1   **Gene family expansion and functional diversification of chitinase and chitin synthase genes in**
2   **Atlantic salmon (*Salmo salar*)**

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9   **Running head:** chitinase and chitin synthase in Atlantic salmon

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11

12 **ABSTRACT**

13 **Background:** Chitin is one of the most abundant polysaccharides in nature, forming important
14 structures in insects, crustaceans, and fungal cell walls. Vertebrates on the other hand are generally
15 considered "non-chitinous" organisms, despite having highly conserved chitin metabolism associated
16 genes. Recent work has revealed that the largest group of vertebrates, the teleosts, have the potential to
17 both synthesize and degrade endogenous chitin. Yet little is still known about the genes and proteins
18 responsible for these dynamic processes. Here we used comparative genomics, transcriptomics, and
19 chromatin accessibility data to characterize the repertoire, evolution, and regulation of genes involved
20 in chitin-metabolism in teleosts, with a particular focus on Atlantic salmon.

21 **Results:** Reconstruction of gene family phylogenies provide evidence for an expansion of teleost and
22 salmonid chitinase and chitin synthase genes after multiple whole-genome duplications. Analyses of
23 multi-tissue gene expression data demonstrated a strong bias of gastrointestinal tract expression for
24 chitin metabolism genes, but with different spatial and temporal tissue specificities. Finally, we
25 integrated transcriptomes from a developmental time series of the gastrointestinal tract with chromatin
26 accessibility data to identify putative transcription factors responsible for regulating chitin-metabolism
27 gene expression (CDX1 and CDX2) as well as tissue-specific divergence in the regulation of gene
28 duplicates (FOXJ2). These transcription factors are also potential regulators of multiple
29 glycosyltransferases being co-expressed with the chitin remodeling genes.

30 **Conclusion:** The findings presented here add support to the hypothesis that chitin metabolism genes in
31 teleosts play a role in developing and maintaining a chitin-based barrier in the teleost gut and provide a
32 basis for further investigations into the molecular basis of this barrier.

33 **INTRODUCTION**

34 Chitin is one of the most abundant polysaccharides in nature, serving as the main building block in

35 insect and crustacean exoskeletons as well as forming structural and protective components in fungi.

36 Chitinases and chitin synthases (CHS) are the two major groups of enzymes that have evolved to

37 degrade and synthesize chitin. Decades of work on these enzymes has revealed that bacterial genes

38 encode chitinases that enable bacteria to degrade and utilize chitin as a nutrient source (Cohen-

39 Kupiec and Chet 1998; Beier and Bertilsson 2013), while eukaryotes rich in chitin (i.e. insects,

40 crustaceans and fungi) depend on endogenous chitinases and CHS for normal growth and development

41 (Gooday 1992; Merzendorfer and Zimoch 2003; Zhu *et al.* 2008; Zhang, Zhang, *et al.* 2011; Zhang, L

42 iu, *et al.* 2011; Eichner *et al.* 2015). Curiously, large and highly conserved repertoires of chitinase and

43 CHS genes are also found in vertebrates that do not rely on chitin as a source of nutrition nor possess

44 obvious chitinous body structures such as exoskeletons. Recent experimental work has shown that

45 teleost fish produce chitin in the gastrointestinal tract (GIT) similar to those found in the insect gut

46 epithelium (peritrophic matrix) (Tang *et al.* 2015; Nakashima *et al.* 2018). This realization contradicts

47 the generally held belief that vertebrates are non-chitinous and questions the dogma that chitin does not

48 play an important role in vertebrate physiology.

49 The function of chitinases in fish has received attention for several reasons. Firstly, chitin is a major

50 component in the natural diets of many fish species, and speculation exists as to whether chitinases

51 could aid in the degradation of chitin to digestible carbohydrates. While fish tissues are known to

52 possess chitinase activity and several fish chitinases have been identified

53 (Fänge *et al.* 1976; Lindsay 1984; Gutowska *et al.* 2004; Zhang *et al.* 2012; Koch *et al.* 2014; Teng *et*

54 *al.* 2014; Gao *et al.* 2017; Ikeda *et al.* 2017), the activity of these enzymes does not seem to correlate

55 with the ability of fish to digest chitin nor with the amount of chitin in their natural diet

56 (Buddington 1980; Lindsay *et al.* 1984; Kono *et al.* 1987; Danulat 1987; Karlsen *et al.* 2017).

57 Secondly, chitin is present in many fish tissues and structures, such as the developing gut of zebrafish

58 (*Danio rerio*) (Tang *et al.* 2015), the blenny cuticle of *Paralipophrys trigloides* (Wagner *et al.* 1993),

59 the Ampullae of Lorenzini of Chondrichthyes (Phillips *et al.* 2020) and in the scales of parrotfish

60 (*Chlorurus sordidus*), red snapper (*Lutjanus argentimaculatus*), common carp (*Cyprinus carpio*) and

61 Atlantic salmon (*Salmo salar*) (Zaku *et al.* 2011; Tang *et al.* 2015; Rumengan *et al.* 2017), but the role

62 of chitin in these structures is not known. Thirdly, salmonid fish chitinases have been linked to host-

63 parasite interactions during an infestation of salmon louse (*Lepeophtheirus salmonis*), a small

64 crustacean that feeds on the skin, mucus, and blood of salmonids. For example, resistance to salmon

65 lice in Pink salmon (*Oncorhynchus gorbuscha*) has been suggested to be linked to an increased response

66 of host chitinase in larger fish (Sutherland *et al.* 2011), and upregulation of chitinase gene expression

67 together with genes involved in tissue repair and wound healing in lice-infected skin of Atlantic salmon

68 (Robledo *et al.* 2018).

2

69   Our understanding of the repertoire and function of chitin degrading enzymes in vertebrates is mostly
70   derived from studies of mammalian genes and proteins. These genes all belong to the glycoside
71   hydrolase 18 family (GH18), which is an ancient multigene family with a conserved *DXXDXDXE*
72   catalytic motif where glutamate represents the catalytic acid (Terwisscha van Scheltinga *et al.* 1996).
73   In mammals, genes encoding these enzymes can be further subdivided into five main groups. Three of
74   these groups have demonstrated enzymatic activity that enables them to break down chitin;
75   chitotriosidase (CHIT1), acidic mammalian chitinase (CHIA), and di-*N*-acetyl-chitobiase (CTBS).
76   CHIT1 and CHIA are hypothesized to have evolved from one common ancestor gene through whole-
77   genome duplication (WGD) in a common vertebrate ancestor (Hussain and Wilson 2013) and can
78   hydrolyze longer chains of chitin into shorter fragments (chitobiose and chitotriose)
79   (Renkema *et al.* 1995; Boot *et al.* 2001). The CTBS group is more distantly related to CHIT1/CHIA
80   and has evolved to hydrolyze shorter, soluble chitooligosaccharides into *N*-acetyl glucosamine
81   monomers (GlcNAc) allowing for complete degradation of chitin. Chitinase-domain containing protein
82   1 (CHID1) is another chitinase-related group of proteins that is highly conserved in all vertebrates,
83   although the sequence similarity to other GH18 chitinases is low. Human CHID1 (stabilin-1 interacting
84   protein) lacks essential catalytic residues but contains conserved aromatic residues potentially important
85   for saccharide binding (Meng *et al.* 2010). In mammals, but not in all vertebrates, other saccharide-
86   binding chitinases are termed chitinase-like lectins (CHIL). CHIL are non-enzymatic chitinase-like
87   proteins very similar to CHIA and CHIT1, but with active site mutations that render the proteins
88   catalytically incompetent. Human CHIL (OVGP1, CHI3L1, and CHI3L2) are according to phylogenetic
89   analyses of mammalian CHIL predicted to have evolved from gene duplications of ancestral CHIA and
90   CHIT1 (Funkhouser and Aronson 2007; Bussink *et al.* 2007). A newly identified group of vertebrate
91   chitinases that does not fit into any of the five mammalian groups is a group called CHIO
92   (Hussain and Wilson 2013). Like CHIL, CHIO is also hypothesized to have evolved from ancestral
93   CHIA and/or CHIT1. Two rounds of whole-genome duplication events specific for teleost (Ts3R) and
94   salmonid fish (Ss4R) have resulted in an amplification of genes that are closely related to this group.
95   There is, however, a lack of systematic effort to characterize the potential for teleost genomes to encode
96   chitin degrading and synthesizing enzymes.

97   In this paper, we have characterized the evolution and diversification of genes involved in chitin
98   breakdown and synthesis in Atlantic salmon. Using a comparative approach that combines both
99   comparative and functional genomics, we provide an improved understanding of putative protein
100 functions and gene regulation of chitinase and CHS genes in Atlantic salmon. Our results provide a
101 knowledge base for further functional studies of chitin-biology in teleost fish and support the idea that
102 chitin plays a major role in GIT function and physiology.

103

3

104 **MATERIALS AND METHODS**

105 **Phylogenetic analysis**

106 Orthofinder (v.0.3.1) was used to construct orthogroups using the longest protein isoform sequence

107 from gene. Species included in the orthogroups computation were spotted gar (*Lepisosteus oculatus*,

108 LepOcu1), zebrafish (*Danio rerio*, GRCz10), stickleback (*Gasterosteus aculeatus*, BROADS1),

109 Japanese medaka (*Oryzias latipes*, HdrR), pike (*Esox Lucius*, Eluc_V3), rainbow trout (*Oncorhynchus*

110 *mykiss*, Omyk_1.0), coho salmon (*Oncorhynchus kisutch*, Okis_V1), Atlantic salmon (*Salmo salar*,

111 ICSASG_v2), human (*Homo sapiens*, GRCh38), and house mouse (*Mus musculus*, GRCm38). For each

112 orthogroup, protein sequences were then aligned using MAFFT (v.7) (Katoh and Standley 2013). A

113 maximum likelihood phylogenetic tree was constructed in MEGA7 (Kumar *et al.* 2016) using a

114 neighbor-joining algorithm with a JTT substitution model and 100 bootstrap replicates.

115

116 **Tissue expression profiles**

117 See Supplementary Table 1 for more information about species, tissues examined, number of

118 individuals, and where the data are available. Tissue expression profiles from Atlantic salmon (except

119 stomach, pyloric caeca, and midgut), rainbow trout, zebrafish and pike (n = 1 for all tissues except the

120 liver where n = 3 for rainbow trout and n = 4 for zebrafish) were generated from RNA-sequencing

121 (RNA-seq) data as described previously (Lien *et al.* 2016; Gillard *et al.* 2021). In brief, the STAR

122 aligner with default settings (Dobin *et al.* 2013) was used to map RNA-seq reads to the annotated

123 reference genomes and RSEM (Li and Dewey 2011) was used to estimate read counts. Tissue

124 expression data from the stomach, pyloric caeca and midgut of Atlantic salmon (n = 15 for stomach and

125 pyloric caeca, n = 167 for midgut) were generated from previously published RNA-seq data

126 (Gillard *et al.* 2018; Jin *et al.* 2018) following the described method. The RNA-seq data were mapped

127 to the annotated genome (ICSASG_v2) using the STAR aligner, and the read counts were estimated

128 with HTSeq-count (Anders *et al.* 2015). The read counts were transformed to Transcript Per Million

129 Reads (TPM) values normalized for average transcript length and sample size. To get TPM values, the

130 raw gene counts were first divided by the transcript length before dividing by the total library count

131 number. The mean gene expression value was used for the liver, and the median gene expression value

132 was used for the stomach, pyloric caeca, and midgut. The gene expression values were log-transformed

133 (Log_2(TPM + 1) before further analysis. The RNA-seq data analysis was performed using R (v.3.6.0).

134

135 **Gene expression in pyloric caeca of Atlantic salmon spanning the transition from endogenous to**

136 **exogenous feeding**

137 The expression profiles of chitinases and CHS in pyloric caeca of Atlantic salmon spanning the

138 developmental transition to external feeding were obtained from an RNA-seq dataset available through

4

139 ArrayExpress under the project number E-MTAB-8306 and was generated as described previously

140 (Jalili *et al.* 2019). Differences in expression levels compared to day 0 was tested by comparing means

141 of expression using a Wilcoxon test with the function "stat_compare_means" in the R-package

142 "Ggpubr" using the default "wilcox.test" parameter. The p-values were adjusted for multiple testing.

143 Genes with low expression (TPM < 1) were removed before the co-expression analysis and quality

144 control of the resulting genes was conducted using the function "goodSamplesGenes" in the "WGCNA"

145 package in R (Langfelder and Horvath 2008) with the argument "verbose = 3". The co-expression

146 analysis was carried out using the minimum biweight midcorrelation ("bicor") function in the

147 "WGCNA" package with the argument «maxPOutliers = 0.05» and genes with a correlation value above

148 0.69 was referred to as co-expressed genes. Gene enrichment of the co-expressed genes was done using

149 KEGG enrichment with the function "kegga" from the "limma" package in R (Ritchie *et al.* 2015) and

150 the argument «species.KEGG = "sasa"» and the universe specified to be only expressed genes. The p-

151 values returned by "kegga" were not adjusted for multiple testing.

152

Chromatin accessibility in pyloric caeca

154 ATAC-seq reads from pyloric caeca of Atlantic salmon were downloaded from ArrayExpress (E-

155 MTAB-9001). Read mapping and ATAC-peak calling was done using BWA (v.0.7.17)

156 (Li and Durbin 2009) and Genrich v.06 (https://github.com/jsh58/Genrich) as described in detail in

157 Bertolotti *et al.* (2020).

158

Transcription factor motif enrichment

160 DNA sequences from open chromatin (i.e. within ATAC-seq peaks) around TSS (1000bp upstream to

161 200bp downstream) of chitinase- and CHS genes were used for transcription factor motif scan and

162 enrichment. The scan and enrichment was carried out using SalMotifDB (Mulugeta *et al.* 2019), a tool

163 for analyzing putative transcription factor binding sites in Atlantic salmon. Consensus motifs were

164 obtained using the "ggseqlogo" package in R.

165

RESULTS

Phylogenetic analysis of chitinase protein sequences

168 The annotated Atlantic salmon genome (ICSASG_v2) includes 12 genes with strong homology to

169 mammalian chitinase genes (see Supplementary Table 2 for gene IDs, proteins accession numbers, and

170 names given in this paper) belonging to the family 18 of the glycoside hydrolases, as classified by the

171 carbohydrate-active enzyme (CAZy) database (Drula *et al.* 2022). To investigate the evolutionary

172 history of the chitinase gene family in fish we reconstructed phylogenetic trees of genes within the

5

173 glycoside hydrolase family 18 orthogroup. The species selection was designed to include vertebrates
174 that have experienced different numbers of whole-genome duplications. All species share the two
175 whole-genome duplications occurring in the ancestor of all vertebrates, and except gar, all fish species
176 share an additional whole genome duplication at the base of the teleost lineage (Ts3R), while Atlantic
177 salmon, rainbow trout and Coho salmon share an additional fourth salmonid-specific whole-genome
178 duplication event (Ss4R) (Figure 1A).

179 Our analysis revealed that the 12 Atlantic salmon chitinase proteins are distributed among six major
180 clades (Figure 1B). These six clades formed two major "superclades" (supported by a high bootstrap
181 value of 83), one containing CTBS and CHID1 type proteins (two from Atlantic salmon) and the other
182 containing CHIT1, CHIA, and CHIO (10 from Atlantic salmon). Of the 10 Atlantic salmon proteins
183 annotated as acidic mammalian chitinases (CHIA) in the NCBI RefSeq annotation (release 100) all
184 salmon proteins share the following chitinase characteristics: a signal peptide, a glycoside hydrolase 18
185 family catalytic domain, and a chitin-binding domain (CBM14; identified by dbCAN2 annotation
186 (Zhang *et al.* 2018)) at the carboxyl-terminus. Three salmon CHIA proteins (namely Chia.3, Chia.4,
187 and Chia.7) fell into a monophyletic clade (descending from a common ancestor) containing the human
188 acidic mammalian protein AMCase, whereas the remaining seven CHIA protein sequences were
189 distributed among two teleost-specific monophyletic clades. One salmon CHIA protein (Chia.5) will
190 hereafter be referred to as a CHIT1-member. This protein is the only salmon chitinase protein with loss-
191 of-function mutations in the catalytic motif and a truncated chitin-binding domain. The remaining six
192 salmon chitinase proteins (Chia.1, Chia.2, Chia.6, Chia.8, Chia.9, and Chia.10) belong to two clades
193 (termed CHIO I and II) forming a larger monophyletic group.

194 To make interferences about how ancient whole-genome duplication and other duplication events have
195 contributed to the present diversity of chitinase proteins in teleost fish, the protein sequence phylogeny
196 was compared with the species tree topology. The CHID1 and CTBS clades only contain one protein
197 sequence per species, and the protein trees resemble to a large extent the species topology except for
198 the polytomy in the CTBS clade that fails to place the mammals as a sister clade to the teleost species.
199 This is in agreement with the hypothesis that CTBS and CHID1 genes resulted from an ancient gene
200 duplication before the vertebrate diversification
201 (Funkhouser and Aronson 2007; Hussain and Wilson 2013), possibly the whole genome duplication at
202 the base of all vertebrates. The two distinct fish-specific CHIA subclades are more closely related to
203 each other than to their sister subclade containing the mammalian CHIA proteins. Furthermore, since
204 both fish subclades contain a predicted gar protein it is likely that these fish-specific duplicates arose
205 through a duplication event prior to the divergence of teleosts. In the fish-specific CHIO clade,
206 comprising 39 protein sequences, the three gar-specific proteins cluster closely together and, because
207 of low bootstrap values (< 70) for key splits in the tree, we cannot firmly place these in relation to the
208 remaining teleosts. However, based on the sequence relationships between the teleost CHIO species it

6

209   is likely that Ts3R has contributed to at least one CHIO-duplication event as previously hypothesized

210   (Hussain and Wilson 2013). Two nodes reflecting the Ss4R event can be inferred in the CHIO I and II

211   clades, including the branches containing Chia.1+10, and Chia.2+9. These proteins are located on

212   homologous regions of different chromosomes (22 and 12) in Atlantic salmon (Supplementary Figure

213   1).

214
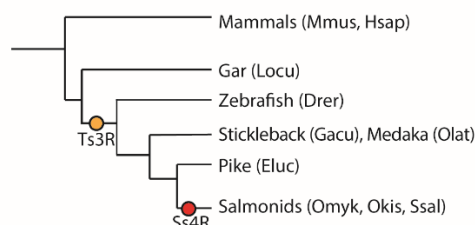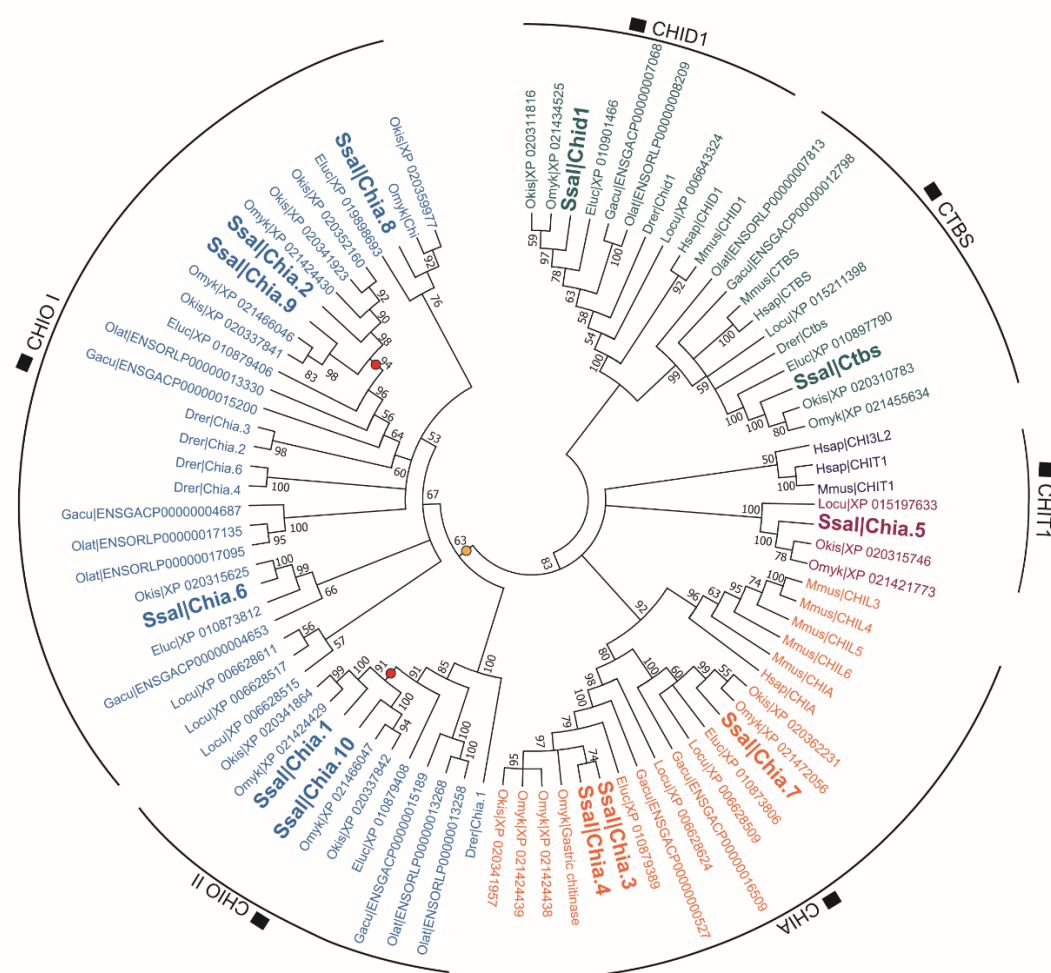
Figure 1. Comparative analysis of GH18 proteins. A) Whole-genome duplication (WGD) events experienced by species included in the phylogenetic comparison. B) Phylogenetic tree of GH18 chitinase proteins in spotted gar (locu), zebrafish (drer), stickleback (gacu), Japanese medaka (olat), pike (eluc), rainbow trout (omyk), coho salmon (okis), Atlantic salmon (ssal), human (hsap) and house mouse (mmus). The colors represent different monophyletic clades in the phylogenetic tree.

220

7

**Tissue-specific regulatory divergence of chitinase genes in the gastrointestinal tract**

A comparative analysis of tissue expression in zebrafish, pike, rainbow trout, and Atlantic salmon (Figure 2) was performed to characterize the divergence of gene regulation encoding chitinase enzymes. Members of the CHIT1 group were not included in this analysis as their expression levels were low, indicating that they may represent pseudogenes encoding non-functional enzymes. Across all species, the results showed a clear bias towards gene expression in GIT and revealed both conserved expression divergence among orthologs in different species as well as lineage-specific regulatory divergence.

CHIA genes displayed the most conserved tissue expression regulation across all species with stomach-specific expression. A similar stomach bias is also observed for CHIA in mice, bats, pigs, chickens, and humans indicating that CHIA enzymes share an important gastric function that is conserved across fish, mammals, and birds (Boot *et al.* 2001; Strobel *et al.* 2013; Ohno *et al.* 2016; Tabata, Kashimura, Wakita, Ohno, Sakaguchi , Sugahara, Kino, *et al.* 2017; Tabata, Kashimura, Wakita, Ohno, Sakaguchi, Sugahara, Imamura, *et al* . 2017; Tabata *et al.* 2019). Notably, the agastric (stomach-less) zebrafish do not express genes related to gastric functions, including CHIA genes.

The tissue expression profiles of CHIO- I and II genes show different patterns compared to the CHIA genes. Although CHIO genes also display GIT expression dominance, these genes are not stomach-specific but rather expressed in other GIT sections such as pyloric caeca and midgut. Additionally, CHIO gene expression is generally less tissue-specific and has larger inter- and intra-tissue specific variations in tissue expression patterns. For example, while both salmon CHIO II genes are expressed almost exclusively in the GIT, the CHIO I clade contains salmon genes expressed in the GIT and a gene (*chia.8*) that is lowly expressed in all tissues examined. We also observe some less striking, but clear, cases of regulatory divergence of CHIO II genes following the more recent Ss4R, with one duplicate being mostly expressed in pyloric caeca (*chia.10*), while the other is expressed in both pyloric caeca and gut (*chia.1*).

Finally, the CTBS and CHID1 gene groups show a different tissue regulation pattern than the other chitinases. Both gene groups are generally expressed at low levels compared to their CHIA and CHIO counterparts. CHID1 orthologs in zebrafish, pike, and rainbow trout are ubiquitously expressed in all tissues, but the salmon *chid1* gene is expressed at two-fold higher levels in the midgut compared to the stomach and pyloric caeca, and four-fold higher in the midgut compared to non-GIT tissues indicating some tendency to GIT specific expression. Similar to CHID1, fish CTBS are expressed across all tissues, but with two-fold higher expression in the GIT of Atlantic salmon.
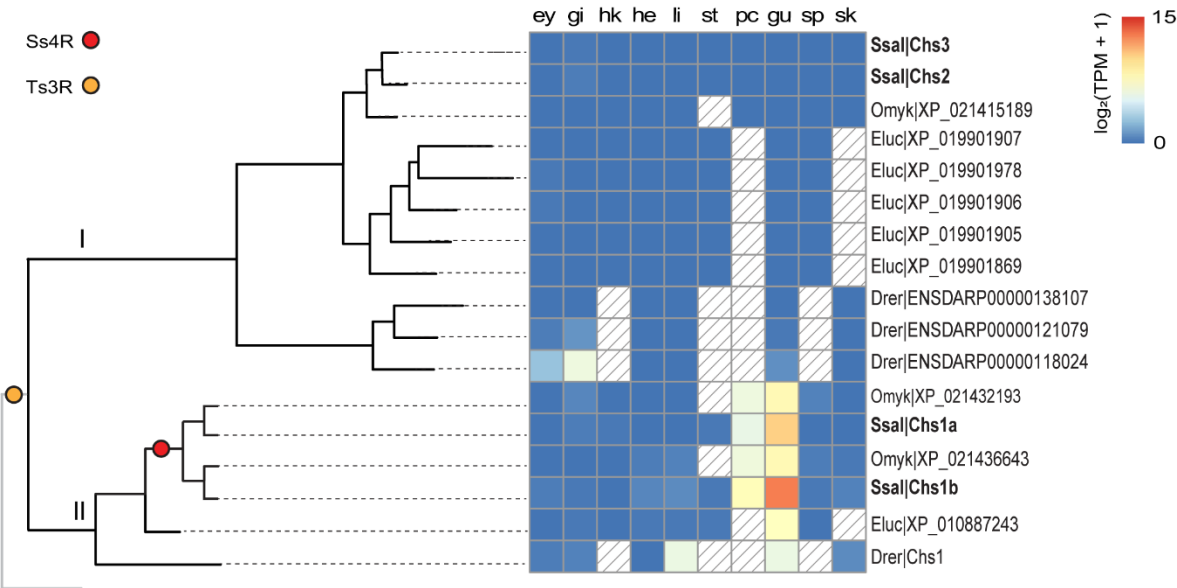
8
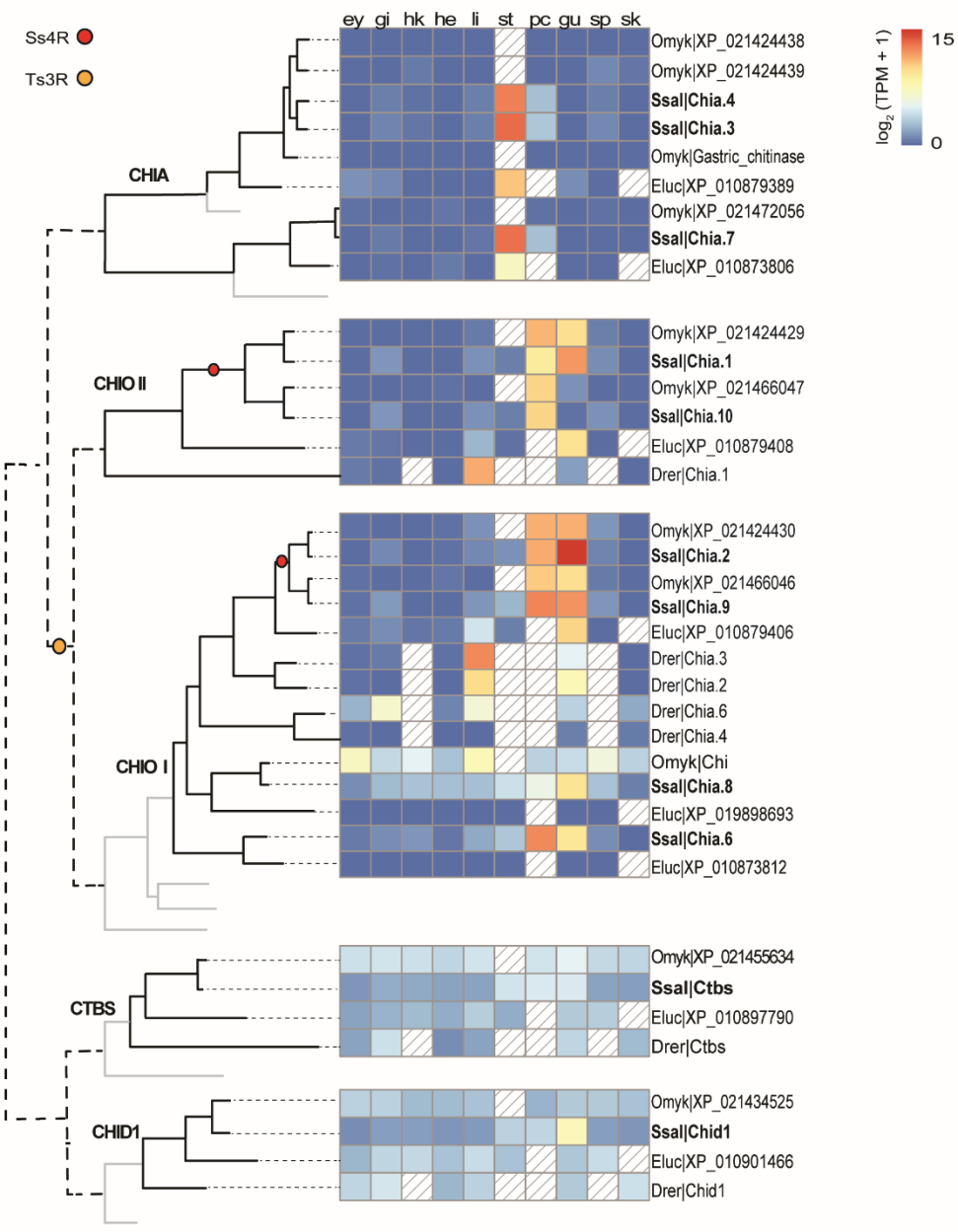
Figure 2. Comparative tissue expression of GH18 genes in zebrafish (drer), pike (eluc), rainbow trout (omyk), and Atlantic salmon (ssal). n≥1. The phylogenetic tree is a simplified version of figure 1B and the lines do not represent real evolutionary distances. Yellow and red circles represent the teleost-specific whole-genome duplication (Ts3R) and salmonid-specific whole-genome duplication (Ss4R), respectively. Solid light grey lines indicate the phylogenetic position of a spotted gar, but expression data were not analyzed for this outgroup. The tissue expression panel shows gene expression of GH18 genes in the following tissues: ey = eye, gi = gill, hk = head kidney, he = heart, li = liver, st = stomach, pc = pyloric caeca, gu = midgut, sp = spleen and sk = skin. Colored boxes indicate gene expression in the range of 0 to 15 $\log_2(\text{TPM} + 1)$ values, while diagonal lines represent missing data.

9

264 **Chitin synthase genes are mainly expressed in pyloric caeca and midgut of teleost fish**

265 The phylogenetic analysis of the gene family containing genes encoding CHS proteins showed a split

266 into two major subclades (I and II) which, since gar has a single gene copy, likely arose in the Ts3R

267 whole-genome duplication event (Figure 3). Furthermore, the salmonid-specific gene copies in subclade

268 II (i.e. *chs1a* and *chs1b* from Atlantic salmon) likely originate from the Ss4R as they are located on

269 chromosomes (28 and 1 respectively) matching the well described synteny within the duplicated

270 Atlantic salmon genome (Lien *et al.* 2016) (Supplementary Figure 1). The tissue expression pattern

271 shows that the CHS genes in subclade I are expressed in low abundance in all tissues, whereas CHS

272 genes in subclade II follow the same expression pattern as CHIO- I and II genes (Figure 2), with

273 expression specific to pyloric caeca and gut (Figure 3).

274 Notably, predicted protein sequences of teleost CHS genes contain similar conserved amino acid

275 sequence motifs as found in insect CHS proteins. The motifs *EDR* and *QRRRW* are common for all

276 CHS, while *CATMWHXT* and *QKFEY* are signatures of insect CHS (Merzendorfer and Zimoch 2003).

277 *EDR*, *QRRW,* and *QKFEY* are motifs found in all predicted fish CHS protein sequences examined, but

278 the *CATMWHXT* motif is present in the CHS subclade II only.

279

281 **Figure 3.** Comparative tissue expression of CHS (chitin synthase) genes in zebrafish (drer), pike (eluc),
282 rainbow trout (omyk), and Atlantic salmon (ssal). n≥1. Yellow and red circles in the illustrative
283 phylogenetic tree represent the teleost-specific whole-genome duplication (Ts3R) and salmonid-
284 specific whole-genome duplication (Ss4R), respectively. The lines do not represent real evolutionary
285 distances. Solid light grey lines indicate the phylogenetic position of a spotted gar, but expression data
286 were not analyzed for this outgroup. The tissue expression panel shows gene expression of CHS genes
287 in the following tissues: ey = eye, gi = gill, hk = head kidney, he = heart, li = liver, st = stomach, pc =
288 pyloric caeca, gu = midgut, sp = spleen and sk = skin. Colored boxes indicate gene expression in the
289 range of 0 to 15 log$_2$(TPM + 1) values. Boxes with diagonal lines represent missing data.

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10

Gene regulation of chitinases and chitin synthases in pyloric caeca of Atlantic salmon

To be able to better understand the regulation of chitin metabolism genes we leveraged a RNA-seq dataset from pyloric caeca of Atlantic salmon (ArrayExpress, E-MTAB-8306) that spans the developmental transition from endogenous to exogenous nutrition (Jalili *et al.* 2019). The changes in gene expression observed across the developmental time series show two major trends; 5 genes significantly increased expression following external feed intake (p < 0.01), while 3 genes did not (Figure 4A).
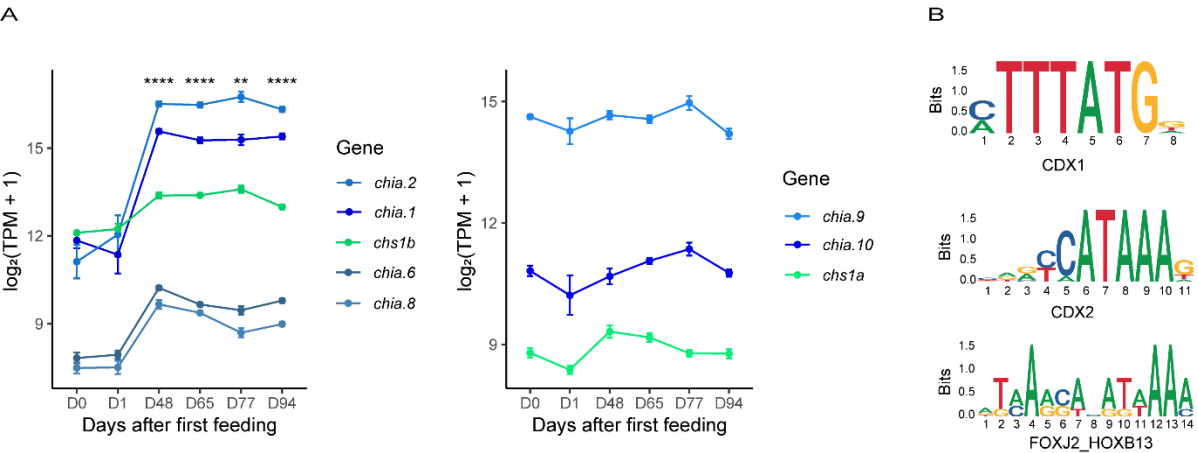
Figure 4. A) Gene expression levels of *chia.1*, *chia.2*, *chia.6*, *chia*.8, *chia.9*, *chia.10*, *chs1a* and *chs1b* before and after external feeding. Chitinase and CHS gene expression in the pyloric caeca of Atlantic salmon days before (D0) and days after external feeding (D1-D94). Please note that the y-axis does not extend to 0. The asterisks (**, ****) indicate a significant difference in expression compared to D0 (p.adj<0.01 and p.adj<0.0001, respectively, n≥4. B) Consensus motifs for binding of transcription factors in promoters of chitinase- and CHS genes. CDX1 and CDX2 motifs are found in promoters of all chitinase- and CHS genes. The FOXJ2_HOXB13 motif is only present in promoters of genes with a significant increase in expression upon transition to external feeding. The numbers indicate the consensus site position of each base, and the vertical axes (Bits) indicate the information content of the base frequency at the given base position.

Co-expressed genes (genes with correlated expression profiles) are often controlled by the same regulators and involved in the same biological processes. To better understand the mechanisms underlying the regulation of genes involved in chitin metabolism, and particularly drive increased expression of some chitinases and CHS following feed ingestion we used a co-expression approach. We first used biweight midcorrelation (bicor) to identify genes with similar expression patterns in the pyloric caeca across the developmental time series. Based on KEGG (Kyoto Encyclopedia of Genes and Genomes) gene enrichment analysis, co-expressed (bicor > 0.69, n = 36) with salmon *chia.1*, *chia.2*, *chia.6*, *chia.8,* and *chs1b* were genes involved in metabolic processes like amino sugar and nucleotide sugar metabolism (p-value = $8.79 \cdot 10^{-10}$) and glycosphingolipid biosynthesis (p-value = $5.92 \cdot 10^{-4}$) (Supplementary Table 3). We found the chitinase and CHS genes to be associated with the amino sugar

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321   and nucleotide sugar metabolism KEGG pathway, together with a UDP-*N*-acetylhexosamine

322   pyrophosphorylase-like gene (*uap1*) which most likely codes for an enzyme that converts uridine

323   triphosphate (UTP) and N-acetylglucosamine-1-phosphate (GlcNAc-1-P) into uridine diphosphate *N*-

324   acetylglucosamine (UDP-GlcNAc) (Mio *et al.* 1998). This is known to result in an activated substrate

325   required for chitin synthesis by CHS. UDP-GlcNAc can also be transferred by beta-1,3-galactosyl-O-

326   glycosyl-glycoprotein beta-1,6-*N*-acetylglucosaminyltransferase (GCNT1) to form mucin-type O-

327   glycan structures. *Gcnt1* is one of the multiple glycosyltransferases being co-expressed with chitinase-

328   and CHS genes. Associated with the glycosphingolipid biosynthetic pathway we found two additional

329   glycosyltransferase genes coding for alpha-2,8-sialyltransferase-like proteins, involved in the transfer

330   of sialic acid to produce sialyl glycoconjugates.

331   To further dissect out putative transcription factors involved in the regulation of chitinase and CHS

332   genes, we performed a transcription factor binding (TFBS) scan for two classes of genes: (1) all CHIO-

333   and CHS genes being highly expressed in pyloric caeca, and (2) those that we find induced during the

334   developmental transition to external feeding exclusively (Figure 4A). We based the scan on open-

335   chromatin regions of promoter sequences of chitinase– and CHS genes. The data show that all chitinase-

336   and CHS genes had open chromatin regions spanning the TSS in pyloric caeca except CHIA-genes and

337   *chia.8*. Furthermore, the transcription factor motif scan revealed that two motifs were common for all

338   CHIO- and CHS genes. These motifs were two homeodomain (HOX) related motifs: a caudal type

339   homeobox 1 (CDX1) motif and a caudal type homeobox 2 (CDX2) motif (Figure 4B). For the CHIO-,

340   CHS- and co-expressed genes induced during the transition to external feeding the FOXJ2_HOXB13

341   motif associated with binding of the forkhead box (FOX) transcriptional factor family (Figure 4B) were

342   enriched (p-value < 0.01).

343

DISCUSSION

345   Our results suggest that Ts3R and Ss4R duplication events resulted in the expansion of chitinase- and

346   CHS genes in fish (Figure 1) and that these genes generally encode proteins with conserved residues in

347   active motifs. This is strikingly different from mammals, which have lost their genes for CHS and where

348   mutations in the active site of chitinases followed by mammal specific gene duplications have resulted

349   in the expansion of non-enzymatic chitinase-like lectins. In general, teleost and salmonid chitinase- and

350   CHS genes share a clear expression bias towards the gastrointestinal tract (Figure 2 & 3). This

351   expression bias may explain the presence of chitin in the gut of zebrafish (Tang *et al.* 2015) and rainbow

352   trout (Nakashima *et al.* 2018), and adds support to a chitin-based mucosal barrier in the gut of teleost

353   fish previously hypothesized to have evolved from the chitin-based barrier we find in invertebrates

354   (Nakashima *et al.* 2018). This is also in line with the increased expression of chitin metabolism genes

355   during development (Figure 4), which coincides with the development of a pyloric caeca with increased

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356   complex intestinal mucosal structures (Sahlmann *et al.* 2015). The transition from endogenous to

357   external feeding involves exposure to both larger food particles and new microbial communities. We

358   predict that this exposure boosts the expression of chitinases and CHS needed to synthesize and remodel

359   a chitinous layer that surrounds the intestinal mucosa and protects the intestinal epithelium, in addition

360   to other genes related to intestinal differentiation and mucus production. Co-expression of *uap1* and

361   glycosyltransferases like *gcnt1* supports this assumption, as UAP1 can produce the activated UDP-

362   GlcNAc used by both CHS to produce chitin and by GCNT1. GCNT1 is important for the production

363   of mucins and mice deficient in related genes have been shown to have increased permeability of the

364   mucosal barrier which can alter the mucosal immune homeostasis (Stone *et al.* 2009). Furthermore, the

365   co-expressed alpha-2,8-sialyltransferase orthologs are, in humans, linked to the production of

366   gangliosides; glycosphingolipids that contain one or multiple residues of sialic acid and that is localized

367   in the brush border membrane of intestinal enterocytes. Such gangliosides are known to be important

368   for maintaining intestinal integrity and reducing inflammation (Miklavcic *et al.* 2012).

369   Little is known about the regulatory networks of chitinases and chitin synthetases. The presence of

370   FOX, CDX1, and CDX2 motifs in the promoter regions of CHIO- and CHS genes presented here

371   support the hypothesis of a possible role in the formation of a chitin-based mucosal barrier. Both CDX1

372   and CDX2 are known to be major regulators of intestine-specific genes and are crucial for intestinal

373   differentiation. In zebrafish, for example, knockdown and overexpression experiments show that

374   CDX1B, homologous to mammalian CDX1, is responsible for terminal differentiation of goblet cells,

375   cells that are responsible for the secretion of mucins into the intestinal mucosa (Chen *et al.* 2009).

376   Moreover, it is plausible that the FOXJ2_HOXB13 motif could be bound by intestinal FOX proteins

377   like FOXA1 and FOXA2. These proteins are also linked to intestinal goblet cell mucus production

378   (van der Sluis *et al.* 2008). Synergetic transcription factor binding of CDX and FOXA transcription

379   factors has previously been shown to regulate intestine-specific mucins (Jonckheere *et al.* 2007).

380   Similar synergetic effects can possibly explain the difference in gene expression of the chitinase- and

381   CHS genes lacking the FOX motif in their promoter. That said, the chromatin accessibility is likely to

382   change during development and it is important to take into consideration that the ATAC-seq data used

383   to guide the search for transcription factor binding sites in this study was derived from adult fish.

384   Nevertheless, CDX1, CDX2 and FOX are interesting candidates for futures studies into the regulation

385   of chitin metabolism genes in fish GIT.

386   Functional diversification is observed for the different fish chitinases. We find that, unlike the CHIO-

387   and CHS genes, CHIA genes are exclusively expressed in the stomach and have no open chromatin

388   regions in their promoters in the pyloric caeca of Atlantic salmon. Thus, these genes are likely to be

389   regulated by other transcription factors than the CHIO- and CHS genes. The high degree of sequence

390   similarity to mammalian stomach chitinases suggests that the fish-specific CHIA proteins share an

391   ancestral function related to the presence of an acidic stomach (Krogdahl *et al.* 2015). This hypothesis

13

392 is strengthened by the loss of CHIA genes in agastric zebrafish. As various fish stomach CHIA proteins

393 have shown to be able to break down chitin structures typically found in the natural diet of Atlantic

394 salmon, like shrimp, squid, and insects (Ikeda *et al.* 2017), we cannot rule out a possible role of teleost

395 CHIA proteins in digestion. The results presented here thus imply that some fish species like Atlantic

396 salmon do have the genetic toolbox needed to tolerate and digest chitin-containing feed.

397

CONCLUSION

399 There has been an expansion of chitinase and chitin-synthase like proteins in Atlantic salmon and

400 different groups of chitinases have evolved to be expressed in different tissues. Based on our results we

401 hypothesize two different roles of Atlantic salmon chitinases and CHS: (1) that stomach-related CHIA

402 proteins aid in the digestion of chitin, whereas (2) CHIO- and CHS proteins are involved in remodeling

403 of chitinous structures surrounding mucosal membranes of pyloric caeca and gut. To verify this,

404 functional characterization of chitinous structures and the enzymes that remodel these are needed.

405 However, this work provides a basis for future functional studies identifying the underlying mechanisms

406 for the presence of chitinous structures in Atlantic salmon.

407

DATA AVAILABILITY STATEMENT

409 The authors declare that the data supporting the results in this study are accessible in the paper and the

410 supplementary file.

411

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