



# Pentraxins CRP-I and CRP-II are post-translationally deiminated and differ in tissue specificity in cod (*Gadus morhua* L.) ontogeny

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## ABSTRACT

Pentraxins are fluid phase pattern recognition molecules that form an important part of the innate immune defence and are conserved between fish and human. In Atlantic cod (*Gadus morhua* L.), two pentraxin-like proteins have been described, CRP-I and CRP-II. Here we show for the first time that these two CRP forms are post-translationally deiminated (an irreversible conversion of arginine to citrulline) and differ with respect to tissue specific localisation in cod ontogeny from 3 to 84 days post hatching. While both forms are expressed in liver, albeit at temporally differing levels, CRP-I shows a strong association with nervous tissue while CRP-II is strongly associated to mucosal tissues of gut and skin. This indicates differing roles for the two pentraxin types in immune responses and tissue remodelling, also elucidating novel roles for CRP-I in the nervous system. The presence of deimination positive bands for cod CRPs varied somewhat between mucus and serum, possibly facilitating CRP protein moonlighting, allowing the same protein to exhibit a range of biological functions and thus meeting different functional requirements in different tissues. The presented findings may further current understanding of the diverse roles of pentraxins in teleost immune defences and tissue remodelling, as well as in various human pathologies, including autoimmune diseases, amyloidosis and cancer.

## 1. Introduction

Pentraxins are ancient pattern recognition molecules that evolved alongside the complement system and are conserved throughout phylogeny from arthropods to mammals. They play important roles in innate immunity, homeostatic regulation and the acute phase response, which is set off by injury, infection or other trauma and involves the immune system as well as other biological and physiological processes (Pepys et al., 1978; Robey and Liu, 1981; Martinez de la Torre et al., 2010). Pentraxins have been classified into four groups, two belonging to the group of long pentraxins (PTX3 and neural pentraxins), while C-reactive protein (CRP) and serum amyloid protein (SAP) are the short pentraxins and prototypical mammalian acute phase proteins of hepatic origin present in serum (Ballou and Kushner, 1992; Martinez de la Torre et al., 2010). Human CRP and SAP share 51% amino acid identity and are believed to be products of a gene duplication event over 500 million years ago (Shrive et al., 1999; Bayne and Gerwick, 2001).

CRP are pentameric non-covalently associated globular protomers, with approximately 206 amino acids folded into two anti-parallel beta-sheets, with each subunit of 23 kDa molecular mass. Each of the five subunits is linked by disulphide bonds (Shrive et al., 1996; Thiele et al., 2015). There is some variation in CRP structure as while CRP in zebrafish has been found to form trimers (Chen et al., 2015), cod CRP was revealed to have a pentameric structure by electron microscopy (Gísladóttir et al., 2009), as does human CRP. CRP binds to phosphorylcholine on pathogen surfaces and can also bind to nuclear histones, chromatin and small nuclear ribonucleoproteins (Du Clos, 1996; Ansar and Ghosh, 2013). CRP activates the complement pathway via C1q binding and has thus roles both in the clearance of bacteria as well as of altered and dying cells (Mihlan et al., 2011; Thiele et al., 2015). While CRP is not pro-inflammatory under physiological conditions, its hepatic synthesis is increased in response to injury and can aggravate existing tissue injury in a complement-mediated manner, for example in myocardial infarction and ischemic cerebral injury (Griselli et al., 1999;

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Gill et al., 2004). CRP also binds to low-density lipoprotein in a  $\text{Ca}^{2+}$  dependent manner and is linked to atherosclerosis (Reynolds and Vance, 1987; Sun et al., 2005). While changes in CRP levels are linked to various autoimmune diseases and cancer, the effects of structural changes in CRP are gaining increased interest (Ji et al., 2007; Eisenhardt et al., 2009; Thiele et al., 2014; Braig et al., 2017; Bello-Perez et al., 2017a).

SAP is a 25 kDa pentameric glycoprotein and recognises carbohydrates, amyloid fibrils and nuclear substances (Xi et al., 2015). While it is not an acute phase protein in human, it serves as an acute phase protein in mice, where CRP does not display acute phase protein function (Cathcart et al., 1965). SAP binds to lipopolysaccharide (LPS) on various bacteria and can prevent LPS-mediated complement activation and LPS-toxicity (De Haas et al., 1999; De Haas et al., 2000). SAP is associated to systemic amyloidosis, Alzheimer's disease and transmissible spongiform encephalitis (Pepys et al., 1994), and has been suggested to serve as a chaperone in amyloidosis by binding to the pathological amyloid cross-beta-sheet structures (Agrawal et al., 2009). SAP is also associated to chromatin degradation and can bind to early apoptotic cells (Gershov et al., 2000; Lu et al., 2012).

In fish, both CRP and SAP-like pentraxins have been detected in serum and while some species have both types, such as rainbow trout (*Oncorhynchus mykiss*) (Murata et al., 1994, 1995), dogfish (*Mustelus canis*) (Robey et al., 1983) and plaice (*Pleuronectes platessa*) (White et al., 1981), others have either CRP or SAP-like pentraxins. For example in channel catfish (*Ictalurus punctatus*), Japanese eel (*Anguilla japonica*), murrel (*Channa punctatus*), carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*), CRP forms are found (Szalai et al., 1994; Nunomura, 1991; Mitra and Bhattacharya, 1992; Cartwright et al., 2004; Kovacevic et al., 2015), while SAP proteins are found in Arctic char (*Salvelinus alpinus* L.), Atlantic salmon (*Salmo salar*), halibut (*Hippoglossus hippoglossus* L.), wolffish (*Anarhichas lupus*) and snapper (*Pagrus auratus*) (Jensen et al., 1997; Lund and Olafsen, 1998, 1999; Cook et al., 2005; Lee et al., 2017). These classifications have generally been based on N-terminal amino acid sequence analysis and calcium-dependent ligand binding specificity to either phosphorylcholine (for CRP) or phosphoethanolamine, agarose, zymosan, glycans, DNA and chromatin (for SAP) (Tennent and Pepys, 1994). In zebrafish, 7 isoforms of CRP have been identified and to date zebrafish is the only fish CRP crystallized and has been shown to form trimers rather than pentamers (Chen et al., 2011).

Both CRP and SAP are resistant to proteolysis, the resistance to proteolysis of human CRP is  $\text{Ca}^{2+}$  dependent, and human CRP has a slightly shorter half-life of 19 h compared to 24 h for SAP (Hawkins et al., 1990; Vigushin et al., 1993; Agrawal et al., 2009). In addition, pentraxins have been shown in some cases to be under hormonal control (Coe and Ross, 1990; Szalai et al., 1998). While the glycosylation of CRP and SAP has been studied, and CRP has for example been shown to vary in glycosylation patterns and binding characteristics in different pathological conditions (Das et al., 2004), other post-translational modifications, including deimination, have not been studied before and may further understanding of the functional repertoire of CRP and SAP depending on tissue type and environmental factors. Post-translational protein deimination is receiving increasing attention in the medical field due to emerging critical roles in a variety of pathologies, including autoimmune diseases, central nervous system insult and degeneration, as well as cancer (Vossenaar et al., 2003; György et al., 2006; Wang and Wang, 2013; Lange et al., 2014, 2017; Witalison et al., 2015a). Protein deimination is caused by peptidylarginine deiminases (PADs), a family of calcium dependent enzymes, which cause irreversible conversion of protein arginine to citrulline in target proteins in a  $\text{Ca}^{2+}$  - dependent manner, leading to structural and functional changes of target proteins (Fig. 1; Vossenaar et al., 2003; György et al., 2006; Bicker and Thompson, 2013). Each conversion of an arginine into a citrulline leads to a loss in charge and decreased molecular mass of 1 Da. This can affect protein-protein interactions, protein structure and hydrogen bond

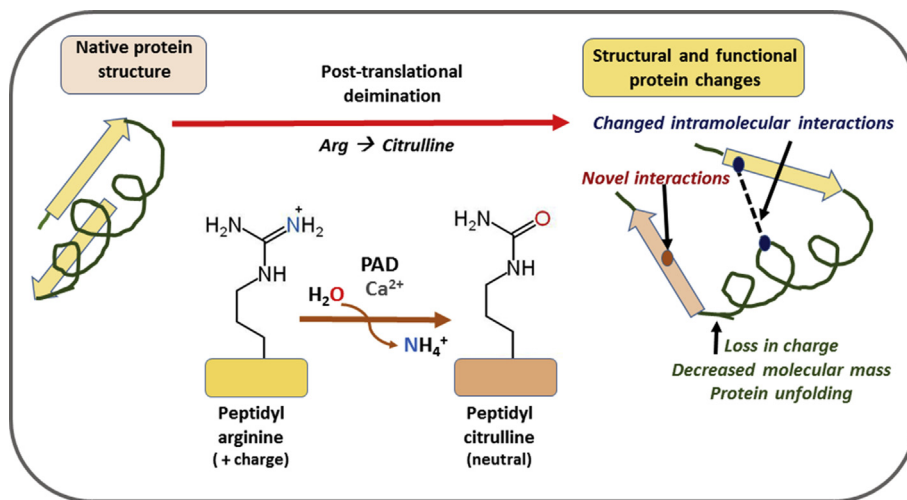
formation, as well as cause denaturation (Tarcza et al., 1996; Witalison et al., 2015a). Structures most prone to deimination are beta-sheets and intrinsically disordered proteins, while the position of the arginine is also important; arginines sitting next to aspartic acid residues are most prone to citrullination, arginines next to glutamic acid residues are rarely citrullinated and those flanked by proline are poorly citrullinated (Nomura, 1992; Tarcza et al., 1996; György et al., 2006). PADs are conserved through phylogeny from bacteria to mammals, and while five tissue-specific isozymes are present in mammals, only one is present in fish (Vossenaar et al., 2003; Rebl et al., 2010). PAD is found in the cod genome (Star et al., 2011) and was recently verified at the protein level and shown to have deiminating activity in both cod serum and mucosa, where 38 deiminated mucosal target proteins were identified, including nuclear, immune-related, metabolic and cytoskeletal proteins. In addition, deiminated proteins, including histone H3, were detected in various organs and mucosal tissues during early cod ontogeny and in immunostimulated cod larvae (Magnadóttir et al., 2018).

The two CRP forms in cod under study here were previously classified as belonging to the CRP type pentraxins due to their binding specificity to phosphorylcholine, while N-terminal amino-acid analysis showed higher similarity for CRP-I to SAP. The two forms were previously shown to vary in overall charge, glycosylation, pentameric and subunit molecular size (Gisladóttir et al., 2009). In relation to our previous studies on innate immune factors during cod ontogeny, including CRP and complement factors (Magnadóttir et al., 2004; Lange et al., 2004, 2005), we set out to identify whether the two cod CRP forms differed in tissue specific localisation during early cod ontogeny. While both pentraxin forms were strongly detected in liver, extrahepatic detection of both forms was found. CRP-I was dominant in nervous tissue of brain and eye, while CRP-II showed strong specificity to mucosal surfaces of gut and skin throughout early ontogeny. To gain further understanding of putative functional differences due to post-translational modifications, that can affect structural changes (Fig. 1) and facilitate protein moonlighting, an evolutionary acquired phenomenon allowing proteins to exhibit more than one physiologically relevant biochemical or biophysical function within one polypeptide chain (Henderson and Martin, 2014; Jeffrey, 2018), we set out to identify whether the two cod CRP forms were post-translationally deiminated. Due to the conserved function of pentraxins throughout phylogeny, this would be of high importance also for human pathologies as protein deimination has previously not been described for either CRP or SAP in fish or mammals. We show here for the first time deiminated forms of CRP and SAP-like pentraxins, also varying in deimination between mucus and serum. This highlights novel roles for post-translational deimination in pentraxin protein moonlighting and may shed novel light on the differing responses of CRP and SAP in various associated pathologies, such as autoimmune diseases, amyloidosis and cancer.

## 2. Materials and methods

### 2.1. CRP-I and CRP-II sequence alignment and phylogenetic reconstruction

CRP and SAP sequences were retrieved from Ensembl (<http://www.ensembl.org/index.html>) and NCBI (<https://www.ncbi.nlm.nih.gov/>), translated to protein and multiple sequence alignment was performed using the MUSCLE sequence alignment tool (<https://www.ebi.ac.uk/Tools/msa/muscle/>). Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Phylogenetic relationships of the pentraxin proteins were inferred using the Neighbour-Joining method under the conditions of the Poisson correction distance model and pairwise deletion of gaps. Bootstrap analysis with 10,000 replicates was used to assess nodal support. The analysis involved only full length protein sequences of short pentraxins (CRP and SAP) from species representing a range of taxa: teleost represented by zebrafish (*Danio rerio* CRP (pentraxin fusion protein) AET80950.1), rainbow trout (*Oncorhynchus*



**Fig. 1. Molecular scheme of post-translational protein deimination.** Peptidylarginine deiminase (PAD) causes deimination/citrullination of arginine in a calcium-dependent manner. Deimination/citrullination is the catalysis of peptidyl arginine to peptidyl citrulline residues, using oxygen from water and releasing nitrogen as ammonia.

mykiss, CRP NP\_001118193.1; SAP XP\_021435889.1), common carp (*Cyprinus carpio carpio* CRP-like-1: AEU04518.1; CRP-like-2: AEU04520.1), goldfish (*Carassius auratus* CRP AKO22072.1), Arctic char (*Salvelinus alpinus* L. CRP XP\_023864349.1; SAP XP\_023862668.1), Atlantic salmon (*Salmo salar* SAP CAA67765.1), Northern pike (*Esox Lucius* SAP NP\_001290956.1); and Atlantic cod (*Gadus morhua* L. pentraxin ACZ06557.1; Seppola et al., 2009, which was similar as CRP-I P86688.1 isolated by Gísladóttir et al., 2009), CRP-II P86689.1 (full length sequence used from Gísladóttir et al. (2009) (Supplementary Fig. 1); amphibian xenopus (*Xenopus laevis* CRP NP\_001165686.1; SAP NP\_001008175.1); reptilian python (*Python bivittatus* SAP: XP\_007444909.1) and alligator (*Alligator mississippiensis* SAP XP\_006272649.1); avian red junglefowl (*Gallus gallus*, CRP NP\_001034653.1); Syrian hamster (*Mesocricetus auratus*, CRP AAB19893.2; SAP P07629.2), mouse (*Mus musculus* CRP NP\_031794.3; SAP EDL39002.1), rat (*Rattus norvegicus* SAP EDL94729.1); and man (*Homo sapiens*, CRP AAL48218.2; SAP NP\_001630). Arthropod horseshoe crab (*Limulus Polyphemus*; CRP AAA28268.1 and SAP AAL55404), was used as outgroup. CRP and SAP sequences were further analysed for putative disordered regions using FoldIndex® (Uversky et al., 2000; Prilusky et al., 2005; <https://fold.weizmann.ac.il/fldbin/findex>).

### 2.1.1. Larval sampling

Experimentally farmed cod (*Gadus morhua* L) larvae were obtained from the Marine Research Institute Mariculture Laboratory, Stadur, Grindavik, Iceland; reared as described before (Steinarsson and Björnsson, 1999; Lange et al., 2004). Cod larvae were collected during the hatching season from the same hatching batch at 3–5, 7, 14, 21, 28, 35, 50 and 84 days post hatching (d.p.h; for the relationship between days after hatching and body length in mm see Supplementary Fig. 2). Four larvae for each date were collected, fixed in 4% formalin in phosphate buffered saline (PBS) at 4 °C for 24 h, followed by embedding in paraffin for tissue sectioning and histological analysis.

### 2.1.2. Mucus sampling and preparation

A pool of cod mucus was carefully collected from the dorsal side of the body of 10 individual adult fish (2–3 year old; 400–1000 g, reared at 4–9 °C), gently using a glass slide to avoid contamination with blood or epithelium cells. The mucus pool was immediately frozen on dry ice and protein extracted according to Al-Harbi and Austin (1993). In brief, mucus was homogenized and dialyzed in PBS at 4 °C, protein extracted from the isolated protein pellet using 50% saturated ammonium sulphate for 1 h at room temperature and thereafter dialysed in saline for 48 h at 4 °C. Precipitated protein isolates were quantified by Bradford assay (Bradford, 1976) and reconstituted in 2 × Laemmli buffer for Western blotting analysis.

### 2.2. Immunoprecipitation

Cod pentraxins CRP-I and CRP-II were immunoprecipitated from the protein extract of cod mucus (2.2.3) and a pool of serum from 5 individual adult cod respectively. Immunoprecipitation was performed using the Catch and Release® v2.0 Reversible Immunoprecipitation System (Merck, U.K.) according to the manufacturer's instructions, using monospecific polyclonal mouse anti-CRP-I and CRP-II antibodies previously described (Gísladóttir et al., 2009). Bound proteins were eluted and analysed by Western blotting.

### 2.3. Western blotting

Immunoprecipitated CRP-I and CRP-II from cod mucus and serum, as well as crude mucus and serum protein extracts, were heated to 100 °C in 2 × Laemmli buffer containing 5% β-mercaptoethanol before separation by SDS-PAGE using 4–20% Mini-Protean TGX protein gels (BioRad, U.K) and thereafter analysed by Western blotting using the monospecific mouse anti-cod CRP-I and CRP-II antibodies, as well as the monoclonal F95 mouse IgM antibody, that was raised against a deca-citrullinated peptide and specifically detects protein citrulline (Nicholas and Whitaker, 2002), for the detection of putative deiminated/citrullinated sites in CRP-I and CRP-II. The detection of deiminated forms of CRP-I and CRP-II was thus assessed by using the F95 antibody to blot the immunoprecipitated CRP fractions, isolated from mucus and serum using the monospecific CRP-I and CRP-II antibodies respectively, as described in 2.3. Approximately 5 µg of protein was loaded per lane, even load was assessed using Ponceau S staining (Sigma, U.K.), membranes were thereafter blocked in 5% bovine serum albumin (BSA) in Tris buffered saline with 0.01% Tween20 (TBS-T) for 1 h, followed by incubation at 4 °C overnight with the primary antibodies (anti-CRP-I and anti-CRP-II 1/1000; F95 1/5000). Membranes were then washed three times in TBS-T, incubated at room temperature for 1 h with HRP-conjugated secondary antibodies (anti-mouse IgG or anti-mouse IgM; BioRad, U.K.), followed by six washes in TBS-T before visualisation with ECL (Amersham, U.K.). Membranes were imaged using the UVP transilluminator (UVP BioDoc-IT™ System, U.K.).

### 2.4. Immunohistochemistry

Paraffin blocks were kept at room temperature and 5 µm serial tissue sections cut and placed on SuperFrost®/Plus microscope slides (Manzel Gläser, U.S.A.) and stored at room temperature until used. Immunohistochemistry was performed as previously described (Lange et al., 2004), with slight modifications. The primary antibodies used were anti-CRP-I and CRP-I (1/100; monospecific polyclonal mouse) and



F95 (1/100; monoclonal mouse-IgM); for detection of CRP forms and deiminated proteins respectively. Sections immunostained with CRP-I and CRP-II antibodies were visualised with fast red solution and background stained with 1% methylene blue, while tissue sections immunostained with F95 were visualised with diaminobenzidine/hydrogen peroxide (DAB) and background stained with haematoxylin blue. As a negative control, normal mouse ascitic fluid was used, which contained IgG1, IgG2a, IgG2b, and IgG3 as verified with the ISOSTrip Mouse Monoclonal Antibody Isotyping Kit, following the manufacturer's instructions (Boehringer Mannheim, Germany). Four larvae were analysed for each developmental stage.

### 3. Results

#### 3.1. Phylogenetic comparison of pentraxin (CRP and SAP) protein sequences, arginine sites and disordered regions

Phylogenetic analysis showed that both cod CRP forms grouped closer with human CRP than human SAP, while cod CRP-I grouped closest with SAP-like pentraxins from rainbow trout (*Oncorhynchus mykiss*) and Arctic char (*Salvelinus alpinus*) and cod CRP-II grouped most closely with carp (*Cyprinus carpio carpio*), goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*) CRP-like pentraxins (Supplementary Fig. 3A). The two cod CRP forms differed in number of disordered regions as CRP-II had one while CRP-I contained none as assessed by FoldIndex<sup>®</sup> (Supplementary Fig. 3B). Based on multiple sequence alignment of the whole amino acid sequences (using Clustal Omega), cod CRP-I and CRP-II showed close identity to both human CRP and SAP, while the two cod CRP forms differed in number of arginine residues, with CRP-I containing 8 arginines (at sites 53, 60, 86, 128, 133, 158, 187, 228) and CRP-II containing 11 arginines (29, 38, 46, 48, 57, 62, 96, 104, 113, 116, 118). Notably only 4 of these arginines occupied the same amino acid location (Supplementary Fig. 3C). Some of the cod arginine residues are conserved between the two CRP forms, zebrafish and human (Supplementary Fig. 3C).

#### 3.2. Western blotting of deiminated forms of CRP-I and CRP-II and proteins in mucus and serum

Immunoprecipitated CRP-I and CRP-II proteins from cod serum and mucus, as well as crude cod serum and mucus protein isolates, were analysed by Western blotting for presence of CRP-I, CRP-II and post-translational deimination (Fig. 2). All protein samples were heated to 100 °C in 2 × Laemmli buffer containing 5% β-mercaptoethanol before separation by SDS-PAGE. In crude mucus and serum extracts both CRP-I and CRP-II showed differing banding patterns. CRP-I was strongly detected in mucus with two prominent bands in the 37 and 45 kDa regions, one band around 75 kDa and another band at 100 kDa, while in serum, the 37 kDa band was hardly detectable, the 45 kDa band was present as well as the 75 and 100 kDa band, but an additional prominent band at 50 kDa was observed in serum, that was not present in the mucus sample (Fig. 2A). For CRP-II, in mucus some prominent bands were seen at 50 kDa and just below 75 kDa, and these were also detected in serum, while in serum an additional thick band (possibly representing a few bands close together) was prominent just below 50 kDa (Fig. 2B). Both CRP-I and CRP-II immunoprecipitated proteins showed signs of post-translationally deiminated forms as detected by the pan-deimination F95 antibody, which detects deiminated proteins by binding to protein citrulline (Fig. 2A and B (F95)). Deimination positive bands differed somewhat between mucus and serum. For CRP-I two single deiminated bands were observed in mucus at 50 and 75 kDa respectively, while in serum the 50 kDa band was much broader, indicating the presence of several F95 positive bands in close proximity in this region. These deimination positive bands corresponded with CRP-I positive bands detected in whole serum and mucus protein extract at 75 kDa and in the 50 kDa range in serum, while CRP-I positive bands in

mucus were around 37 and 45 kDa. As deimination changes protein conformation (Vossenaar et al., 2003; Witalison et al., 2015a) it may possibly have affected the migration observed here of deiminated CRP compared to non-deiminated forms in SDS-PAGE. For CRP-II a similar difference in deiminated binding pattern was observed between mucus and serum with two deiminated bands at 50 and 75 kDa in mucus, albeit the 50 kDa band was fainter, while in serum, the deiminated band in the 50 kDa region was very strong, indicating also the presence of several deiminated bands in this size range. The deimination positive bands detected around 50 kDa correlated with the narrow CRP-II positive bands detected at 50 kDa in both mucus and serum, and the further strong CRP-II positive bands detected just below 50 kDa in serum, while the 75 kDa deimination positive band migrated higher compared to the CRP-II band detected just below 75 kDa, both in serum and mucus. Thus there seemed some difference in migration of putative deiminated CRP subunits in this size range. The presence of deiminated total proteins in crude mucus and serum protein extract, using the pan-deimination F95 antibody, revealed considerably higher levels of total deiminated proteins in serum compared to mucus (Fig. 2C).

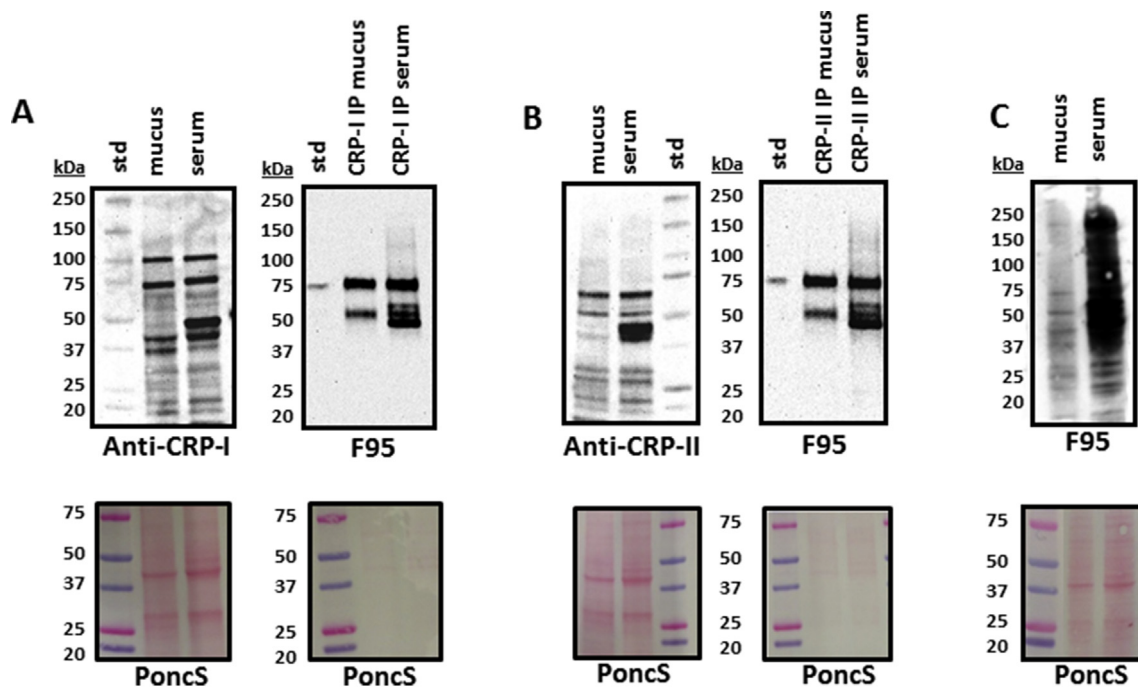
#### 3.3. Histological analysis of CRP-I, CRP-II and deiminated proteins in cod ontogeny

##### 3.3.1. CRP-I and CRP-II in hepatic tissue in cod larvae ontogeny 3–84 days post hatching

The main detection site for both CRP forms was the liver, where they were clearly detected in hepatocytes from 7 d.p.h. onwards. A temporal difference in hepatic detection of the two forms was observed (Fig. 3). While CRP-I detection diminished at 50 d.p.h. and was hardly visible at 84 d.p.h., CRP-II remained strongly detectable at 50 d.p.h. and slightly reduced, but still clearly present, at 84 d.p.h. CRP-I showed a stronger specificity for brain and eye while CRP-II showed a stronger mucosal association, particularly in gut and skin. This difference was particularly prominent at 35 d.p.h. (Fig. 3; Table 1). The temporal and spatial pattern of protein detection for CRP-I and CRP-II was consistent in all 4 fish analysed for each developmental stage.

##### 3.3.2. Detection of CRP-I in cod larvae 3–84 days post hatching – hepatic and extrahepatic detection

CRP-I specific immunodetection in nervous tissue is highlighted in Fig. 4a, also showing deiminated proteins in corresponding regions. During ontogeny CRP-I was detected as following: at 3–7 d.p.h. a faint response was seen for CRP-I in the plexiform layers of eye and a faint detection in liver. At 11 d.p.h. a strong response was seen in the liver as well as in the inner and outer plexiform layers and in rods and cones (photoreceptor layer) of the eyes. By 14 d.p.h. CRP-I was detected in the axons and photoreceptors of the eyes and positive signal was increased in the liver. On day 17 some response was also detected in the cell fibrils of the brain and increased on day 21, where a reaction was also seen in the liver and in pancreas (Fig. 5A). At 28 d.p.h. and 35 d.p.h. the detection in the liver was strong. At 35 d.p.h. very strong reaction was seen in cell fibrils of brain (Fig. 4a-i), in the inner plexiform layer and inner nuclear layer of the eyes (Fig. 4a-ii), and in the spinal cord (Fig. 5B). Some positive response was seen in myofibrils of skeletal muscle (Fig. 5C). Some faint positive detection was seen in the brush border in wall of the intestines at 17–50 d.p.h. By day 50 a strong reaction was seen in the liver, in chondrocytes (Fig. 5D) and the head kidney was positive while at 84 d.p.h. only the liver showed a faint positive reaction. Some unspecific staining, mainly defined to the brush border, was seen in the intestines on day 84 (Table 1). The characteristic tissue distribution identified for the two CRP forms as described above was consistent in all 4 cod larvae tested for each developmental stage.



**Fig. 2.** Western blotting showing CRP-I and CRP-II and deiminated CRP forms in cod mucus and serum. **A)** CRP-I was immunoprecipitated from adult cod mucus and serum using the mono-specific mouse anti-cod CRP-I antibody. Eluted CRP protein samples were heated to 100 °C in 2 × Laemmli buffer containing 5% β-mercaptoethanol before separation by SDS-PAGE using 4–20% Mini-Protean TGX protein gels. CRP-I elutes were thereafter analysed for post-translational protein deimination using the monoclonal mouse IgM pan-deimination F95 antibody, which detects protein citrulline (Nicholas and Whitaker, 2002), for detection of putative deiminated forms of CRP-I. Deimination positive bands (F95) were seen at 75 and 50 kDa in mucus, while in serum a deimination positive band was also at 75 kDa but in the 50 kDa range several bands were F95 immunopositive. These deimination positive bands correspond with CRP-I positive bands detected in whole serum and mucus protein extract at 75 kDa and in the 50 kDa range in serum, while CRP-I bands in mucus were around 37 and 45 kDa. As deimination changes protein conformation it may possibly affect migration of deiminated CRP compared to non-deiminated forms in SDS-PAGE. Also, a difference in CRP subunit patterns was observed between mucus and serum while CRP-I positive bands were detected in mucus and serum at 100, 75, and 45 kDa; a 50 kDa band was strongly expressed in serum, but not in mucus, which had a 37 kDa band that was stronger expressed compared to serum. All protein samples were heated to 100 °C in 2 × Laemmli buffer containing 5% β-mercaptoethanol before separation by SDS-PAGE. **B)** CRP-II was immunoprecipitated from adult cod mucus and serum using a mono-specific mouse anti-cod CRP-II antibody. Eluted samples were heated to 100 °C in 2 × Laemmli buffer containing 5% β-mercaptoethanol before separation by SDS-PAGE using 4–20% Mini-Protean TGX protein gels. CRP-II protein elutes were blotted against the pan-deimination antibody F95, for detection of putative post-translationally deiminated forms of CRP-II. Deimination positive bands were detected at 50 and 75 kDa in mucus, while in serum several bands in the 50 kDa region were strongly detected as well as a 75 kDa band. The deimination positive bands detected around 50 kDa correlate with the narrow CRP-II positive bands detected at 50 kDa in both mucus and serum, and the further strong CRP-II positive bands detected just below 50 kDa in serum, while the 75 kDa deimination positive band migrates higher compared to the CRP-II band detected just below 75 kDa, both in serum and mucus. Thus there seems some difference in migration of putative deiminated CRP subunits in this size range. **C)** Total deiminated proteins, as detected by the pan-deimination F95 antibody, are detected at lower levels in mucus compared to serum, while protein load, as assessed by Ponceau red staining, indicates even protein load. Protein standard (std) is indicated in all blots and the corresponding PonceauS (PoncS) staining is shown as a loading control beneath each Western blot. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

### 3.3.3. Detection of CRP-II in cod larvae 3–84 days post hatching – hepatic and extrahepatic detection

CRP-II specific immunodetection in mucosal tissue is highlighted in Fig. 4b, also showing deiminated proteins in corresponding regions. At 3–7 d.p.h. some positive detection for CRP-II was seen in the liver, the intestines and in photoreceptors of the eyes at 7 d.p.h. At 11 d.p.h. a relatively weak response was seen in the inner and outer plexiform layer of the eyes, a reaction was also seen in the liver and a sporadic response in the brain. By day 14 the liver detection was strong and the sacciform cells of skin showed a positive reaction as well as axons and photoreceptors of the eyes. At 17 d.p.h. some response was seen in the inner and outer plexiform layers of the eyes, albeit not as strong a reaction as seen with the anti-CRP-I antibody. At 21 d.p.h. the liver detection had become very strong and positive reaction was also seen in the pancreas (Fig. 5E). At 28 d.p.h. a strong reaction in the liver was maintained and reaction was also seen in the small intestines and the skin as well as in chondrocytes (Fig. 5H). On day 35 the liver detection was very strong (Figs. 3 and 4B) and a strong reaction was seen in intestines, particularly in goblet-like cells of the small intestines (Fig. 4b-i), as well as in sacciform cells of epidermis and mucus

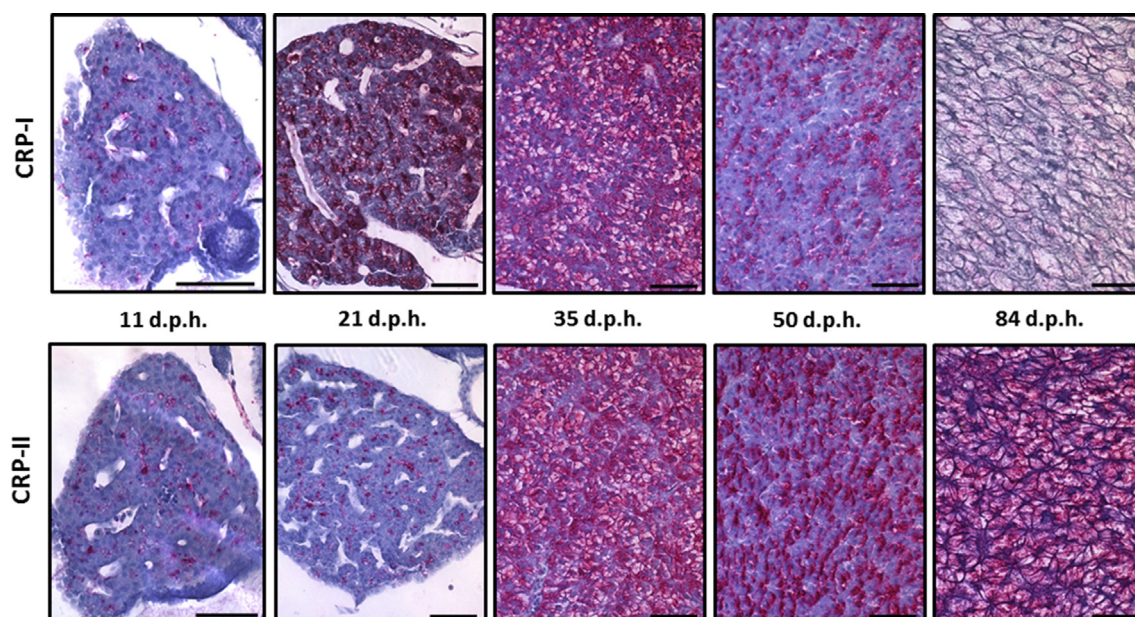
(Fig. 4b-ii and Fig. 5G). Chondrocytes also showed strongly immunopositive. On day 35, neither the eyes nor the brain showed any reaction (Fig. 4b). At 84 d.p.h. the liver showed a strong positive detection, as well as fibrous layer in notochord (Fig. 5F), while no positive staining showed in brain or eyes (Table 1).

### 3.3.4. Detection of deiminated proteins in cod larvae

Deiminated proteins, as identified by the pan-deimination antibody (F95) were detected in corresponding sites to CRP-I and CRP-II in cod ontogeny, namely in brain (Fig. 4c), eye (Fig. 4d), gut (Fig. 4e), liver (Fig. 4f), pancreas (Fig. 5I), spinal cord and notochord (Fig. 5J), muscle and sacciform cells in epidermis (Fig. 5K), chondrocytes of gills (Fig. 5L) and kidney (not shown).

## 4. Discussion

For the first time, deiminated forms of CRP and SAP-like pentraxins have been identified and are shown in cod serum and mucus. Both cod CRP forms differ in number of arginine residues, which are putative candidates for post-translational deimination, some of which are



**Fig. 3. Immunohistological analysis of hepatic CRP-I and CRP-II in cod ontogeny.** Representative histological figures of CRP-I and CRP-II protein detection in liver are shown in cod ontogeny at 11, 21, 35, 50 and 84 d.p.h. Both forms are detected in hepatocytes at all stages examined. While the levels of CRP-I and CRP-II were similar at 11 d.p.h.; CRP-I detection was relatively stronger in liver hepatocytes at 21 d.p.h. than CRP-II, but thereafter CRP-I levels reduced on day 50 and CRP-I immune-detection was very low in hepatocytes at 84 d.p.h. Protein-levels of CRP-II was lower at 21 d.p.h. compared to CRP-I, but similar to CRP-I at 35 d.p.h.; thereafter CRP-II protein detection was visible stronger in hepatocytes at both 50 and 84 d.p.h. All figures are photographed using a 40× objective and scale bars represent 50 µm in all figures.

**Table 1**

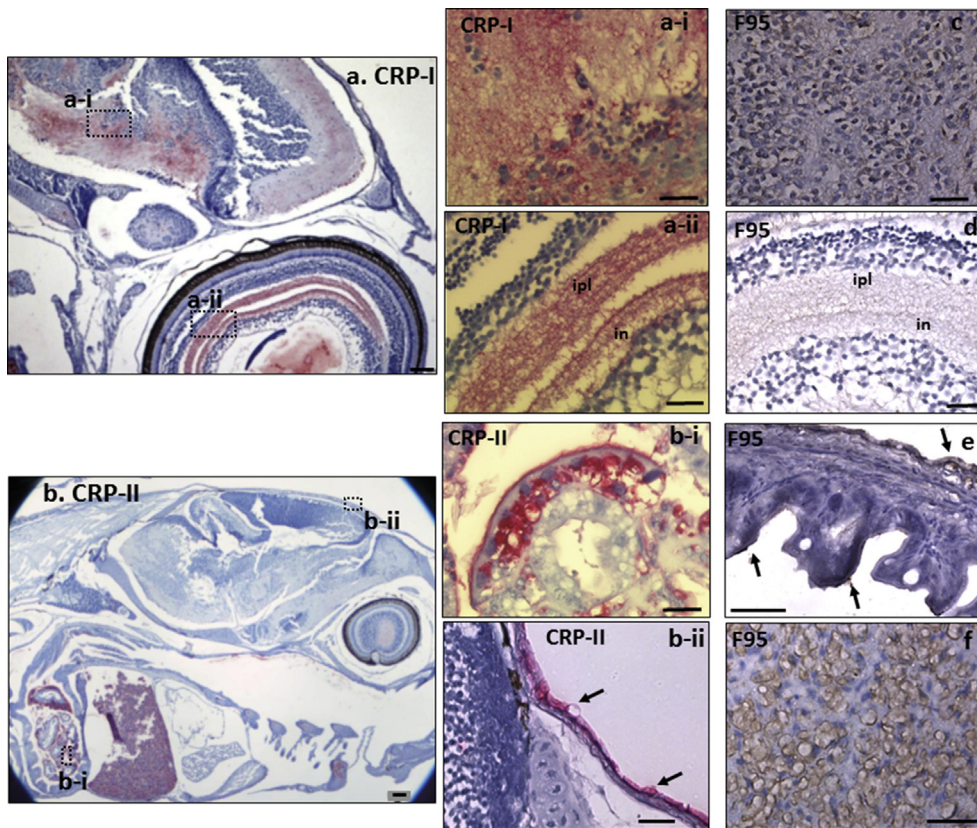
**CRP-I and CRP-II in organs and tissues during early cod ontogeny.** A schematic overview of immunohistochemical detection of CRP-I and CRP-II in various organs of cod larvae from 3 to 84 days post hatching (d.p.h.).

Organ	CRP-I	CRP-II
	Detection at days post hatching:	Detection at days post hatching:
Brain	17–35	negative
Eye	3–35 (prominent)	3–17 (faint)
Spinal cord	35	negative
Notochord	negative	84
Intestines	17–50 (faint and restricted to brush border)	11–50 (prominent in goblet cells)
Kidney	50	negative
Pancreas	21	21
Liver	7–84	3–84
Skin	7–84 (low at all stages)	7–84 (prominent at all stages)
Chondrocytes of gills	28–84	28–84
Muscle	21–35	7–84

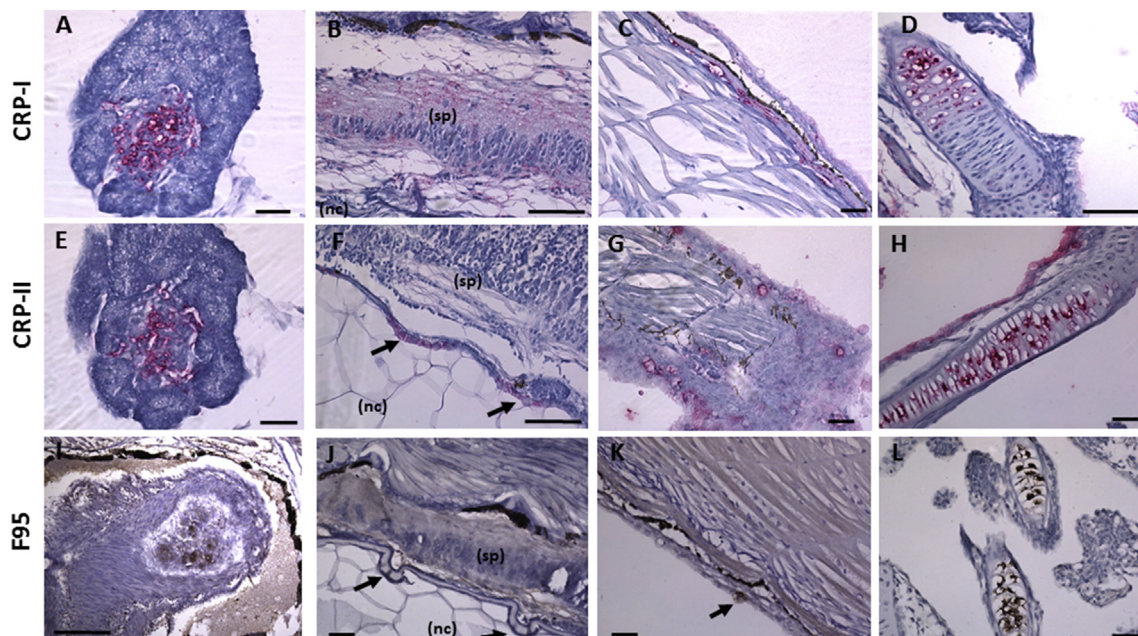
conserved between the two isoforms and also throughout phylogeny (Supplementary Fig. 3C). Disordered regions, which are the most prone structures for undergoing post-translational protein deimination, are revealed here in a variety of pentraxins throughout phylogeny, including in cod CRP-II (Supplementary Fig. 3B). Phylogenetic analysis, based on amino acid sequence alignment, showed that both cod CRPs grouped closer to human CRP than SAP, while CRP-I grouped more with some teleost SAP-like pentraxins and CRP-II grouped closer with some teleost CRP-like pentraxins. This correlates with previous findings where, albeit both forms were classified as CRP pentraxins based on binding specificity, N-terminal sequence analysis of cod CRP-I showed closer similarity to SAP while cod CRP-II was closer to CRP (Gisladóttir et al., 2009). This trend was further reflected in the immunohistochemical detection during cod ontogeny, revealing some

distinctive differences in tissue localisation of the two forms. CRP-I dominated in brain, spinal cord, eye and kidney, while CRP-II was significantly more prominent in mucosal tissues of skin and gut. Both temporal and spatial differences were observed between the two forms in the various tissues. CRP-I showed considerably higher detection in parts of the nervous system i.e. white matter of brain, and inner and outer plexiform layers and photoreceptors of the eye from 5 d.p.h. onwards, peaking at 35 d.p.h., while only very faint levels of CRP-II were detected in the photoreceptor layer of the eye between 7 and 14 d.p.h. which were thereafter was not detected at older stages. A particularly striking difference was observed between the two CRP forms in nervous tissue of brain, spinal cord and eye at 35 d.p.h., where a very strong positive response was seen for CRP-I, while CRP-II showed no positive reaction (Figs. 4 and 5). CRP-I was also detected in head kidney, which did not show positive for CRP-II in these samples. A prominent strong mucosal association for CRP-II was evident in mucosal tissues of intestines and skin, showing positive in goblet cells and sacciform cells (Fig. 4b). Both forms showed strong hepatic detection, indicating the liver to be the main production site of both CRP types, albeit CRP-II appeared sooner and showed a stronger reaction at the older stages tested (50 and 84 d.p.h.; Fig. 3). These findings are consistent with that the liver is the main production site of CRP and SAP (Hutchinson et al., 1994), while extrahepatic pentraxin detection has previously been described (Murphy et al., 1991), including in muscle (Rees et al., 1988), smooth muscle cells in atherosclerotic plaques (Yasojima et al., 2001), kidney (Jabs et al., 2003), respiratory tract (Gould and Weiser, 2001), lung epithelia (Dong and Wright, 1996; Ramage et al., 2004), cervical mucus (Raffi et al., 1977) and in brain (Yasojima et al., 2000; Mulder et al., 2010). The strong nervous tissue detection of Cod CRP-I shows thus a more similar tissue localisation to human SAP, which for example binds to fibrillary deposits in dementia brains (Rostagno et al., 2007) and to amyloid plaques in Alzheimer's disease brains, where it is also associated with wound repair (Mulder et al., 2010). SAP is also associated to other amyloidosis, as well as liver and renal fibrosis where SAP inhibits proteolytic cleavage and stabilises fibril aggregates (Xi et al., 2015). As a pattern recognition receptor, SAP removes opsonised





**Fig. 4. Histological immunostaining of CRP-I and CRP-II.** a) CRP-I in brain and eye. Immunohistochemical detection of CRP-I in the brain and eye at 35 d.p.h.: CRP-I was clearly seen in the brain in cell fibrils of the medulla oblongata (a-i); The forebrain and optic tectum fibrils were also positive. CRP-I was also strongly expressed in the eye (a-ii): the internal plexiform layer (ipl), consisting of the dense reticulum of fibrils of retinal ganglion cells and cells of the inner nuclear (in) layer are strongly positive. b) CRP-II in mucosal tissues. A strong detection was seen in liver and intestines at 35 d.p.h. while brain and eye were negative. b-i) A distinctively strong detection was observed in goblet cells of the intestines; b-ii) Strong positive was seen in mucosal layer and sacciform cells (arrows) of epidermis. c-d) Deiminated proteins (F95) were detected in corresponding sites to CRP-I and CRP-II using the pan-deimination F95 antibody in: c) medulla oblongata of brain; d) eye; e) mucosal layer of gut and epidermis (arrows); f) liver; note positive hepatocytes. Scale bars represent 100  $\mu$ m (a; b) and 50  $\mu$ m (a-i; a-ii; b-i; b-ii; c-f).



**Fig. 5. Examples showing CRP-I and CRP-II extrahepatic immunopositive detection, as well as deiminated proteins at different developmental stages. A-D) CRP-I in:** A) Pancreas, strong positive in Island of Langerhans at 21 d.p.h.; B) Spinal cord at 35 d.p.h., note positive in neuronal tissue of spinal cord (sp) while notochord (nc) is negative; C) Muscle at 21 d.p.h., positive in striated myofibril in muscle cells and fibroblasts covering muscle cells; D) Chondrocytes of gill arches at 28 d.p.h.; **E-H) CRP-II in:** E) Pancreas at 21 d.p.h., strong positive in Island of Langerhans; F) CRP-II in fibrous layer of notochord (arrows) is positive at 84 d.p.h – note negative in spinal cord for CRP-II; G) Sacciform cells are strongly positive in mucosal epidermis at 50 d.p.h.; H) Chondrocytes of gill arches at 28 d.p.h., note also strong positive in mucosal epidermis; **I-L) Deiminated proteins (F95) in:** I) Island of Langerhans in pancreas at 57 d.p.h.; J) Spinal cord is positive (sp) and peripheral and fibrous layer (arrow) of notochord (nc) at 28 d.p.h.; K) Muscle fibres, epidermis and sacciform cells (arrow) of skin at 28 d.p.h.; L) Chondrocytes of gills at 28 d.p.h.; . All scale bars represent 50  $\mu$ m.

and apoptotic cells (Ma et al., 2011) and is thus an important factor in tissue remodelling after tissue damage. Structural conformation of SAP, for example via post-translational deimination, may play roles in its function and this is also interesting in relation to findings on horseshoe crab SAP-like pentraxin, which contains four disordered regions (Supplementary Fig. 3B) and has been shown to have two molecular aggregations with different calcium binding sites; while sequence homology with human SAP is low, the structural homology is high and binding specific preferences similar (Shrive et al., 2009).

The deimination and mucosal detection of both cod CRP forms is of considerable interest as teleost skin mucosa is representative of human mucosal surfaces I of respiratory tract, gut and uterus (Gomez et al., 2013). In several fish species, either a single pentraxin type or both short types (CRP and SAP), have been isolated and characterised in serum (White et al., 1981; Szalai et al., 1994; Jensen et al., 1995, 1997; Lund and Olafsen, 1998; Kovacevic et al., 2015; Lee et al., 2017; Shi et al., 2018), while pentraxins have also been shown to form part of the humoral defence in mucosa of some fish (Yano, 1996; Jones, 2001). Mucosal pentraxins have for example been described in skin mucosa in the common skate (*Raja kenoi*) (Tsutsui et al., 2009), lumpsucker (*Cyclopterus lumpus*) (Patel and Brinchmann, 2017) and Atlantic salmon (*Salmo salar*) (Valdenegro-Vega et al., 2014). In zebrafish (*Danio rerio*), a multigene family of CRP-like proteins has been identified and structural conformations studied, albeit not post-translationally modified forms, while mucosal forms were not described (Chen et al., 2011, 2015; Falco et al., 2012; Bello-Perez et al., 2017a). Some studies on pentraxin involvement in the acute phase response of fish have been carried out. For example, turpentine injection resulted in an eighteen fold increase serum level of CRP in channel catfish (*Ictalurus punctatus*) (Szalai et al., 1994) while reduced serum levels of CRP were seen in rainbow trout (*Oncorhynchus mykiss*) and both CRP and SAP levels were reduced in plaice (*Pleuronectes platessa* L.) (White et al., 1981). The change in the gene expression of pentraxins during acute phase response has also been studied in some fish species following infection, inflammation or stress induction with varying results (Cairns et al., 2008; Talbot et al., 2009; Kovacevic et al., 2015; Bello-Perez et al., 2017b; Shi et al., 2018). In previous studies on the two cod CRP forms, infection or acute phase induction appeared to have non-significant effect on the serum levels of CRP-I or II (Magnadóttir et al., 2010, 2011), while increased gene expression of both pentraxins was observed in anterior kidney, inducing cortisol release and cytokine (IL-1 $\beta$ ) stimulation (Audunsdóttir et al., 2012). In granulomatous disease in cod, CRP-II was detected at stronger protein level than CRP-I and thus seems to be the more immune-related form in cod (Magnadóttir et al., 2013; Gudmundsdóttir et al., 2014). CRP has been identified at the protein level in gill and skin mucus of Atlantic salmon (*Salmo salar*) affected by amoebic gill disease (Valdenegro-Vega et al., 2014) and in trematode infection of English sole (*Parophrys vetulus*) (Moore et al., 1994), while goldfish (*Carassius auratus*) CRP was shown to enhance complement-mediated lysis of trypanosomes *in vitro* (Kovacevic et al., 2015), and ayu (*Plecoglossus altivelis*) CRP/SAP has been shown to agglutinate bacteria and to inhibit complement-mediated opsonophagocytosis (Shi et al., 2018). However, changes in post-translational modifications have hitherto received little attention, besides a recent study identifying significant increase in total deiminated proteins and deiminated histone H3 in gut-associated mucosal tissue of LPS immunostimulated cod larvae (Magnadóttir et al., 2018). The role of such post-translational modifications, besides increased CRP protein levels *per se*, may be of pivotal importance for protein function, protein-protein interactions and protein moonlighting, an evolutionary acquired phenomenon allowing proteins to exhibit multifunctional physiological or biophysical functions within one polypeptide chain (Henderson and Martin, 2014; Jeffrey, 2018). This may further expand the repertoire of immune recognition and may modify the binding specificity and pattern recognition properties of both CRP forms, depending on environment and tissue localisation. Heterogeneity in the two cod CRP forms

has previously been shown with respect to glycosylation (Gisladóttir et al., 2009) and in Indian carp (*Labeo rohita*) heterogeneity of glycosylated pentraxin subunits has been shown depending on environment (Mandal et al., 1999), while other post-translational modifications, such as deimination revealed here, remain to be investigated in further detail and may be of considerable importance.

The detection of deimination positive CRP forms found in the present study is maybe not surprising when considering the presence of arginines in both CRP-I and CRP-II (Supplementary Fig. 3C). The arginines present in both CRP forms are putative candidates for post-translational deimination by irreversible conversion into citrulline, in a calcium dependent manner by peptidylarginine deiminases (PADs), and may result in changed protein structure and function (Vossenaar et al., 2003; György et al., 2006) of CRP. This may also explain in part the slightly different migration observed for deimination positive (F95) CRP bands, when compared to CRP-I and II detection in crude mucus and serum protein preparations. The banding pattern for deiminated CRP-I and CRP-II positive bands varied somewhat between mucus and serum, possibly reflecting differently post-translationally deiminated forms of CRP present depending on tissue type. In addition, CRP may also be complexed with or bound to deiminated neo-epitopes of other proteins, including histones; possibly representing some of the deimination positive bands detected at lower levels. As pattern-recognition molecules, pentraxins form part of the tissue remodelling machinery, via DAMPS and clearance of apoptotic cells, and can also contribute to clearance and processing of nuclear antigens. This may be of particular importance in tissues with ongoing neurogenesis such as the brain and eye and the presence of deiminated histone H3 and deiminated proteins was recently described in these tissues throughout early cod ontogeny (Magnadóttir et al., 2018).

As PADs are conserved throughout phylogeny from bacteria to mammals, arginine deiminases of commensals, pathogenic bacteria and parasites may contribute to deiminated protein generation in the host. Arginine deiminases have indeed been identified in various pathogens of cod such as *Vibrio anguillarum* (YP\_004567339.1), *Aeromonas salmonicida* (YP\_001140162.1) and *Photobacterium damsela* (VDA\_002926). In human oral mucosa, *Porphyromonas gingivalis* has been shown to contribute to deiminated auto-antigens and associated autoimmune pathologies (Rosenstein et al., 2004; Stobernack et al., 2016; Potempa et al., 2017). While commensals might modulate CRP via changes in deimination for regulation of tissue remodelling and homeostasis, pathogens may possibly also use their arginine deiminase activity as a mechanism to aid immune evasion by modifying CRP via deimination-mediated structural changes and manipulate CRP-mediated inflammatory responses.

Protein deimination is involved in an array of human pathologies, including autoimmune diseases and cancer (Witalison et al., 2015a, 2015b; Crevecoeur et al., 2017), where CRP has also been implicated either through raised levels, or other hitherto unexplained pathways (Ansar and Ghosh, 2013). While pentraxins are known glycoproteins, post-translational deimination has not been studied in CRP before and it may well be possible that CRP is structurally modified via deimination in these diseases, exposing deiminated neo-epitopes which contribute to inflammatory responses. In addition, CRP is known to bind to damage associated molecular patterns (DAMPs), some of which may contain deiminated neo-epitopes, leading to C1q binding and activation of the complement system. Indeed, the presence of pentraxins and circulating deiminated autoantibodies correlates in various autoimmune and inflammatory diseases as well as fibrosis (Gitlin et al., 1977; Robey et al., 1984; Breathnach et al., 1989; Du Clos, 1996; Butler et al., 1999; Li et al., 2010; Acharya et al., 2012; Martinoid et al., 2016). Tissue deposited CRP is thought to be structurally different from circulating pentameric CRP (Eisenhardt et al., 2009; Thiele et al., 2014; Braig et al., 2017) and such conformational change of CRP is associated with proinflammatory properties (Braig et al., 2014; Strang et al., 2012). Monomeric forms of CRP are increasingly being linked to various



diseases (Thiele et al., 2015) and are for example indicated to be a key driver of Alzheimer's disease development (Slevin et al., 2015). Interestingly, circulating pentameric CRP localised to damaged tissue has recently been shown to bind to cell-derived microvesicles, introducing structural changes in CRP, with the CRP-microvesicle complexes enhancing leukocyte recruitment (Braig et al., 2017). Importantly, protein deimination has been shown to be crucial for cellular microvesicle release (Kholia et al., 2015; Kosgodage et al., 2017). As these are calcium-mediated pathways, the regulatory role of PAD-mediated microvesicle release on CRP function in different tissues and pathologies may be of great interest. The newly identified deimination of CRP here, reveals a putatively novel mechanism that may affect structural changes in CRP and thus be of great relevance for understanding CRP function in various pathologies. This may also offer novel ways of modulating CRP-mediated inflammation using inhibitors of deimination. Indeed, pharmacological PAD inhibitors have been shown to be effective in neuroinflammation (Lange et al., 2011, 2014) and autoimmune animal models (Chumanevich et al., 2011; Witalison et al., 2015b; Willis et al., 2011); as well as having anti-cancer effects through modulation of microvesiculation (Kholia et al., 2015; Kosgodage et al., 2017). Due to the phylogenetic conservation of pentraxins, the findings presented here in teleost cod may bring novel insights into CRP and SAP function in various human pathologies. In the light of established and newly developed PAD inhibitors (Bicker and Thompson, 2013; Mondal et al., 2018), the attuning of CRP and SAP deimination may offer novel therapeutic approaches in pathologies where PAD activation and pentraxin function may be intertwined.

## 5. Conclusion

This is the first ontogeny study on pentraxin forms CRP-I and CRP-II in early cod development, revealing tissue specificity for nervous *versus* mucosal tissue for the two different forms, reflecting tissue localisation of human SAP and CRP respectively. For the first time deiminated forms of pentraxins are described. CRP-I and CRP-II were found to differ in deimination in mucus and serum, indicating protein moonlighting through this post-translational modification in different tissue types. This study provides novel insights into tissue specific localisation and putative novel effects on structural changes of pentraxins, mediated through post-translational protein deimination. Our findings may further current understanding of CRP and SAP function in homeostasis and disease.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.dci.2018.05.014>.

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