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# **The Role of Whey Acidic Protein Four-Disulfide-Core Proteins in Respiratory Health and Disease**

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**Running Head:** The Role of WFDC Proteins in Respiratory Health and Disease

## **Abstract**

Members of the whey acidic protein (WAP) or WAP four-disulfide-core (WFDC) family of proteins are a relatively under-explored family of low molecular weight proteins. The two most prominent WFDC proteins, secretory leukocyte protease inhibitor (SLPI) and elafin (or the precursor, trappin-2), have been shown to possess multiple functions including anti-protease, anti-bacterial, anti-viral and anti-inflammatory properties. It is therefore of no surprise that both SLPI and elafin/trappin-2 have been developed as potential therapeutics. Given the abundance of SLPI and elafin/trappin-2 in the human lung, most work in the area of WFDC research has focused on the role of WFDC proteins in protecting the lung from proteolytic attack. In this review, we will outline the current evidence regarding the expanding role of WFDC protein function with a focus on WFDC activity in lung disease as well as emerging data regarding the function of some of the more recently described WFDC proteins.

## **Keywords**

Host defence; inflammation; lung disease; protease inhibitors

## Introduction

The whey acidic protein (WAP) or WAP four-disulfide-core (WFDC) protein family is a small family of human secreted proteins that possess a wide repertoire of activities, including the ability to inhibit proteases. They are distinguished by the presence of WAP domains as detailed in Table 1 (Insert Table 1). Murine WAP is recognised as the prototype WFDC protein containing two WAP domains of approximately 50 amino acids which include 8 conserved cysteine residues that form the four-disulfide core (FDC) (Drenth et al., 1980; Piletz et al., 1981). The WFDC signature was initially determined from analysis of a large number of FDC domain cysteine arrangements in a range of species (Ranganathan et al., 1999) ((C1-(X<sub>n</sub>)-C2-(X<sub>n</sub>)-C3-(X5)-C4-(X5,X6)-C5-C6-(X2,X3)-C7-(X3,X4)-C8)) where C represents cysteine and X is the number of intervening amino acids. This WFDC signature was then further characterised in humans and FDC-domains have been found in 18 different human proteins to date (Bingle and Vyakarnam, 2008). The WAP domain sequence has been deposited in the PROSITE database (entry number PS51390), where the domain can be detected in 123 different sequences available via UniProtKB/Swiss-Prot (Sigrist et al., 2013).

## WFDC Gene Evolution

Advancements in genomics have allowed the identification of rapidly evolving gene regions in a number of species including humans. One such region, located at 20q13, contains 14 WFDC genes and hence has been named the WFDC locus (Clauss et al., 2002). The WFDC locus can be divided into the centromeric and telomeric sub-loci, both of which contain WAP proteins. Genes for *WFDC5*, *WFDC12*, *PI3* and *SLPI* are found on the centromeric sub-locus, whereas the genes for *WFDC2*, *WFDC6*, *EPPIN*, *WFDC8*, *WFDC9*, *WFDC10A*, *WFDC11*, *QFDC10B*, *WFDC13* and *WFDC3* are found on the telomeric end of this locus as shown in

Figure 1. (Clauss et al., 2011; Ferreira et al., 2013). A number of the WFDC genes on chromosome 20q including *EPPIN*, *WFDC6* and *WFDC8* contain a Kunitz domain in addition to WAP domains, making them WAP/Kunitz-type protease inhibitors (Clauss et al., 2002). The WFDC genes that fall outside of the WFDC locus, including *ANOS1*, *WFIKKN1*, *WFIKKN2* and *WFDC1*, encode larger multi-domain proteins with the exception of *WFDC1*, which consists of a single WAP domain (Larsen et al., 1998). The genes located outside of the WFDC locus are randomly spread in the human genome and are more highly conserved when compared to the 14 WFDC genes at 20q13, which exhibit adaptive evolution, a characteristic of genes involved in reproduction and immunity (Ferreira et al., 2013).

The clustering of the majority of WAP genes on the WFDC locus would suggest that they are potentially derived from an ancestral gene through multiple duplications. Evolution of these WFDC/Kunitz genes alongside the semen coagulum proteins on the WFDC locus may indicate the importance of these genes in male reproduction as many of these genes are expressed in the epididymis and some are known to possess anti-protease and anti-microbial functions which are important for sperm maturation and function (Hurle et al., 2007; Lundwall and Clauss, 2011).

### **The Function of WFDC Proteins**

Using Gene Expression Atlas (EMBL-EBI) and the publicly available mRNA-Seq expression datasets (Petryszak et al., 2016), the expression of the WFDC proteins was assessed in human tissues (Table 1). Some of the WFDC proteins such as SLPI *WFDC1* and *WFDC2* were widely expressed in human tissues. Expression of the WFDC proteins was found to be prevalent in human testis, whereas 16 out of the 18 analysed were found to be expressed in

human lung tissue. SLPI, WFDC2, PI3 and ANOS1 showed the largest expression scores in pulmonary tissue, which may indicate roles for these WFDC proteins in lung homeostasis and disease.

Secretory leukocyte protease inhibitor (SLPI, WFDC4) and trappin-2 or elafin (WFDC14) are among the best characterised WFDC proteins to date, while many of the other family members have undefined biological roles. For the purpose of this review, trappin-2 (also known as pre-elafin) refers to the full length product encoded by the *PI3* gene, whereas elafin refers to the mature processed form of trappin-2, and for discussions involving both molecules, elafin/trappin-2 will be used. WFDC proteins were classically viewed as a family of proteins with roles as protease inhibitors and anti-microbial agents. While serpins like alpha-1 antitrypsin (AAT) bind covalently to cognate proteases and irreversibly distort the structure of the catalytic site to inhibit function (Farady and Craik, 2010), WAP proteins bind non-covalently to the catalytic cleft of the protease like a substrate, thus inhibiting substrate binding (Krowarsch et al., 2003). This mode of action is shared with Kunitz and Kazal-type anti-proteases. The WAP domain consists of a central  $\beta$ -sheet with two external segments linked by a loop that connects the protease binding site (Francart et al., 1997). SLPI has two homologous WAP domains, but the anti-protease active site for chymotrypsin, neutrophil elastase (NE) and trypsin is located in a loop within the COOH-terminus (residues 67-74) with leucine at position 72, the active residue for NE inhibition (Eisenberg et al., 1990; Grutter et al., 1988). Although SLPI can bind and inhibit a range of proteases, it is most potent at inhibiting NE, which is indicated by its low dissociation constant for this protease (Boudier and Bieth, 1992). Elafin/trappin-2 has a more restricted inhibitory spectrum of proteases when compared to SLPI, which is outlined in subsequent sections in more detail.

SLPI and elafin/trappin-2 are reported to exert anti-bacterial activity against a range of Gram positive and Gram negative bacteria including *Pseudomonas aeruginosa* and *Staphylococcus aureus*, however, the biochemical mechanism to explain this function has not been fully elucidated. One hypothesis is that the cationic nature of WFDC proteins allows them to interact and disrupt anionic bacterial cell membranes (Baranger et al., 2008; Zani et al., 2009). Conversely, SLPI and elafin/trappin-2 are also up-regulated in the presence of bacterial infections and bacterial products such as lipopolysaccharide (LPS) most likely due to increased inflammation (Kammouni et al., 1997; Jin et al., 1998; Meyer-Hoffert et al., 2003; Vos et al., 2005). In addition, both SLPI and elafin/trappin-2 have been shown to have roles in mediating responses to pathogenic fungi including *Aspergillus fumigatus* and *Candida albicans* (Tomee et al., 1997; Baranger et al., 2008), and to have anti-retroviral properties towards the human immunodeficiency virus (HIV) in human saliva (McNeely et al., 1995). More recently, Drannik and colleagues demonstrated that elafin and trappin-2 possess anti-viral properties toward the genital herpes virus herpes simplex virus 2 (HSV-2). They report that elafin/trappin-2 have multifaceted activities that can target virus-cell interactions, viral attachment/entry and enhanced inflammatory responses to HSV-2 (Drannik et al., 2013).

Very little data exists on the role/function of the WFDC family as a whole, though some evidence suggests that these proteins have functions beyond the inhibition of proteases. Emerging data highlights specialised immunomodulatory roles, which is especially true for SLPI and elafin/trappin-2, but more research is required to determine if the other WFDC family members demonstrate similar immunomodulatory roles as SLPI and elafin/trappin-2. The remainder of this review will consider the current literature on WFDC proteins, with a

focus on SLPI and elafin/trappin-2, and discuss the functions of this family of proteins in respiratory health and associated diseases.

### **Secretory Leukocyte Protease Inhibitor (SLPI)**

SLPI is an 11.7 kDa protein member of the family (Thompson et al., 1986). It is a 107 amino acid, single polypeptide chain which contains two FDC domains that are cysteine rich (Thompson et al., 1986; Ranganathan et al., 1999). The tertiary structure of SLPI resembles a boomerang shape with the gene located on chromosome 20q12-13.2 (Grutter et al., 1988; Kikuchi et al., 1998). It is a multi-system protein, which is secreted by the mucosal epithelial cells as well as by macrophages and neutrophils (Sallenave et al., 1994; Mihaila et al., 2001). In normal homeostasis, it has several important roles including anti-bacterial, anti-viral, anti-inflammatory and anti-protease activities.

An important function of SLPI in the lung is thought to be the neutralisation of key neutrophil serine protease activity, such as NE, cathepsin G, trypsin, and the mast cell protease, chymase, thereby preventing excessive tissue damage and inflammation (Figure 2). The levels of SLPI increase in response to cellular infiltration and bacterial infection associated with chronic lung inflammation, but may be cleaved by proteases such as NE and cysteine cathepsins and thus downregulated (Taggart et al., 2001; Weldon et al., 2009). Cleavage of SLPI at Thr<sup>67</sup>-Tyr<sup>68</sup> along with protein trimming alters the NE inhibitory site of SLPI (Leu<sup>72</sup>-Met<sup>73</sup>) thereby suppressing the inhibitory effect of SLPI (Taggart et al., 2001; Rudolph et al., 1991). Recent research suggests that, while SLPI does not have a cementin domain, it can be cross-linked to extracellular matrix (ECM) proteins such as fibronectin and elastin by tissue transglutaminase via reactive lysine and glutamine residues



located in its NH<sub>2</sub>-terminal domain, while retaining its anti-protease functions (Baranger et al., 2011). SLPI may play a significant role in the regulation of the NF-κB signalling pathway with effects on IκB degradation reported *in vitro* and *in vivo* (Jin et al., 1997; Lentsch et al., 1999; Taggart et al., 2002; Mikami et al., 2015). In addition, SLPI has been shown to enter the nuclei of monocytic cells and compete with p65 for binding to NF-κB consensus binding sites thus inhibiting the transcription of pro-inflammatory genes such as TNF-α and IL-8 (Taggart et al., 2005).

The anti-fungal and anti-bacterial role of SLPI has been attributed to the NH<sub>2</sub>-terminal domain of the protein (Hiemstra et al., 1996; Tomee et al., 1997). It has the ability to decrease the association between *C. albicans* and epithelial cells as well as diminishing the proteolytic activity from proteases released from *C. albicans* (Curvelo et al., 2014). It is thought that at least some of the anti-bacterial effect of SLPI may be due to its ability to bind and interfere with mRNA translation and bacterial replication (Miller et al., 1989). SLPI has also been shown to possess anti-HIV activity and can prevent the HIV virus from entering immune cells and replicating in these cells (McNeely et al., 1995; Hocini et al., 2000).

### **Elafin (WFDC14)**

Elafin is a 6 kDa serine protease inhibitor derived as a cleavage product from its precursor trappin-2 (pre-elafin) by proteases including the mast cell-derived protease tryptase (Guyot et al., 2005b). Trappin-2, but not elafin, contains an NH<sub>2</sub>-terminal cementin domain and a COOH-terminal WAP domain which is homologous to the second domain of SLPI (Zeeuwen et al., 1997; Schalkwijk et al., 1999). Elafin interacts with NE and proteinase-3 (PR3) through its active site centered at Ala<sup>24</sup>-Met<sup>25</sup> (Ala<sup>62</sup>-Met<sup>63</sup> in trappin-2), however, the NH<sub>2</sub>-terminal

region in trappin-2 was found to be non-essential for inhibitory activity but plays a key role in disulfide bond formation (Tsunemi et al., 1992). Elafin/trappin-2 was initially isolated from human psoriatic skin and bronchial secretions and it was found to be expressed on the surface of epithelial cells, though there are no orthologues found in mice and rats (Schalkwijk et al., 1990; Wiedow et al., 1990; Sallenave & Silva, 1993). It is also expressed by immune cells such as macrophages and neutrophils (Sallenave et al., 1992; Mihaila et al., 2001). Various studies have revealed that elafin/trappin-2 is a multifaceted host defence protein with anti-microbial, anti-protease and immunomodulatory properties (Figure 3) (Simpson et al., 1999; Zani et al., 2009; Butler et al., 2006; Baranger et al., 2008; Williams et al., 2006). Similar to SLPI, elafin and trappin-2 are also transglutaminase substrates, mediated by reactive lysine and glutamine residues (elafin) and the repeating Gly-Gln-Asp-Pro-Val-Lys motifs of the cementin domain in trappin-2 thus permitting cross-linking to ECM proteins (Molhuizen et al., 1993; Nara et al., 1994; Guyot et al., 2005b; Baranger et al., 2011).

Elafin/trappin-2 expression is upregulated at sites of inflammation by an array of pro-inflammatory mediators and it is well-documented to act as an effective but specific inhibitor of the human serine proteases, NE and PR3, and also porcine pancreatic elastase (PPE) as denoted in Figure 3 (Schalkwijk et al., 1991; Wiedow et al., 1991; Sallenave et al., 1994; Pfundt et al., 2000; Bingle et al., 2001; Mihaila et al., 2001; Zani et al., 2009). Unlike SLPI, it has no effect on cathepsin G, trypsin and chymase. However, in a similar manner to SLPI, elafin/trappin-2 is proposed to exert anti-bacterial properties via its capacity to disrupt bacterial cell membranes (Simpson et al., 1999; Baranger et al., 2008; Zani et al., 2009).

In a healthy lung environment, anti-proteases such as SLPI and elafin/trappin-2 are often present at higher concentrations than proteases where they act as potent anti-inflammatory regulators, screening the airways from potential damage. However, when the lung endures excessive inflammatory conditions, associated with diseases such as adult respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD) or Cystic Fibrosis (CF), the protease/anti-protease balance is tilted in favour of protease activity. This elevated protease burden leads to dysregulated extracellular protease activity thus contributing to inflammation via degradation of host defence proteins, increasing susceptibility to infection, as well as pulmonary structural damage. It is interesting to note that one of the most abundant anti-proteases in the lung is AAT which belongs to the serpin family. AAT is largely produced in the liver and it is a major inhibitor of NE, but also has activity against PR3 and cathepsin G (James et al., 1978; Rao et al., 1991). It has been reported that certain mutations in AAT are associated with an increased risk of COPD (Laurel et al., 1963; Foreman et al., 2012). However, more recently, AAT has been shown to possess immunomodulatory activities (reviewed in Stockley et al., 2015), which indicate similarities in functionality to some of the WFDC proteins discussed above. However, as AAT is not part of the WFDC protein family and has no documented interaction with WFDC proteins, its discussion is beyond the scope of this review.

## **Other WFDC Proteins**

### **HE4 (WFDC2)**

In 1991, the gene for WFDC2 (WAP5) which encodes the human epididymis protein 4 (HE4) was cloned (Kirchhoff et al., 1991). The *WFDC2* gene is found on chromosome 20q13.12,

contains two WAP domains and can undergo alternative splicing to generate multiple isoforms of the protein (Bingle et al., 2002). Five mRNA isoforms have been currently identified, 4 as a result of alternative splicing (HE4-V1, V2, V3, and V4) and a further isoform resulting from the use of an alternative promoter (Kirchhoff et al., 1991; Bingle et al., 2002). HE4 expression has been demonstrated in a number of normal and cancerous tissues. Although initially thought to be solely expressed in the epididymis, it has since been shown to be expressed in the epithelia of a number of tissues, including lung, kidney and salivary gland (Bingle et al., 2002; Galgano et al., 2006; Nagy et al., 2016). In addition, HE4 has been found to be frequently overexpressed in respiratory and reproductive malignancies, and has been detected at a lower level in gastrointestinal and renal neoplasms (Schummer et al., 1999; Bingle et al., 2006; Galgano et al., 2006; Kamei et al., 2010; O'Neal et al., 2013).

As with most of the WAP containing proteins, HE4 is small in size (124 amino acids) and secreted. A definitive function for HE4 has not been fully elucidated, nonetheless, a role has been postulated in sperm maturation and it is now known to function as a protease inhibitor. HE4 has been shown to inhibit serine (trypsin, chymotrypsin, prostate specific antigen, and proteinase K) and cysteine proteases (papain) (Chhikara et al., 2012; Hua et al., 2014). As a result, it has been postulated that HE4 is a cross-class anti-protease that may confer protection against microbial virulence factors that possess protease activity (Chhikara et al., 2012). Recombinant HE4 has been shown to bind Gram positive and Gram negative bacterial membranes, and can inhibit the growth of *S. aureus*, albeit weakly (Hua et al., 2014). A recent study reported the presence of HE4 in vaginal secretions and that its expression, which correlated with inflammation, may be induced in response to certain

bacterial species (Orfanelli et al., 2014). In addition, due to its similarities with SLPI and elafin, HE4 is proposed to have a role in innate immune defences of the respiratory system.

### **WFDC12**

The gene for WFDC12 (WAP2) was initially identified on chromosome 20q12-13.1 and has similarities to both elafin and SLPI (Lundwall and Clauss, 2002). The WFDC12 gene has a nucleotide sequence of 774 bp which encodes a 111 amino acid polypeptide. The WAP domain is found at the NH<sub>2</sub>-terminus of WFD12, which without its signal peptide, has a predicted molecular weight of 9.7 kDa (Lundwall and Clauss, 2002). Expression analysis for WFDC12 has shown that it is found in the prostate, skin, lung and oesophagus (Lundwall and Clauss, 2002). Immunostaining for WFDC12 has recently confirmed expression of this protein in human lung tissue (Glasgow et al., 2015). The mouse homologue of WFDC12 known as SWAM2 is found expressed in the tongue and has been shown to elicit anti-bacterial actions against *Escherichia coli* and *S. aureus* (Hagiwara et al., 2003).

### **The Role of WFDC Proteins in Respiratory Diseases**

Evidence suggests that WFDC proteins play an important role in respiratory disease by contributing to the innate immune response as a result of their anti-inflammatory, anti-protease and anti-microbial properties. However, dysregulation of WFDC protein function may also be associated with inflammation in a number of respiratory diseases. As outlined below, research to date suggests that dysregulated expression and/or function of a number of WAP proteins (in particular, SLPI and elafin/trappin-2) may be feature of a number of respiratory diseases, however, further work is required to delineate potential role(s) in disease pathogenesis.

## **Asthma**

Asthma is an airway disease that can present with a number of variable characteristics which varies from patient to patient. The symptoms are often recurring and include wheezing, coughing, dyspnea, reversible airway/flow obstruction and bronchial hyper-responsiveness which emanate from underlying airway inflammation (Desia and Oppenheimer, 2016, Dougherty et al., 2009). Asthma is divided into two main phenotypes; Th2-associated and non-Th2-associated asthma (Wenzel, 2012). Th2-associated asthma includes early-onset allergic asthma, late-onset persistent eosinophilic asthma and exercise-induced asthma, while non-Th2-associated asthma includes obesity-related asthma and neutrophilic asthma (Wenzel, 2012).

While a number of reports indicate that SLPI levels are significantly increased in asthma patients compared to control groups, it is unclear if SLPI plays a protective or detrimental role (Simpson et al., 2005; Hollander et al., 2007; Belkowski et al., 2009). Raundhal et al. recently showed that SLPI expression was significantly lower in bronchial brushings from patients with severe asthma compared to patients with mild-moderate asthma suggesting a potentially protective role (Raundhal et al., 2015). *In vivo*, SLPI decreased airway resistance and IgE concentration, as well as eosinophil and goblet cell infiltration, leading to decreased inflammation and disease pathology (Marino et al., 2011). Furthermore, increased SLPI expression *in vivo* was found to be associated with reduced IL-33; a pro-asthmatic cytokine that may play a role in leukocyte infiltration into the lung (Oboki et al., 2010; Draijer et al., 2016).

Rohde et al. recently reported that elafin levels were elevated in asthmatic patients when compared to healthy controls at day 4 post-experimental rhinovirus infection (Rohde et al., 2014). In this study, elafin levels in asthmatic patients were inversely correlated to a maximal decrease in the peak expiratory flow (PEF) rate. However, no difference in the elafin levels was observed in asthmatic patients at base-line, at day 4 or 6 weeks post-infection (Rohde et al., 2014). Degradation and inactivation of elafin (but not human SLPI) by proteases of the house dust mite *Dermatophagoides pteronyssinus* – a common allergen associated with atopic asthma – may contribute to the pro-inflammatory environment associated with the initiation of an asthmatic episode (Brown et al., 2003).

### **Cystic Fibrosis (CF)**

CF is an inherited autosomal recessive disorder associated with mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, with the delta F508 mutation being the most common mutation associated with a severe pulmonary phenotype (Cutting, 2015). CF leads to defective chloride ion secretion and sodium ion absorption which leads to dehydrated mucus, neutrophil-dominated inflammation, elevated protease activity (including NE) and a progressive decline in lung function. Although Sagel et al. reported no significant difference in SLPI levels between healthy control patients and CF patients, it was noted that SLPI correlated positively with forced expiratory volume in one second (FEV<sub>1</sub>) indicating a potential beneficial effect of elevated SLPI levels in CF (Sagel et al., 2012). In contrast, Muller et al. demonstrated that CF patients had reduced SLPI levels in both the lower and upper airways, which increased following administration of antibiotics in these patients (Muller et al., 2015). Furthermore, it has been reported that both SLPI and elafin are cleaved by NE in CF patients with established *P. aeruginosa* infection (Guyot et al., 2008;

Weldon et al., 2009). Cleaved SLPI and elafin in the CF lung show diminished anti-inflammatory properties including a decreased ability to bind LPS and DNA and reduced immobilization by transglutamination (Guyot et al., 2008; Weldon et al., 2009).

In contrast to SLPI, a significant increase in HE4 protein levels was detected in lung sections from patients with CF who had undergone transplantation compared to normal lung samples (Bingle et al., 2006). HE4 levels were not altered in the large airways, however, in the smaller airways of the peripheral lung, staining for HE4 showed increased and more diffuse staining of epithelia as well as the inflammatory mass in the lumen (Bingle et al., 2006). The upregulation of HE4 in the chronically inflamed CF lung was proposed to result from the phenotypic alteration of cells in the lung and not as a direct result of inflammatory mediators (Bingle et al., 2006). HE4 may also have potential as an inflammatory biomarker as serum levels were found to correlate with CF severity and the level of pulmonary dysfunction (Nagy et al., 2016).

### **Chronic Obstructive Pulmonary Disease (COPD)**

COPD is an umbrella term for a group of chronic lung conditions (including emphysema and bronchitis) that lead to a reduced airflow and inflammation within the lung environment. COPD patients have significantly increased levels of proteases such as NE which can lead to increased tissue damage and progressive worsening of the COPD phenotype (Andelid et al., 2015). Patients with stable COPD presented with significantly higher sputum SLPI levels than patients with COPD experiencing an acute exacerbation (Pant et al., 2009). In addition, lower SLPI levels were detected in COPD patients experiencing frequent exacerbations compared to those with fewer exacerbations (Gompertz et al., 2001). Levels of SLPI in exhaled breath



condensate of COPD patients undergoing a pulmonary exacerbation correlated with forced vital capacity (FVC) (Tateosian et al., 2012). Decreased levels of both SLPI and elafin were detected in the sputum of COPD patients experimentally infected with rhinovirus who presented with secondary bacterial infections (Mallia et al., 2012). As in CF, decreased SLPI (and elafin) levels in COPD may be due, in part, to proteolytic cleavage as a consequence of the altered protease/anti-protease balance (Taggart et al., 2001; Guyot et al., 2008; Weldon et al., 2009). In patients with COPD associated with AAT deficiency, administration of aerosolised AAT resulted in an increase in pulmonary SLPI levels (Geraghty et al., 2008).

### **Acute Respiratory Distress Syndrome (ARDS)**

ARDS is defined as increased capillary permeability and lung inflammation characterized by progressive hypoxemia and pulmonary edema (Yadam et al., 2016). ARDS usually develops after a trauma such as sepsis, pancreatitis or infection, which activates an inflammatory cascade leading to pulmonary damage (Yadam et al., 2016). Similar to findings from chronic lung disease studies outlined previously, altered levels of WFDC proteins have been reported in ARDS. Research into the role of WFDC12 in acute and chronic respiratory disease is still in its infancy, with few publications available for review. However, bronchoalveolar lavage (BAL) fluid from healthy individuals administered LPS and ARDS patient BAL fluid showed significantly elevated levels of WFDC12 protein compared to BAL fluid from healthy controls (Glasgow et al., 2015). Although the role of WFDC12 in the lung is as yet unclear, a role in host defence is hypothesised on the basis of reported immunomodulatory and anti-protease effects (Glasgow et al., 2015).

Increased levels of SLPI were detected in BAL fluid from patients with ARDS (Sallenave et al., 1999; Kerrin et al., 2013). In addition, patients at risk of ARDS, who went on to develop ARDS, had a significantly higher level of SLPI than those at risk who did not progress to ARDS (Sallenave et al., 1999). Altered SLPI levels in ARDS may be limited to the pulmonary compartment as plasma SLPI levels were not significantly altered between critically ill patients at risk for ARDS and ARDS patients (Wang et al., 2009). However, plasma SLPI levels in both the ARDS and at risk groups were higher compared to plasma from healthy individuals (Wang et al., 2009). Research suggests that elafin levels may also be altered in ARDS patients. Although BAL fluid elafin levels were significantly increased at the onset of ARDS compared to samples from healthy volunteers, and intubated and mechanically ventilated patients at risk for ARDS, levels decreased in a temporal fashion at day 3 and day 7 post-onset of ARDS (Kerrin et al., 2013). This decrease was attributed to proteolytic degradation by elevated extracellular 20S proteasome activity (Kerrin et al., 2013). An opposing trend was observed for plasma elafin levels, whereby levels were lower in ARDS patients at onset compared to critically ill patients at risk for ARDS (Wang et al., 2008; Wang et al., 2009). A temporal change was also observed in ARDS patient plasma elafin, as levels were elevated in plasma samples collected pre-ARDS diagnosis compared to ARDS onset (Wang et al., 2009). Whether the alteration in plasma elafin levels reflects redistribution of elafin from the bloodstream to the lung at ARDS onset, altered gene expression or polymorphisms in the PI3/elafin gene remains to be determined (Wang et al., 2008; Wang et al., 2009; Tejera et al., 2009; Tejera et al., 2014).

## **Pulmonary fibrosis**

Pulmonary fibrosis or as it is largely termed idiopathic pulmonary fibrosis (IPF) is a chronic condition in which the lungs become inflamed and undergo persistent scarring leading to a state of fibrosis. Patients with lung fibrosis have difficulty breathing and are often short of breath. The causes of IPF remain unknown though several risk factors including environmental/air pollutants, smoking and certain viral infections are thought to be attributed to the disease (Daccord and Maher, 2016). The number of studies investigating the role of the WFDC protein family in pulmonary fibrosis is very limited to date. Tsoumakidou et al. demonstrated that the levels of SLPI or elafin/trappin-2 did not differ between IPF patients and control patients (Tsoumakidou et al., 2010). However, treatment with SLPI significantly increased survival, and reduced alveolar wall thickness and lung fibrosis in a hamster model of bleomycin-induced lung fibrosis (Mitsuhashi et al., 1996). These beneficial effects were associated with NE inhibition (Mitsuhashi et al., 1996). Habgood et al. noted that bleomycin-exposed SLPI null mice demonstrated a significant increase in active MMP-9 in BAL fluid, but it did not influence the extent of pulmonary fibrosis between WT and SLPI null animals (Habgood et al., 2016). Beyond SLPI and elafin/trappin-2, there is little known about the other WFDC proteins and pulmonary fibrosis. However, a recent study has highlighted a role for HE4 (WFDC2) in renal fibrosis (LeBleu et al., 2013). In this study, HE4 was shown to inhibit serine proteases (Prss23 and Prss35), and the administration of HE4 neutralising antibodies enhanced collagen I degradation and inhibited renal fibrosis. These findings, alongside the fact that HE4 is highly expressed in lung tissue, may suggest a potential role for HE4 in pulmonary fibrosis but further research in this area is needed.

## **Lung Cancer**

The majority of WFDC genes are located on chromosome 20 on a region (q12-q13.1, Table 1) which is frequently altered in a number of cancers (Bingle et al., 2002). Amplifications of this region have been demonstrated in breast, ovarian neoplasms and certain lung cancers (Tanner et al. 1996; Michelland et al., 1999; Sham et al., 2002; Zhu et al., 2007), whereas deletions of this region have been identified in oral squamous cell carcinomas (Imai et al., 2001). This would suggest that genes in this region, which include the majority of WFDC proteins, have some role in carcinogenesis. For the purposes of this review, WFDC research primarily from the lung cancer field is presented.

There are two main types of lung cancer; small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which is further subdivided into adenocarcinoma, squamous cell carcinoma and large cell lung carcinoma. Plasma SLPI levels were found to be significantly higher in lung cancer patients compared to healthy control samples (Ameshima et al., 2000; Zelvyte et al., 2004). Histological analysis further revealed that SLPI levels were significantly increased in NSCLC groups (squamous cell carcinoma and adenocarcinoma) compared to SCLC, and that SLPI increased with the tumour stage and decreased with treatment within the NSCLC groups (Ameshima et al., 2000). SLPI has been shown to significantly increase tumour cell proliferation *in vivo* with SLPI null mice exhibiting reduced lung tumour volume and incidence (Jan Treda et al., 2014). Furthermore, overexpression of SLPI promoted tumour growth and metastasis *in vivo* (Devoogdt et al., 2003 and 2006). SLPI has also been shown to increase spontaneous neoplastic cell metastasis to the lung and lung metastatic relapse in patients as well increasing neo-angiogenesis and vascular mimicry within the tumour *in vivo* (Sugino et al., 2007; Wagenblast et al., 2015). In contrast to this pro-tumourigenic role, SLPI has been reported to reduce tumour cell invasion *in vitro* indicating

that, the pro- or anti-tumour effect of SLPI may be dependent on tumour environment and type (Sugino et al., 2007).

Elafin has also been proposed to have a role in various cancers, including lung cancer and cancers of the upper respiratory tract. Yoshida et al. reported elevated expression levels of elafin in 82.6% of cases of lung squamous cell carcinoma cases and in 73.5% of oesophageal cancer, while low elafin levels were reported in the uninvolved bronchial epithelial tissue and normal oesophageal tissue (Yoshida et al., 2002). Within the upper respiratory tract, elafin was found to be expressed in tumours from larynx, hypopharynx, tonsils, tongue, gingiva and oral cavity where it was noted to be significantly higher in well-differentiated and moderately differentiated carcinomas than in poorly differentiated carcinomas (Westin et al., 2002).

HE4 expression has been detected in a number of lung cancers including adenocarcinomas and mesotheliomas, and to a lesser extent small cell squamous and large cell lung cancers (Bingle et al., 2006; Galgano et al., 2006). Further investigation into the role of HE4 in lung adenocarcinomas revealed that tumours expressing high levels of the HE4-V3 variant had more favourable prognosis than those expressing low levels of this variant (Tokuishi et al., 2012). Studies have shown serum HE4 levels to be elevated in lung cancers (Escudero et al., 2011; Liu et al., 2013) and this has led to serum HE4 being developed as a diagnostic and prognostic marker for lung cancers (Iwahori et al., 2012; Liu et al., 2013; Cheng et al., 2015; Lamy et al., 2015).

## **Clinical Applications of WFDC Proteins**

The development of WFDC proteins (i.e. SLPI and elafin/trappin-2) as potential anti-inflammatory therapies for the treatment of lung disease has been explored in clinical trials over the last 3 decades. Early studies demonstrated that administration of aerosolised SLPI to CF patients resulted in reduced active NE and IL-8 levels in epithelial lining fluid (McElvaney et al., 1992). More recently, SLPI has been evaluated for its ability to reduce elastin levels and inflammation at wound sites (National Institute of Dental and Craniofacial Research, 2000), and as a potential biomarker in the measurement of different treatment outcomes in a range of diseases and innate mucosal immunity (Imperial College London, 2005; Boston Medical Center, 2010). Elafin has been investigated as a potential anti-inflammatory agent in a number of diseases including myocardial injury and inflammation following coronary artery bypass graft-induced ischaemia-reperfusion injury, inflammation due to transthoracic esophagectomy in esophageal cancer surgery cases and pulmonary arterial hypertension (Proteo Inc., 2015). Other trials have reported elafin as a potential biomarker in acute graft-versus-host disease, atopic dermatitis and the development of chronic kidney disease following haematopoietic cell transplant for childhood haematologic malignancies (Assistance Publique - Hôpitaux de Paris, 2014; Icahn School of Medicine at Mount Sinai 2015; Center for International Blood and Marrow Transplant Research, 2015). Focus has also centred on the development of engineered SLPI and elafin/trappin-2 molecules as potential therapeutic inhibitors. The second inhibitory SLPI domain (SLPI2) was fused with the elafin (Elaf) domain in multiple conformations and these new molecules demonstrated improved functional protease inhibition properties compared to parent molecules (Zani et al., 2009). The Elaf-SLPI2 and SLPI2-Elaf chimeras possessed the inhibitory profiles of both elafin (NE and PR3 inhibition) and SLPI2 (NE and cathepsin G inhibition).

Furthermore, Zani et al. produced a trappin-2 variant capable of inhibiting cathepsin G; trappin-2 A62L, in which the P1 residue Ala<sup>62</sup> was replaced by a Leu residue, which is responsible for cathepsin G inhibition in the native SLPI molecule (Zani et al., 2009). Many of these engineered molecules retain the ability to covalently cross-link to extracellular matrices such as fibronectin or elastin by transglutamination while preserving their ability to inhibit serine proteases (Zani et al., 2009). Further work is required to determine whether other biological functions of the parent molecules are retained in these variants.

A number of studies have demonstrated that proteolytic cleavage of SLPI and elafin in various diseases characterized by elevated protease activity may limit their *in vivo* functionality in the lung, and this may be of importance particularly when considering the development of WFDC proteins as potential therapeutic targets. The generation of variants of SLPI and elafin with enhanced resistance to proteolytic cleavage and their successful use in *in vivo* models of acute lung inflammation supports this hypothesis (Small et al., 2015; Camper et al., 2016). A protease-resistant variant of elafin was found to exert a heightened anti-inflammatory effect when compared with the parent elafin molecule in a murine LPS-induced acute lung injury model (Small et al., 2015). Camper et al. showed that a SLPI variant in which Ser<sup>15</sup> and Ala<sup>16</sup> residues were replaced by Gly prevented NE cleavage of SLPI (Camper et al., 2016). Furthermore, the effect of the NE-resistant SLPI variant was investigated in an *in vivo* model of *P. aeruginosa* lung infection and it highlighted the ability of this novel form to exert a more pronounced anti-inflammatory effect compared to the parent SLPI protein (Camper et al., 2016).

## **Conclusion**

Research into the WFDC family and their role in respiratory diseases is very much in its infancy. Whilst a great deal of information has been generated on the role and function of both SLPI and elafin/trappin-2, much remains to be established regarding other WFDC family members as little is known about their expression, activity and roles in pulmonary homeostasis. However, the evidence to date suggests that these proteins may have diagnostic, prognostic and therapeutic potential in respiratory diseases and warrant further investigation.

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## Figure Legends

### **Figure 1: Schematic diagram of the human WFDC locus and the relative locations of the WFDC genes (Located on page 4).**

The WFDC locus spans ~680 kb and is composed two subloci oriented relevant to the centromere (CEN) or telomere (TEL). The 145 kb centromeric subloci consists of four WFDC genes, whereas the telomeric subloci contains a further ten WFDC genes as shown.

### **Figure 2: The role of SLPI in pulmonary inflammation. (Located on page 7)**

SLPI is expressed by macrophages and mucosal epithelial cells during inflammation on the respiratory tract. It is transported to the cell membrane and where it undergoes exocytosis from the cell into the extracellular space. Following uptake by cells, SLPI has the ability to block localised inflammation by preventing p65 binding to nuclear DNA in cells such as monocytes, thereby inhibiting the NF- $\kappa$ B pathway and downstream expression of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-8. Within the extracellular space, SLPI neutralises proteases and pro-inflammatory factors such as cathepsin G, IgE, NE, TGF- $\beta$  and IL-8. Additionally, increased SLPI can also lead to elevated levels of the anti-inflammatory cytokine IL-10. SLPI has also been shown to be increased during bacterial infection and may play a role in controlling neutrophil recruitment during the resolution process.

### **Figure 3. The pleiotropic effects of Elafin/Trappin-2. (Located on page 9)**

Inflammatory mediators such as proteases (NE) and pro-inflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ ) have the ability to induce elafin/trappin-2 expression in human bronchial epithelial cells and various immune cells such as macrophages. Once elafin/trappin-2 is secreted, it has a number of targets. It can eradicate respiratory pathogens like *S. aureus* and *P. aeruginosa* via

indirect opsonisation or modification of the bacterial membrane and it can inhibit vesicular stomatitis virus (VSV) replication and avert HIV attachment to the epithelium. Moreover, elafin/trappin-2 has been shown to be a potent protease inhibitor of NE and proteinase 3, both of which have the ability to act as immunomodulators regulating immune cell infiltration. Elafin/trappin-2 may also modulate the immune response as it can influence neutrophil recruitment and the activation of dendritic cells.



**Table 1:** Human WFDC Proteins: Current nomenclature, number of WAP domains for each WFDC protein, gene location and known function.

Gene Name	Gene Synonyms	Protein Name/Alias	WAP Domains	Gene Loci	Lung Expression level	Tissue expression	Protein Function/Role	Reference
<i>WFDC1</i>	<i>PS20</i>	WAP four-disulphide core domain 1/ Prostate Stromal Protein	1	16q24.1	*	Widely expressed; Highest in testis, prostate, lung	Wound healing, Cell migration, Enhances HIV Infection	Alvarez et al., 2008, Reviewed in (Drannik et al., 2011; Ressler & Rowley.,2011)
<i>WFDC2</i>	<i>HE4, WAP5</i>	WAP four-disulphide core domain 2/Human epididymis protein 4	2	20q13.12	**	Widely expressed; highest in kidney, lung, salivary gland, prostate, testis, fallopian tube	Protease Inhibitor, Anti-microbial, Cancer biomarker	Hellström et al., 2003; Hua et al., 2014
<i>WFDC3</i>	<i>WAP14</i>	WAP four-disulphide core domain 3	4	20q13.12	*	Prostate, Testis	Not defined	
<i>SLPI</i>	<i>WFDC4, ALP, BLPI, WAP4, HUS1, ALK-1</i>	Secretory leukocyte peptidase inhibitor/ Antileukoproteinase	2	20q13.12	***	Widely expressed; highest in salivary gland, lung, skeletal muscle, oesophagus, fallopian tube, prostate, kidney, breast	Protease inhibitor, Anti-microbial, Anti-inflammatory, Wound healing	Reviewed in Majchrzak-Gorecka et al., 2015
<i>WFDC5</i>	<i>WAP1</i>	WAP four-disulphide core domain 5	2	20q13.11	*	Skin, Tonsil, Testis	Not defined	
<i>WFDC6</i>	<i>WAP6</i>	WAP four-disulphide core domain 6	1	20q13.11	*	Testis	Anti-microbial, Protease inhibitor	Yenugu et al., 2004
<i>EPPIN</i>	<i>WFDC7, WAP7</i>	Epididymal peptidase inhibitor	1	20q13.12	*	Testis	Anti-microbial, Protease Inhibitor	McCrudden et al., 2008; Yenugu et al., 2004
<i>WFDC8</i>	<i>WAP8</i>	WAP four-disulphide core domain 8	3	20q13.11	*	Testis	Not defined	
<i>WFDC9</i>	<i>WAP9</i>	WAP four-disulphide core domain 9	1	20q13.12			Not defined	
<i>WFDC10A</i>	<i>WAP10</i>	WAP four-disulphide core domain 10A	1	20q13.12		Testis	Not defined	
<i>WFDC10B</i>	<i>WAP12</i>	WAP four-disulphide core domain 10B	1	20q13.12	*	Testis	Not defined	
<i>WFDC11</i>	<i>WAP11</i>	WAP four-disulphide core domain 11	1	20q13.12	*	Testis	Not defined	
<i>WFDC12</i>	<i>WAP2</i>	WAP four-disulphide core domain 12	1	20q13.12	*	Testis, Lung, Skin, Prostate	Protease inhibitor, Transglutaminase substrate	Glasgow et al., 2015
<i>WFDC13</i>	<i>WAP13</i>	WAP four-disulphide core domain 13	1	20q13.11	*	Testis	Not defined	

<i>PI3</i>	<i>WFDC14, ELAFIN, WAP3, SKALP</i>	Peptidase Inhibitor 3/trappin-2/elafin/skin-derived anti-leucoproteinase	1	20q13.12	**	Tonsil, Oesophagus, Lung, Skin, Breast	Protease inhibitor, Anti-microbial, Anti-inflammatory, Transglutaminase substrate	Reviewed in Verrier et al., 2012
<i>ANOS1</i>	<i>WFDC19, KALIG1</i>	Anosmin 1	1	Xp22.32	**	Testis, Lung, Skin, Adrenal Gland	Adhesion protein, Neuronal development	Reviewed in Choy & Kim., 2010
<i>WFIKKN1</i>	<i>WFDC20A, WFIKKN, RJD2</i>	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 1	1	16p13	*		Protease inhibitor, Growth factor binding	Nagy et al., 2003
<i>WFIKKN2</i>	<i>WFDC20B, WFIKKNRP</i>	WAP, follistatin/kazal, immunoglobulin, Kunitz and netrin domain containing 2	1	17q21.33	*	Testes, Ovary	Protease inhibitor, Growth factor binding	Kondás et al., 2008

**Abbreviations:** PS20 – prostate stromal protein, HE-4 – human epididymis protein 4, ALP - antileukoproteinase, BLPI – bronchial leukocyte proteinase inhibitor, SKALP – skin derived anti-leukoproteinase, KALIG - Kallmann's syndrome interval gene 1.

Key: \* = Detectable expression, \*\*\* = Highly expressed

Figure 1.

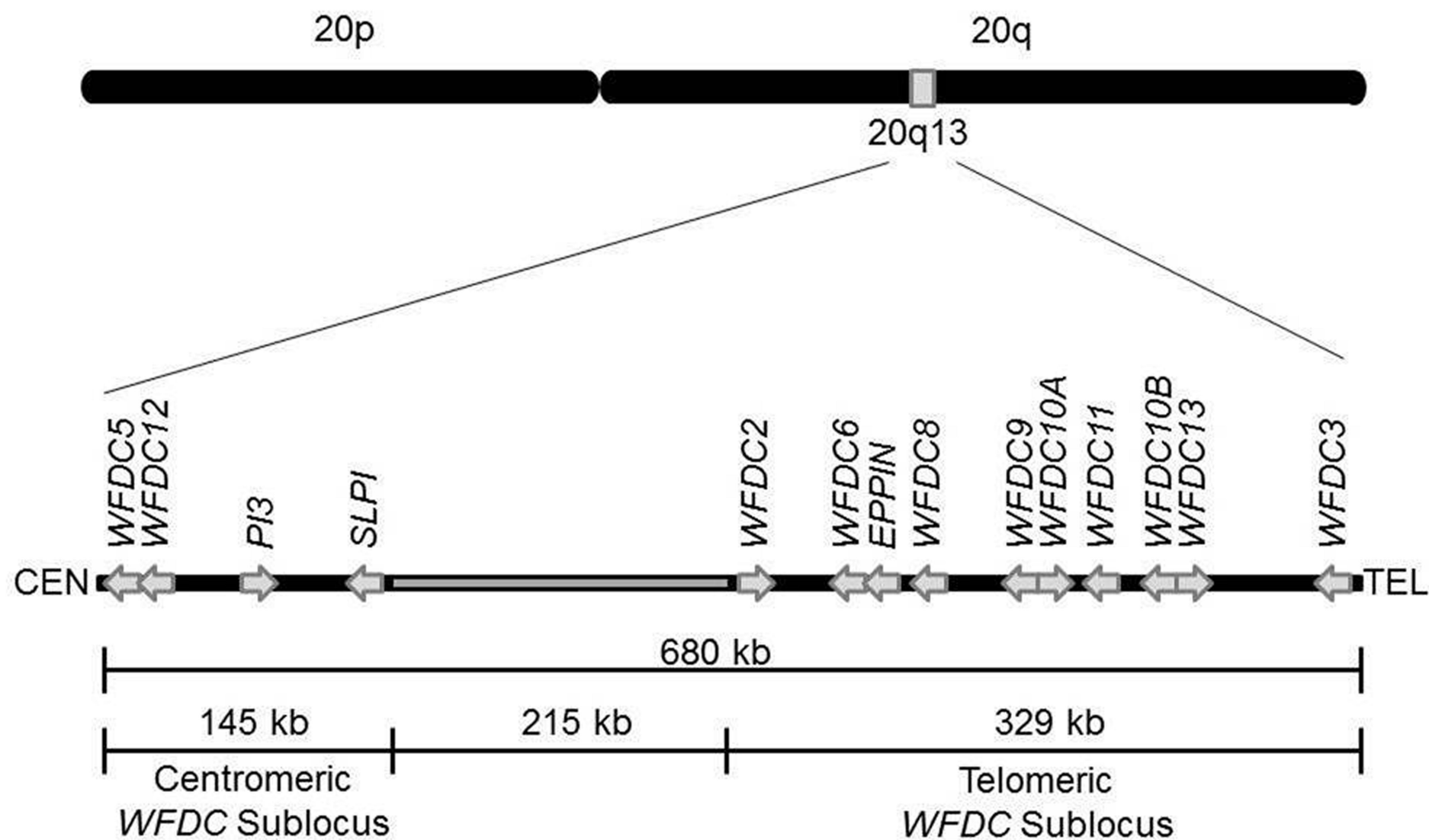


Figure 2.

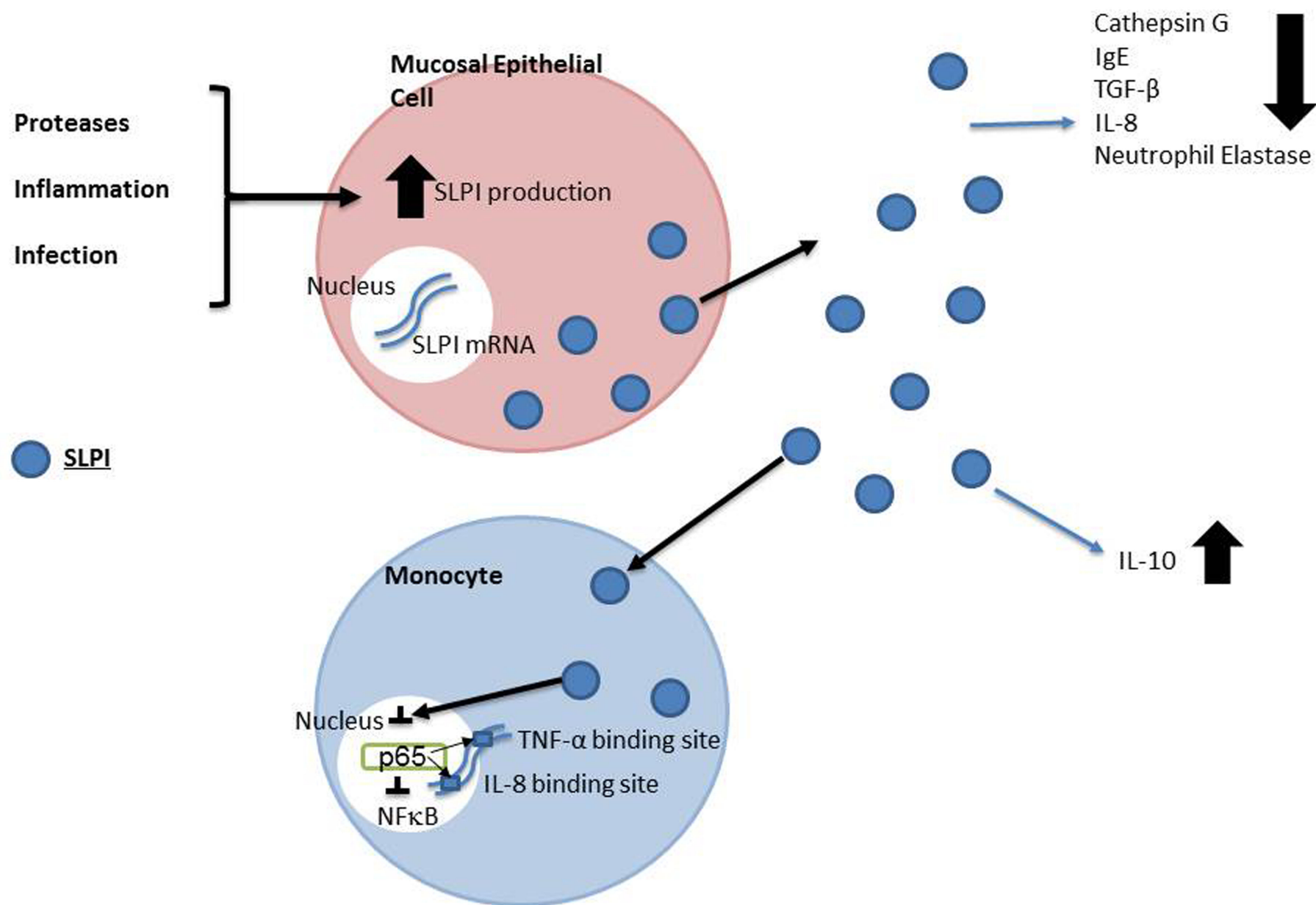


Figure 3.

