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Conservation of members of the free fatty acid receptor gene family in common carp

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ABSTRACT

Accumulating evidence supports the crucial role intestinal microbiota and their metabolites play in the homeostasis of organisms. An important class of metabolites that have been shown to affect the immune system are short chain fatty acids (SCFAs). These SCFAs can affect the host cells via passive diffusion or via ligation to receptors, among others G-protein coupled receptor (GPR) 41 and 43. GPR41 and GPR43 are both part of a family of GPR40-related receptors. Mammalian studies have shown an important role for GPR41 and GPR43 in the modulation of immune responses by SCFAs. However, up till date, no validated coding sequences for orthologues of these SCFA receptors have been published for teleost fish. We used genomic resources and cDNA cloning, to identify and validate ten coding sequences for *gpr40L* genes in common carp. Phylogenetic analysis showed a division into three subclasses, putatively named class a, b and c, and showed the common carp genes had a closer phylogenetic relationship to mammalian GPR43 than to mammalian GPR41. Synteny analysis revealed a clear conservation of syntenic relationships between *gpr40L* in the genomes of spotted gar and common carp with the relevant region in the human genome. This conservation of synteny validates the genes identified, as *gpr40L*. Finally, presence of *gpr40L* genes was investigated in silico for genomes of 25 different, mostly teleost, fish species largely confirming the observations for *gpr40L* of common carp with regards to both, subdivision in three subclasses a-c and conservation of synteny. Our data provide an important first step towards an understanding of the role and function of receptors for SCFAs and immunomodulation in fish.

1. Introduction

Ever increasing evidence suggests that the microbiota of the gastro-intestinal tract of mammals plays a pivotal role in the regulation of a wide range of biological processes with metabolic, endocrine and immune functions [As reviewed by: (Butt and Volkoff, 2019; Rosenbaum et al., 2015; Wu and Wu, 2012)]. Fermentation of dietary fibres by bacteria present in the gastro-intestinal tract, can lead to the production of several metabolites such as short-chain fatty acids (SCFAs) among which acetate, butyrate and propionate. Fermentation of dietary fibres and the resulting SCFA production explains at least part of the regulation of immune functions by dietary fibre supplementation, as SCFAs have been shown in mammals to have clear immunomodulatory effects on leukocyte recruitment, cytokine production, lymphocyte activation, phagocytosis or oxygen radical production [As reviewed by: (Vinolo et al., 2011)].

In mammals, the critical roles of SCFAs in the regulation of cellular function can be indirectly or directly exerted as SCFAs can be absorbed by passive diffusion into cells, a process greatly enhanced by different solute transporters (e.g. monocarboxylate transporter 1 and sodium-coupled monocarboxylate transporter 1) and because SCFAs can act as ligands for G protein-coupled receptors (GPCRs), resulting in an active regulation via receptor activation [As reviewed by: (Parada Venegas et al., 2019; Yang et al., 2018)]. When SCFAs enter cells they can inhibit histone de-acetylases (HDACs), which normally mediate hypo-acetylation associated with closed chromatin structures and repressed gene transcription [As reviewed by: (Li et al., 2018; Ma et al., 2013; Marks et al., 2003)]. The presence of SCFAs can thus result in hyper-acetylation and facilitate the binding of transcription factors to promotor regions, leading to active gene transcription in a process called epigenetic reprogramming. When SCFAs are recognised by certain GPCRs, also knowns as free fatty acid receptors (FFARs), a signalling cascade results in regulation of genes important for intestinal health, which can explain subsequent immunomodulatory effects of SCFAs [As reviewed by: (Rauf et al., 2021)]. Presence and activation of these receptors can be crucial for intestinal homeostasis and immune

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modulation.

Well-known mammalian SCFA receptors include FFAR3 [or G protein-coupled receptor 41 (GPR41)], FFAR2 (GPR43), hydroxycarboxylic acid receptor 2 (HCAR2, also known as GPR109A), all three rhodopsin-like subfamily A11 receptors, and OLFR78 (olfactory receptor 78), an unclassified rhodopsin-like receptor. GPR41 and GPR43 are currently the only receptors that show affinity for acetate, butyrate and propionate, while the other receptors are believed to be more selective [As reviewed by: (Muralitharan and Marques, 2021)]. Both are classified as 'GPR40-related receptors' and positioned in the mammalian genome next to other GPR40-related receptors (e.g. GPR40, and GPR42 specifically in primates). Both GPR41 and GPR43 have affinity for propionate, butyrate and acetate in that order, with GPR41 having a higher overall affinity than GPR43 (Brown et al., 2003). In mice, expression of GPR43 is generally associated with immune tissues and immune cell types, contrasting GPR41 which shows a more widespread expression pattern (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003), while both have been implicated in regulation and induction of inflammation in knockout studies [As reviewed by: (Ang and Ding, 2016)]. Clearly, the presence of GPR41 and/or GPR43 and their function in the recognition of SCFAs plays an important role in immune homeostasis in the gastro-intestinal tract, at least in mammals.

Intensive forms of aquaculture pose an urgent need for fish health feeds containing immunomodulatory additives, to help prevent fish diseases especially in intensive, outdoor systems. Amongst the better known immunomodulatory feed additives are non-starch polysaccharides, with probably the best characterized being the β-glucans [As reviewed by: (Dawood et al., 2018; Petit and Wiegertjes, 2016)]. Despite recent studies showing regulation of C-type lectin receptors by β-glucans, well characterized receptors on fish leukocytes for direct recognition of β-glucans have remained elusive (Kiron et al., 2016; Petit et al., 2019a). It may well be that the mode of action of β-glucans is mainly by bacterial fermentation and subsequent SCFAs production and recognition [As reviewed by: (Petit and Wiegertjes, 2016; Tran et al., 2020)]. Identification and characterization of SCFA receptors in fish thus can be crucial to unravelling the mode of action of fish health feed. Yet, despite the rapid increase in genomic and transcriptomic information for teleost fish, coding sequences for G protein-coupled receptors associated with the recognition of SCFAs have not yet been widely published. Advances in next generation sequencing increasingly provide detailed information on the genetic consequences of whole genome duplication (WGD) events for teleosts (3R), tandem duplications and fish families specific duplications (4R) such as autotetraploid salmonids and allotetraploid cyprinids (Petit et al., 2017). A recent study described FFAR4 (GPR120) in fish and showed a correlation between presence of FFAR4 and immune regulation by long-chain fatty acids, the dogmatic ligands for GPR120 (Wu et al., 2021). Another study, reported on an ortholog of *hcar1* in zebrafish and revealed a reduction in inflammatory responses to wounding after immersing zebrafish in butyrate (Cholan et al., 2020). Furthermore, this reduction in inflammatory responses was specifically abolished in neutrophils and not macrophages through knockdown of *hcar1*. Here, we identify and validate ten coding sequences for common carp *gpr40L* genes, with closest relationship to mammalian GPR43, and identify the presence of multiple *gpr40L* genes in 25 different, mostly teleost, fish species, showing a general subdivision in three subclasses a-c of *gpr40L* genes and conservation of synteny for these G protein-coupled receptors for SCFAs in teleost fish.

2. Methods

2.1. Genome search and full-length cloning of common carp gpr40L sequences

Using human and mouse coding sequences of GPR41 (Hs: NM_005304.5, Mm: NM_001033316.2) and GPR43 (Hs: NM_005306.3, Mm: NM_001168509.1), a BLAST search was performed, using CLC Main workbench v20.0.2[1] and the most recent common carp (*Cyprinus carpio carpio*) genome assembly (PRJEB42985). Following BLAST search with human and mouse sequences, AUGUSTUS gene prediction software (Stanke and Morgenstern, 2005) was used on hit regions and putative coding sequences were identified. Subsequently, putative common carp sequences were used as BLAST query to identify other *gpr40L* sequences. Ten identified coding sequences were aligned and curated with *de novo* transcriptome data from Bioprojects PRJNA73579 (Kolder et al., 2016) and PRJEB42985.

Based on these curated sequences, validation primers were designed for cDNA cloning (Suppl. Table 1). A cDNA pool of stimulated and unstimulated leukocyte samples from previous studies (Petit et al., 2019a, 2019b) were used as template for PCR for sequence validation. Proofreading PCR was prepared as follows: 5 μL 10X Buffer with 15 mM $MgCl_2$, 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), 1 μL dNTP mix (10 mM, Promega), 5 μL cDNA template, 0.3 μL high fidelity taq polymerase (Roche), 36.7 μL DNase free water. PCR amplification was performed in a thermocycler T3000 (Biometra). Following amplification, PCR products were analysed using gel electrophoresis and amplicons of predicted size were cleaned using DNA Clean & Concentrator (D4029, Zymo research) and send for sequence confirmation (Eurofins). Sequence results were analysed using SnapGene viewer v5.3. (Insightful Science[2]).

2.2. Protein analysis and synteny

Translated protein sequences were examined for the presence of a signal peptide using SignalP-5.0[3] and presence of transmembrane regions using TMHMM Server v2.0.[4] Molecular weight and protein pI were calculated using compute pI/Mw tool from Expasy (Wilkins et al., 1999). Analysis of conserved protein domains was performed with InterProScan 5 (Jones et al., 2014).

Synteny analysis relied on genomic location data from NCBI and Ensembl Gene Summary databases. As primary genome assemblies for genomic location data were used: human (GRCh38.p13) and spotted gar (LepOcu1). For genomic location of carp genes the most recent common carp assembly (PRJEB42985) was used.

2.3. Identification of teleost gpr40L genes

Coding sequences for *gpr40L* genes from diverse, mostly teleost, fish species were obtained via BLAST search using several different genomic resources (Suppl. Table 2). BLAST search was performed using CLC Main Workbench v20.0.2.[5] Upon finding homologous sequences, genomic regions were analysed for single exon sequences using AUGUSTUS gene prediction software (Stanke and Morgenstern, 2005) and putative coding sequences were identified translated for further analysis.

2.4. Phylogenetic analysis

Model Selection feature of MEGA-X (Kumar et al., 2016) was used to calculate the most appropriate amino acid substitution model using all sites of the alignment as input data. The evolutionary history of phylogenetic analysis of common carp and selected mammalian protein sequences was inferred by using the Maximum Likelihood (ML) method based on the Jones-Taylor-Thornton (JTT) model (Jones et al., 1992) allowing for Gamma distribution (+G) with four Gamma categories. The bootstrap consensus tree inferred from n = 1000 replicates was taken to represent the evolutionary history of the taxa analysed (Felsenstein,

[1] https://www.qiagenbioinformatics.com.
[2] available at www.snapgene.com.
[3] http://www.cbs.dtu.dk/services/SignalP/.
[4] http://www.cbs.dtu.dk/services/TMHMM/.
[5] https://www.qiagenbioinformatics.com.

1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (+G, parameter = 2,8912). This first alignment included a total of n = 18 GPR40-like sequences.

A second phylogenetic analysis was performed to analyse evolutionary history of all uncovered teleost *gpr40L* sequences and selected other vertebrate genes. A ML method based JTT + G model with four Gamma categories (Gamma parameter for evolutionary rate differences among sites 0,8238) and n = 1000 replicates was used to infer the bootstrap consensus tree. This second alignment included a total of n = 157 gpr40-like sequences from 26 fish species, mouse and human.

3. Results

3.1. Genomic identification of GPR40 family genes of common carp

An initial BLAST search with human and mouse sequences for GPR41 and GPR43, identified ten GPR40-like (gpr40L) sequences and a non-coding remnant of a gpr40L gene divided over four regions of the common carp genome (Suppl. Table 3). For further curation, predicted sequences were aligned with a *de novo* transcriptome from common carp. Conventional cloning (description of primers in Suppl. Table 1) validated the complete cDNAs for all ten GPR40 family member like genes. All coding sequences consist of a single exon and translated into protein sequences ranging from 305 to 329 amino acids. Sequences were named *gpr40L* followed by the number of the relevant linkage group (LG) and numbered relative to their position close to *hpn* as conserved neighbouring gene (Suppl. Table 3, see also Fig. 2).

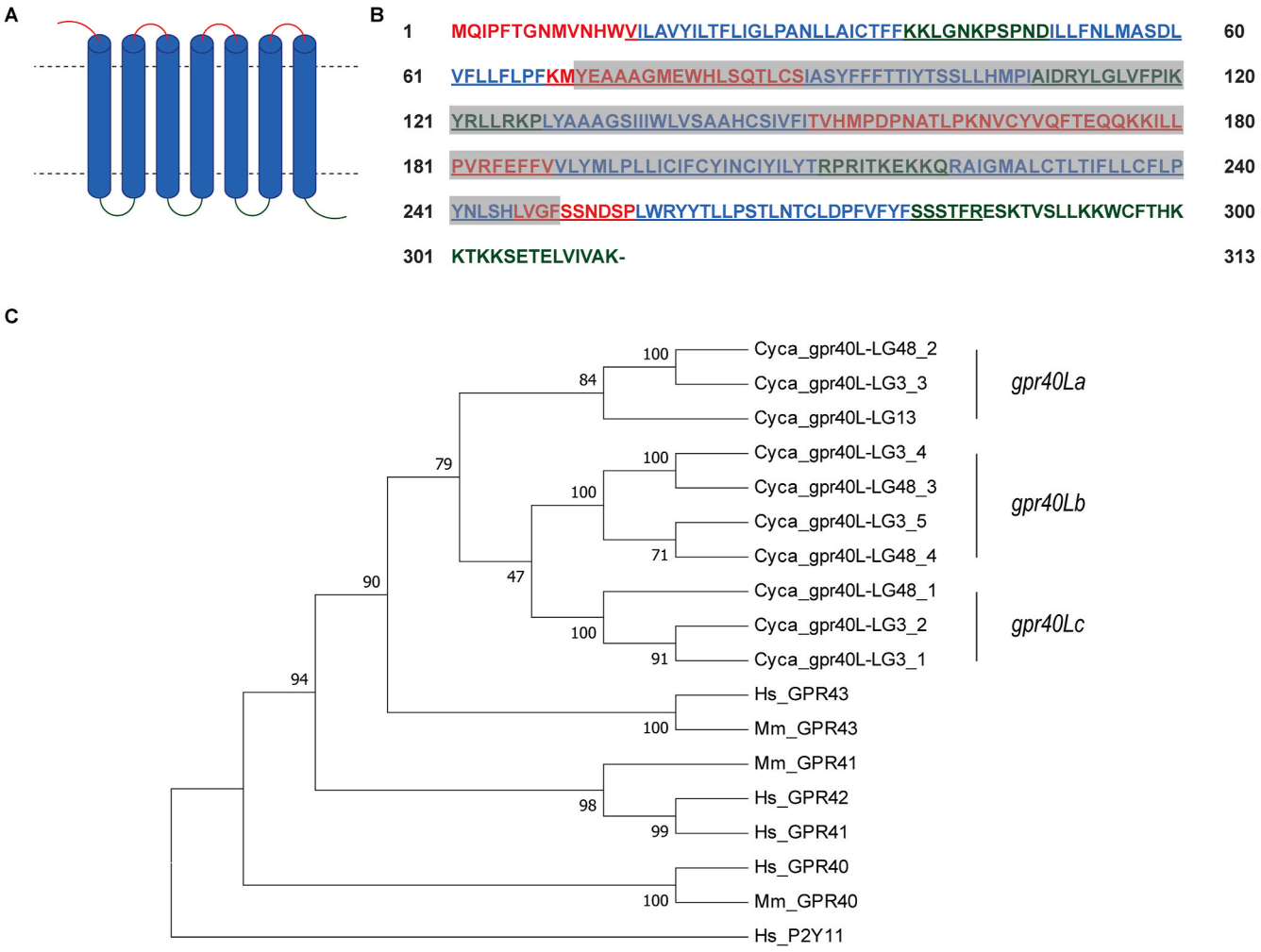

Figure 1. Protein analysis of common carp gpr40L sequences. 1A. Schematic representation of a typical GPR40-related receptor. Blue cylinders indicate transmembrane regions, red lines indicate extracellular regions and green lines indicate intracellular regions. Black dotted lines indicate the cell membranes. 1B. Protein sequence analysis of gpr40L-LG3_1. Red text indicates extracellular regions, blue text indicates transmembrane region and green text indicates intracellular cytoplasmic domain. Underlined text indicates the region that is identified as a conserved 'G protein-coupled receptor, rhodopsin-like' domain (IPR000276) and grey boxed text indicates the region within that domain that is identified as a conserved 'G protein-coupled receptor 40-related receptor' domain (IPR013312). 1C. Phylogenetic analysis of common carp putative gpr40L and human and mouse GPR40 family member protein sequences (GPR40 hs: NP_005294.1, mm: AAN03478.1; GPR41 hs: NP_005295.1, mm: NP_001028488.1; GPR42 hs: NP_001335124.1; GPR43 hs: NP_001357016.1, mm: NP_001161983.1). Species is indicated as Hs (*Homo sapiens*) for human, Mm (Mus musculus) for mouse and Cyca (Cyprinus carpio) for common carp. Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are indicated. Protein sequence of human P2Y11 (AAB88674.1) is used as an outgroup, as it is a Rhodopsin-like receptor Subfamily A11, but not a GPR40-related receptor. Based on the clustering of the common carp sequences a division of gpr40L-a, -b and -c is made.

3.2. Protein alignment and phylogenetic characterization of common carp gpr40L

Analysis of protein sequence architecture and domain structures show a comparable and conserved architecture for all ten sequences. As expected, all putative common carp *gpr40L* proteins lack a signal peptide but have a G protein-coupled receptor rhodopsin-like (IPR000276) protein domain, further specified as G protein-coupled receptor 40-related receptor (IPR013312) domain, and seven transmembrane domains typical of GPR40 family members (Fig. 1A and B). Phylogenetic analysis groups the different *gpr40L* protein sequences of common carp closest to mammalian GPR43, with three distinct subclasses putatively named *a-c* (Fig. 1C).

3.3. Synteny analysis of common carp gpr40L

Mammals generally have three GPR (GPR40, GPR41 and GPR43), in primates such as human GPR41 has tandem duplicated, giving rise to GPR42. The spotted gar provides an excellent evolutionary outgroup because it diverged from teleosts before the teleost WGD (3R), while its genome is organized more similarly to that of humans than teleosts, it is biologically more similar to teleost fish. Comparison of the region on human chromosome 19 that includes GPR40, GPR41, GPR42 and GPR43 revealed a clear conservation of syntenic relationships with the genomic organisation of LG24 of spotted gar and with the relevant regions of LG3 and LG48 of common carp, although the orientation and the relative positions of the *gpr40L* genes could be different (Fig. 2). Comparison of a total of 40 genes neighbouring the GPR cluster on human chromosome 19 indicated conservation of synteny of at least 28 genes on LG24 of spotted gar. Comparison with the gene organisation of common carp indicated conservation of at least 14 genes on both LG3 and LG48. A block of ten genes (*fxyd5 – hsp-like*) relocated (spotted gar versus common carp) to a different linkage group.

Our synteny analysis suggests that the *gpr40L* sequences present on LG3 and LG48 are the result of the common carp-specific WGD (4R). Similarly, LG13 and LG32 can also be considered the result of such 4R duplication event, with remnants of a *gpr40L* gene still present on LG32. Further investigation of genomic organisation of this region in closely related zebrafish (data not shown), suggests *gpr40L-LG3_1* and *gpr40L-LG3_2* to be the result of a tandem duplication, because in zebrafish only a single *gpr40L* gene is located between *hpn* and *hamp*. This hypothesis is supported by the phylogenetic clustering of the *gpr40L* gene sequences of common carp (Fig. 1C). Taken together, synteny analysis reveals conservation of genomic organisation of the region neighbouring the *gpr40L* genes and provides a strong basis for further research into the functions of the different *gpr40L* genes in common carp.

Based on phylogenetic clustering and genomic organisation, we named the different common carp sequences as *gpr40L-a*, *-b* and *-c*. Duplicated sequences were numbered based on suggested evolutionary relationship (Fig. 1C) and in case of more duplications, numbering was based on the relevant linkage group (low to high, e.g. x-x.1 and x-x.2). The proposed nomenclature for the *gpr40L* genes of common carp is detailed in Supplementary Table 4.

3.4. Teleost gpr40L

An extended BLAST search with not only the human and mouse sequences for GPR40, GPR41, GPR42 and GPR43, but also the newly identified common carp *gpr40L* sequences, identified GPR40-like (*gpr40L*) gene sequences in several teleost genomes (Suppl. Table 5). These teleost *gpr40L* sequences were numbered based on their relative location with respect to syntenic genes (as indicated in Suppl. Table 5), or in absence of syntenic genes, based on their 5′-3′ order. Phylogenetic analysis of these gpr40L protein sequences confirms a ubiquitous presence across teleosts of three subclasses (*a-c*), closer related to mammalian GPR43 than to mammalian GPR41 (Suppl. Fig 1). A clear further distinction into two separate clades can be made within subclass *-a* (highlighted in red and separated by a dotted line). Analysis of high-quality genomes confirmed a ubiquitous conservation of synteny with several neighbouring genes, in particular: *hamp, hpn, lsr, mag* and *usf2L* (data not shown). Interestingly, species-specific variation in copy number for *gpr40L* appears evident. For example, the goldfish genome shows evidence of 15 full *gpr40L* sequences, while the genomes of Atlantic cod, three-spined stickleback and haddock only provide evidence for two coding *gpr40L* sequences. The observed teleost species-specific variation in copy numbers of *gpr40L* supports previous suggestions that this genomic region could be a hotspot for tandem-duplications, such as the one that led to the primate-specific GPR42 (Brown et al., 2003; Brown et al., 2005). Based on our finding that all teleost *gpr40L* genes appear closest related to human GPR43, we hypothesize the teleost *gpr40L* family originates from a single ancestral gene multiplied by tandem duplication and subsequent divergence.

4. Discussion

This study identified ten different *gpr40L* genes in common carp. Phylogenetic analysis showed a division into three subclasses, putatively named class a, b and c, and showed the common carp genes had a closer phylogenetic relationship to mammalian GPR43 than to mammalian GPR41. Synteny analysis showed a clear conservation of genomic organisation, further supporting the identity of these genes as *gpr40L*. The presence of multiple *gpr40L* genes in 25, mostly teleost, fish species investigated supports a conserved subdivision in three subclasses a-c.

Analysis of a transcriptome dataset available for common carp head kidney-derived macrophages (Wentzel et al., 2020) confirmed the presence and regulation of expression of most but not all genes in common carp macrophages stimulated with M1/M2 stimuli typically used to polarize (Suppl. Table 6). Although it is interesting to observe the differential regulation of the *gpr40L* genes under influence of M1/M2 stimuli (lipopolysaccharide (LPS) and cyclic AMP (cAMP), respectively), the real interest lies with effects on SCFAs on the polarization of fish macrophages, acting via SCFA receptors. Evidence in mammals shows differential effects of SCFAs on M1 and M2 macrophages. Butyrate affects M1-induced TNFα production but not M1-induced IL1β production, and augments M2-induced IL10 production in a human cell line (Foey, 2011). Mouse macrophages also show distinct responses after GPR43 activation by SCFAs, with TNFα production in SCFA-activated M2 macrophages but not M1 macrophages (Nakajima et al., 2017). Our preliminary observations that typical M1-and M2-stimuli (LPS and cAMP, respectively) can regulate *gpr40L* gene expression is a first step towards more targeted investigations into the functioning of these receptors in common carp, and in other teleosts.

Future studies into the role of fish *gpr40L* by studying the effects of SCFAs on the regulation of these genes can possibly allow for first and interesting insights into immunomodulatory responses induced by different stimuli in, for example, fish health feeds supplemented with additives such as β-glucans. Some of these studies may be performed in retrospect using existing samples and datasets. If *gpr40L* genes indeed can be differentially regulated by the presence of different (concentrations of) SCFA, studies into the effects of prebiotic stimuli on fish health and performance can be greatly aided by such analyses. As the majority of SCFAs produced in the digestive tract is rapidly absorbed by the intestine (Collinder et al., 2003), analysis of *gpr40L* gene regulation might prove informative.

As already shown in studies in mice, knockdown of GPR41 and GPR43 can elucidate some of the mechanisms underlying immunomodulatory effects of SCFAs and their role in, for instance, regulation of inflammation [As reviewed by: (Ang and Ding, 2016; Ulven, 2012)]. The identification of *gpr40L* genes in common carp opens the door for such studies in closely related zebrafish in particular, as established animal model. The value of this approach was already evidenced in a recent study investigating the role of an ortholog of *hcar1* in zebrafish (Cholan

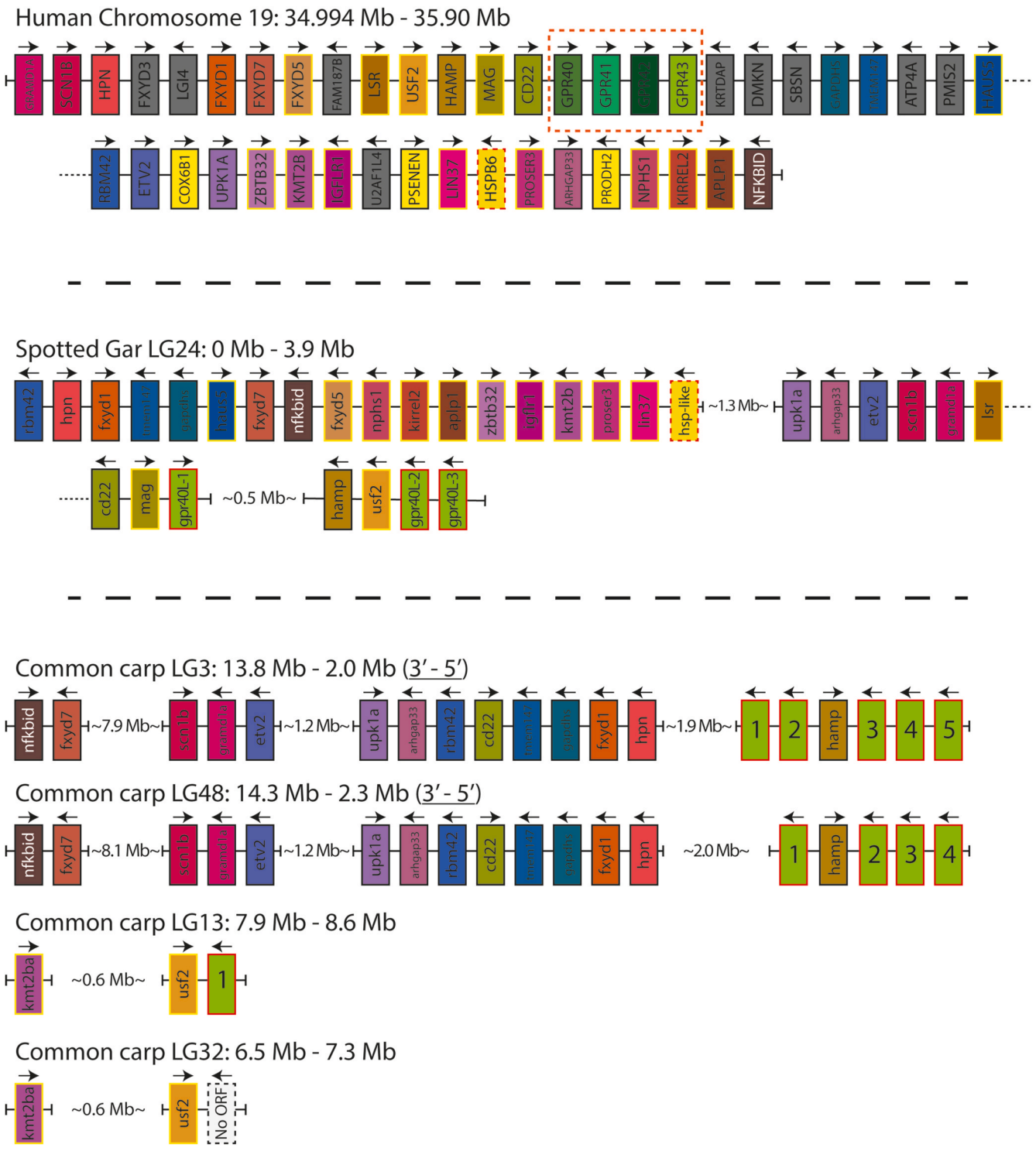

Fig. 2. Synteny analysis of human chromosome 19 (34.994 Mb–35.90 Mb), Spotted gar linkage group (LG) 24 (0 Mb–3.9 Mb) and common carp LG3 (13.8 Mb–2.0 Mb), LG48 (14.3 Mb–2.3 Mb), LG13 (7.9 Mb–8.6 Mb) and LG32 (6.5 Mb–7.3 Mb). Orange dotted box highlights the genomic localization of human GPR40-related receptors, GPR40, GPR41, GPR42 and GPR43. Green squares with red borders represent putative fish gpr40L family members. In spotted gar, putative sequences are designated 1, 2 and 3 based on relative genomic position to hpn. In common carp, sequences are designated 1–5 (LG3), 1–4 (LG48) or 1 (LG13) based on their relative genomic position to hpn (LG3 and LG48) or usf2 (LG13). Yellow bordered squares represent neighbouring genes that have relocated to different linkage groups (other than linkage groups with HPN) in either spotted gar or common carp. Transparent box with dotted line represents a putative remnant of a gpr40L member without ORF. Grey squares with black text represent genes for which no clear ortholog was uncovered in the spotted gar or common carp genome. Yellow square with red dotted border, represents a comparable gene but not orthologous.

et al., 2020). Already established *in vivo* gut enteritis models in zebrafish [As reviewed by: (Brugman, 2016)] could be employed in combination with *gpr40L*-knockout or knockdown zebrafish lines, either or not in combination with prebiotic or direct SCFA administration, to help investigate the role of *gpr40L* receptors in teleost fish.

Even though our identification of *gpr40L* genes in teleost fish allows for further investigation into mechanisms underlying immunomodulatory effects of SCFAs, it should be noted that there are several other genes and mechanisms of importance to be considered. Despite recent identification of *hcar1* in zebrafish, genes homologous to GPR109A (HCAR2) and OLFR78 have remained unidentified in fish. Furthermore, SCFAs can also passively (and aided by transporters) diffuse into cells without activating receptors. Nevertheless, the identification of *gpr40L* genes in fish is an important first step towards a further understanding of the role and functions of receptors for SCFAs and associated immunomodulation in fish.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2021.104240.

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