombinant protein: SDS-PAGE analysis of fusion proteins expressed in *E.coli* BL21 (DE3)pLys. Samples were collected at different timepoints (0 – 4hrs) after induction with 0.1 (Lanes 2,4,5) & 0.4mM (Lanes 3,6,7) IPTG and also samples representing the solubly expressed (Lane 5,7) and the insolubly expressed (Lane 2,3,4,6) protein fractions were collected. All samples were separated by SDS-PAGE (10%) and the gel was stained with Coomassie brilliant blue to detect proteins. Lane M: Precision plus 4 -20% Tris-HCL gel SDS-PAGE marker. The arrow indicates the predicted electrophoretic mobility of intein-ficolin-B fusion protein (~62 kDa).

The calculated size of recombinant ficolin-B including the intein-tag was ~62 kDa. Figure 3.8 shows that the protein expression increased over the time course of 4 hours (Lanes 2h to 4h) as compared to the uninduced stage (Lane 1). Several proteins of lower as well as of higher molecular weight were detected. Prominent bands appeared, indicating proteins of a molecular weight of approximately 25 kDa and 35 kDa. These proteins represented putative degraded products of ficolin-B. A band in the range of ~62 kDa was visible in all lanes except for the uninduced protein fraction (before induc.). This band probably resulted from the recombinant ficolin-B protein. The majority of the proteins seemed to be insolubly expressed as there were more intense bands in this fractions (Lanes 2,3,4,6) as compared to the solubly expressed protein fraction (Lanes 5,7.) at the expected size of ~62 kDa as indicated by arrowhead.

3.1.2.2. Miniprep - Protein expression test for the Intein-ficolin-B fusion protein to check solubility

To further investigate whether the intein-ficolin-B fusion protein was in the insoluble (pellet) or in soluble (supernatant) fraction, a miniprep expression test was done where the bacterial sample collected was lysed and centrifuged to get it separated into insoluble and soluble sample fractions (Materials and Methods – Section 2.3.9). To monitor the protein expression, 12 µL of the soluble and insoluble fractions as well as of each sample taken every hour were loaded on SDS-PAGE gels (10 or 12%), with Coomassie brilliant blue staining. The expression of proteins was monitored at different temperatures 30℃ and 20℃ at 0.1mM IPTG (Figure 3.9). The majority of the proteins seemed to be insolubly expressed as there were more intense bands in these fractions of pellet lysates (P) as compared to the solubly expressed protein fraction in the supernatant of the lysates (S) at the expected size of ~62 kDa as indicated by arrow. Only a weak band was observed in the supernatant lysate (S) of the expected size of fusion proteins.

A) Expression of pTXB1 Ficolin-B at 30℃ B) Expression of pTXB1 Ficolin-B at 20℃

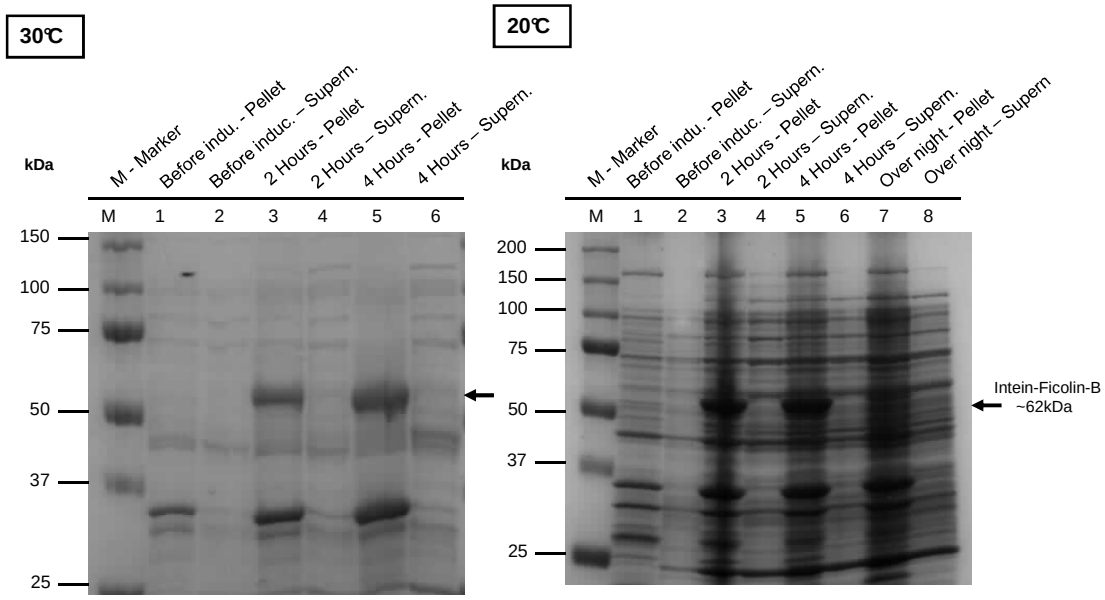


Fig. 3.9: Solubility test expression of pTXB1-Ficolin-B recombinant protein: SDS-PAGE analysis of fusion proteins expressed in *E.coli* BL21(DE3)pLys. Samples were collected at different time points (Lane 1,2 non-induced; Lane 3,4 induced 2hrs, lane 5,6 induced 4hrs; lane 7,8 induced overnight) after induction with 0.1mM IPTG. The samples were lysed and run on gel representing the solubly expressed supernatant and the insolubly expressed pellet protein fractions. All samples were separated by SDS-PAGE (10%) and the gel was stained with Coomassie brilliant blue to detect proteins. Lane M: Precision plus 4 -20% Tris-HCL gel SDS-PAGE marker. The other lanes were applied with pellet and supernatant lysates from non-induced and induced bacteria for 2hrs and 4hrs.

3.1.2.3. Western blot for the Intein-ficolin-B fusion protein

To further confirm the presence of the ficolin-B protein, The expression of intein-tagged proteins over the time period of 4 hours was analysed by immunoblotting (Western blot and Dot blot). We analysed whether the weak bands of approximately ~62 kDa represented the protein of interest. We performed Western and dot blots to specifically detect CBD intein-tagged proteins and ficolin-B protein. Samples separated by SDS-PAGE were transferred onto polyvinyl difluoride (PVDF) membrane. After saturation of non-specific binding sites the sheets were incubated with a rabbit anti-chitin antibody (A) and rabbit anti-ficolin-B antibody (B) (Figure 3.10). Detection was achieved by a secondary antibody, that was HRP-linked and the Lumigen™ detection reagent.

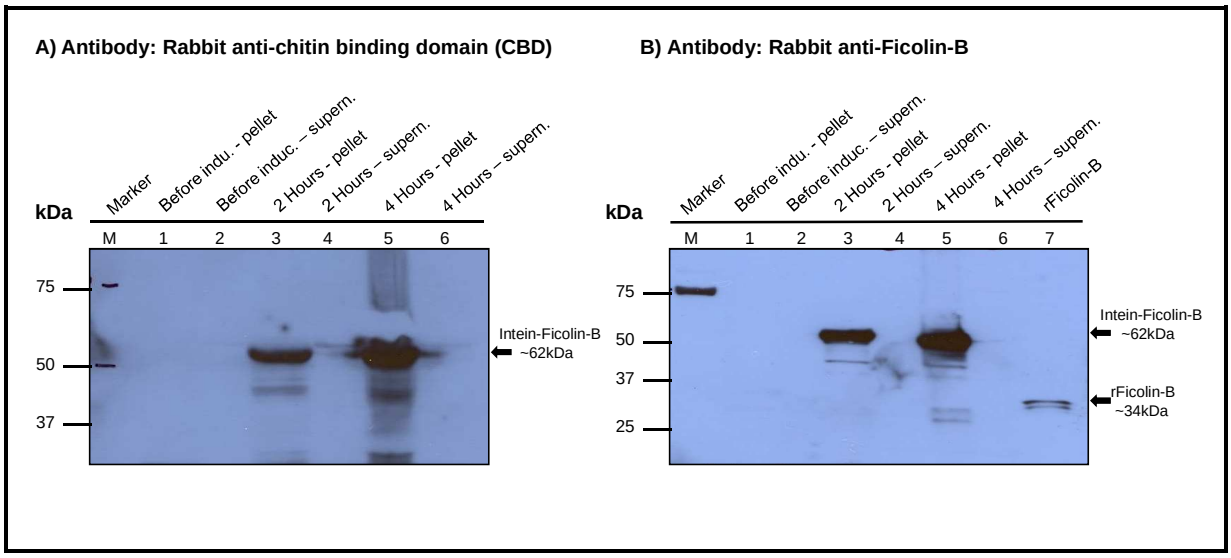


Fig.3.10: Immunoblot for the pTXB1-ficolin-B recombinant protein *:* Western blot analysis of fusion proteins expressed in *E.coli BL 21 (DE3) p Lys.* The same samples collected at different time points (0 – 4hrs) after induction with 0.1mM IPTG was used for blot analysis. The samples representing the solubly expressed supernatant) and the insolubly expressed pellet protein fractions as described for Fig.3.9 were separated by SDS-PAGE (10%), blotted onto a PVDF membrane and finally, detected with the Lumigen™ detection reagent and developed on a hyperfilm. Figure 3.10 A was detected with anti-chitin binding domain (CBD) antibody and Figure 3.10 B with anti-ficolin-B antibody. Lane M: Protein molecular weight marker; lane 1,2 *E.coli*/pTXB1 ficolin-B uninduced pellet and supernatent as negative control; lane 3, 4 *E.coli*/pTXB1 ficolin-B induced 2 Hrs pellet and supernatent for ; Lane 5,6 *E.coli*/pTXB1 Ficolin-B induced 4 hrs pellet and supernatant; lane 7, recombinant ficolin-B as positive control.

3.1.2.4. Dot Blot for the Intein-ficolin-B fusion protein

In order to confirm that the antibody is able to detect the native form of the recombinant protein, a dot blot assay was performed. For dot blot analysis, different volumes of the protein samples (1.0, 2.5 and 5µl) were spotted on the membrane. Recombinant ficolin-B and ficolin-B fusion protein samples (pellet and supernatant) were detected with anti-CBD (Fig 3.11 A) and anti-ficolin-B (Fig 3.11 B) and DS2 cells purified ficolin-B as positive control and PBS as negative control.

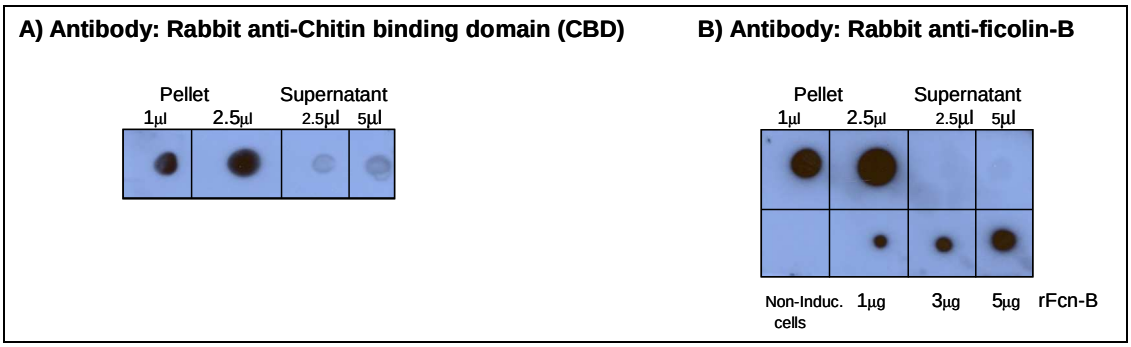


Fig. 3.11: Dot blot for the detection of non-reduced ficolin-B with the rabbit anti-chitin binding domain (CBD) and rabbit anti-ficolin-B antibody. Recombinant ficolin-B was spotted on a nitrocellulose membrane at the specified volumes and incubated with the a anti-CBD antibody (Figure-A) and anti-ficolin-B antibody (Figure-B) in a 1:2000 dilution. An HRP conjugated anti-rabbit antibody was used for detection. Non-induced cells was spotted as a negative control.

Both the Western blot and dot blot analysis showed that the protein was only to be detected in the insoluble fractions.

Protein expression at lower temperatures decreases the rate of protein expression. This decreased rate of protein synthesis results in an enhanced capacity to fold proteins correctly. We induced protein expression by addition of 0.1 mM IPTG and incubated the cells at 16℃ as *E. coli* cells still grow and express proteins at this temperature. Finally, we checked protein expression over the investigated time course of expression by SDS-PAGE and staining with Coomassie brilliant blue. We also tried a short induction time with very low IPTG (0.05mM) to check for the expression of proteins (data not shown).

Neither expression at 16℃ nor short induction time led to improved expression of the ficolin-B protein. So we decided to continue with a large scale protein expression under standard conditions to purify the protein. We thought the expression of proteins under large scale conditions might help to get some proteins expressed in the soluble form and if not decided to purify the proteins from inclusion bodies since we already showed in SDS-PAGE and Western blot the expression of proteins in the insoluble fraction.

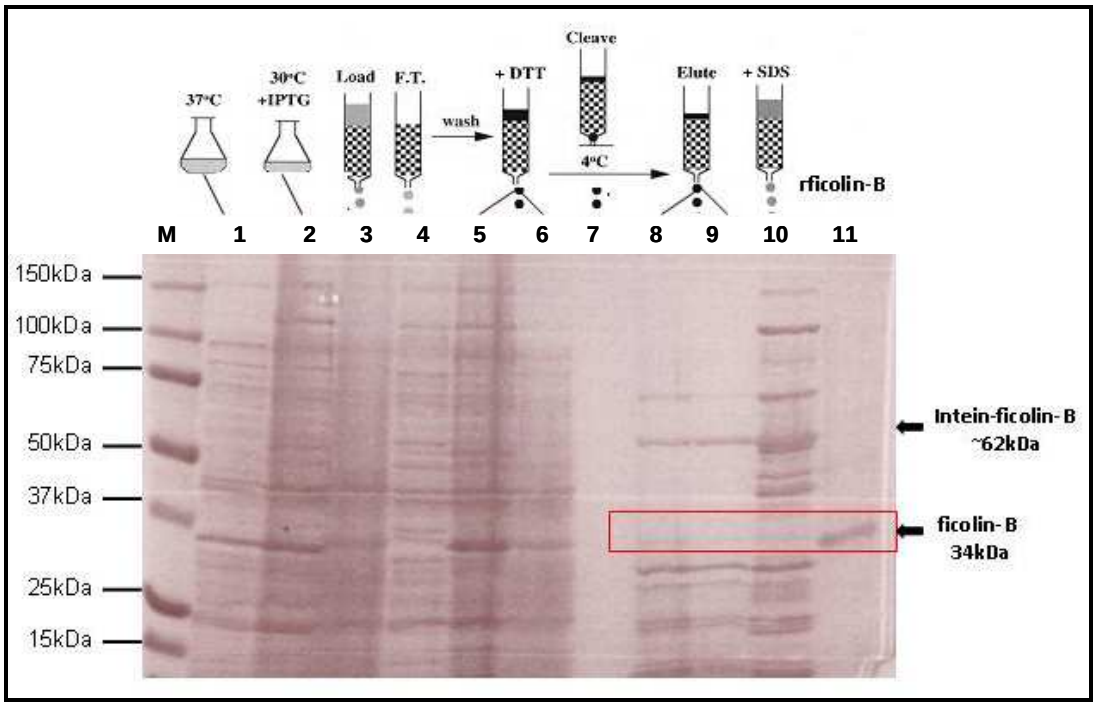
3.1.3. Large scale purification of Intein-ficolin-B fusion protein

3.1.3.1. Purification of pTXB1-ficolin-B fusion protein from cell lysates

The expression of recombinant proteins with intein fusion tags is widely used to facilitate their purification from a variety of prokaryotic expression systems. The main concept works via the intein-mediated protein purification with a self-cleavable affinity tag. A target protein is fused to the C-terminus of a modified protein splicing element (intein) which is in turn fused to an affinity tag (CBD). The fusion protein from a crude cell extract is purified by adsorption to a chitin affinity column. The intein is then induced to undergo on-column self-cleavage by a chemical reagent (DTT). The target protein is specifically released from the column and eluted as a pure protein (Fig 3.12) (Materials and Method – Section 2.4.1.1).

I Native Purification -

A) 10% SDS- Coomasie gel



B) Western blot

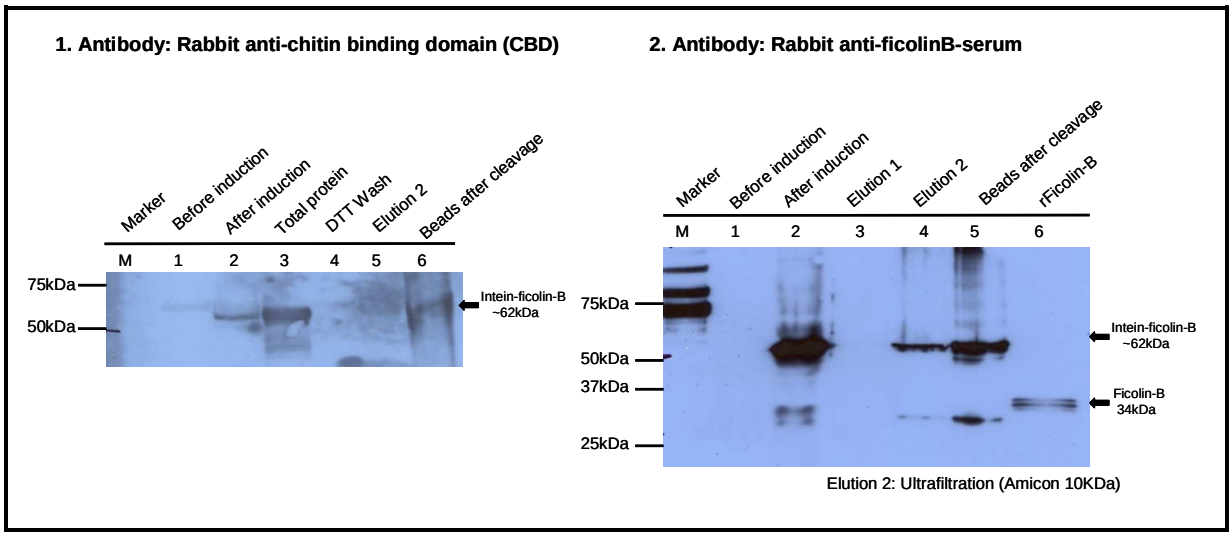


Fig. 3.12: Single column native purification of recombinant proteins from *E. coli* using pTXB1 vectors. **A)** Schematic representation of the expression and purification of ficolin-B using a pTXB1 vector. Samples taken from different steps during the expression and purification procedures were separated by SDS-PAGE and the gel was stained with Coomassie blue. Lanes: M, protein molecular weight standards (kDa, NEB); 1, uninduced cell extract; 2, induced cell extract; 3,4, flow through (F.T.) from the load. After loading, the column is washed with column buffer until the protein content of the eluate reached a minimum; 5,6, flow through from the quick DTT flush; 7–9, the first three fractions of the elution after the 4℃ overnight (o.n.) incubation in the presence of DTT; 10, a fraction from the SDS elution (to look for cleavage efficiency).11, rficolin-B as positive control. **B)** Western blot analysis of fusion proteins expressed in *E.coli BL 21 (DE3) p Lys.* The large scale purified samples were tested with rabbit anti-CBD antibody (1) and rabbit anti-ficolin-B antibody (2). The samples as described for Fig.3.12A were separated by SDS-PAGE (10%), blotted onto a PVDF membrane and finally, detected with the Lumigen™ detection reagent and developed on a hyperfilm. Lane M: Protein molecular weight marker; lane 1,2 *E.coli*/pTXB1 ficolin-B uninduced sample and induced sample; lane 3, ficolin-B elution fractions,lane 4, ficolin-B elution fractions concentrated with ultrafilter;lane 5, sample beads after cleavage; lane 6, recombinant ficolin-B as positive control.

However, these data further confirmed that even the expression of proteins under large scale conditions had no effect on the production of proteins into the supernatant fraction.

3.1.3.2. Purification of the Intein-ficolin-B protein from inclusion bodies

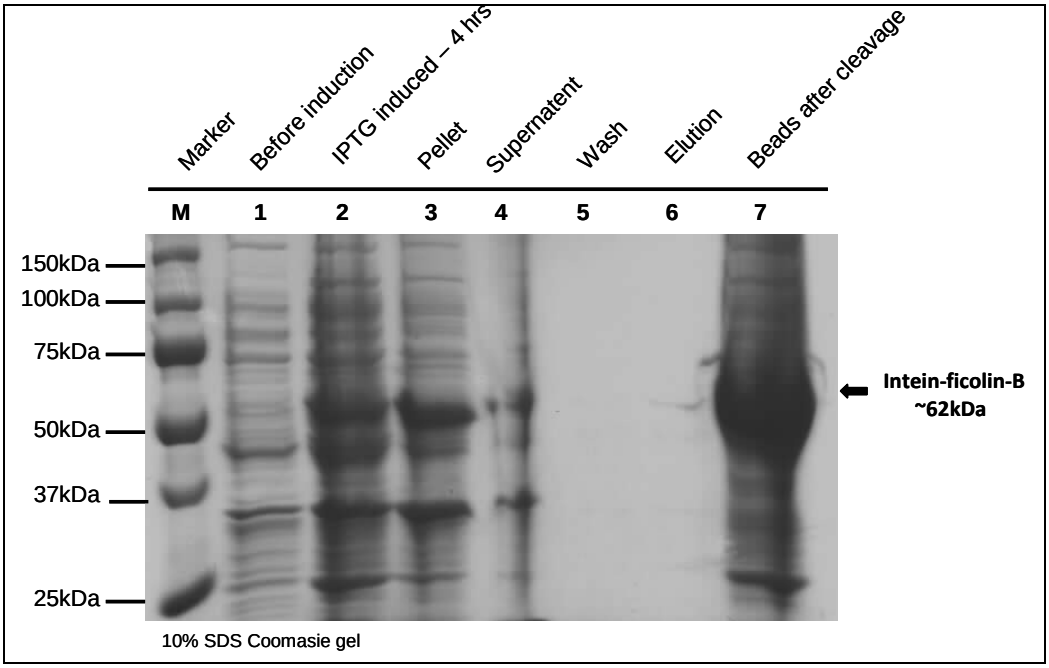
Since we observed a good expression of proteins in the insoluble fraction, we tried to purify the ficolin-B proteins from the inclusion bodies. When purifying a protein that may be insoluble, several factors have to be considered:

1. The binding efficiency of the intein-tag to the chitin resin is lower at 4 M urea or higher
2. The intein-mediated cleavage reaction may be carried out at 0 - 2 M urea
3. The higher the urea concentration, the better the chance to solubilize a target protein. However, the cleavage reaction should be performed in 0 - 2 M urea

Using a standard protocol for chitin chromatography and using the cleavage conditions recommended for the specific intein-tag (Materials and Methods – Section 2.2.2) we eluted the protein and analyzed both the eluate and chitin beads for cleavage and protein solubility (Fig 3.13).

Protein Purification from Inclusion bodies

A) 10% SDS - Coomasie gel



10% SDS Coomasie gel

B) Western blots

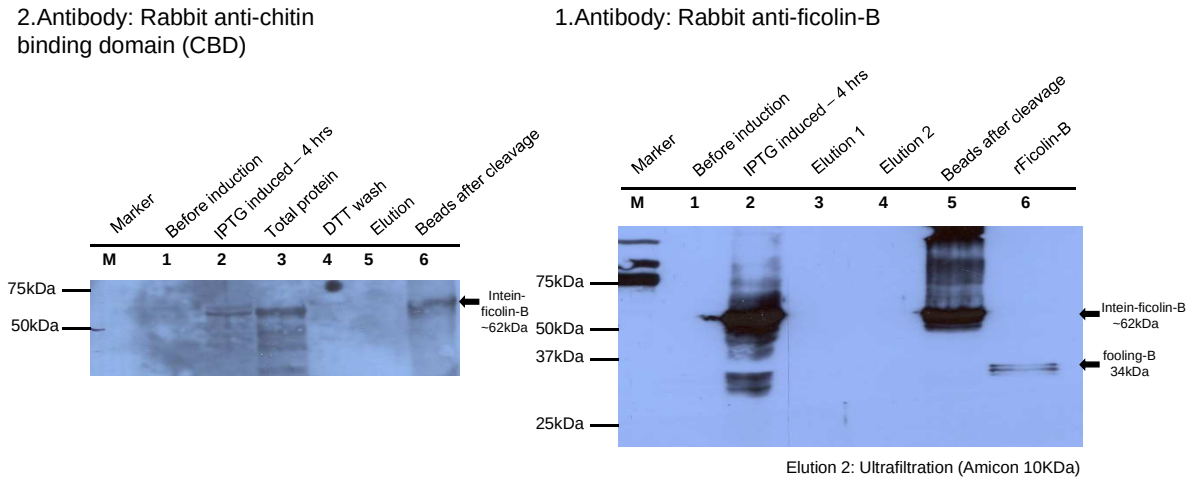


Fig. 3.13. Single column purification of recombinant proteins from Inclusion bodies A) Schematic representation of the expression and purification of ficolin-B using a pTXB1 vector. The pellets are lysed with guanidine hydrochloride and dialysed against different concentrations of urea. Samples taken from different steps during the expression and purification procedures were separated by SDS-PAGE and the gel was stained with Coomassie blue. Lanes: M, protein molecular weight standards (kDa, NEB); 1, uninduced cell extract; 2, induced cell extract; 3, lysed pellet and 4, supernatant. After denaturation with guanidine chloride and slow renaturation with urea, the column is washed with column buffer until the protein content of the eluate reached a minimum; 5, flow through from the quick DTT flush; 6, the fractions of the elution after the 4°C overnight (o.n.) incubation in the presence of DTT; 7, a fraction from the SDS elution (to look for cleavage efficiency). **B)** Western blot analysis of fusion proteins expressed in *E.coli BL 21 (DE3) p Lys.* The large scale purified samples were tested with rabbit anti-CBD antibody (1) rabbit anti-ficolin-B antibody (2). The samples as described for Fig.3.13A were separated by SDS-PAGE (10%), blotted onto a PVDF membrane and finally, detected with the Lumigen™ detection reagent and developed on a hyperfilm. Lane M: Protein molecular weight marker; lane 1,2 *E.coli*/pTXB1 ficolin-B uninduced sample and induced sample; lane 3, ficolin-B elution fractions,lane 4, ficolin-B elution fractions concentrated with ultrafilter;lane 5, sample beads after cleavage; ; lane 6, recombinant ficolin-B as positive control.

The conclusion from the attempts with the IMPACT kit expression system is that ficolin-B shows a good expression, but the protein is insoluble and remains aggregated with the chitin beads even upon purification. Ficolin-B from the intein fusion protein is not cleaved-off as ficolin-B still seems to be associated with chitin.

3.2. Binding studies

3.2.1. Binding studies of the intein-ficolin-B fusion protein from *E.coli* lysates to chitin

It was theoretically possible that the intein-ficolin-B fusion protein loaded onto the chitin-column would be properly cleaved, but that the ficolin-B itself would re-bind to the chitin by its carbohydrate recognition site. Therefore, as a next approach, I wanted to test whether ficolin-B has binding affinity for chitin. It was shown that human ficolins as well as mouse ficolins share binding specificity for N-acetylglucosamine (GlcNAc) (Le *et al.,* 1998 ; Fujimori *et al.,* 1998 ; Teh *et al.,* 2000). Therefore, It was of particular interest to assess the ficolin-B specificity for this molecule. The assay was performed as Fujimori and co-workers reported in 1998 for ficolin-A.

DS2 expressed ficolin-B on GlcNAc agarose beads served as a positive control and non-induced intein-ficolin-B was used as a negative control. But no binding activity was observed with the *E.coli* expressed material (data not shown). Since the purification of recombinant ficolin-B from *E.coli* BL21(DE3)pLysS cells did not result in soluble ficolin-B, we tested crude bacterial lysates of DS2 insect cells that had been transfected with the vector containing the ficolin-B insert for their ability to bind to GLcNAc agarose or Chitin (Fig 3.14).

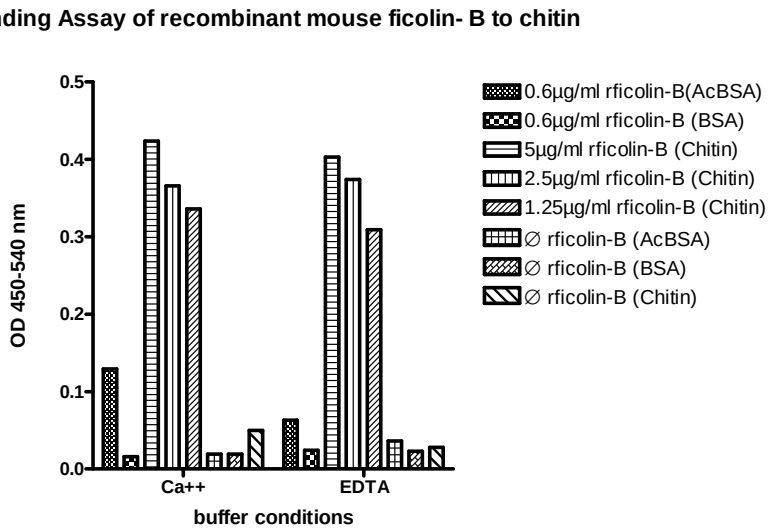


Fig. 3.14. Binding of ficolin-B to various ligands: Plates were coated with either BSA, AcBSA or chitin beads, as indicated. Ficolin-B binding (at different concentrations) was detected by ELISA. The binding was tested under different buffer conditions (e.g. Ca2+ or EDTA) to know whether the binding is Ca2+ dependent. DS2-ficolin-B binding to AcBSA served as a positive control and AcBSA, BSA and chitin in the absence of ficolin-B as negative controls. Binding was detected with monoclonal antibodies directed aganist the ficolin-B protein.The absorbance values measured at 450nm.

We also tested the binding activity to chitin on the *A.fumigatis* cell wall (kindly gifted by Prof. Dr. Frank Ebel, Max-von-Pettenkofer-Institut, München). These experiment however, did not lead to reproducible results. These experiments were performed in collaboration with Katja Hunold and Dorothea Weber-Steffens.

The binding studies show that there was an dose-dependent binding of ficolin-B to chitin and that the binding was Ca2+ independent. EDTA had no significant effect on ficolin-B binding to chitin. Binding of ficolin-B to *A.fumigatis* could not be shown due to technical problems.

3.3. Cloning and expression of ficolin-B with His-tagged vector

Since the intein vector system failed to express the fusion protein in a soluble form, we decided to continue with a His-tagged vector system which is a more convenient system for purification of proteins by affinity chromatography. We analysed mficolin-B protein expression with an N-terminal His tag vector (pET28) and a C-terminal His tag vector (pPROEXHTb).

3.3.1. Cloning of ficolin-B with N-terminal and C-terminal His-tagged vectors

Based on the known mouse ficolin-B cDNA sequence (Gen-Bank Accession No. M 070497) the specific primers (numbered from 3 and 4 in section 2.1.9) for PCR amplification were used. Appropriate restriction sites, absent in the target gene were incorporated in the forward and reverse primers. PCR was done as described in the materials and methods (Section 2.2.1.1.1). The amplification products were analyzed by electrophoresis on a 1% agarose gels stained with ethidium bromide (Fig 3.15). The amplification product (500ng), digested with BamHI and XhoI, after isolation using Gel-Out kit (Promega).

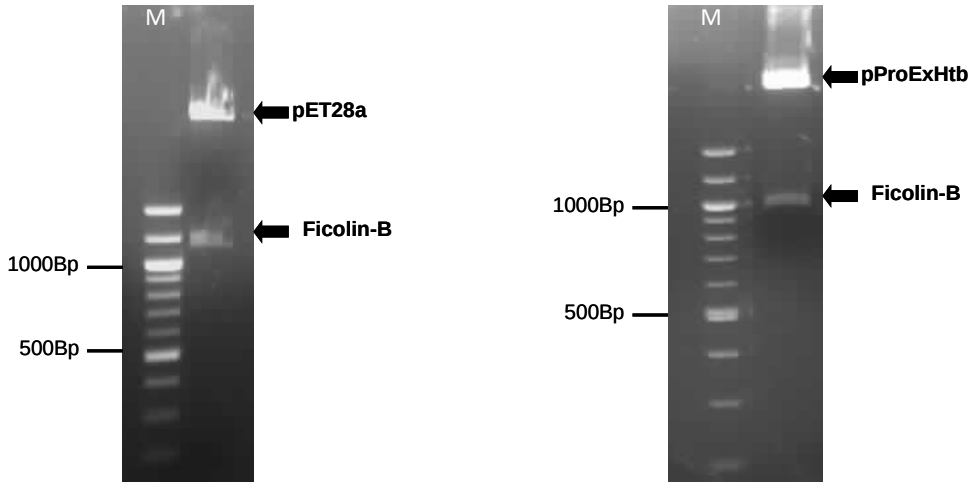


Fig. 3.15: mficolin-B in pET28 and pProExHtb vector: Plasmid DNA (Fig. left and right) was digested with restriction enzymes BamHI/XhoI and checked on a 1.5% (w/v) agarose gel containing EtBr. Lane M: NEB 100 bp DNA ladder marker and the digestion of pET28 and pProExHtb ficolin-B recombinant with BamHI/XhoI.

3.3.2. Miniprep for protein expression as a test to check solubility

(Protocol followed as described for GST section 3.2.3.1)

1) N-Terminal His-tag protein expression 2) C-Terminal His-tag protein expression

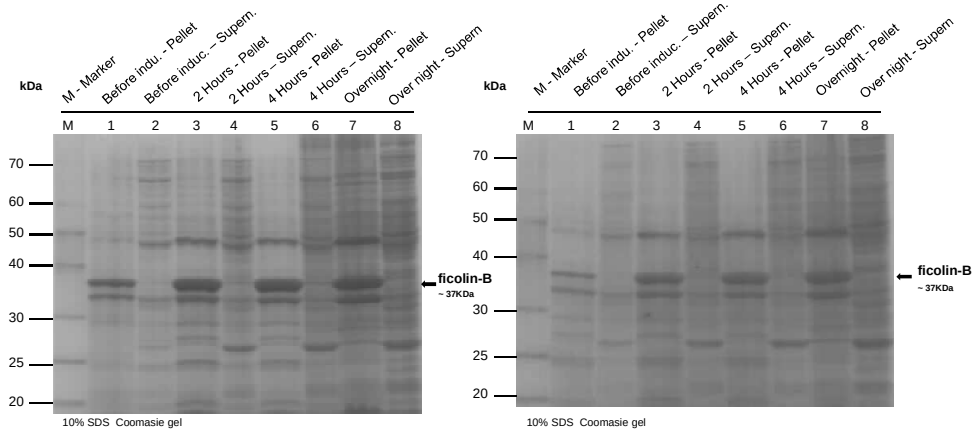


Fig. 3.16: Solubility test expression of N- and C-terminal ficolin-B recombinant protein: SDS-PAGE analysis of fusion proteins expressed in *E.coli* BL21 (DE3)pLys. Samples were collected at different time points (0 – overnight) after induction with 0.1mM IPTG. The samples were lysed and run on gel representing the solubly expressed supernatant) and the insolubly expressed pellet protein fractions. All samples were separated by SDS-PAGE (10%) and the gel was stained with Coomassie brilliant blue to detect proteins. Lane M: Precision plus 4 -20% Tris-HCL gel SDS-PAGE marker. The other lanes were applied with pellet and supernatent samples from non-induced and induced for 2hrs, 4hrs and overnight. The arrow indicates the predicted electrophoretic mobility of His-ficolin-B fusion protein.

However the His-tagged protein expression again was only detected in the insoluble fractions (Fig 3.16). The different expression vector system showed that the ficolin-B cannot be expressed in the soluble form which might be due to its complex higher oligomers and multimers that might be dependent on glycosylation which cannot happen in *E. coli.*

3.4. Cloning and expression of ficolin-B with GST tagged vector

3.4.1. Cloning of a ficolin-B expression construct in pGEX vector

As a next approach, we decided to go for the GST expression vector system. The use of GST as a fusion tag is desirable because it can act as a chaperone to facilitate protein folding, and frequently the fusion protein can be expressed as a soluble protein. Here we used a modified pGEX vector which has a TEV cleavage site (Fig 3.15). The advantage of TEV (Tobacco etch virus) is its specifity and that the cleavage efficiency is not much altered by salt or temperature .

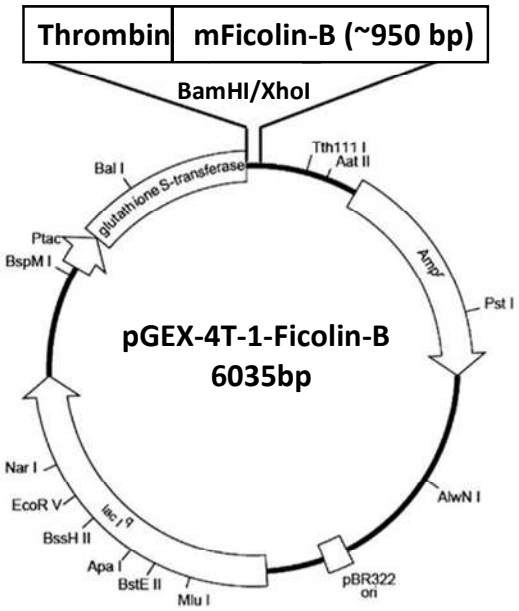


Fig. 3.17. Diagram describing the construction of the recombinant expression plasmid pGEX4T-1-NTEV.The arrow indicates the cleavage sit of TEV which allows the release of the target protein from the tag.

Based on the known mouse ficolin-B gene sequence (Gen-Bank Accession No. M 070497) specific primers (numbered from 3 to 4 in section 2.7) for PCR amplification were used as described in (section 2.2.1.1.1). BamHI and XhoI restriction sites absent in the target gene were incorporated in the forward and reverse primers. Cloning into the BamHI and the XhoI sites will place the ficolin-B cDNA in frame with the GST coding sequence (Fig 3.17). PCR

was done as described in the Materials and Methods (Section 2.2.1.1.1). The amplification products were analyzed by electrophoresis on a 1% agarose gels stained with ethidium bromide. A Specific 950-bp PCR product was obtained. (Figure 3.18). The amplification product (500ng), digested with BamHI and XhoI, was isolated from an agarose gel bands using Gel-Out kit (Promega).

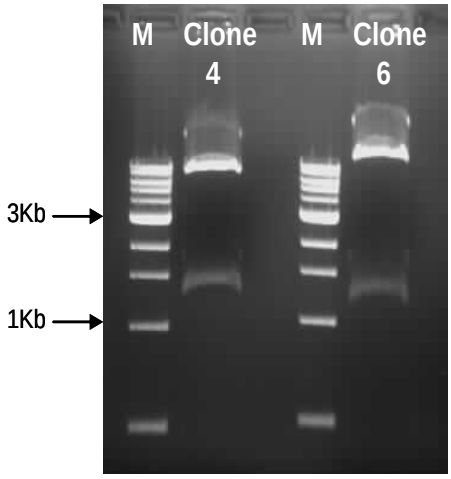


Fig. 3.18: mficolin-B in pGEX vector: Plasmid DNA (Clone 4, 6) was digested with restriction enzymes BamHI/XhoI and checked on a 1.5% (w/v) agarose gel containing EtBr. Lane M: NEB 100 bp DNA ladder marker and the digestion of pGEX ficolin-B recombinant with BamHI/XhoI

3.4.2. Expression of the GST ficolin-B fusion protein

In order to determine wether the expression of GST ficolin-B fusion protein was either in the insoluble (pellet) or in the soluble (supernatant) fraction, an expression test was done with bacteria collected by centrifugation (as described in section 3.1.2.1) followed by a miniprep expression where the bacterial sample collected was lysed and centrifuged to get it separated into soluble and insoluble fractions (as described in section 3.1.2.2).

To monitor protein expression over time, 12 µL of the soluble and insoluble fractions as well as of each sample taken every hour were loaded on SDS-PAGE gels (10 or 12%), with Coomassie brilliant blue staining. The expression of proteins was monitored at temperatures 30℃ and at 0.1mM IPTG (Figure 3.19).

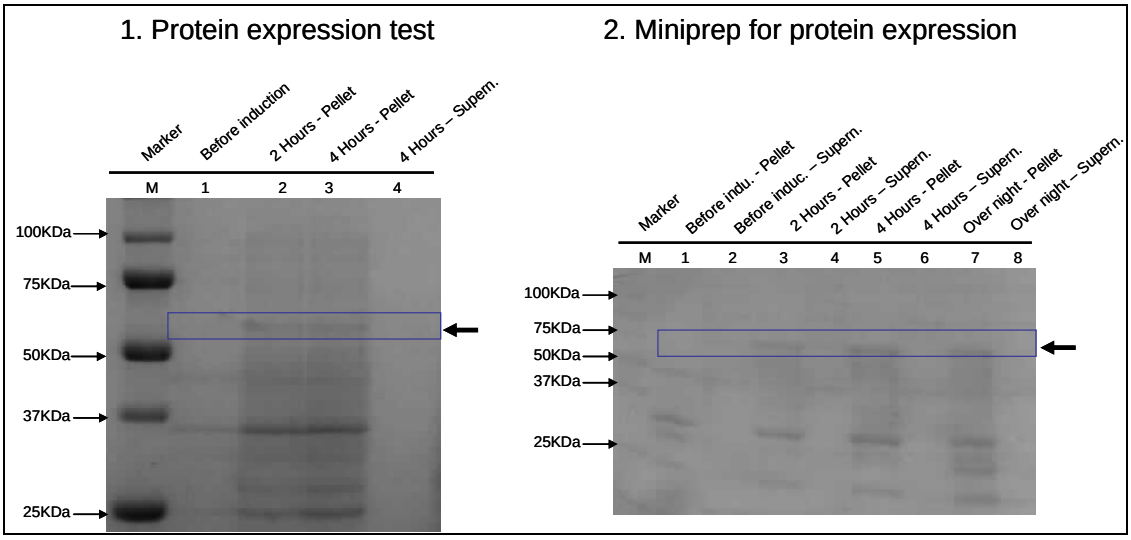


Fig. 3.19: Test expression of pTXB1-ficolin-B recombinant protein: SDS-PAGE analysis of fusion proteins expressed in *E.coli* BL21(DE3)pLys. Samples were collected at different time points (0 – 4hrs and Overnight) after induction with 0.1 mM IPTG (Gel 1) and also samples representing the solubly expressed and the insolubly expressed (Gel 2) protein fractions were collected. All samples were separated by SDS-PAGE (10%) and the gel was stained with Coomassie brilliant blue to detect proteins. Lane M: Precision plus 4 -20% Tris-HCL gel SDS-PAGE marker. The arrow indicates the predicted electrophoretic mobility of GST-ficolin-B fusion protein.

However, also the GST fusion protein expression was only in inclusion bodies and neither expression at different temperatures nor at different IPTG concentrations had any effect on protein expression in the supernatant fraction.

3.5. Cloning and expression of the collagen domain of ficolin-B fused to GST

As our attempts did not lead to successful high level expression of the full-length ficolin-B, we decided to individually express the conserved regions of the ficolin-B. The gene sequence alignment of all ficolins and MBL had shown the presence of a putative MASP binding motif residing at the collageous domain (Wallis et al., 2007). For this purpose we cloned the collagen domain (180 bp) of ficolin-B into the pGEX-4T-1 vector and expressed it in *E.coli* BL21(DE3)pLysS cells (as shown in fig.3.20)

PCR was done as described in Materials and Methods – (Section 2.2.1.1.1).

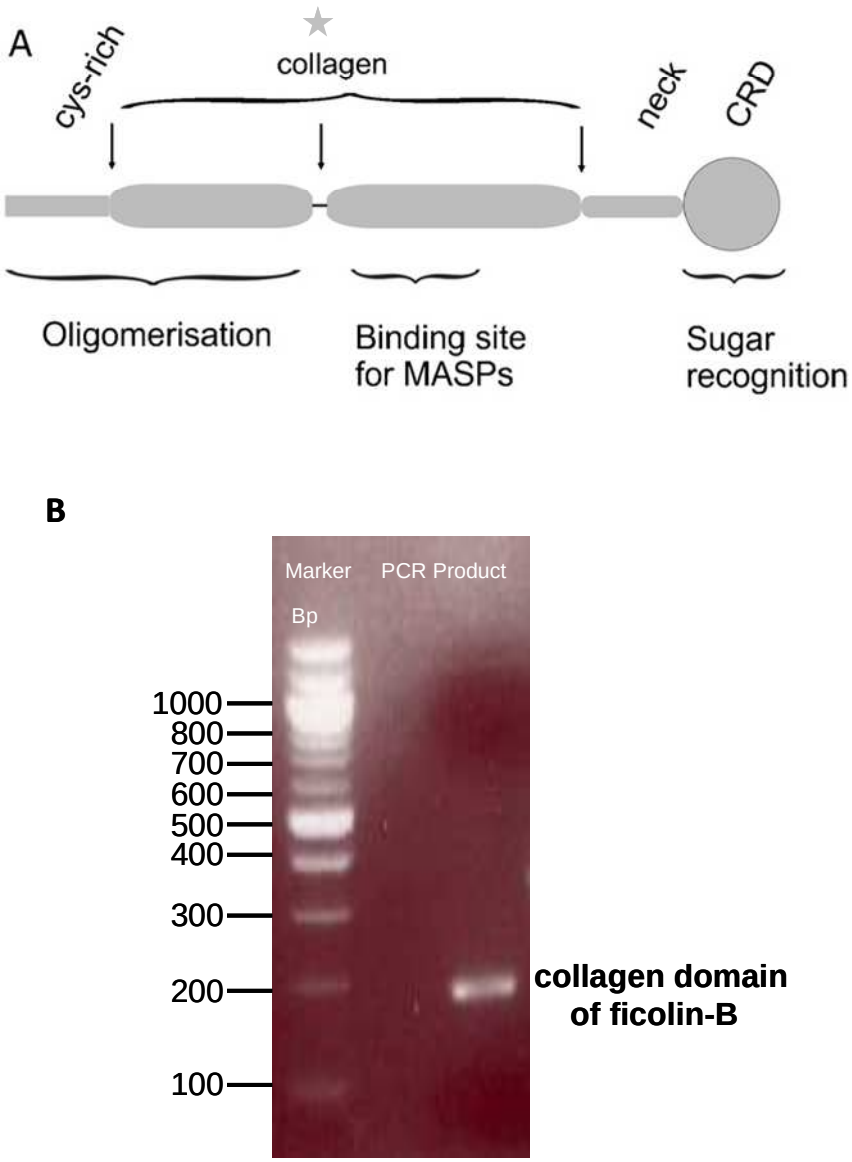


Fig. 3.20: The PCR products of the target genes : A) Domain organisation of mouse ficolin-B. The collagenous domain (*) is shown as two segments, which are separated by the interruption in the Gly–Xaa–Yaa collagen repeat (Wallis., 2007). Arrows show potentially flexible regions. The aligned sequences of the collagenous domain of ficolin-B is shown below **B)** DNA samples were separated on a 1.5% (w/v) agarose gel containing EtBr Lane M: NEB (1Kb) DNA high range marker ready to use and lane 2 PCR product.

3.5.1. Miniprep for protein expression as a test to check solubility

To further investigate whether GST-Collagen of ficolin-B fusion protein expression was in the insoluble or in soluble fraction, a miniprep expression test was done where the bacterial sample collected was lysed and centrifuged to get it separated into pellet and supernatant (as described in section 3.1.2.2). The expression of proteins was monitored at temperature 30℃ and at 0.1mM IPTG (Figure 3.21).

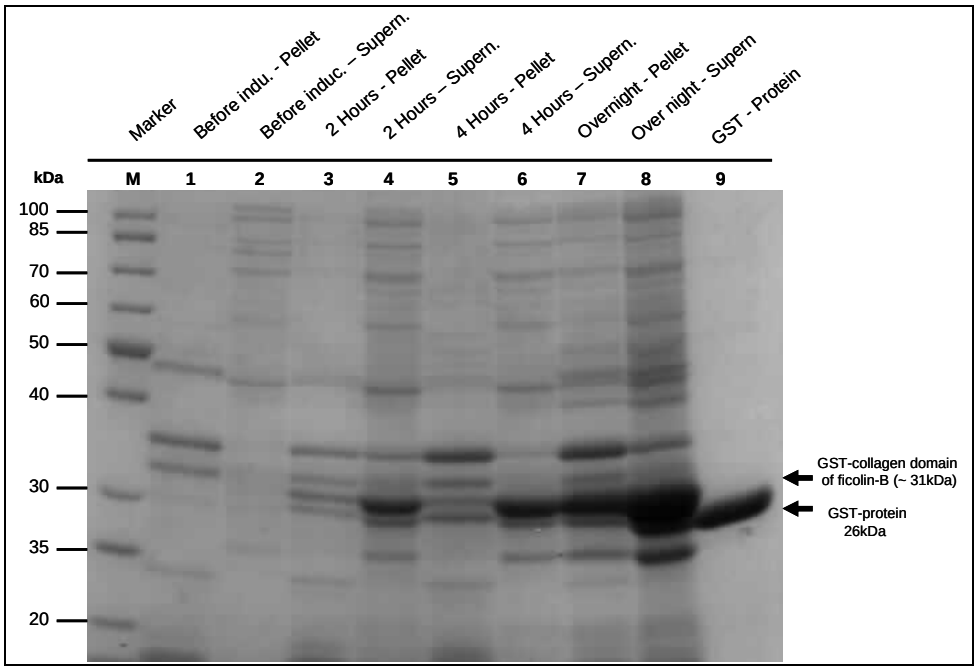


Fig. 3.21: Solubility test expression of pTXB1-ficolin-B recombinant protein: SDS-PAGE analysis of fusion proteins expressed in *E.coli* BL21(DE3)pLys. Samples were collected at different time points (0 – overnight) after induction with 0.1mM IPTG. The samples were lysed and run on gel representing the solubly expressed supernatant and the insolubly expressed pellet protein fractions. All samples were separated by SDS-PAGE (10%) and the gel was stained with Coomassie brilliant blue to detect proteins. Lane M: Precision plus 4 -20% Tris-HCL gel SDS-PAGE marker. lane 1,2 uninduced sample; lane 3, 4, -2 hrs induced sample; lane 5, 6, -4 hrs induced sample; lane 7, 8, -overnight induced sample; lane 9, GST protein as positive control.

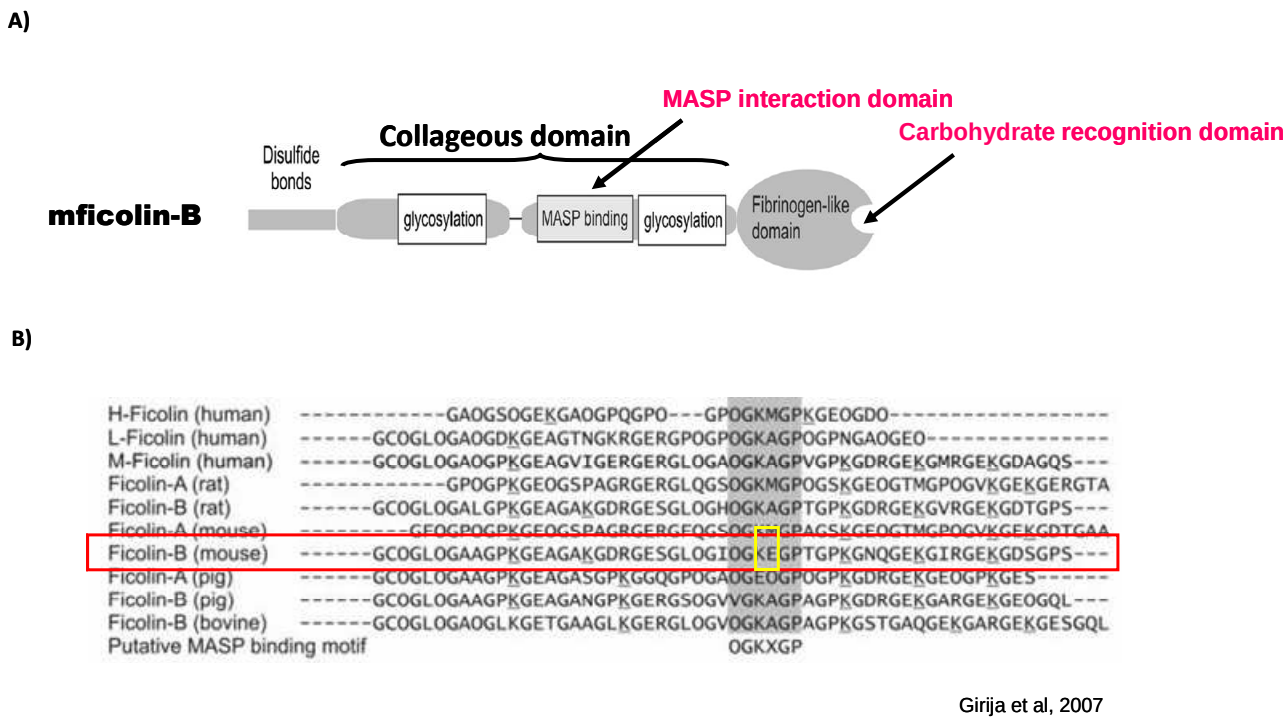
The GST collagen domain of ficolin-B fusion protein can be expressed in the soluble form (supernatant).

Part - 2

Mutational analysis of mouse ficolin-B and biochemical characterization

3.6. Mutational analysis of ficolin-B specificity and biological function

The goal of this work was to look, if any mutational changes in the collageous domain of ficolin-B will lead to a change of binding of mouse ficolin-B to MASP2 and modulate or affect activation of the lectin pathway of the complement system. Here with ficolin-B, we mutated the glutamic acid (in X (E72A) position of putative MASP binding motif) to alanine (which is a common amino acid in other lectins).



Girija et al, 2007

Fig. 3.22: Domain organization of the ficolins. A) Aligned sequences of the collagenous domains of ficolins. **B)** The putative MASP-binding motif is shaded. Lysine residues within the Y position of the Gly-X-Y repeat are underlined. All such residues are at least partially hydroxylated and glycosylated. Glutamic acid (E) (Yellow box) in the mouse ficolin-B is mutated and replaced by alanine.

3.6.1. Generation of ficolin-B expression constructs

The ficolin-B gene contains a 942bp-base pair (bp) open reading frame (ORF) encoding 314 amino acids. In order to clone the sequences into the pMT/BiP/V5-His A – Hygro expression vector, primer pairs (numbered 6 to 7 in materials and methods section 2.7) were designed to amplify the mficolin-B cDNA without endogenous leader peptide region because the plasmid pMT/BiP/V5-His A contains the *Drosophila* BiP signal sequence for secretion of the recombinant protein into the culture medium. Therefore, a 882 bp fragment was amplified by PCR from spleen cDNA. The fragment was first cloned into the pCR2.1-TOPO vector, which

is an activated plasmid ready to accept PCR products as inserts, to generate the “TOPO-ficolin-B” constructs. Since the primers also contained endonuclease restriction sites at the 5’ ends these constructs were further digested with *BamH*I/*Xho*I and the ficolin–B cDNA was subcloned into the pMT/BiP/V5-His A –Hygro expression vector, which was previously digested with the same enzymes to generate the constructs called “pMTA-Hygro-ficolinB”. Figure 3.23 shows a scheme of the described cloning process. The resulting expression constructs were evaluated by restriction analysis and sequenced to confirm that the inserts were cloned in-frame with the V5/His-tags and lack any mutations before proceeding to transfection. Johann Röhrl is acknowledged for the pMT-pCoHygro expression construct.

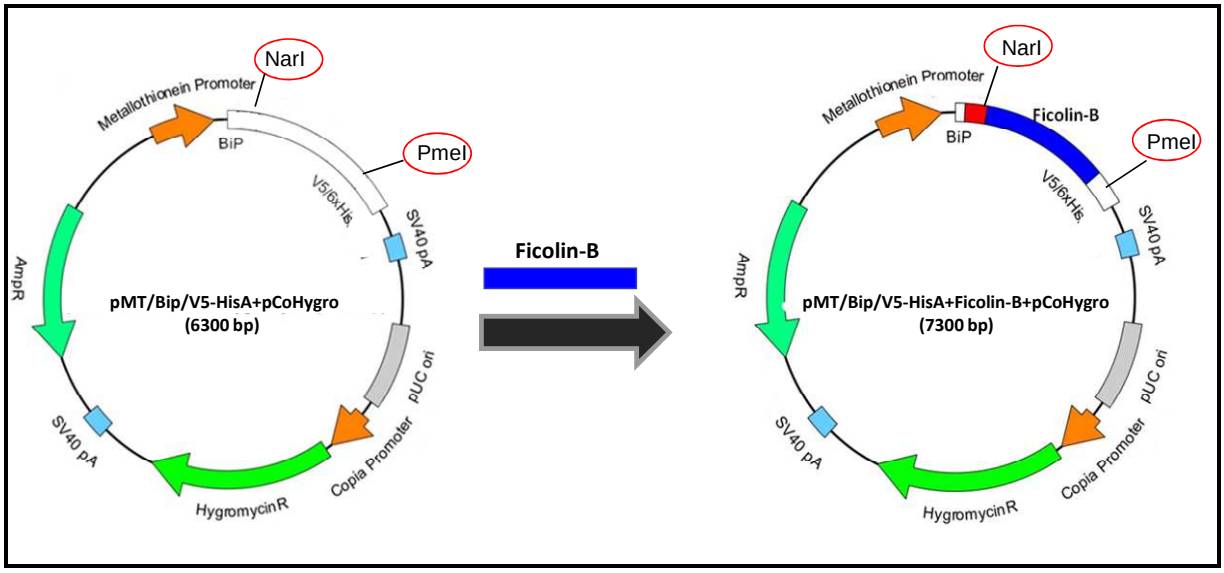


Fig. 3.23: Scheme for the cloning of mficolin–B genes into the pMT/BiP/V5-His A-Hygro expression vector. The ficolin–B genes were amplified by PCR, constructs were then digested with *BamH*I/*Xho*I and further subcloned into the pMT/BiP/V5-HisA –Hygro engineered expression plasmid. **AmpR**: ampicillin resistance gene; **LacZ**: α-fragment of the ß-galactosidase gene; **MCS:** multiple cloning site; **P Lac**: LacZ promoter; **P MT**: metallothionein promoter; **SV40 pA**: Simian virus 40 polyadenylation signal.

3.6.2. Site-directed Mutagenesis

Mutations (E72A) were constructed using the QuickChange Site-directed Mutagenesis kit (Stratagene) following the instructions provided by the manufacturer. Primers were designed as shown in table 1.The mutations were verified by DNA sequencing. *pMT*/BiP/V5-*His* A - Hygro (Ficolin-B fusion full length) was used as the template.

Table 1. Primers used for Site directed mutagenesis of pMT-ficolin-B-pCoHygro

Mutation name	Primer sequence (5´to 3´)
E72A	cttcctggaattcctggaaaa**gca**ggaccaactggacccaaagga

Introduced sites of mutagenesis are in bold and underlined. Mutations were done taking the pMT-ficolin-B-pCoHygro (full length) as template.

3.7. Biochemical characterization of the recombinant ficolin-B

3.7.1. Reducing and non-reducing SDS-PAGE

We looked for the oligomerization of ficolin-B expressed in DS2 cells by SDS-PAGE. For this purpose ficolin-B was separated by SDS-PAGE (10% gel) under reducing (Fig.3.24 A) and non-reducing (Fig.3.24 B) conditions. Then immunoblot analysis was performed using a polyclonal rabbit anti-ficolin-B antiserum.

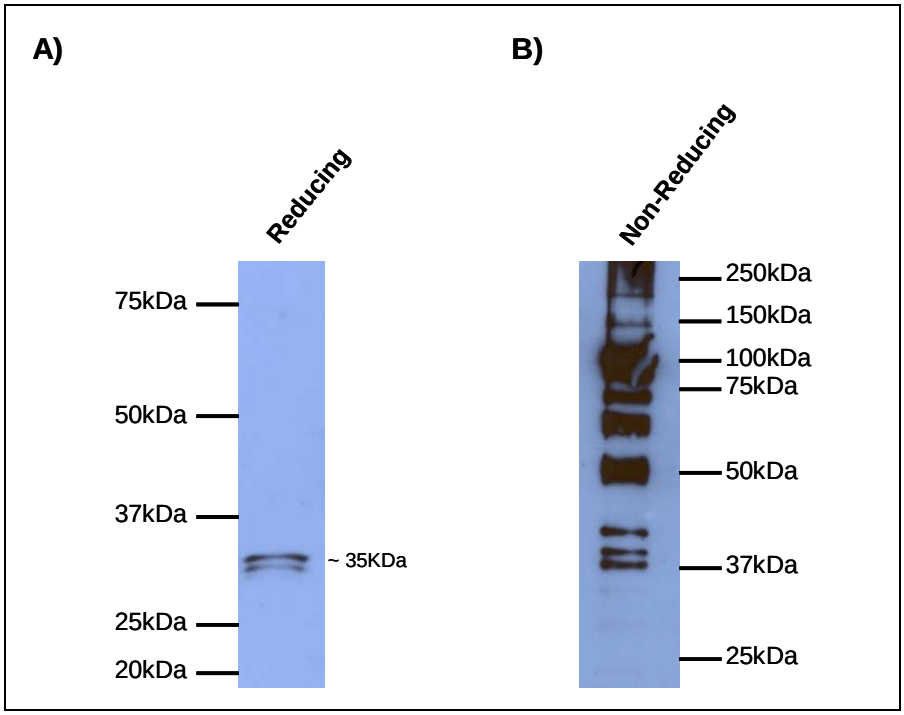


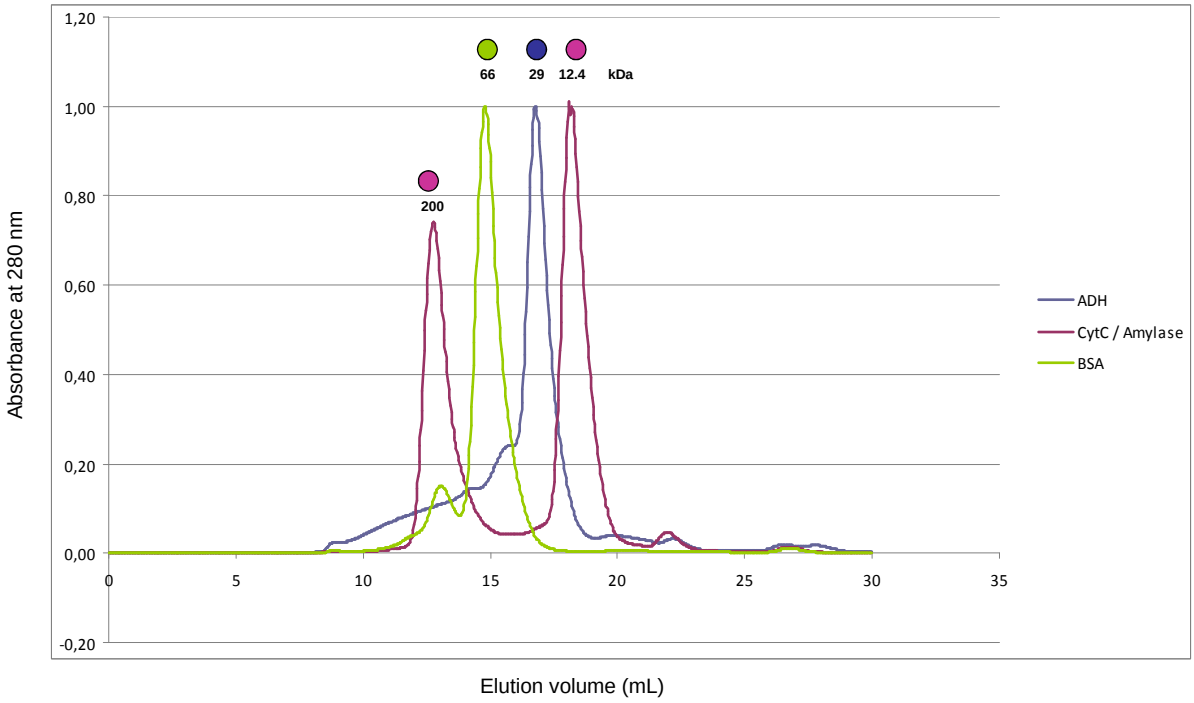
Fig. 3.24: Oligomerization of mficolin-B in DS2 cells: Purified ficolin-B was separated by SDS-PAGE (10% gel) under reducing (A) and non- reducing (B, C) conditions. Then immunoblot analysis was performed using rabbit anti-ficolin-B antiserum. Molecular weight markers are indicated.

Purified ficolin-B migrated as a double band on SDS-polyacrylamide gels under reducing conditions, with apparent molecular masses of 35 and 36 kDa (Figs.3.24A). Higher oligomers of ficolin-B were observed in the non-reducing conditions (3.24B).

3.7.2. Analytical gel filtration

In order to analyze the role of the multimerization in ficolin-B for effective biological activity, recombinant ficolin-B produced by DS2 cells was used for gel filtration analysis. I analysed two types of ficolin-B: one called "Old ficolin-B" which was produced a year before and the other one is "Fresh ficolin-B" which was produced immediately before the experiment. Fig.3.25 shows the elution profiles obtained for the two recombinant ficolin-B preprations. Compared to the elution profile of standard molecular weight marker on the superdex 200 gel filtration column, the majority of "Fresh ficolin-B" migrated with an apparent molecular mass of 200-36 kDa.

Elution profile for the marker proteins



Elution profile of “Fresh” ficolin-B and “old” ficolin-B

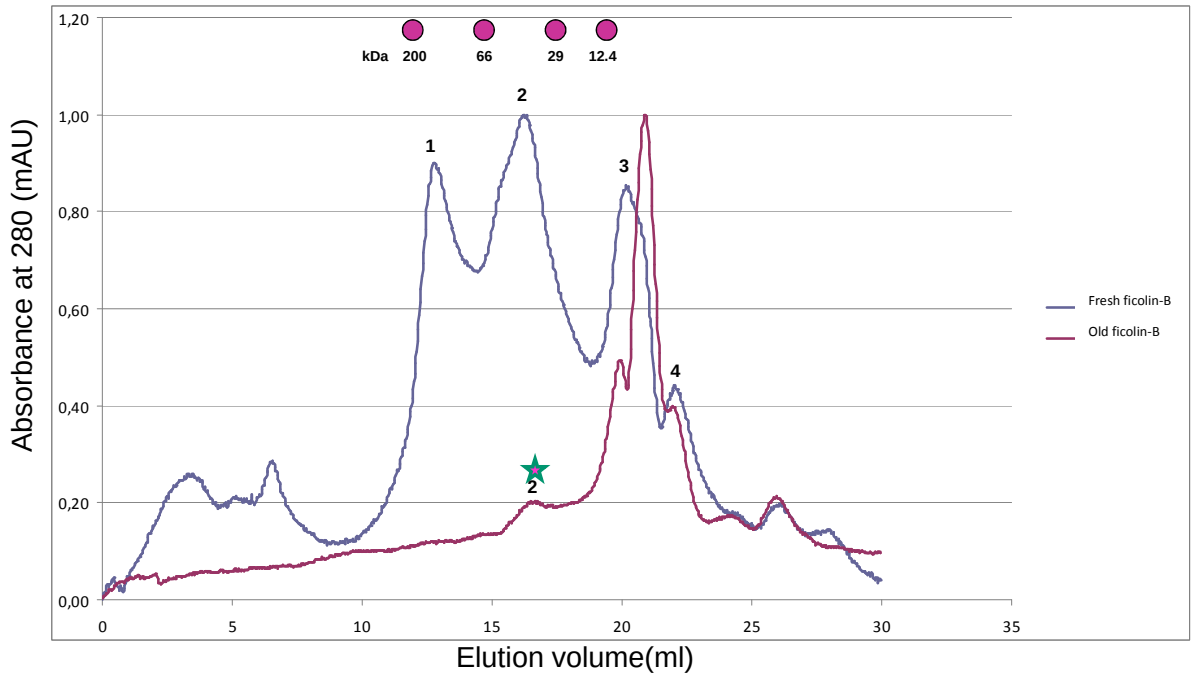


Fig.3.25: Gel filtration of ficolin-B on a Superdex-200 column. The elution positions of monomers (2), and tetramers (1) of mouse ficolin-B are indicated. Elution positions (3,4) might be the degraded or proteolized products of ficolin-B. *Blue elution curve*, Fresh ficolin-B; pink *elution curve*, Old ficolin-B; Masses of molecular weight standards: 12.4 kDa. cytochrome *c*; 29 kDa, carbonic anhydrase; 66 kDa, bovine serum albumin; 200 kDa, b-amylase.

The elution profile clearly shows that the “Fresh ficolin-B” elutes as monomers (peak 2) and tetramers (peak 1) wheras the “Old ficolin-B”, shows a small peak of monomers (peak 2) and more of the degraded or proteolized products of ficolin-B (peaks 3,4). From the results it seems that the “old ficolin” is degraded.

3.8. Biochemical and functional characterization of the mouse ficolin-B mutant

Since wild type ficolin-B and mutant ficolin-B expression took longer than expected, I took the supernatant of DS2 cells expressing the protein and analyzed the biochemical and functional characteristics of the ficolin-B. The following experiments were performed in collabration with Katja Hunold and Dorothea Weber-Steffens.

3.8.1. Dot blot

As a first step we performed a dot blot assay to check if the V5-HRP antibody could equally detect the wild type and mutant ficolin-B.

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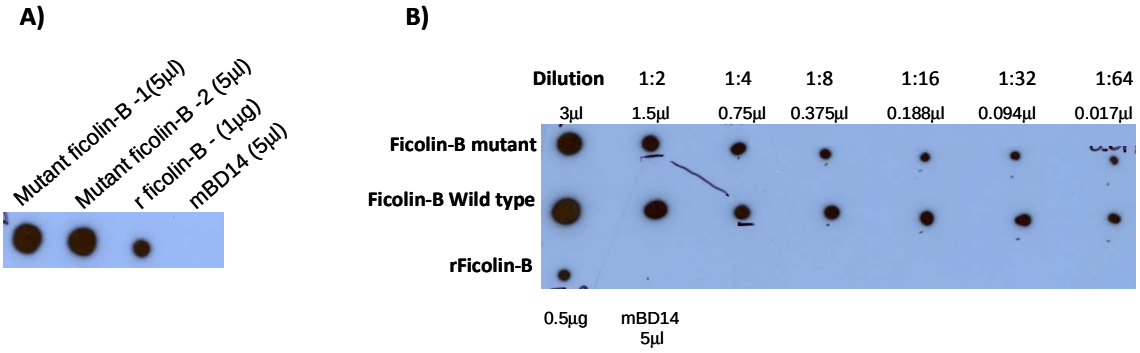


Fig. 3.26: Dot blot for the detection of wild type mficolin-B and mutant (supernatants) with the anti-(V5) tag antibody. A) Recombinant wildtype ficolin-B and mutant (supernatant) was spotted on a nitrocellulose membrane at the indicated volumes and incubated with the anti-(V5) tag antibody. DS2 expressed mBD14 was spotted as a negative control. **B)** Recombinant wild type ficolin-B and mutant (supernatant) was spotted on a nitrocellulose membrane for a dose response and incubated with the anti-(V5) tag antibody. DS2 expressed mBD14 was spotted as a negative control.

For dot blot analysis, we also used supernatants from wild type ficolin-B spotted on the membrane. Mutant as well as wild type ficolin-B protein was detected by anti-(V5) tag antibody (Fig 3.26A). DS2 cells purified ficolin-B was a positive control and mBD14 was used as negative control. We checked in dot blot analysis different volumes of wild type and mutant ficolin-B supernatant with anti- (V5) tag antibody (Fig 3.26B) and used purified recombinant ficolin-B as positive and DS2 expressed mBD14 as negative control.

The dot blot confirmed the presence of the wild type and mutant ficolin-B protein detected by the ani-(V5) tag antibody.

3.8.2. Western blot

Ficolin-B is a oligomeric protein (Erickson *et al.,* 1997). To further look for the oligomerization difference between wild type and mutant ficolin-B, the supernatant of the mutant protein was analysed by Western analysis. We performed reduced and non-reduced gel runs that was followed by Western blot to detect the difference in oligomerization of wild type and mutant ficolin-B. Samples separated by SDS-PAGE were transferred onto polyvinyl difluoride (PVDF) membrane. After saturation of non-specific binding sites the membrane were incubated with anti-(V5) tag antibody (Figure 3.27).

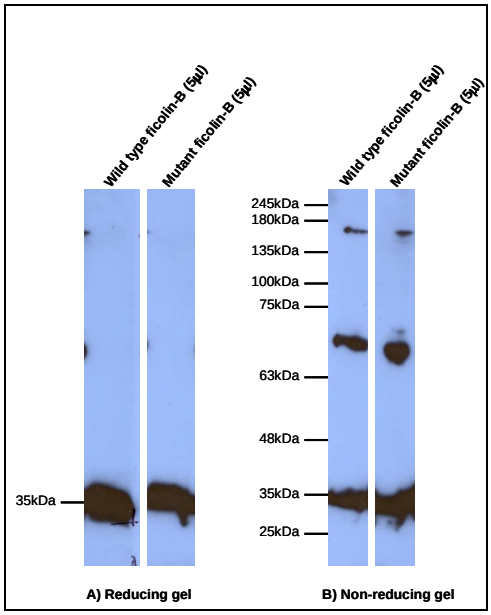


Fig.3.27: Immunoblot for the wild type and mutant mficolin-B protein ***:*** Western blot analysis of the wild type and mutant supernatant proteins expressed in DS2 cells **A)** shows the reducing gels and **B)** shows the non-reducing gels. Samples were separated by SDS-PAGE (7.5%), V5 tagged proteins detected by the anti-(V5) tag antibody.

Western blot analysis showed that there was no difference in oligomerization between the wild type and mutant ficolin-B proteins.

3.8.3. ELISA

Mouse ficolin-A and ficolin-B bind to GlcNAc and GalNAc. Ficolin-B, like M-ficolin, additionally recognizes sialic acid residues (Endo *et al.,* 2005). As a next step we tested the binding activity to AcBSA with wildtype and mutant ficolin-B (Fig. 3.28). The test was done in the same way as shown in (Fig.3.14).

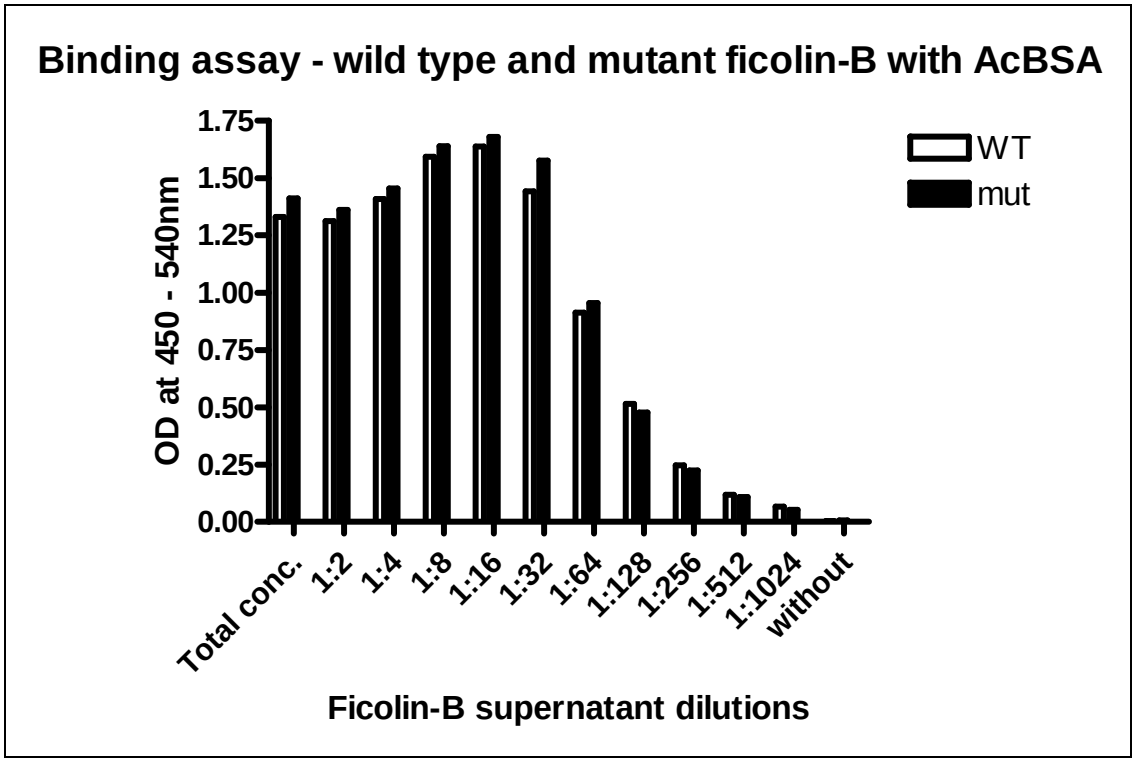


Fig.3.28. Binding of wild type and mutant ficolin-B to AcBSA: Plates were coated with AcBSA (10µg/ml). Wild type and mutant ficolin-B (supernatant) binding (at the different dilutions as indicated) was detected by ELISA. The binding buffer condition was 5mM Ca2+/TBST. Binding was detected with anti- (V5) HRP antibodies (1:5000 dilution).

The binding study shows that there was no difference in binding to AcBSA of mutant ficolin-B when compared to wild type ficolin-B.

4. Discussion

Ficolins are becoming more important in innate immunity as they are shown to have many functions. Ficolins activate the complement system through their association with MASPs which in turn leads to pathogen phagocytosis, aggregation, and lysis.

The first part of my project was to generate a tag-free recombinant ficolin-B in order to further characterize the protein and gain insights into its functions. Therefore, It was important to find a good expression system. An empirical approach is often necessary to find the best expression system for a protein of interest. Though the aim was to produce a tag-free ficolin-B recombinant protein, comparison of the *E.coli* system with the DS-2 cell system clearly showed the advantages and problems of each system.

Among the many systems available for heterlogous protein production, the use of the gram-negative bacterium *E.coli* is widely used because of its ability to grow rapidly and at high density on inexpensive substrates. It is genetically well-characterized and available with an increasingly large number of cloning vectors and mutant strains. Moreover, the quality of the final product is higher than that of any non-recombinant method. With the DS-2 system (already an established system in our lab), the cells grow slow with low yields, but the main advantage is that they produce proteins with a glycosylation pattern similar to mammalian proteins.

The recognition between protein partners is a fundamental molecular mechanism by which proteins execute their biological functions. In the second part of my project, I used some biochemical approaches to study the molecular recognition mechanisms between proteins to study their biological functions.

To rationalize the results, I will discuss the two parts of the projects separately.

4.1. Expression and purification of the ficolin-B-intein fusion protein from *E.coli*

4.1.1. IMPACT kit for the production of tag-free recombinant proteins

The first aim of the present work was the production of recombinant ficolin-B by using the IMPACT kit system. We used this novel protein purification IMPACT system, because, it has several distinct advantages. It is rapid, and yields are comparable to those obtained by other expression methods. The IMPACT system utilizes the controlled C-terminal cleavage activity of the protein splicing elements (termed inteins) which can lead to better expression of proteins without the use of proteases. It can separate the target protein from the affinity tag in a single chromatographic step without any vector derived amino acids (Evans, T.C., *et al.*

(1998). The system has a chitin binding domain (CBD) incorporated which has extremely high affinity for the chitin beads, allowing better purification of fusion proteins (Chong et al., 1997). High salt concentration of detergents can be used during washing steps which will reduce non-specific binding, thus increasing purity (Szweda et al., 2001). A recent report has shown a successful intein-mediated expression and purification as an effective approach in the study of β-defensins (Diao et al., 2007).

For our experiments, we amplified cDNA from HoxB8 cells encoding full length mouse ficolin-B and cloned it into the PTXB1 vector (C-terminus IMPACT vector; Fig 3.7). Cloning at the C-terminus of the vector will result in a better expression of the protein. The target protein is fused at its C-terminus to a self-cleavable intein tag (~28 kD) that contains the (CBD, ~6 kDa) allowing for affinity purification of the fusion precursor on a chitin column.

4.1.2. Analysis of differential expression for the intein-ficolin-B fusion protein

Next, we tried to optimize the expression conditions for ficolin-B under different conditions including low-temperature, IPTG, and induction time (Fig 3.8). Intrestingly, we were able to see a good expression of the ficolin-B protein as revealed by the SDS-PAGE, but the expression at the expected size (~62kDa) was seen only in the insoluble fraction. We could not detect any leaky expression in the post-induction sample, which further demonstrates a controlled high level induction by the T7 expression system. Gadgil *et al.,* 2005 had reported that lower temperatures have the advantage of decreasing the growth rate of bacteria, which indirectly facilitates correct protein folding and brings the expression of protein in the soluble fraction. Since the expression was only in the insoluble fraction, we tried to bring the expression in the soluble form with different approaches like short time induction, low IPTG, or lowering the temperature (data not shown). Unfortunately a combination of these approaches was unable to induce sufficient levels of protein in the soluble fraction. It has been shown in this work that 0.1mM IPTG is used to induce expression and slightly higher or lower concentration of IPTG or temperature did not have any impact on the production of the protein in the soluble fraction (Fig 3.9). The majority of the protein seemed to be insolubly expressed as seen with more intense bands with the pellet lysates at the expected size of the fusion protein (~62kDa) and rapid expression of recombinant ficolin-B protein resulted in accumulation of insoluble protein. Arie *et al*., 2006 have reported that the protein aggregates into inclusion bodies in either the cytoplasm or the periplasm, depending on the target protein being produced. However in this case, it might be that ficolin-B being a complex oligomeric protein might depend on an increased capacity of post-translational modifications to express the protein in the soluble fraction. Production of “complex proteins” that require further modification or secretion in the soluble fraction is a challenging task. Miroux and Walker

(1996) reported that by over-expression plasmids themselves are intrinsically toxic to *E.coli* strain BL21(DE3). In order to check that point, I also tried to express ficolin-B in the Rosetta strain which is said to be a good expression strain for the mammalian proteins. But, nevertheless the expressed protein remained insoluble.

4.1.3. Protein identification

Although I could detect the intein fusion protein by coomassie brilliant blue by its expected size (~62kDa), I wanted to confirm the same through the immunoblot detection system specific for CBD intein-tagged proteins and ficolin-B protein. Both the Western blot (Fig 3.10) and dot blot (Fig 3.11) again confirmed the nature of the fusion protein and that it was insoluble. I also used Bradford and Lowrys assays to measure the lysate concentrations, but accurate determination of protein concentrations could not be done (data not shown).

4.1.4. Purification with the IMPACT system

Nevertheless, I tried large scale purification of proteins with the IMPACT system, but was not able to purify detectable amounts of the protein. The large scale purification again confirmed by SDS-PAGE and Western blot (Fig 3.12) that the protein was only insolubly expressed. I also tried to purify the protein from inclusion bodies. First I tried with 8M urea as denaturating agent, but this procedure was not successful to denature the protein completely (data not shown). So as a next step, I tried to lyse with a harsh denaturant like 7 M guanidine-HCl and the data (Fig 3.13) shows that the protein tends to get aggregated at the chitin beads. The inclusion bodies are mainly composed of unfolded and misfolded polypeptides that have aggregated as a result of non-specific interactions. The results presented here are in strong agreement with results reported from previous research, which demonstrated by gel filtration heterogenous oligomers due to improper folding of ficolins (Erickson *et al.,* 1997). Bhuyan, 2002 has shown that, in contrast to urea, guanidine-HCl is a highly charged molecule, hence the electrostatic interaction of guanidine-HCl and the *E.coli* protein with this high overall charge could explain the effect of strong aggregation. Gracia-Fruitos et al., 2005 have reported that inclusion bodies are composed of proteins that are correctly folded and have biological activity. Maybe in this case, a small part of the polypeptide of ficolin-B gets purified from inclusion bodies to be biologically active and that again strongly associates with the chitin beads in the chitin column. I also tried by increasing the concentration of cleavage buffer dithiothreitol (DTT) and cleavage time, but in vein. Ficolin-B as a multimeric protein is composed of inter- and intra-chain disulphides and may not be possible for protein expression in the reducing cytoplasm of wild-type *E.coli* which results in aggregation. In addition *E.coli* does not have the ability to support all post-translational modifications that a protein requires for proper folding.

4.1.5. Binding studies with the intein-ficolin-B fusion protein to chitin

It was theoretically possible that the intein-ficolin-B fusion protein loaded onto the chitin column would be properly cleaved, but that the ficolin-B itself would re-bind to the chitin by its carbohydrate recognition site. Therefore, I wanted to check the binding efficiency of recombinant ficolin-B from *E.coli* BL21 (DE3) pLysS cells. All ficolins bind to GlcNac (Teh *et al.,* 2000, Matsushita *et al.,* 1996). The molecular basis of this binding has been shown by X-ray crystallography (Garlatti *et al.*, 2007a, 2007b; Tanio *et al*., 2007). The cell wall analysis of *A. fumigates* has shown that chitin, mannans glycoproteins, and importantly β-1, 3-glucan is extensively distributed on the surface of the fungus (Goldman *et al.,* 1999, Bowmann *et al.,* 2006). Recent studies have shown that L-ficolin binds to β-1, 3-glucan of *A. fumigates* and the binding was not significantly affected by EDTA (Garred *et al*., 2009).

I tested the IPTG-induced crude bacterial lysates of *E.coli* cells that had been transformed with the vector containing the ficolin-B insert for their ability to bind GLcNAc agarose or chitin. But no binding activity was observed (data not shown). This might be due to the improper folding of proteins leading to inclusion bodies. Tanio *et al*., 2009 has shown that multimerization of ficolins is important for effective biological activity and here in this case may not happen in *E.coli.*

Since no binding activity could be observed with the *E.coli* lysate, in collaboration with Katja Hunold, we tested the binding activity to chitin and *A.fumigatis* with the DS2-expressed ficolin-B. The binding studies showed (Fig 3.14) that there was an increased binding to chitin with increasing concentration of ficolin-B independently of Ca2+. EDTA had no significant effect on ficolin-B binding to chitin beads. The binding of ficolin-B to *A.fumigatis* needs to be tested further.

Therefore it can be concluded that with the IMPACT kit system, polypeptides of ficolin-B might be expressed under denaturing conditions but these aggregate and make the intein system useless for ficolin-B purification.

4.1.6. Protein expression in other vector systems

Since we had problems in the expression and purification of ficolin-B with the IMPACT kit system, the next step was to test ficolin-B expression in *E.coli* with different vectors. As a first step we chose a His-tagged vector system as target proteins can be purified by immobilized metal affinity chromatography (IMAC) and immunodedected with His-tagged antibodies. Erickson *et al.,* (1997) previously reported the His-tagged purification of the fibrinogen domain of ficolin protein. About 40% of the ficolin fibrinogen was recovered in the soluble

supernatant. However the protein was bound to the Q column and could not be eluted as it gets aggregated.

Here I tried to express the full length ficolin-B protein with N- or C-terminal His-tags as I was not sure which tag will facilitate better expression of the protein. It was also observed that the molecular weights of the His-tagged ficolin-B (Fig. 3.16) were slightly higher (~37kDa). This difference was expected and is due to the presence of the His-tag at the N- or C-terminal end of the protein (approximately 3.6kDa). However the expression was again shown to be only in the insoluble fraction.

As a next step I chose a GST-tagged vector system, since the use of GST as a fusion tag is desirable because it can act as a chaperone to facilitate protein folding, and frequently the fusion protein can be expressed as a soluble protein (Rancour *et al.,* 2010). We used the pGEX4T1-NTEV vector which has a TEV-cleavage site. As TEV-protease is His-tagged we can easily remove it after cleavage by Ni-NTA agarose. The advantage of TEV is its specifity and that the cleavage efficiency is not much altered by salt or temperature. The full length ficolin-B coding sequence was cloned (Fig 3.18) and expressed (Fig 3.19) in *E.coli* cells. But again ficolin-B was expressed as an insoluble protein. The next step was to express the N-terminal collagen domain of ficolin-B as an individual protein. Girija *et al*., 2007 have shown the gene sequence alignment of ficolins and mannose binding lectin (MBL) and had shown the presence of the putative MASP binding motif within the N-terminal collagen domain of ficolin. I cloned (Fig 3.20) and expressed (Fig 3.21) the N-terminal domain of ficolin-B. The resulting fusion protein had an expected calculated molecular weight of (~31kDa) along with the GST- protein (26 kDa). We were now able to express the N-terminal domain of ficolin-B in fusion with GST in a soluble form. This could be further purified in large scale and tested for its biological activity by protein interaction studies with MASP-2. This would help us to know whether multimerization is important for biological activity.

Therefore it can be concluded from the different expression vector systems shown that full-length ficolin-B cannot be expressed in the soluble form in *E.coli* and this might be due to its complex higher oligomer and multimer structure. In addition, protein conformation might dependent on glycosylation which cannot happen in *E. coli.*

4.2. Mutational analysis of the ficolin-B specificity and biological function

This part of the experimental research was aimed to know the mutational sensitive sites on ficolin-B for its effective biological functions. Since I was unable to express and purify ficolin-B from *E.coli* cells, we decided to do this set of experiments with the ficolin-B expressed in the DS2 cell system, which is already an established system in our lab. Recent point

mutational studies have revealed the essential role of Lys^{55} in the collagenous region of MBL in the interaction with the MASPs and calreticulin (CRT) (M. Lacroix *et al.*, 2009). Endo *et al.,* 2007 showed that the mouse ficolin-B cannot activate the complement system and this may be due to this single amino acid shift (alanine or methionine replaced by glutamic acid in the case of ficolin-B). Girija *et al.,* 2011 provided the proof for the lack of complement activity of mouse ficolin-B being due to the glutamate residue in place of the usual aliphatic/hydrophobic residue in the MASP-binding site. Substitution of the glutamate for the aliphatic alanine residue in rat ficolin-B completely abolished MASP-binding and complement activation, so the acidic residue is particularly disruptive within the ficolin-B which might disturb complement activation.

I undertook to mutate the glutamic acid of ficolin-B lack to alanine and see if this could lead to MASP2-binding and activation of the complement system. The mutants were constructed by the the QuickChange Site-directed Mutagenesis kit (Stratagene) to be tested for biological effects. Weak adjacent sites may enhance or decrease binding affinity and mutants will help to find these difficulties in fine specificity. Recent work by Cymer *et al* 2010 has shown that a single glutamate residue controls the oligomerization, function, and stability of the aquaglyceroporin GlpF.

4.2.1. Production of recombinant wild type ficolin-B and mutants

I expressed and purified the recombinant wild type ficolin-B and the mutant in the DS2 expression system. In our lab, we have the DS2 expression system which utilizes a co-transfection for generation of stably transfected S2 cell lines producing high quantities of recombinant mouse ficolin-B. But the draw back with this method was to get more positive clones during selection with hygromycin. So I cloned the wild type ficolin-B and the mutant gene in a fusion vector (pMT-pCoHygro) (Fig 3.23) that will allow for single transfection for production of more positive clones.

4.2.2. Functional characterization of wild type ficolin-B and mutant ficolin-B

Protein biochemical and chromatographic studies were used to characterise the protein. First I checked for the oligomerization of ficolin-B proteins by SDS-PAGE and gel filtration. Ficolins are highly oligomeric proteins with inter- and intra-chain disulfide bridges (Erickson *et al.*, 1997) and multimerization is important for the protein to be biologically active (Tanio *et al.*, 2007: Garlatti *et al.,* 2009). I found that the mouse ficolin-B migrated as two bands on the SDS-PAGE reducing gel (10%) with an apparent molecular mass of 35 and 36 kDa (Fig. 3.24 A), both of which are greater than the molecular mass of 34 kDa calculated from the amino acid sequence. This was in agreement with the recently published data on rat ficolin-B (Girija

et al., 2010). The reason for the double bands in SDS-PAGE is due to the difference in the glycosylation which was also observed for recombinant pig ficolin-α expressed in a mammalian system (Ohashi and Erickson, 2004). Girija *et al.,* 2010 has reported that the double bands seen in SDS-PAGE is due to the fact that there is one potential N-linked glycosylation site in the fibrinogen domain and five potential O-linked sites in the collagen-like domain. So the two species observed on gels probably reflect different glycoforms. Under non-reducing conditions, ficolin-B migrated as mixture of polymers on SDS-PAGE (10%) gel consisting of more than 8 to 10 chains (Fig 3.24 B).

Next I tested for the oligomerization and stability for ficolin-B in gel filtration. Here I used, "Old ficolin-B" (ficolin-B which was prepared a year before) and "Fresh ficolin-B" which was prepared just before the experiments. With "fresh ficolin B" we observed several overlapping peaks from the gel filtration column, indicating the presence of different oligomeric forms (subsequently called monomer or tetramer of subunits). With "Old ficolin-B" only trace amounts on monomers were observed. Oligomers were identified by comparison with the elution positions of the marker run. The results (Fig 3.25) indicate that the polypeptide (11-13) formed a complex by self-association with an apparent molecular mass of more than 190 kDa, most likely representing tri- or tetramers rather than dimers, whereas the polypeptide (15-17) migrated with an apparent mass less than 40 kDa, suggesting a monomer. Each of the eluted fractions was tested in Western blot for ficolin-B using the rabbit anti ficolin-B antiserum, however, the antibodies did not detect any ficolin-B. The protein content was probably too low (at background levels with the elution profile). The profile indicated only slightly that there might be a protein eluting within the molecular weight range of ficolin-B.

Girija *et al.,* (2011) also reported that the rat ficolin-B eluted as several overlapping peaks from a gel filtration column, indicating the presence of different oligomeric forms. They identified two major oligomers consisting of six and nine polypeptides (subsequently called dimers and trimers of subunits), together with smaller amounts of monomers and tetramers of subunits. Trace amounts of even larger oligomers were observed, probably comprising pentamers and/or hexamers of subunits. They used the eukaryotic expression system for protein production. In our case, I could not detect higher oligomers and this might be due to the difference in post translational modifications in the DS2 system.

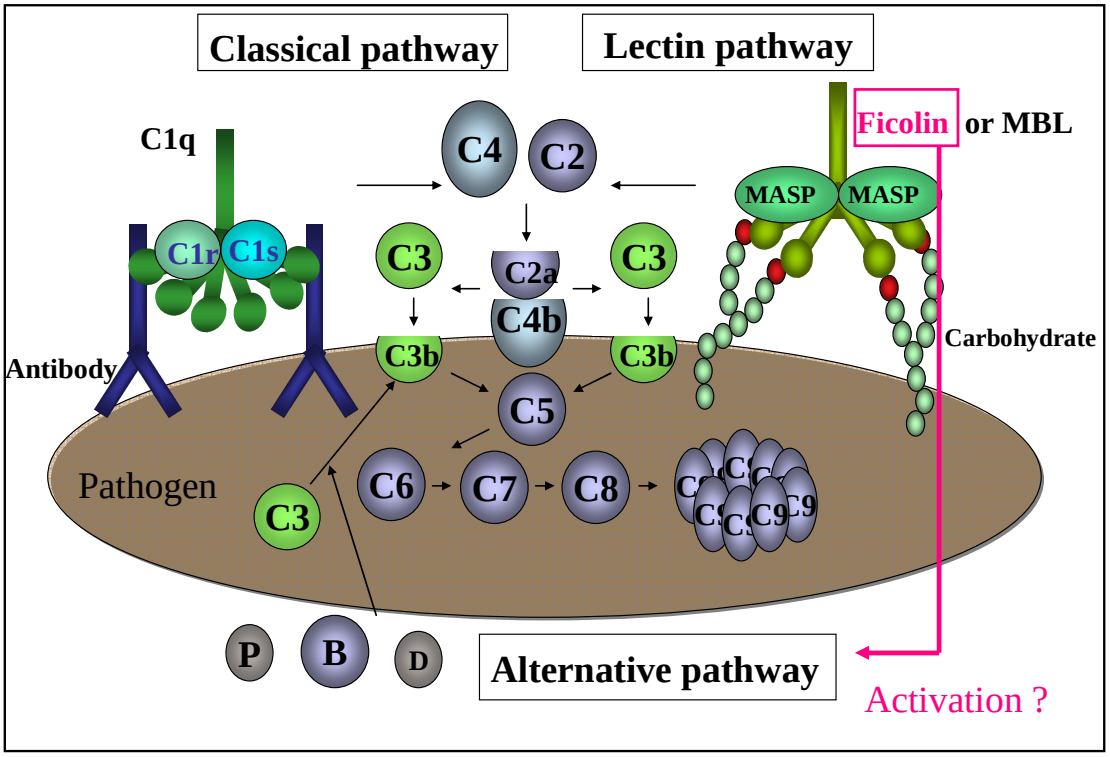
The expression of wild type and mutant ficolin-B expression is still going on. Therefore, in collaboration with Katja Hunold, I was only able to do the intial characterization of the protein regarding size and biological avtivity using DS2- expressed mutant ficolin-B supernatant. We tested for the mutant in supernatant for wild type ficolin-B characteristics in dot blot, Western blot and ELISA. The dot blot (Fig. 3.26) confirmed equally the presence of wild type and

mutant ficolin-B and the Western blot showed that there was no difference in oligomerization between the two proteins (Fig 3.27). Binding studies show that ficolin-B binds to GlcNAc and GalNAc (Endo *et al*., 2005). As already described above, we tested the mutant and wild type ficolin-B binding activity for AcBSA in ELISA (Fig 3.28). We found that there was not much difference in the binding activity between the two proteins and this may be due to the fact that the fibrinogen-like domains of ficolin is responsible for carbohydrate and pathogen recognition which is not affected by the mutation. X-ray crystallography studies on ficolins had demonstrated the molecular basis of GlcNAc-recognition in the fibrinogen-like domains (Garlatti *et al*., 2007a, 2007b; Tanio *et al.,* 2007). The most interesting functional study will be the MASP-2 binding and C4 deposition assay since it is required for activation of the complement system.

5. Conclusion and perspectives

Expression and purification of ficolin-B in the *E.coli* system was not possible because of formation of high oligomeric complex due to inter- and intra-chain disulfide bridges. With the DS2-expressed ficolin-B, some preliminary biochemical characterization was performed. The DS2-expressed recombinant ficolin-B was shown to be at least a trimer by gel filtration analysis, which is a low oligomeric form possibly due to the posttranslational modifications differing from other mammalian expression systems. Multimerization of ficolins is important for the protein to be biologically active. The use of an eukaryotic expression system will allow to produce proteins with the right glycosylations and posttranslational modifications. The wild type and mutant DS2-expressed ficolin-B will be tested for their oligomeric confirmation, complex formation with MASPs and subsequent C4 cleavage activity. It will be interesting to do mass spectrometry analysis of the recombinant proteins to know the details on the glycosylation motifs and potential glycosylation sites in the ficolin-B protein. In addition, Glycan array screening could be performed to know binding specificities for a broad screen of endogenous and exogenous carbohydrates (Emory glycomics lab, US. http://www.functionalglycomics.org/). This will provide a detailes insight into the binding affinities and possible function for ficolin-B.

It will also be interesting to know the effect of the single amino acid mutational change (Glutamate to Alanine) in the collagen domain (which differs from other ficolins) of ficolin-B. The mutant ficolin-B can be used for comparision with wild type ficolin-B in respect to the strength of interaction with MASP-2 by surface Plasmon resonance (SPR). C4-deposition assay will let us know the binding intensity with MASPs and activation capacity for the complement pathway. In the lectin pathway, upon binding of the lectin-MASPs complexes to carbohydrates on the surface of pathogens, MASPs are activated to acquire proteolytic activity for the complement components C4 and C2, resulting in the elimination of pathogens after a chain reaction of proteolysis of complement components and protein assembly. It was recently reported that MBL activates complement component C3 without involvement of C2, implying that it also activates the alternative pathway (Selander *et al.,* 2006). It would be intresting then to know a possible function for ficolin-B in the alternative pathway (Figure 5.1).



Matsushita et al., 2009

Fig. 5.1 Working model for alternative pathway activation by ficolins

APPENDIX

Appendix - I - Abbreviations and acronyms

#	Number
Δ	heat aggregated
A_x	absorbance at a wavelength of x nm
aa	amino acid
Amp^R	ampicilin resistance cassette
AP	alkaline phosphatase or alternative pathway
APS	ammonium persulphate
bp	base pairs
CBD	Chitin binding domain
cDNA	complementary DNA
CP	classical pathway
DEPC-H_2O	diethylpyrocarbonated water
DES	*Drosophila melanogaster* expression system
DNA	deoxyribonucleic acid
dATP = A	deoxyadenosine triphosphate
dCTP = C	deoxycytidine triphosphate
dGTP = G	deoxyguanosine triphosphate
DMEM medium	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
dNTP	deoxyribonucleotide
dTTP = T	deoxythymidine triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminiscence
EDTA	ethylenediaminetetraacetic acid

ELISA	enzyme linked immunosorbant assay
FACS	fluorescence activated cell sorter
fbg	fibrinogen
FCS	fetal calf serum
g	grams
gDNA	genomic DNA
GlcNAc	N-acetyl-D-glucosamine
GPC	gel permeation chromatography
GST	Glutathione –S-transferase
H_2O_2	destilled water
His	histidin
HPLC	High-performance liquid chromatography
HRP	horseradish peroxidase
HSA	human serum albumin
Ig	immunoglobulin
IMAC	ion-metal affinity chromatography
IMP	inosine monophosphate
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobases
kD	kilodalton
LB medium	Luria Bertani medium
LP	lectin pathway
LPS	lipopolysaccharyde
mAb	monoclonal antibody
Mb	megabases
MCS	multiple cloning site
min	minutes
ml	millilitre

mM	millimolar
µM	micromolar
M	molar
MP	millipore water
mRNA	messanger RNA
MurNAc	N-acetylmuramic acid
MW	molecular weight
MWCO	molecular weight cut off
Ni-NTA	nickel-nitrilotriacetic acid
NTA	2-naphthoyltrifluoracetate
OD	optical density
ORF	open reading frame
PAA	polyacrylamide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PECs	peritoneal exudate cells
Pen/Strep	penicillin/streptomycin
PMNs	polymorphonuclear cells
PMSF	phenylmethylsulphonylfluoride
PVDF	polyvinylidene fluoride
RBCs	red blood cells
RIPA buffer	radioimmunoprecipitation buffer
RNA	ribonucleic acid
RT	room temperature
RT-PCR	retrotranscription PCR
S2	Drosophila Schneider-2 cells
sec	seconds

SAP	shrimp alkaline phosphatase
SDS	sodium dodecylsulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SP-A	surfactant protein A
SP-D	surfactant protein D
SPR	surface plasmon resonance
TAE buffer	Tris acetate EDTA electrophoresis buffer
TBE buffer	Tris borate EDTA electrophoresis buffer
TBS	Tris buffered saline
TE buffer	Tris EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylendiamin
TK	thymidine kinase
TOPO	tri-o-octylphosphine oxide
TRITC	tetramethyl rhodamine isothiocyanate
Tween	Tween 20
U	units
vol	volume(s)
WB	Western Blot
wt	wildtype
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
℃	degree celcius

Appendix – II – mouse ficolin-B sequence

```
 29 atggccctgggatctgctgcactattcgtcttgaccctgactgtc
    M  A  L  G  S  A  A  L  F  V  L  T  L  T  V
 74 catgcggctggcacatgcccagaactgaaggtcctagatctggaa
    H  A  A  G  T  C  P  E  L  K  V  L  D  L  E
119 ggctacaagcagctcaccatccttcaaggttgccctggcttgcct
    G  Y  K  Q  L  T  I  L  Q  G  C  P  G  L  P
164 ggagctgcagggcccaagggagaggcaggtgccaaaggagataga
    G  A  A  G  P  K  G  E  A  G  A  K  G  D  R
209 ggagagagtggccttcctggaattcctggaaaagaaggaccaact
    G  E  S  G  L  P  G  I  P  G  K  E  G  P  T
254 ggacccaaaggaaaccaaggagaaaaaggaatacgtggagaaaaa
    G  P  K  G  N  Q  G  E  K  G  I  R  G  E  K
299 ggagactctgggccctctcagtcatgtgctacaggacctcggacc
    G  D  S  G  P  S  Q  S  C  A  T  G  P  R  T
344 tgcaaggagttgctcacccagggccactttctcactggctggtat
    C  K  E  L  L  T  Q  G  H  F  L  T  G  W  Y
389 accatctatctgccagactgcaggcccctgactgtgctgtgtgac
    T  I  Y  L  P  D  C  R  P  L  T  V  L  C  D
434 atggacacagatggtggaggctggaccgtcttccagaggaggctt
    M  D  T  D  G  G  G  W  T  V  F  Q  R  R  L
479 gacggctctgtggacttctttcgggactggacctcatacaagagg
    D  G  S  V  D  F  F  R  D  W  T  S  Y  K  R
524 ggctttggcagccaactaggggagttctggctggggaatgataat
    G  F  G  S  Q  L  G  E  F  W  L  G  N  D  N
569 atccacgctctaaccacccagggaaccagtgagctgcgggtggat
    I  H  A  L  T  T  Q  G  T  S  E  L  R  V  D
614 ctttcagacttcgaaggcaagcatgactttgccaagtacagctcc
    L  S  D  F  E  G  K  H  D  F  A  K  Y  S  S
659 ttccagatccagggagaggccgagaaatacaagcttatcctggga
    F  Q  I  Q  G  E  A  E  K  Y  K  L  I  L  G
704 aacttccttggcggtggtgctggtgactctctgacaccccataac
    N  F  L  G  G  G  A  G  D  S  L  T  P  H  N
749 aacaggttattctccaccaaagaccaagacaatgacggcagtact
    N  R  L  F  S  T  K  D  Q  D  N  D  G  S  T
794 tccagctgtgccatgggttaccatggagcctggtggtactcccag
    S  S  C  A  M  G  Y  H  G  A  W  W  Y  S  Q
839 tgccacacttccaacctgaatggcctctacctgaggggtccccat
    C  H  T  S  N  L  N  G  L  Y  L  R  G  P  H
884 aagagctatgcaaatggtgtcaactggaagtcatggagagggtac
    K  S  Y  A  N  G  V  N  W  K  S  W  R  G  Y
929 aactacagctgcaaggtttctgagatgaaggtgcggctcatctag
    N  Y  S  C  K  V  S  E  M  K  V  R  L  I  *
```

Appendix – III – Construct overview

Construct	Description	Restriction sites	Purpose
pTXB1	Intein tag	SapI / NdeI	*E.coli* expression and purification
pET28a	N-terminal His tag	BamHI / XhoI	*E.coli* expression
pProEXhtb	C-terminal His tag	BamHI / XhoI	*E.coli* expression
pGEX-4T-1	GST tag	BamHI / XhoI	*E.coli* expression
pGEX-4T-N-TEV	GST and His tag	NdeI / XhoI	*E.coli* expression
pMT-Bip-V5-Hygro	V5 His tag	NarI / PmeI	DS2 cell expression

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