REVIEW

Phylogeny and evolution of class-I helical cytokines

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Abstract

The class-I helical cytokines constitute a large group of signalling molecules that play key roles in a plethora of physiological processes including host defence, immune regulation, somatic growth, reproduction, food intake and energy metabolism, regulation of neural growth and many more. Despite little primary amino acid sequence similarity, the view that all contemporary class-I helical cytokines have expanded from a single ancestor is widely accepted, as all class-I helical cytokines share a similar three-dimensional fold, signal via related class-I helical cytokine receptors and activate similar intracellular signalling cascades. Virtually all of our knowledge on class-I helical cytokine signalling derives from research on primate and rodent species. Information on the presence, structure and function of class-I helical cytokines in non-mammalian vertebrates and non-vertebrates is fragmentary. Consequently, our ideas about the evolution of this versatile multigene family are often based on a limited comparison of human and murine orthologs. In the last 5 years, whole genome sequencing projects have yielded draft genomes of the early vertebrates, pufferfish (*Takifugu rubripes*), spotted green pufferfish (*Tetraodon nigroviridis*) and zebrafish (*Danio rerio)*. Fuelled by this development, fish orthologs of a number of mammalian class-I helical cytokines have recently been discovered. In this review, we have characterised the mammalian class-I helical cytokine family and compared it with the emerging class-I helical cytokine repertoire of teleost fish. This approach offers important insights into cytokine evolution as it identifies the helical cytokines shared by fish and mammals that, consequently, existed before the divergence of teleosts and tetrapods. A 'fish–mammalian' comparison will identify the class-I helical cytokines that still await discovery in fish or, alternatively, may have been evolutionarily recent additions to the mammalian cytokine repertoire.

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Introduction

Class-I helical cytokines are considered a monophyletic group, although they share little primary sequence identity (Bazan 1990*b*). This implies that a single ancestral gene that expanded by successive gene duplication events (largely in the vertebrate lineage) is at the basis of the contemporary multigene class-I helical cytokine family. All class-I helical cytokines fold into a bundle of four α-helices and signal via related receptors that share molecular signatures (Bazan 1990*a*, Thoreau *et al.* 1991); moreover, following ligand binding, these receptors activate similar intracellular signalling pathways (Taga & Kishimoto 1997, Gadina *et al.* 2001, Vosshenrich & Di Santo 2002, Heinrich *et al.* 2003). Our knowledge about class-I helical cytokines is largely based on mammalian studies and therefore offers only a limited view on the evolutionary history of this large and important family of protein signals. Spurred by the recently acquired knowledge about the genome sequences of different bony fish species such as pufferfish (*Takifugu rubripes*), spotted green pufferfish (*Tetraodon nigroviridis*) and zebrafish (*Danio rerio*) (Aparicio *et al.* 2002, Jaillon *et al.* 2004), several fish class-I helical cytokines have recently been discovered. This now allows us to compare the class-I helical cytokine repertoires of different vertebrate classes. All fish class-I helical cytokines described to date share limited primary sequence identity with their mammalian orthologs. In contrast, the gene structure of orthologous cytokines is conserved throughout vertebrates, a characteristic that is instrumental in the identification of orthologous relationships within the multigene cytokine family that is characterised by poor primary sequence conservation. The instrumental importance of conserved exon size and intron phase is also true for class-I and -II helical cytokine receptor genes, as was already recognised over

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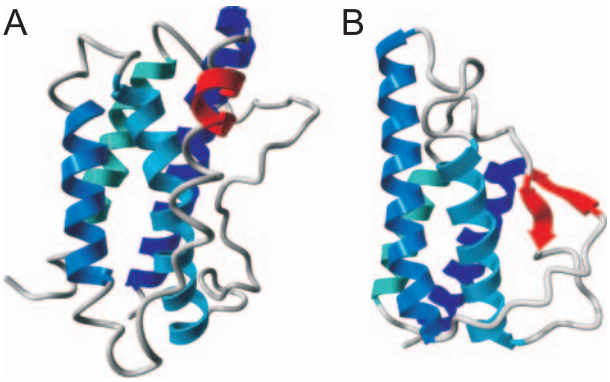


Figure 1 Class-I helical cytokines fold into a bundle of four tightly packed α-helices. On the basis of their helix length, class-I helical cytokines are characterised as (A) long chain, such as IL-6, or (B) short chain, such as IL-4. Many long-chain class-I helical cytokines are also recognisable by an additional short α-helix in the AB or CD loop, indicated in red in (A). In some short-chain class-I helical cytokines, these loops both contain a stretch of β-strand that connect to form a β-sheet (B).

a decade ago (Lutfalla *et al.* 1992, Nakagawa *et al.* 1994). Intron/exon structures have also been used as a criterion for the identification of fish orthologs to mammalian class-II helical cytokines and their receptors (Lutfalla *et al.* 2003). Even though the fish helical cytokine repertoire is as yet not completely known, among the fish helical cytokines characterised to date are several examples of conspicuous differences between fish and mammalian orthologs that illustrate the eventful history of vertebrate class-I helical cytokines. Following an introduction on the principles of class-I helical cytokine receptors and their intracellular signalling that is based on mammalian studies, we separately introduce each mammalian class-I helical cytokine and discuss the evidence for their presence in early vertebrates.

Class-I helical cytokines share a common fold

The tertiary structure of class-I helical cytokines is characterised by a bundle of four tightly packed α-helices, designated helix A–D (Fig. 1); their primary amino acid sequences share little to no sequence similarity. Their common fold separates class-I helical cytokines from other cytokines. For example, class-II helical cytokine molecules (interferons (IFNs), interleukin (IL)-10 and IL-20) contain over four α-helices each (Ealick *et al.* 1991, Zdanov *et al.* 1995). The IL-1 family, which includes IL-1β and IL-18, is characterised by a fold rich in β-strands (Veerapandian *et al.* 1992). A unique aspect of the class-I helical cytokine fold is that the four α-helices are arranged in an 'up-up-down-down' fashion as a result of the anti-parallel orientation of two consecutive pairs of helices. In most class-I helical cytokines this four-helix bundle fold is stabilised by up to three disulphide bridges. In IL-11, which lacks conserved cysteine residues, the four-helix bundle is stabilised solely by hydrophobic interactions that result from buried hydrophobic and exposed charged residues (Czupryn *et al.* 1995).

On the basis of the length of their α-helices, class-I helical cytokines are subdivided into either 'long chain' or 'short chain' (Boulay *et al.* 2003). The long-chain class-I helical cytokines such as growth hormone (GH), prolactin (PRL), leptin, erythropoietin (EPO) and the 'gp130 cytokines' (see below) are made up of 170–250 amino acid residues; the 'short-chain' helical cytokines (among which are IL-2, IL-3, IL-4 and IL-13) typically do not exceed 160 amino acids. An additional difference between the long- and short-chain class-I helical cytokines resides in the loops that connect α-helices A, B, C and D. Given the anti-parallel orientation of the A–B and C–D helix pair, the loop connecting helix B and C (the BC loop) is invariably short. In contrast, the AB and CD loops are much longer, as they span the entire length of the helix bundle. In short-chain helical cytokines, the AB and the CD loops often connect to form a small section of β-sheet (Fig. 1). In contrast, the AB or CD loop in several long-chain class-I helical cytokines contains an additional short α-helix.

The modular make-up of helical cytokine receptors

Class-I helical cytokines exert their actions via cell surface receptors that share a similar modular make-up. The extracellular domain of all class-I helical cytokine receptors includes at least one cytokine-binding domain of approximately 200 amino acids (made up of a tandem of fibronectin type-III (FnIII) domains) and is often associated with additional FnIII or immunoglobulin domains (Fig. 2). The FnIII domain that makes up the membrane proximal half of the cytokine-binding domain usually contains a characteristic WSXWS signature (Bazan 1990*a*, Thoreau *et al.* 1991, Taga & Kishimoto 1997). The WSXWS motif has in some cases been shown to be required for proper receptor folding, but is not directly involved in ligand binding (Yawata *et al.* 1993, Taga & Kishimoto 1997). Whereas some class-I helical cytokine receptors have long intracellular domains, the cytoplasmatic tails of others are short, and incapable of intracellular signalling. The receptor complexes for all class-I helical cytokines contain at least one receptor chain with a long intracellular domain (Fig. 2). Some class-I helical cytokines share a single signalling receptor chain. The best example is provided by gp130, which is a class-I helical cytokine receptor chain that participates in the receptor complexes of several helical cytokines (Ernst & Jenkins 2004). For that reason, the corresponding class-I

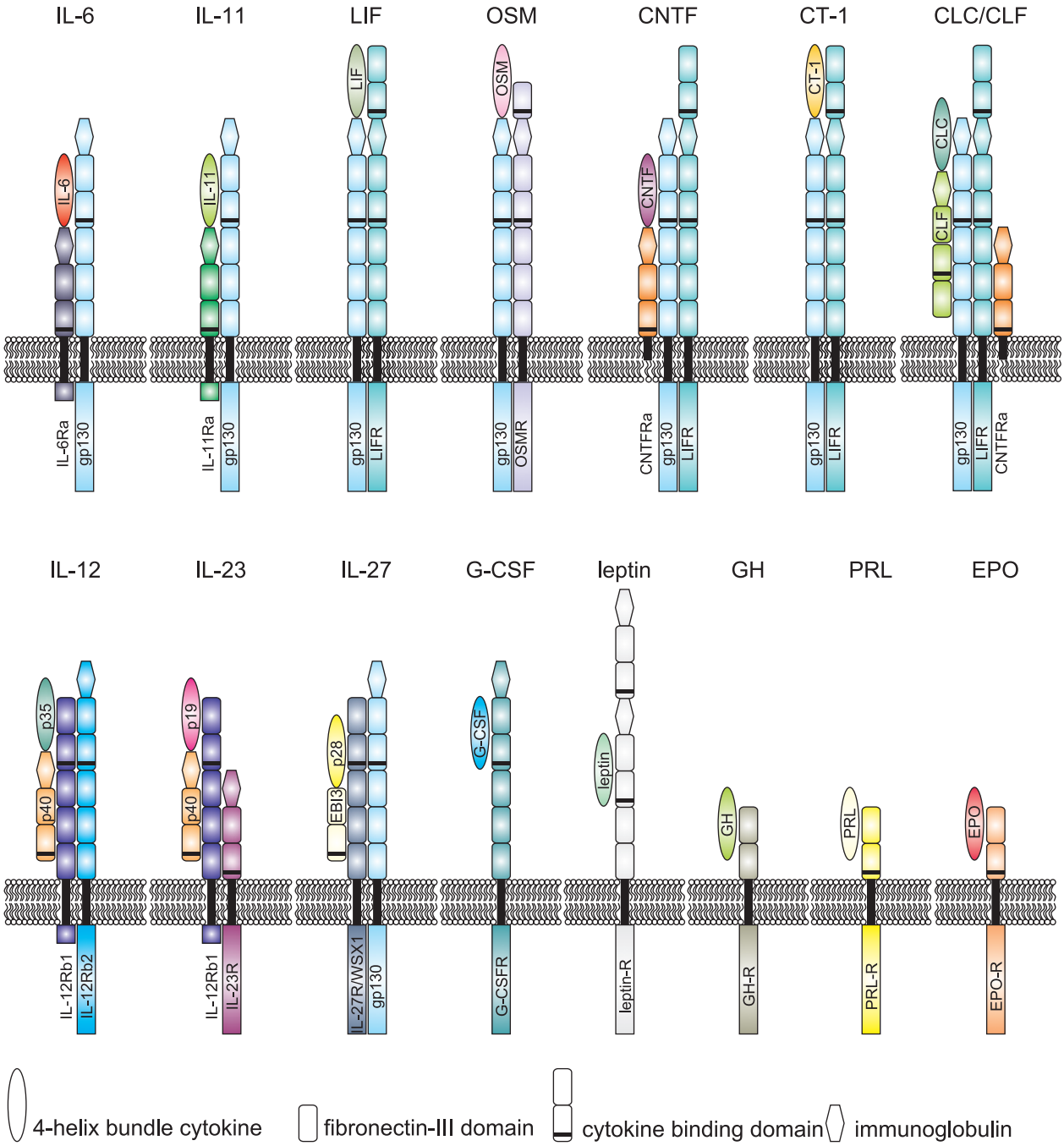


Figure 2 Cytokine receptors share a modular make-up. Class-I helical cytokines signal via a multimeric receptor complex that usually consists of several cytokine receptor chains. All receptor chains contain at least one cytokine-binding domain, formed by two fibronectin type-III (FnIII) domains, of which the membrane-proximal one in most cases contains a consensus WSXWS motif. Many cytokine receptor chains contain an immunoglobulin domain as well as several FnIII domains in addition to the pair that forms the cytokine-binding domain. The typical cytokine receptor complex is composed of a ligand-specific receptor chain which in some cases lacks intracellular signalling capacity, and a second receptor chain which is shared by multiple receptor complexes and is responsible for the transduction of an intracellular signal.

helical cytokines are collectively referred to as 'gp130 cytokines'. This group includes IL-6, IL-11, ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1) and cardiotrophin-like cytokine (CLC) (Fig. 2). The ligand specificity of the receptor is in these instances determined by a second receptor chain that participates in the formation of the receptor complex (Bravo & Heath 2000, Heinrich *et al.* 2003). The IL-6 receptor complex provides a prototypical example: IL-6 binds with high affinity to its specific receptor chain (IL-6Rα) and the IL-6/IL-6Rα complex subsequently recruits gp130 to convey an intracellular signal (Simpson *et al.* 1997). The stoichiometry of this receptor complex indicates a hexamer consisting of doublets of the IL-6, the IL-6Rα-chain and gp130 (Boulanger *et al.* 2003). The IL-11Rα and CNTF receptor α (CNTFRα) resemble the IL-6Ra in that they are short and cannot signal intracellularly. A characteristic feature of the CNTFRα is the lack of a transmembrane domain and its anchoring via a glycosylphosphatidylinositol (GPI) anchor. The IL-11 and CNTF receptor complexes are hexamers, similar in conformation to the IL-6 receptor (Hirano *et al.* 1997, Barton *et al.* 2000, Heinrich *et al.* 2003). The CNTF receptor complex contains a single gp130 chain. Instead, the second gp130 chain is replaced by the LIF receptor (LIFR) (Fig. 2), a signalling chain similar to gp130. The other gp130 cytokines, OSM, LIF and CT-1, bind directly and with high affinity to receptor chains that possess long intracellular domains. Their receptor complexes consist of a combination of gp130 and LIFR (for LIF and CT-1), or gp130 and OSM receptor (OSMR) (for OSM) (Fig. 2) (Heinrich *et al.* 2003).

Some members of the class-I helical cytokine receptor family lack a transmembrane domain, effectively making them soluble rather than membrane bound. IL-12 provides the prototypical example of such a soluble receptor. IL-12 is a heterodimeric cytokine that consists of two disulphide-linked subunits, designated p35 and p40. Whereas p35 is a typical four-helix bundle class-I helical cytokine, p40 is effectively a short soluble class-I helical receptor with a cytokine-binding domain and an immunoglobulin domain (Fig. 2). The receptor for IL-12 is a heterodimer that consists of IL-12Rβ1 and IL-12Rβ2, receptor chains that both resemble gp130 (Holscher 2004). IL-12 is usually not listed as a gp130 cytokine, yet the recent characterisation of several novel heterodimeric cytokines, including IL-23 and IL-27 and their receptors, illustrates that the emerging IL-12 family of heterodimeric cytokines is a mere variation on the common gp130 theme. IL-23 is a disulphide-linked heterodimer of the p40 subunit and the newly identified p19 class-I helical cytokine subunit (Oppmann *et al.* 2000). Its receptor complex is a combination of the IL-12Rβ1 chain with the newly identified IL-23R (Parham *et al.* 2002). Another novel heterodimeric cytokine, IL-27, is a covalently linked combination of the soluble helical cytokine receptor Epstein–Barr virus-induced protein-3 (EBI-3) and the class-I helical cytokine p28 (Pflanz *et al.* 2002). The IL-27 receptor complex consists of the IL-27R (also known as WSX1) and gp130 (Pflanz *et al.* 2002, Trinchieri *et al.* 2003). The shared use of the latter receptor chain illustrates the close parallels between the original gp130 cytokines and the growing family of heterodimeric cytokines. These parallels are further evidenced by the class-I helical cytokine CLC that circulates as a dimer with the soluble receptor molecule cytokine-like factor (CLF). The CLC/CLF complex signals via the CNTF receptor complex (Fig. 2). The heterodimer conformation of CLC/CLF is similar to that of IL-12, although both subunits are not linked via a disulphide bridge (Elson *et al.* 2000). The non-covalently linked heterodimer conformation of CLC/CLF is not unique among the gp130 cytokines. Similar heterodimers of IL-6, IL-11 and CNTF with truncated, soluble versions of each of their specific receptor chains occur (Heinrich *et al.* 2003). This adds an extra level of complexity to gp130 signalling, since these circulating heterodimers convey signals to cells that express only the gp130 chain and lack the endogenous ligand-specific receptor chain (Taga & Kishimoto 1997).

The remaining long-chain class-I helical cytokines (granulocyte-colony stimulating factor (G-CSF), leptin, EPO, thrombopoietin (TPO), GH and PRL) all signal via homodimers of receptor chains that combine high affinity ligand binding with intracellular signalling. The receptors for G-CSF and leptin are similar to gp130 in size and extracellular composition. In contrast, the receptors for GH, PRL, EPO and TPO are notably shorter and consist of a single cytokine-binding domain connected to a long cytoplasmatic tail (Fig. 2). Despite these differences, the early studies that addressed the complex of GH and its receptor (GHR) are at the basis of our knowledge of class-I helical cytokine receptor complex formation and stoichiometry (Simpson *et al.* 1997, Bravo & Heath 2000), as the crystal structure of the GH–GHR complex was the first helical cytokine/receptor complex that was solved (de Vos *et al.* 1992).

Intracellular signalling pathways: phosphorylation is the message

Intracellularly, the recruitment of the multimeric cytokine receptor complex upon cytokine binding brings together the long intracellular signalling domains of two receptor chains, leading to the activation of several distinct intracellular signalling cascades. Each cytoplasmatic domain of gp130 and related signalling chains is constitutively associated with a tyrosine kinase of the JAK family (Janus kinase; after the two-faced, Roman god Janus, referring to JAK's dual (pseudo)-catalytic domains). Dimerisation of class-I helical cytokine receptor chains with an intracellular

signalling domain leads to JAK activation via the transphosphorylation of both JAKs (Chen *et al.* 2004). Phosphorylated JAKs, in turn, phosphorylate several intracellular membrane-distal tyrosine residues in the cytoplasmatic domain of the receptor chain. The phosphorylated tyrosines serve as a docking site for members of the signal transducer and activator of transcription (STAT) family that bind to phosphorylated tyrosine through their Src homology-2 (SH2) domain (Gadina *et al.* 2001). The mammalian STAT family consists of seven members (STAT1, 2, 3, 4, 5a, 5b and 6) that are phosphorylated upon docking to a phosphorylated tyrosine. Phosphorylated STATs dissociate from the receptor chain and form STAT homo- or heterodimers that are translocated into the nucleus to initiate transcription (Darnell 1997, Levy & Darnell 2002, O'Shea *et al.* 2002). Which genes are transcribed depends on the composition of the STAT dimer. As untimely or prolonged JAK/STAT signalling can shift the balance from adequate immune regulation and host defence to a derailed immune response with potentially serious consequences, multiple inhibitory mechanisms control JAK/STAT activity.

One of these inhibitory mechanisms is formed by the SH2 domain containing tyrosine phosphatases (SHP), such as SHP1 and SHP2. These phosphatases dephosphorylate the tyrosines of key signalling components such as JAKs, STATs and helical cytokine receptors, thereby abrogating cytokine signalling (Symes *et al.* 1997, Heinrich *et al.* 2003, Shuai & Liu 2003, Chen *et al.* 2004, Wormald & Hilton 2004).

The protein inhibitors of activated STAT (PIAS) constitute a second class of JAK/STAT signalling inhibitors. Currently, five PIAS members have been identified (PIAS1, PIAS3, PIASxα, PIASxβ and PIASy). By binding to activated STAT dimers, PIAS1 and PIAS3 directly prevent association with DNA, whereas PIASx or PIASy prevent activated STAT from transcription without directly interfering with DNA binding (Chen *et al.* 2004).

Among the many target genes that are transcribed in response to JAK/STAT activation is one particular group of genes that encodes members of the suppressor of cytokine signalling (SOCS) family. Currently the SOCS family consists of eight members (SOCS1–7 and cytokine-inducible SH2-containing domain (CIS)); the role of SOCS4–7 in cytokine signalling has received limited attention. The SOCS proteins exert a classical negative feedback. Like STATs, SOCS proteins contain a central SH2 domain that allows them to bind phosphorylated tyrosine residues (Heinrich *et al.* 2003). SOCS proteins suppress in several ways: SOCS1 directly binds to phosphorylated JAKs and inhibits JAK catalytic activity. CIS, SOCS2 and SOCS3 bind directly to phosphorylated helical cytokine receptor tyrosines, effectively blocking STAT binding (Cooney 2002, Larsen & Ropke 2002, Chen *et al.* 2004, Wormald & Hilton 2004).

Redundancy and specificity in helical cytokine signalling

Many class-I helical cytokines, such as IL-6 and IL-11, and LIF and OSM, display considerable overlap in their spectrum of biological actions. This redundancy is explained by the shared use of the gp130 signalling chain that funnels the intracellular response that follows receptor activation along the same signalling cascade. The cell's response is eventually determined by integration of a plethora of biological signals that may differ among cell types. The redundancy that stems from the shared use of gp130 is illustrated by the embryonically lethal phenotype of gp130 knockout mice, which is considered to result from the cumulative effects of the loss of signalling capacity for a large group of helical cytokines (Yoshida *et al.* 1996).

Besides the overlap in cytokine receptor conformation, redundancy in helical cytokine signalling is achieved by the promiscuity of components of the JAK/STAT signalling pathway. Four JAKs (JAK1–3 and tyrosine kinase 2 (Tyk2)) and seven STATs have been identified in mammals. Together they constitute the intracellular mechanism that is responsible for signalling in response to at least 30 different class-I helical cytokines. This can only be achieved when different cytokines share an intracellular signalling cascade. The involvement of the various JAKs and STATs in signalling in response to specific (groups of) class-I helical cytokines has been defined by assessing the phenotypes of mice lacking specific components of JAK/STAT signalling. The absence of JAK1 leads to a complex perinatally lethal phenotype that is characterised by neurological as well as lymphoid defects (Chen *et al.* 2004). JAK1$^{-/-}$ mice fail to respond to IFNs and lack signalling capacity via the γc-receptor chain, which is the receptor chain responsible for intracellular signal transduction in the receptor complex of many short-chain class-I helical cytokines (Gadina *et al.* 2001). The phenotype of JAK3 knockout mice in part resembles that of the JAK1 knockout, but is less severe. JAK3$^{-/-}$ mice are viable, although they suffer from severe combined immunodeficiency (Shuai & Liu 2003, Chen *et al.* 2004). This immunological defect is caused by abrogated γc-receptor signalling and, since the γc-receptor$^{-/-}$ and JAK3$^{-/-}$ phenotypes are identical, it follows that JAK3 is only employed by the short-chain class-I helical cytokines that signal via this common receptor chain (Suzuki *et al.* 2000). JAK2 is employed by all class-I helical cytokines that signal via a homodimeric receptor. This group includes leptin, PRL, GH, EPO, TPO and G-CSF (Gadina *et al.* 2001, Vosshenrich & Di Santo 2002). Phenotypically, JAK2$^{-/-}$ mice are characterised by defective erythropoiesis as a consequence of silenced EPO signalling (Shuai & Liu 2003, Chen *et al.* 2004). This renders JAK2 knockouts embryonically lethal, before a phenotype of defective signalling by any of the other helical cytokines

that depend on JAK2 becomes manifest. The Tyk2 knockout is viable; this is a mild phenotype compared with the phenotypes described earlier for JAK1–3. Tyk2 can be activated in response to IFN-α/β, IL-10 and various gp-130 cytokines, but appears only to be critically required for IL-12 signalling (Gadina *et al.* 2001, Vosshenrich & Di Santo 2002, Shuai & Liu 2003). Due to this defective IL-12 signalling and possibly diminished IFN signalling capacity, $Tyk2^{-/-}$ mice are susceptible to (viral) pathogen infection, but appear to be otherwise normal (Karaghiosoff *et al.* 2000, Shimoda *et al.* 2000).

STAT3 is the principle signal transducer that is activated in response to gp130 cytokines as well as leptin and G-CSF. The class-I helical cytokines that bind receptors with short extracellular domains (GH, PRL, EPO and TPO) all engage STAT5 (Gadina *et al.* 2001, Vosshenrich & Di Santo 2002). Duplicated and highly similar STAT5 genes, designated STAT5a and STAT5b, are encoded in both the human (locus 17q11·2) and the mouse (locus 11D) genome (Ambrosio *et al.* 2002). Although both are activated by the helical cytokines that depend on STAT5 *in vitro*, $STAT5a^{-/-}$ and $STAT5b^{-/-}$ mice each have a specific phenotype. Female STAT5a knock-outs have impaired mammary gland development and fail to lactate as a consequence of abrogated PRL signalling (Liu *et al.* 1997). In contrast, $STAT5b^{-/-}$ mice display growth defects due to defective GH signalling (Udy *et al.* 1997).

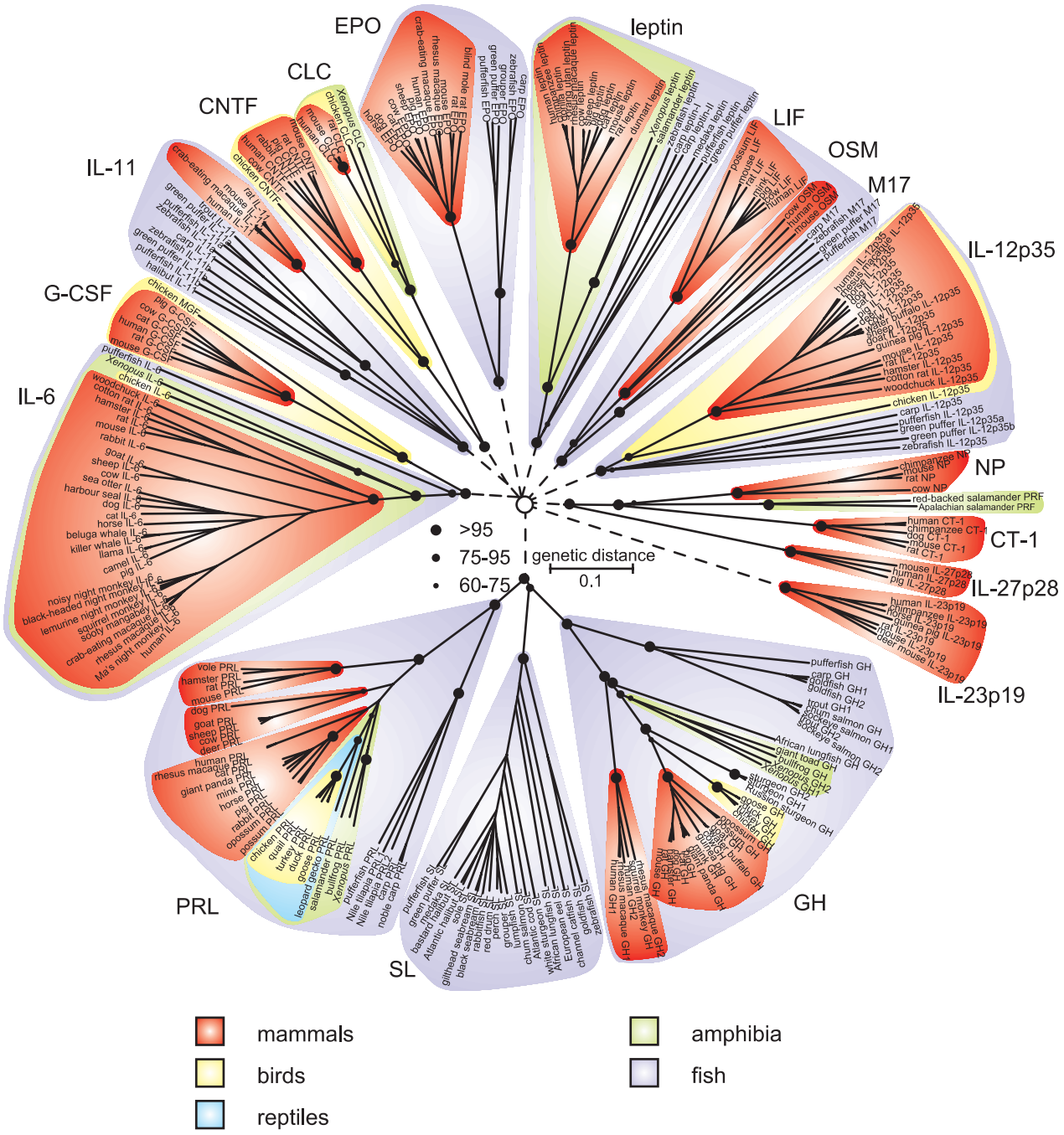
Despite the general redundancy of intracellular signalling pathways, the use of some STATs is highly restricted to a single helical cytokine or a small group of related class-I helical cytokines. These restrictions are so specific that activation of these STATs has become synonymous for the activation of distinct immunological pathways. This is pre-eminently illustrated by the specific role of STATs in the determination of T helper 1 (Th1) and Th2 immune responses. The Th1/Th2 dogma states that the modality of an immune response depends on and is tailored for the effective eradication of the eliciting pathogen. Th1 and Th2 refer to the extremes of a continuous spectrum of immune responses and are, as a rule of thumb, associated with intracellular and extracellular pathogens respectively. Both Th1 and Th2 responses are associated with a panel of 'signature' cytokines (Mosmann *et al.* 1986, Mosmann & Coffman 1989). IL-12 is a key cytokine in the determination of Th1 responses that, when released from activated macrophages, induces IFNγ production in T cells (Agnello *et al.* 2003, Watford *et al.* 2003). A Th2 response, in contrast, is associated with the release of IL-4, IL-5 and IL-13, a group of related short-chain class-I helical cytokines that utilises similar receptors (Jarnicki & Fallon 2003, O'Byrne *et al.* 2004). Th1 cytokines inhibit the expression of Th2 cytokines and vice versa, thus the balance between Th1 and Th2 cytokines determines the outcome of the overall immune response. And whereas most STATs are promiscuous to some degree, Th1 and Th2 signalling each involve a unique STAT: IL-4 and IL-13 activate STAT6 whereas IL-12 relies solely on STAT4 (Gadina *et al.* 2001, O'Shea *et al.* 2002, Vosshenrich & Di Santo 2002). Consequently, the activation of STAT4 or STAT6 is a tell-tale for the type of immune response unfolding (Agnello *et al.* 2003).

Class-I helical cytokines in teleosts

In the following paragraphs we separately introduce the long-chain class-I helical cytokines and discuss evidence for orthologous non-mammalian cytokines. Helical cytokines that are found on the same locus in mammals (e.g. LIF and OSM) are discussed together, as are helical cytokines that share distinct structural characteristics and/or functional properties such as the heterodimeric cytokines. Also the order in which the various (pairs of) helical cytokines are covered is loosely determined by shared features such as gene structure and shared cysteine residues.

IL-6

The pleiotropic nature of IL-6 is aptly illustrated by the history of its discovery. The cloning and characterisation of IL-6 by four separate groups (Haegeman *et al.* 1986, Hirano *et al.* 1986, May *et al.* 1986, Zilberstein *et al.* 1986) revealed that a variety of factors known as B-cell stimulatory factor 2, B-cell differentiation factor, hybridoma-plasmacytoma growth factor, IFN-β2, hepatocyte-stimulating factor, T cell replacing factor-like factor and monocyte-granulocyte inducer type 2 were in fact one and the same. IL-6 is an important inducer of antibody production, although the finding that IL-6-deficient mice display reduced IgG, but normal early IgM responses to *Vaccinia* virus infection indicates that IL-6 is not equally important for the production of all Ig isotypes (Kopf *et al.* 1994). IL-6 also has the capacity to stimulate T cells, and participates in haematopoiesis by inducing the proliferation of pluripotent progenitor cells. In addition, IL-6 stimulates the differentiation of myeloid progenitors into granulocytes and macrophages and promotes megakaryocyte maturation (Taga & Kishimoto 1997). In the liver, IL-6 is one of the major inducers of the acute phase reaction, in concert with IL-1β and tumour necrosis factor-α (TNFα) (Suffredini *et al.* 1999). An interesting property of IL-6 is that it is one of several class-I cytokines that can modulate the hypothalamic–pituitary–adrenal axis, thus directly influencing the stress response (Chesnokova & Melmed 2002). The IL-6 gene in humans and mice consists of five exons (Tanabe *et al.* 1988) and the four-helix bundle topology of IL-6 is stabilised by two internal disulphide bridges. The elucidation of the pufferfish genome has revealed the existence of IL-6 in fish (Fig. 3). The pufferfish IL-6 gene is similar in organisation to that of mammalian IL-6 and G-CSF genes. Pufferfish IL-6 shares two conserved cysteine residues



with mammalian IL-6 and G-CSF sequences but lacks their N-terminal cysteine pair (Fig. 4). *In vitro* stimulation of pufferfish head kidney cells with the T cell activator phytohaemagglutinin rapidly upregulates IL-6 expression, demonstrating its expression and upregulation within the leukocyte compartment (Bird *et al*. 2005*b*).

Figure 3 Phylogeny of the vertebrate class-I helical cytokines. For most mammalian class-I helical cytokines, non-mammalian orthologs have been identified to date. The amino acid sequence alignment obtained by T-Coffee (Notredame *et al.* 2000) was refined by hand to correct for overt alignment mismatches. Phylogenetic analysis of such a large number of amino acid sequences (>250 sequences), which are only weakly similar both within a single species (paralogs) as well as between distantly related species (orthologs), is sensitive to artifactual topologies. To prevent this from occurring, constraints (indicated by dashed lines) were introduced for the deep topology of the tree based on the information regarding gene organisation, chromosomal location and conserved cysteine pairs, which is summarised in Fig. 4. The phylogeny was reconstructed on the basis of an amino acid differences (p-distance) in MEGA3 (Kumar *et al.* 2004) using the neighbour-joining algorithm. The tree obtained via the minimum evolution algorithm had an essentially identical topology (not shown). Branch lengths reflect the extent of the genetic distance between sequences. The confidence level of 1000 bootstrap replications is indicated by the size of the dots at the branch nodes. Clusters of mammalian, avian, reptilian, amphibian, and teleostean cytokines are indicated in red, yellow, blue, green and lilac respectively. Note that some clusters are notably more compact than others, reflecting a higher degree of primary sequence conservation. Accession numbers are as follows: human IL-6, P05231; rhesus macaque IL-6, P51494; crab-eating macaque IL-6, P79341; sooty mangabey IL-6, P46650; Ma's night monkey IL-6, AF014510; squirrel monkey IL-6, AF294757; lemurine night monkey IL-6, AF097323; black-headed night monkey IL-6, AF097322; noisy night monkey IL-6, AF014505; mouse IL-6, P08505; rat IL-6, P20607; rabbit IL-6, AAF86660; hamster IL-6, BAA78766; cotton rat IL-6, AF421389; woodchuck IL-6, O35736; cow IL-6, P26892; pig IL-6, P26893; sheep IL-6, P29455; horse IL-6, Q95181; camel IL-6, AB107656; llama IL-6, AB107647; goat IL-6, Q28319; cat IL-6, P41683; dog IL-6, P41323; killer whale IL-6, Q28747; beluga whale IL-6, AAD42929; harbour seal IL-6, Q28819; sea otter IL-6, AAB01428; chicken IL-6, CAC40812; *Xenopus* IL-6, AW637075; pufferfish IL-6, Q6 L6X6; mouse G-CSF, P09920; rat G-CSF U37101; human G-CSF, P09919; cat G-CSF, NM_001009227; cow G-CSF, AF092533; pig G-CSF, U68481; chicken myelomonocytic growth factor, NM_205279; human IL-11, P20809; crab-eating macaque IL-11, P20808; mouse IL-11, P47873; rat IL-11, AAK29623; carp IL-11, AJ632159; halibut IL-11, AU090873; pufferfish IL-11a, BN000713; pufferfish IL-11b, BN000714; green puffer IL-11a, BN000715; green puffer IL-11b, AY374508; zebrafish IL-11a, BN000717; zebrafish IL-11b, BN000718; trout IL-11, AJ535687; horse EPO, AB100030; cow EPO, P48617; dog EPO, P33707; cat EPO, P33708; human EPO, P01588; crab-eating macaque EPO, P07865; rhesus macaque EPO, Q28513; mouse EPO, P07321; rat EPO, P29676; sheep EPO, P33709; blind mole rat EPO, AJ715792; pig EPO, NM_214134; pufferfish EPO, AY303753; zebrafish EPO, DQ278896; carp EPO, AJ831393; green puffer EPO, AY374507; grouper EPO, AY735012; human IL-12p35, P29459; rhesus macaque IL-12p35, P48091; mouse IL-12p35, P43431; rat IL-12p35, AAD51364; hamster IL-12p35, AB085791; woodchuck IL-12p35, X97018; guinea pig IL-12p35, AB025723; cotton rat IL-12p35, AF421396; cow IL-12p35, P54349; water buffalo IL-12p35, AY232819; deer IL-12p35, U57751; pig IL-12p35, Q29053; horse IL-12p35, Q9 XSQ6; sheep IL-12p35, Q9TU27; goat IL-12p35, O02814; dog IL-12p35, Q28267; cat IL-12p35, O02743; carp IL-12p35, AJ580354; zebrafish IL-12p35, AB183001; chicken IL-12p35, AY262751; pufferfish IL-12p35, AB096265; green puffer IL-12p35a, AY374509; green puffer IL-12p35b, AY374510; human IL-23p19, AF301620; chimpanzee IL-23p19, AY412450; mouse IL-23p19, AF301619; rat IL-23p19, NM_130410; deer mouse IL-23p19, AY259629; guinea pig IL-23p19, AB058509; horse IL-23p19, AY704416; mouse IL-27p28, AY099297; human IL-27p28, AY099296; pig IL-27p28, AY788913; human CT-1, Q16619; chimpanzee CT-1, XM_523348; mouse CT-1, Q60753; rat CT-1, Q63086; dog CT-1, XM_843979; chimpanzee neuropoitin (NP), Q6R2R2; mouse NP, P83714; rat NP, AY518205; cow NP, XM_609151; red-backed salamander plethodontid receptivity factor (PRF), AY926884; Appalachian salamander PRF, AY926937; human leptin, P41159; chimpanzee leptin, O02750; gorilla leptin, Q95189; orang utan leptin, Q95234; rhesus macaque leptin, Q28504; mouse leptin, P41160; rat leptin, P50596; cow leptin, P50595; sheep leptin, Q28603; pig leptin, Q29406; dog leptin, O02720; cat leptin, AB041360; dunnart leptin, AF159713; *Xenopus* leptin, AY884210; salamander leptin, CN054256; medaka leptin, AB193548; zebrafish leptin, BN000830/GENSCAN00000007598; pufferfish leptin, AB193547; green puffer leptin, AB193549; carp leptin-I, AJ830745; carp leptin-II, AJ830745; human CLC, AF176912; mouse CLC, Q9QZM3; rat CLC, NM_207615; chicken CLC, XM427323; *Xenopus* CLC, CR762259; human CNTF, P26441; rat CNTF, P20294; mouse CNTF, P51642; pig CNTF, O02732; cow CNTF, XM_607445; rabbit CNTF, P14188; chicken CNTF, Q02011, carp M17, AY102632; green puffer M17, CAF99247; zebrafish M17, NW_634687; pufferfish M17, GENSCANSLICE00000010963/CAAB01000021·1; cow LIF, Q27956; human LIF, P15018; mouse LIF, P09056; rat LIF, P17777; mink LIF, O62728; pig LIF, Q9 GKZ8; hamster LIF, AY171245; possum LIF, AF303448; cow OSM, P53346; human OSM, P13725; mouse OSM, P53347; human GH1, P01241; human GH2, P01242; mouse GH, P06880; rat GH, P01244; rhesus macaque GH1, P33093; rhesus macaque GH2, Q07370; squirrel monkey GH, AF339060; cow GH, V00111; dog GH, Z23067; cat GH, U13390; water buffalo GH, X72947; mink GH, X56120; giant panda GH, AF540936; guinea pig GH, AF233853; hamster GH, S66299; possum GH, AF052192; opossum GH, AF312023; pig GH, AY536527; goat GH, Y00767; chicken GH, P08998; turkey GH, M33697; duck GH, X07079; goose GH, AY149895; *Xenopus* GH1, P12855; *Xenopus* GH2, P12856; bullfrog GH, AY251538; giant toad GH, AF062746; carp GH, P10298; pufferfish GH, O12980; goldfish GH1, O93359; goldfish GH2, O93360; trout GH1, P09538; trout GH2, P20332; sockeye salmon GH1, Q91222; sockeye salmon GH2, Q91221; chum salmon GH, P07064; Russian sturgeon GH, AY941176; sturgeon GH1, P26773; sturgeon GH2, P26774; African lungfish GH, AF062745; human PRL, P01236; rhesus macaque PRL, U09018; mouse PRL, P06879; rat PRL, P01237; hamster PRL, AAB20367; vole PRL, AF178933; cat PRL, U25974; rabbit PRL, Q28632; deer PRL, AY373035; sheep PRL, X13483; goat PRL, X76049; cow PRL, V00112; horse PRL, AY373339; dog PRL, AY741405; mink PRL, P29234; giant panda PRL, AY161285; pig PRL, NM_213926; opossum PRL, AF067726; possum PRL, AF054634; chicken PRL, P14676; quail PRL, AB162003, duck PRL, AB158610; turkey PRL, U05952; goose PRL, AY993962; leopard gecko PRL, AB182277; bullfrog PRL, X16063; salamander PRL, AY332494; *Xenopus* PRL, BC075216; pufferfish PRL, newsinfrut00000132442/CAAB01004482·1; carp PRL, X12543; Nile tilapia PRL1, A07820; Nile tilapia PRL2, A07824; noble carp PRL, X61049; zebrafish somatolactin (SL), AJ867249/ENSDARP00000023510; goldfish SL, P79697; green puffer SL, AY374504; pufferfish SL, GENSCANSLICE00000000291/CAAB01000976·1; red drum SL, AF062520; rabbitfish SL, AB026186; black seabream SL, AY714370; gilthead seabream SL, L49205; sole SL, U06753; medaka SL, AY530202; Atlantic cod SL, D10639; European eel SL, U63884; channel catfish SL, AF267991; lumpfish SL, L02118; grouper SL, AY129310; perch SL, AY332490; chum salmon SL, D10640; Atlantic halibut SL, L02117; bastard halibut SL, M33696; African lungfish SL, O73847; white sturgeon SL, AB017200.

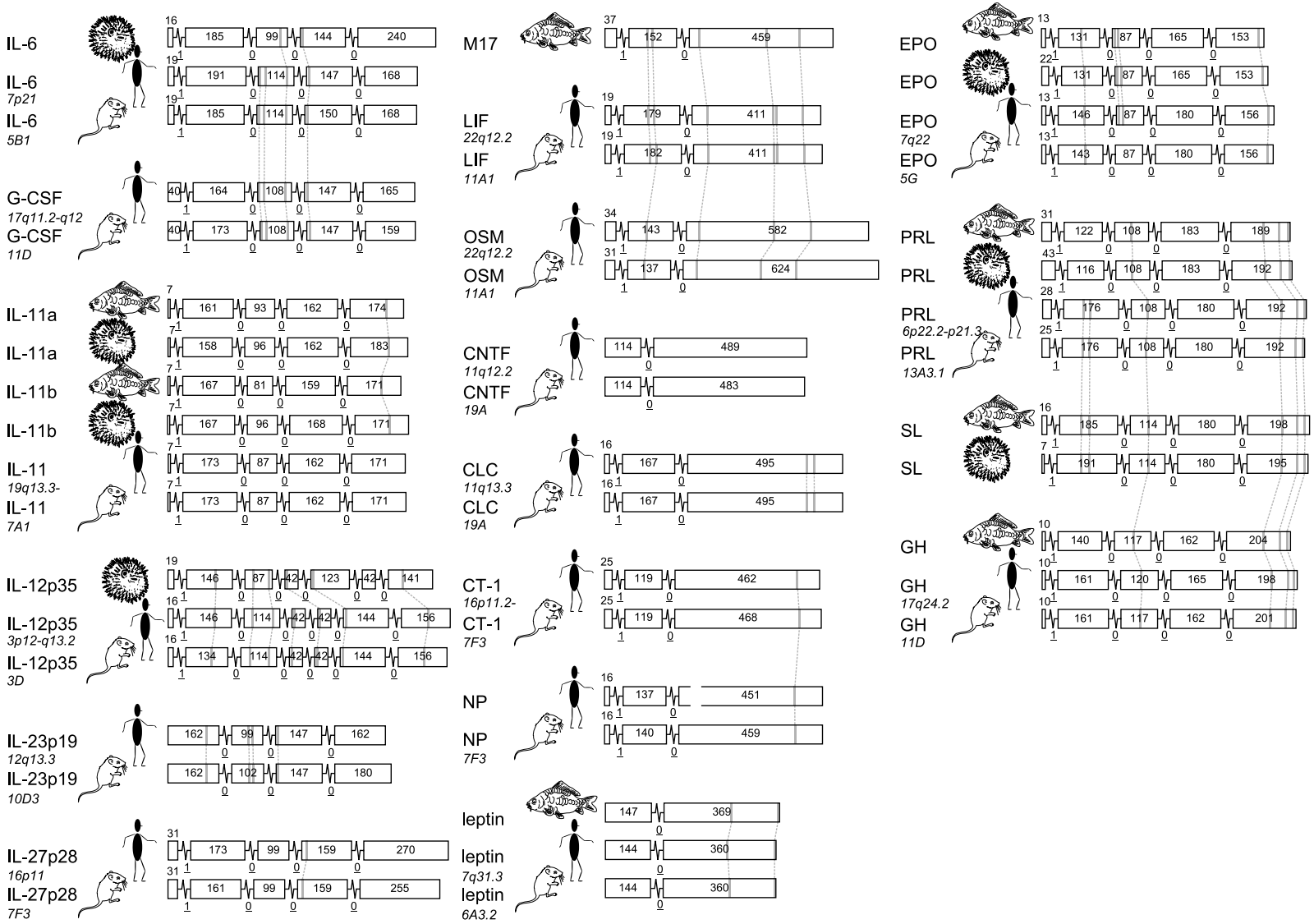


Figure 4 The gene structure of orthologous cytokines is conserved throughout vertebrates, a feature that is instrumental in the identification of orthologous relationships within the multigene cytokine family, which is characterised by limited primary sequence conservation. Boxes represent exons and are drawn to scale; numbers indicate their size in nucleotides. The underlined numbers underneath each intron indicate intron phase. Note that all introns are phase 0, except when the coding part of the first exon is small (<43 nucleotides), then the first intron is invariably phase 1. Shaded lines indicate conserved cysteine residues, which are generally conserved in their presence and spacing between orthologous cytokines. The italic lettering beneath the human and mouse cytokine names reflect their chromosomal locations. The carp and pufferfish icons reflect the gene structure of cyprinid (*Cyprinidae*) and puffer (*Tetraodontidae*) species respectively. Note that the human NP gene is a pseudogene due to an eight-nucleotide deletion in its third exon, indicated by the gap in the third exon.

G-CSF

Circulating neutrophilic granulocytes constitute an essential component of innate immunity and respond rapidly to local pathogenic insults by local margination. The circulating neutrophil population is short lived and is continuously replenished from the bone marrow haematopoietic precursor population. G-CSF is one of the principle regulators of neutrophil differentiation and activation. In the bone marrow, stromal cell-derived G-CSF stimulates the proliferation and maturation of bone marrow granulocytic progenitors and promotes the release of neutrophils into the circulation (Stull 2002). Moreover, peripherally administered G-CSF induces a rapid (minutes) margination of circulating neutrophils, resulting in acute neutropenia that is gradually (hours) resolved by the release of neutrophils from the bone marrow (Katoh *et al.* 1992). The main phenotypic aberrations of G-CSF$^{-/-}$ mice are a reduced number of bone-marrow neutrophil progenitor cells accompanied by moderate neutropenia, which is in line with the actions of G-CSF described above (Lieschke *et al.* 1994). Although G-CSF is often not included in the list of gp130 cytokines (since its receptor complex lacks the gp130 chain), the organisation of the G-CSF gene is similar to that of IL-6 (Simpson *et al.* 1997). Not only do both genes consist of five exons of similar length, the four conserved cysteine residues that make up the two disulphide bridges of IL-6 are identically spaced in G-CSF (Fig. 4). This suggests that IL-6 and G-CSF are related within the large class-I helical cytokine family. Chicken myelomonocytic growth factor (MGF) represents an avian G-CSF ortholog that shares limited similarity with mammalian IL-6 and G-CSF sequences (Fig. 3) (Leutz *et al.* 1989). Its gene structure is also reminiscent of that of G-CSF, and chicken MGF expression is upregulated in response to lipopolysaccharide (LPS) *in vitro* and *in vivo* (Sterneck *et al.* 1992, Sijben *et al.* 2001). To date, no G-CSF-like molecule has been described outside the mammalian and avian species.

IL-11

The discovery of IL-11 as a growth factor that drives the proliferation of an 'IL-6-dependent' plasmacytoma is illustrative of the redundancy within the class-I helical cytokine family, but also indicates the close relation between IL-6 and IL-11 (Paul *et al.* 1990). Like IL-6, IL-11 is encoded by five exons, although it lacks the conserved cysteine pairs that stabilise the four-helix bundle conformation of other class-I cytokines, including IL-6. Instead, IL-11 is stabilised solely by the hydrophobic interactions that result from the convergence of hydrophobic residues at the buried side of each α-helix, whereas charged residues abound at the solvent-exposed surface of each helix (Czupryn *et al.* 1995). IL-11 is no exception with regard to the pleiotropy characteristic of class-I helical cytokines. It is an important growth factor of haematopoietic lineages that give rise to neutrophilic granulocytes, erythrocytes and megakaryocytes (Quesniaux *et al.* 1992, Du *et al.* 1993, Neben *et al.* 1993). The stimulatory effects on the megakaryocytic lineage, in particular, have led to the approval of IL-11 by the Food and Drug Administration USA as a therapeutic agent in the prevention of thrombocytopenia resulting from severe bone-marrow ablative chemotherapy (Zheng *et al.* 2001). IL-11 also exerts protective effects on the mucosa of respiratory organs and the gastrointestinal tract. The inhibitory actions of IL-11 on macrophage reactive oxygen species production and pro-inflammatory cytokine release are considered to be the underlying mechanism of the protective effects on mucosa (Redlich *et al.* 1996, Waxman *et al.* 1998, 2003).

IL-11 has been considered as 'evolutionarily conserved' based on the primary sequence conservation of a limited number of primate and rodent orthologs. IL-11 orthologs have been characterised recently in carp (Huising *et al.* 2005) and rainbow trout (Wang *et al.* 2005) that share only limited primary sequence identity (maximally 31%) with mammalian IL-11. In fish, IL-11 is ubiquitously, albeit not very abundantly, expressed in most organs and tissues examined (Huising *et al.* 2005, Wang *et al.* 2005). Differences in the IL-11 expression between organs are less pronounced in carp than in trout, where gills and intestine express more IL-11 than any other organ. This may reflect protective effects of IL-11 at mucosal surfaces throughout the vertebrates. IL-11 expression in carp head kidney primary macrophages or a trout RTS-11 monocyte/macrophage cell line is reproducibly upregulated in response to various pro-inflammatory stimuli such as LPS, concanavalin A, polyI:C and recombinant IL-1β (Huising *et al.* 2005, Wang *et al.* 2005), establishing the involvement of IL-11 in the immune response from fish to mammals.

In addition to the orthologs of carp and trout IL-11 that are discussed above, a second IL-11 gene is present in the genome of evolutionarily distantly related fish species such as spotted green pufferfish (*Tetraodon nigroviridis*) (Jaillon *et al.* 2004), pufferfish (*Takifugu rubripes*) and zebrafish (*Danio rerio*) (Fig. 3) (Huising *et al.* 2005). Both fish IL-11 paralogs, referred to as IL-11a (which includes carp and trout IL-11) and IL-11b, share limited amino acid identity (27–29%), but have similar gene structures that closely resemble those of mammalian IL-11 genes (Fig. 4). In line with mammalian IL-11, neither fish IL-11 paralog contains a pair of conserved cysteines, although all fish IL-11 peptides do contain a single conserved C-terminal cysteine of unknown significance (Fig. 4). The extensive sequence dissimilarity of both paralogous fish IL-11 peptides, coupled with their presence in phylogenetically disparate species, pinpoints their origin to early in the teleostean lineage (Huising *et al.* 2005). The genome duplication that has occurred around this time

(Taylor *et al.* 2003) provides a plausible explanation for the fish-specific duplication of IL-11 genes.

LIF and OSM

The genes encoding LIF and OSM are located in close proximity on the same locus in humans and mice (Rose *et al.* 1993). The same is true for the LIFR and OSMR, suggesting that LIF and OSM as well as the genes encoding their receptors arose through gene duplication (Fig. 4). Both helical cytokines were initially identified as potent regulators of tumour cell proliferation, but have since been implicated in an impressive number of diverse and partially redundant functions (Metcalf 2003). OSM was originally identified as a product of phorbol myristate acetate-stimulated macrophage-like cells that inhibited the proliferation of a human melanoma cell line (Zarling *et al.* 1986). LIF was initially discovered for its potential to limit self-renewal and induce macrophage maturation of an undifferentiated murine myeloid cell line (Gearing *et al.* 1987). Subsequently LIF and OSM were both found to be essential for the maintenance of embryonic stem cell pluripotency (Smith *et al.* 1988, Williams *et al.* 1988, Rose *et al.* 1994). Although these observations are intuitively opposite from a functional perspective, they do illustrate that LIF and OSM are important factors in the regulation of proliferation and differentiation of cells of diverse origins. Additional actions of OSM and LIF include the induction of hepatocyte acute phase protein production (Baumann & Wong 1989, Richards *et al.* 1997) and the stimulation of various haematopoietic lineages, mostly in concert with other growth factors (Metcalf 2003, Tanaka & Miyajima 2003). LIF and OSM also stimulate the production of adrenocorticotrophic hormone (ACTH) production in the pituitary (Kim *et al.* 2000, Chesnokova & Melmed 2002, Auernhammer *et al.* 2004) and affect the secretion of PRL and GH (Tomida *et al.* 2001). Moreover, LIF is required for blastocyst implantation (Chen *et al.* 2000, Song *et al.* 2000) and exerts a multitude of proliferative and differentiating effects on various neuronal cell populations (Metcalf 2003). The extensive redundancy between LIF and OSM is at least in part explained by the fact that human (but not murine) OSM can signal via the LIF receptor complex in addition to its own receptor (Tanaka & Miyajima 2003).

LIF and OSM genes both have three exons and share four conserved cysteine residues that form a pair of intra-chain disulphide bridges (Fig. 4) (Kallestad *et al.* 1991). LIF has an additional third disulphide bridge, but the most conspicuous structural difference between LIF and OSM is the observation that the latter helical cytokine is synthesised as a pre-pro-peptide: in addition to an N-terminal signal peptide (pre) OSM possesses a C-terminal (pro) region of 31 amino acids (57 in the mouse) that is removed to yield a mature OSM with increased biological activity (Linsley *et al.* 1990).

M17

In 2003, a novel class-I helical cytokine was reported in common carp that shares features with several different mammalian class-I helical cytokines. As this resemblance to multiple mammalian helical cytokines precludes the assignment of one-to-one orthology, this carp cytokine was designated M17 after the original clone number assigned to different clones of a carp leukocyte cDNA library (Fujiki *et al.* 2003). The M17 peptide shares its highest sequence identity with chicken CNTF, although at 25% this percentage is relatively low compared with that observed for orthologous avian–teleostean pairs of class-I helical cytokines. The amino acid identity shared between carp M17 and human CT-1, IL-11, OSM and LIF ranges from 19 to 15%. M17 is predominantly expressed in brain and to some extent in peripheral blood leukocytes, a pattern that resembles the expression of CNTF. Nevertheless, in contrast to CNTF, M17 has a predicted signal peptide and is encoded by three exons of a size comparable with the exons of OSM and LIF (Fujiki *et al.* 2003). Moreover, M17 contains five cysteine residues that are similarly spaced compared with the conserved cysteine residues of LIF and OSM (Fig. 4). Taken together, these observations suggest that M17 is closely related to the mammalian LIF/OSM locus and likely represents the teleost ortholog of LIF and OSM. Since its discovery in carp, genes that encode M17 have been found in the genomes of several phylogenetically distantly related fish species (Fig. 3) (Huising *et al.* 2005), indicating that M17 is a class-I helical cytokine common to most teleost fish. The recent discovery of a single LIFR-like gene in goldfish that clusters outside of the cluster of mammalian LIFR and OSMR sequences (Hanington & Belosevic 2005) (which are both located at chromosome 5p13 in the human) suggests that LIFR and OSMR may have originated from a duplication event specific to the tetrapod lineage. The discovery in the spotted green pufferfish of duplicate class-I helical cytokine receptor genes that both resemble the human LIFR (Jaillon *et al.* 2004) and are situated in each other's proximity on *Tetraodon* chromosome 12 slightly complicates this scenario. Whether both LIFR-like genes reflect, in fact, the *Tetraodon* orthologs to the mammalian LIFR and OSMR genes (ruling out the possibility that separate LIFR and OSMR genes are unique to the tetrapod lineage) or, alternatively, are the result of an independent gene duplication event that was specific to the teleostean lineage is an issue that requires further attention.

CNTF and CLC/CLF

The genes that encode CNTF and CLC, which is the class-I helical cytokine component of the heterodimeric CLC/CLF complex, are located in close proximity on the same locus (Fig. 4) (Senaldi *et al.* 1999, Shi *et al.* 1999),

suggestive of a common origin through gene duplication. In addition, although the CNTF gene is shorter than the CLC gene and lacks a signal peptide (as well as the short first exon that partially encodes it in most other class-I helical cytokines), the second exon of CNTF and the third of CLC closely resemble each other in length (Fig. 4). Unlike most class-I helical cytokines, the activity of CNTF was first described in a non-mammalian vertebrate prior to its cloning in rabbit (Lin *et al.* 1989). It was initially characterised for its ability to support the survival of chicken neurones that were isolated from embryonic ciliary and sensory ganglia (Nishi & Berg 1977). Since then, CNTF has emerged as a pleiotropic neurotrophic factor that supports survival and growth of diverse cells of neuronal origin (Ip & Yancopoulos 1996). CNTF is also expressed in a variety of mammalian peripheral tissues but, in those cases, is usually associated with peripheral nerves. Its non-neuronal effects include the induction of hepatocyte acute phase protein expression (Espat *et al.* 1996), a property shared with several other class-I helical cytokines. CNTF$^{-/-}$ mice display only a mild loss of motor neurone numbers, which is accompanied by increased muscle atrophy later in adult life, despite the prominent effects of CNTF on neurone growth and survival (DeChiara *et al.* 1995). Also in the human population, CNTF null mutations occur rather frequently (2·5% of the Japanese population) without overt consequences for the bearer (Takahashi *et al.* 1994). In contrast, mice lacking the CNTFRα die perinatally due to severe motor neurone loss that prevents jaw movement resulting in a failure to suckle (DeChiara *et al.* 1995). The phenotypic discrepancy between CNTF$^{-/-}$ and CNTFRα$^{-/-}$ mice is explained by the observation that CNTFRα is also an essential component of the receptor complex for several other class-I helical cytokines, including CLC/CLF (Fig. 2) (Lelievre *et al.* 2001, Vergara & Ramirez 2004). Heterodimerisation of CLC with the truncated helical CLF is required for the secretion of this non-covalently coupled soluble complex from the cell (Elson *et al.* 2000). The requirement of the CNTFRα for CLC/CLF signalling is supported by the observation that CLF$^{-/-}$ mice, like CNTFRα$^{-/-}$ mice, die shortly after birth due to a similar suckling defect (Alexander *et al.* 1999). CLC/CLF acts as another neurotrophic factor for motor neurones (Senaldi *et al.* 1999, Forger *et al.* 2003), but also initiates pro-opiomelanocortin expression and ACTH release from murine pituitary AtT20 cells (Auernhammer *et al.* 2004) and potently stimulates B cells (Senaldi *et al.* 1999).

Despite its early discovery in chicken, no orthologs for CNTF have been described in poikilothermic vertebrates to date. The same is true for CLC, although orthologs of its obligatory binding partner CLF have been identified in spotted green pufferfish (Jaillon *et al.* 2004) as well as in zebrafish (accession number BE016629). Moreover, these fish CLF genes are considerably better conserved compared with related soluble class-I helical cytokine receptors such as the IL-12p40 and others (Jaillon *et al.* 2004, Huising *et al.* 2006).

CT-1 and neuropoietin (NP)

CT-1 and NP provide yet another example of a pair of mammalian class-I helical cytokines that is located on a single locus (Fig. 4). CT-1, like CNTF, lacks an N-terminal signal peptide, but is nevertheless readily secreted by an as yet unknown mechanism. As its name suggests, CT-1 promotes cardiac myocyte survival and is expressed only in heart cells during murine embryonic development (Pennica *et al.* 1996*a*). Additionally, CT-1 shares several functions with other class-I helical cytokines, such as the induction of hepatocyte acute phase protein production (Richards *et al.* 1997) and the release of ACTH (Auernhammer *et al.* 2004). The CT-1 receptor complex contains gp130 as well as LIFR, and there is evidence to suggest that a third, GPI-anchored receptor chain distinct from the CNTFRα participates in the CT-1 receptor complex (Pennica *et al.* 1996*b*, Robledo *et al.* 1997). To date, no orthologs for CT-1 have been described in non-mammalian vertebrates.

NP, also known as cardiotrophin-2, was discovered in 2004 and is the latest addition to the class-I helical cytokine family. It is the third helical cytokine, in addition to CNTF and CLC/CLF, which is known to use the tripartite CNTF receptor complex that consists of CNTFRα, LIFR and gp130. In the mouse, NP is expressed only during embryonic development in the brain. Moreover, the embryonic expression of NP and CNTFRα, but not CLC or CNTF, overlap and this indicates that defective NP signalling may partially contribute to the perinatally lethal phenotype observed in CNTFRα$^{-/-}$ mice (Derouet *et al.* 2004). Curiously, the human NP gene contains an eight nucleotide deletion within the third exon that causes a frame-shift, which renders human NP a pseudogene (Derouet *et al.* 2004). This observation seems to preclude a critical role for NP signalling during early mammalian ontogeny.

No NP has been described in non-mammalian species to date. Interestingly, in certain species of terrestrial salamander that belong to the *Plethodontidae* family, the males produce a proteinaceous pheromone substance in a specialised gland under the male chin which, when applied directly to the female nostrils, enhances female receptivity. This factor is called plethodontid receptivity factor (PRF) and displays modest amino acid identity to human CT-1 and CNTF (20 and 16% respectively) (Rollmann *et al.* 1999). However, comparison of PRF with the newly reported mammalian NP gene reveals a 28% overall amino acid identity, which is considerably higher than the identity between PRF and any other mammalian class-I helical cytokine, suggesting that PRF may represent the amphibian ortholog of the mammalian NP gene.

IL-12 and other heterodimeric cytokines

IL-12 is composed of the class-I helical cytokine subunit p35, covalently linked to the soluble helical cytokine receptor p40. Together, they constitute a 70 kDa bioactive heterodimeric cytokine, IL-12p70, which is usually plainly referred to as IL-12. It is secreted from antigen-presenting cells in response to stimulation by microbial agents such as LPS, lipoteichoic acid or prokaryotic DNA via Toll-like receptors. IL-12 induces the release of other cytokines, in particular IFNγ, from T cells and natural killer cells and by doing so is an early and key factor that drives an immune response towards a Th1 phenotype. In recent years, IL-12 has emerged as the founding member of a larger family of composite cytokines which share involvement in the Th1 response (Trinchieri *et al.* 2003, Holscher 2004). IL-23 is formed by the covalent association of the p40 subunit with the novel class-I helical cytokine subunit p19 (Oppmann *et al.* 2000). Besides the p40 subunit, IL-23 shares the IL-12Rβ1 chain with IL-12 (Fig. 2). Furthermore, the IL-23R lies in close proximity to IL-12Rβ2, the receptor chain it substitutes in the IL-23 receptor complex, on human chromosome 1 (Parham *et al.* 2002). The third composite cytokine, IL-27, is constituted by the non-covalently associated class-I helical cytokine p28 with EBI-3, and which has all the characteristics of a soluble helical cytokine receptor (Devergne *et al.* 1996, Pflanz *et al.* 2002). IL-23 and IL-27, like IL-12, are involved in the Th1 response and their roles differ qualitatively (Brombacher *et al.* 2003). IL-27 precedes the early actions of IL-12 in the initiation of a Th1 response and consequently acts on naïve $CD4^+$ T cells (Takeda *et al.* 2003). IL-23, on the other hand, acts much later in the Th1 response and predominantly activates memory T cells (Frucht 2002, Lankford & Frucht 2003). Although, in contrast to IL-12 and IL-23, the IL-27 subunits are not linked by an interchain disulphide bridge, co-expression of both subunits by the same cell is required for the secretion of all three composite cytokines (Gubler *et al.* 1991, Oppmann *et al.* 2000, Pflanz *et al.* 2002).

The p35 and p40 subunits of IL-12 have recently been identified in chicken (Balu & Kaiser 2003, Degen *et al.* 2004), pufferfish (Yoshiura *et al.* 2003), spotted green pufferfish (Jaillon *et al.* 2004) and carp (Huising *et al.* 2006) (Fig. 3), but the amino acid identity of these non-mammalian IL-12 peptides compared with mammalian orthologs is invariably low at around 20–25%. Interestingly, the resolution of the gene structure of non-mammalian IL-12p35 genes (Yoshiura *et al.* 2003, Degen *et al.* 2004) revealed that although the p35 gene structure is conserved throughout vertebrates, mammalian IL-12p35 genes possess an extra exon (Fig. 4). This additional exon may have resulted from an exon duplication specific to the mammalian lineage: in mammals, exons four and five both consist of 42 nucleotides, contain an identically spaced conserved cysteine residue and are similar throughout their coding as well as their flanking non-coding regions (Huising *et al.* 2006).

Pufferfish IL-12p35 was induced in the head kidney as well as the spleen in response to i.p. injection of polyI:C but not LPS (Yoshiura *et al.* 2003). In contrast to IL-12p35, pufferfish IL-12p40 is constitutively expressed in both organs, but was not detectably upregulated in response to injection of either stimulus. This suggests that IL-12p35 expression is the determining step in the production of pufferfish IL-12p70 (Yoshiura *et al.* 2003), a situation that is comparable to the regulation of IL-12 release from human monocytes (Snijders *et al.* 1996). We have investigated the expression of carp IL-12p35 and p40 expression in head kidney macrophages *in vitro* and found that their constitutive expression was barely detectable. Following LPS stimulation, however, the expression of both subunits was upregulated several 100-fold within hours (Huising *et al.* 2006).

To our surprise we identified two more paralogous carp IL-12p40 genes, which we designated IL-12p40b and IL-12p40c, in addition to the p40a subunit discussed above. These three carp p40 paralogs are maximally 32% identical at the amino acid level, but all residues that are critically required for the formation of the interlocking topology between p35 and p40 in human IL-12 (Yoon *et al.* 2000) are conserved in all three carp p40 subunits (Huising *et al.* 2006). Moreover, their identification as IL-12p40 is supported by phylogenetic analyses, where all three carp p40 genes cluster within the vertebrate IL-12p40 cluster and separate from other soluble class-I helical cytokine receptors such as CLF, EBI-3 and soluble CNTFRα (Huising *et al.* 2006). The constitutive expression of each carp p40 paralog in head kidney macrophages varies profoundly from barely detectable (p40a) to abundant (p40c) and their inducibility by *in vitro* LPS stimulation is inversely proportional to the level of their constitutive expression (Huising *et al.* 2006). Although no non-mammalian orthologs of any of the subunits that constitute IL-23 and IL-27 (other than IL-12p40) have been identified to date, the discovery of multiple, substantially different p40 subunits in carp suggests that considerable differences exist between the subunit repertoires involved in the formation of composite cytokines in mammals and bony fish. This notion is corroborated by the presence of two genes for IL-12p40 as well as IL-12p35 in the spotted green pufferfish (Jaillon *et al.* 2004).

Leptin

Leptin is a soluble factor which, in mammals, circulates in proportion to body fat mass. It traverses the blood–brain barrier to reach the hypothalamus, where it evokes satiety and regulates energy metabolism (Schwartz *et al.* 2000). The involvement of a soluble, circulating factor in the morbidly obese, diabetic and hyperphagic phenotype of

the obese mouse strain was already established in the 1970s by parabiosis. A circulating factor from a wild-type mouse was capable of suppressing food intake and weight gain in the obese parabiont (Coleman 1973). Nevertheless, this factor was not identified until 1994 with the cloning of leptin as the product of the obese gene (Zhang *et al.* 1994). Since leptin does not share considerable sequence identity with other proteins, it took another three years (until the crystal structure of leptin was resolved) before leptin was recognised as a member of the class-I helical cytokine family (Zhang *et al.* 1997). Leptin is a 167 amino acid protein encoded by two exons. Its four-helix bundle conformation is stabilised by a single conserved disulphide bridge that connects the C-terminal ends of helix C and D. Consequently, the final amino acid before the stop codon is a cysteine residue (Fig. 4), a distinctive feature among class-I helical cytokines. Leptin's important role in food intake, a process that is considered to be a key in the etiology of obesity, has spurred an enormous boost in leptin research. Despite the immense attention given to leptin, no *bona fide* leptin ortholog from any non-mammalian species had been reported in the decade that followed leptin's discovery in the mouse. A leptin molecule from chicken was reported by two groups in the late 1990s (Taouis *et al.* 1998, Ashwell *et al.* 1999). This chicken leptin sequence was 97% identical to murine leptin at the amino acid level, which would signify an incredible evolutionary conservation. Since its discovery, several research groups have reported their inability to repeat the amplification of chicken leptin (Friedman-Einat *et al.* 1999, Pitel *et al.* 2000). The chicken leptin receptor has been cloned, but displays only modest amino acid identity of around 50% to its mammalian orthologs (Horev *et al.* 2000, Ohkubo *et al.* 2000). The difference between the degree of conservation of chicken leptin and its cognate receptor is counterintuitive, since ligands and the ligand-binding domain of their receptors tend to co-evolve. Also, the recently published draft chicken genome does not contain a gene that encodes chicken leptin as it was originally reported. Collectively, these considerations raise doubts about the validity of the published chicken leptin sequence and warrant a careful re-evaluation of chicken leptin.

Very recently, we reported the presence of two obese genes that encode duplicate leptins in common carp (MO Huising, EJW Geven, CP Kruiswijk, SB Nabuurs, EH Stolte, FAT Spanings, BM Verburg-van Kemenade & G Flick, unpublished observations). Both carp leptins are 83% identical and are likely the result of a recent duplication event. In line with our prediction that cytokines are generally poorly conserved from fish to mammals, we found that the amino acid identity of the carp leptins with their mammalian orthologs is relative low at 22–25%. Recently, the leptin sequences of two species of pufferfish have also become available (Kurokawa *et al.* 2005). Among the amino acid residues that are conserved between fish and mammalian leptins are the two characteristically spaced cysteines that make up leptin's single disulphide bridge. Leptin's gene structure is also conserved, as the first and second exons of both carp leptin genes extend merely one and three triplets respectively, compared with those of human and murine leptins (Fig. 4). Their leptin identity is further supported by phylogenetic analyses that place the carp leptins in a single cluster with mammalian orthologs, separate from other class-I helical cytokines (Fig. 3). In contrast to mammalian species, where leptin is predominantly expressed in the subcutaneous adipose compartment, the liver is the major site for leptin expression in carp and pufferfish (Kurokawa *et al.* 2005, MO Huising, EJW Geven, CP Kruiswijk, SB Nabuurs, EH Stolte, FAT Spanings, BM Verburg-van Kemenade & G Flick, unpublished observations). Moreover, the liver mRNA expression of both carp leptins displays a postprandial peak in response to a single meal at a scheduled feeding time (MO Huising, EJW Geven, CP Kruiswijk, SB Nabuurs, EH Stolte, FAT Spanings, BM Verburg-van Kemenade & G Flick, unpublished observations). Prolonged (six days) fasting followed by refeeding for three days did not affect liver leptin expression. This contrasts with that reported for rodents, where food deprivation for a short period results in a more considerable weight loss and a drop in leptin expression (de Vos *et al.* 1995, Bertile *et al.* 2003). The divergent responses of leptin to fasting in fish and mammals may well relate to the overt physiological differences that are associated with homoeothermic and poikilothermic vertebrates, and further experiments are ongoing to further unravel the role of leptin in early vertebrates and the primary site of its production.

EPO

EPO is the principle regulator of the proliferation and differentiation of progenitors of the erythroid lineage. It is mainly produced in the fetal liver and adult kidney in response to hypoxia and stimulates bone-marrow erythropoiesis (Jacobs *et al.* 1985). Central to the oxygen-sensing mechanism that is responsible for the transcription of EPO is the transcription factor complex hypoxia-inducible factor-1, which is activated in response to hypoxia (Fandrey 2004). The human EPO gene consists of five exons and contains two conserved cysteine bridges (Jacobs *et al.* 1985, Lin *et al.* 1985). One of these disulphide bridges is lost in the murine EPO peptide (Shoemaker & Mitsock 1986) (Fig. 4). The EPO gene of several fish species has recently been resolved (Fig. 3). The genes encoding carp and pufferfish EPO contain five exons of a size comparable with those of human EPO. Moreover, both fish EPO peptides share four conserved cysteine residues with human EPO (Fig. 4). The amino acid identity of EPO of either fish species is higher than 30%, which ranks EPO among the best conserved vertebrate

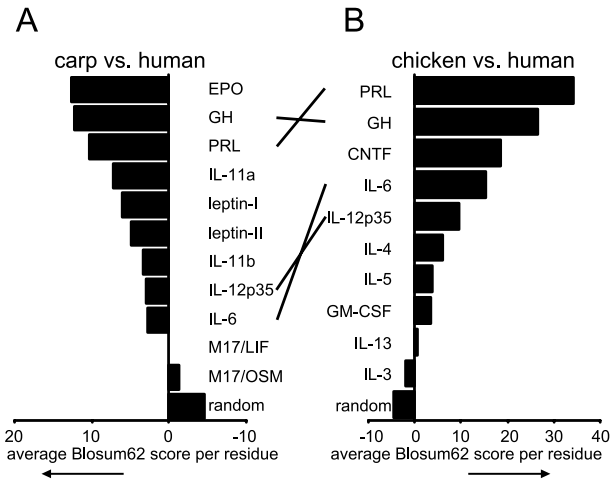


Figure 5 Comparison of the degree of evolutionary conservation of various class-I helical cytokines. The score assigned to a pairwise amino acid alignment (via the T-Coffee algorithm) of orthologous cytokines on the basis of the blocks substitution-62 matrix (Henikoff & Henikoff 1992), divided by the length of the alignment to correct for the differences in sequence length, is used to quantify their degree of conservation (identity and similarity). The average score of pairwise alignments of three randomly generated amino acid sequences of 200 residues is included to provide a baseline value (random). (A) The conservation scores of all known carp class-I helical cytokines are ranked from high (EPO) to low (M17/OSM) to provide a direct comparison of their evolutionary conservation. Since the IL-11b and IL-6 from carp have not yet been identified, their conservation scores are the result of the comparison of human with zebrafish (IL-11b) and pufferfish (IL-6) respectively. In (B), the conservation scores of all known chicken class-I helical cytokines compared with their human orthologs are ranked. Note that the five members of the Th2 cytokine cluster are the five most poorly conserved chicken cytokines. In carp as well as chicken, the sequences of the pituitary hormones GH and PRL rank among the best conserved class-I helical cytokines. Pairwise comparisons of carp and chicken with mouse instead of human results in very similar rankings (not shown).

class-I helical cytokines (Fig. 5). The heart is the major site of EPO expression in the pufferfish, where modest expression is also observed in liver and brain (Chou *et al.* 2004). In contrast to mammalian species, the pufferfish (head) kidney does not express EPO, which may relate to the fact that in fish the head kidney and kidney are major haematopoietic sites, analogous to mammalian bone marrow. Transfection of the pufferfish EPO gene into a fish hepatoma cell line shows that the pufferfish EPO gene is upregulated under hypoxic conditions (Chou *et al.* 2004), which would suggest that EPO is involved in hypoxia-induced erythropoiesis throughout vertebrates.

PRL and GH

In contrast to most other class-I helical cytokines, PRL and GH are both secreted as classical hormones: they are released into the circulation from the anterior pituitary gland and act peripherally in an endocrine fashion. GH is released in response to hypothalamic stimulation via GH-releasing hormone and exerts negative feedback onto the hypothalamus. Its most prominent and best-known effect throughout vertebrates is the stimulation of post-natal growth by inducing the growth and differentiation of bone, cartilage and muscle. Many of the growth-promoting effects of GH are mediated via insulin-like growth factors produced in the liver (Mauras & Haymond 2005). In addition to the effects of GH on somatic growth, GH and GH receptors are expressed in many peripheral sites, including the immune system, where GH promotes cellular growth and survival (Jeay *et al.* 2002).

The actions of PRL were already recognised in the 1920s as an anterior pituitary hormone capable of inducing milk secretion in female rabbits (Stricker & Grueter 1928). Not much later, PRL was shown to induce the growth of the pigeon crop sac, an organ that is used by these birds to feed their chicks with crop milk released under PRL control (Riddle *et al.* 1933). The similarity of the actions of PRL in birds and mammals alike demonstrates the involvement of PRL in reproduction in different vertebrate classes. In addition, in early vertebrates such as fish, PRL also induces parental behaviour (Blum & Fiedler 1965). However, in the decades that have passed since PRL's discovery as an inducer of lactation, over 300 distinct functions for PRL have been described, indicating that PRL is extremely pleiotropic and arguable the most versatile class-I helical cytokine of all (Nicoll & Bern 1972, Bole-Feysot *et al.* 1998). PRL is also the only class-I helical cytokine to date where orthologs have been identified in fish, amphibia, reptiles, birds and mammals (Fig. 3). Its actions have been classified into six major categories: osmoregulation; growth and development; endocrinology and metabolism; brain and behaviour; reproduction; and immune regulation (Bole-Feysot *et al.* 1998). Osmoregulatory actions of PRL are particularly important for, although not exclusive to, fish. Fish are mostly exposed to an aqueous environment of different osmolarity compared with the body fluids (with the exception of some brackish waters) and at the same time lack the protective barrier of a water-impermeable epidermis. Euryhaline fish (fish that migrate from salt to fresh water or vice versa) especially rely on PRL for osmoregulation in fresh water. When seawater-adapted fish migrate to fresh water, their pituitary and plasma PRL levels rise. PRL decreases the permeability of the integumental surfaces to water and ions and increases ion retention, properties that both favour life in a freshwater environment (Manzon 2002). This principle is aptly demonstrated by the discovery that hypophysectomised killifish (*Fundulus heteroclitus*) are only able to survive in fresh water following PRL replacement (Burden 1956, Pickford & Phillips 1959). The important osmoregulatory properties of PRL in fish as well as more recent vertebrates have led to the postulation that osmoregulation may reflect

PRL's original function; the vertebrate lineage stems from an aquatic environment (Manzon 2002). The water–land transition was followed by many physiological changes, including, amongst others, the development of a keratinous skin, decreased litter size and increased parental care, and PRL might have expanded its repertoire by taking on these additional roles.

PRL and GH are generally considered to share a direct common ancestor (Bole-Feysot *et al.* 1998, Manzon 2002), as they are both encoded by five exons of comparable size and signal via related receptors (Fig. 2). Moreover, the four conserved cysteine residues in vertebrate GH peptides are also present and identically spaced in PRLs (Fig. 4). Mammalian PRLs have an additional N-terminal cysteine pair that is lost in actinopterygian fish due to a partial deletion of exon two (Manzon 2002). In addition to PRL and GH which are present throughout vertebrates, several lineage-specific members of the PRL/GH subfamily of helical cytokines exist. Somatolactin (SL) is a helical cytokine that is considered specific to the fish lineage with approximately equal amino acid identity to PRL and GH. SL is secreted from the pars intermedia of the pituitary gland and has been implicated in a variety of functions such as background adaptation, stress responses, energy metabolism, acid-base regulation and the control of reproduction (Amemiya *et al.* 1999, Company *et al.* 2001). Interestingly and in contrast to teleostean PRLs, SLs share all three conserved cysteine residues with mammalian PRLs (Fig. 4). No SL ortholog has been discovered in a non-piscine vertebrate to date. However, the presence of SL in the sarcopterygian lungfish indicates that the SL gene has been present in the early tetrapod ancestor and may have been lost prior to the origin of most classes of present-day vertebrates (Amemiya *et al.* 1999, Forsyth & Wallis 2002). Another example of a lineage-specific helical cytokine within the GH/PRL subfamily is provided by the placental lactogens (PL) that are the result of gene duplications that are specific to the mammalian lineage. Mammalian PLs do not form a monophyletic group. Instead, they arose independently by duplication of the PRL gene in rodents and ruminants and by a duplication of the GH gene in primates (Forsyth & Wallis 2002). PLs are expressed mainly in the placenta and serve as a luteotrophic factor and regulate fetal growth (Forsyth 1994). Collectively this indicates that, although GH and PRL are present throughout the vertebrate lineage, their subfamily of class-I helical cytokines underwent lineage-specific duplication and deletion in different classes of vertebrates. Perhaps the latest example of such a lineage specific gene duplication is formed by the discovery of a second PRL gene, designated PRLb, which is distinctly different from its paralogous PRLa gene in *Tetraodon* and zebrafish, suggesting an origin early in the fish lineage (Jaillon *et al.* 2004).

In contrast to other class-I helical cytokines that cluster according to the accepted patterns of vertebrate evolution, mammalian GH and PRL sequences form respectively two and three separate clusters (Fig. 3). This slightly erratic branching pattern of GH and PRL has been observed previously and has been attributed to variability in the evolutionary rate of GH and PRL between different mammalian lineages (Forsyth & Wallis 2002).

'Short-chain' class-I helical cytokines: the Th2 example

Class-I helical cytokine genes are scattered over the genome of mice and men, where they are found mostly isolated. Some occur in small aggregations of two or sometimes three genes. Examples include pairs of class-I helical cytokines, such as LIF/OSM and CNTF/CLC, and the threesome of CT-1, NP and IL-27p28 (Fig. 4). Although these clusters of helical cytokine genes residing on a single locus may reflect recent gene duplication events, it is likely that the distribution of class-I helical cytokine genes over multiple loci occurred earlier in the vertebrate lineage. A similar situation is encountered within the CXC chemokine family. This subclass of chemokines that is defined by its characteristic CXC pattern of cysteine residues (X denoting any amino acid) currently has 16 mammalian representatives, of which 13 reside on a single locus (human 4q21), the remaining three each occupy a separate locus. Representatives of at least three of these loci are found in fish, indicating that the distribution over multiple loci occurred prior to the fish–tetrapod divergence (Huising *et al.* 2003*a*). The total repertoire of fish CXC chemokines differs profoundly from that of mammalian species, and even primate and rodent species differ substantially with regard to their total number of chemokine genes. Collectively, this indicates that the expansion of the number of chemokine genes on a single locus occurred much later in evolution and in a lineage-specific way (Huising *et al.* 2003*a*).

In contrast to the long-chain class-I helical cytokines which, in the majority, occur isolated in the genome, many of the genes for short-chain four-helix bundle helical cytokines are concentrated in a cluster on human chromosome 5q31·1 (chromosome 11B1 in the mouse). This gene cluster is also referred to as the Th2 cluster, as it contains the genes encoding for IL-4, IL-5 and IL-13, which are important Th2 cytokines. In addition to these Th2 cytokines, this cluster also contains the genes that encode IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF). Their close proximity indicates that the genes that form the Th2 cluster expanded from a single gene by gene duplication, similar to the CXC chemokines on human locus 4q21. The next question is when these gene duplications occurred or, in other words, is the Th2 cluster restricted to the mammalian lineage or a feature shared by mammals and earlier vertebrates alike?

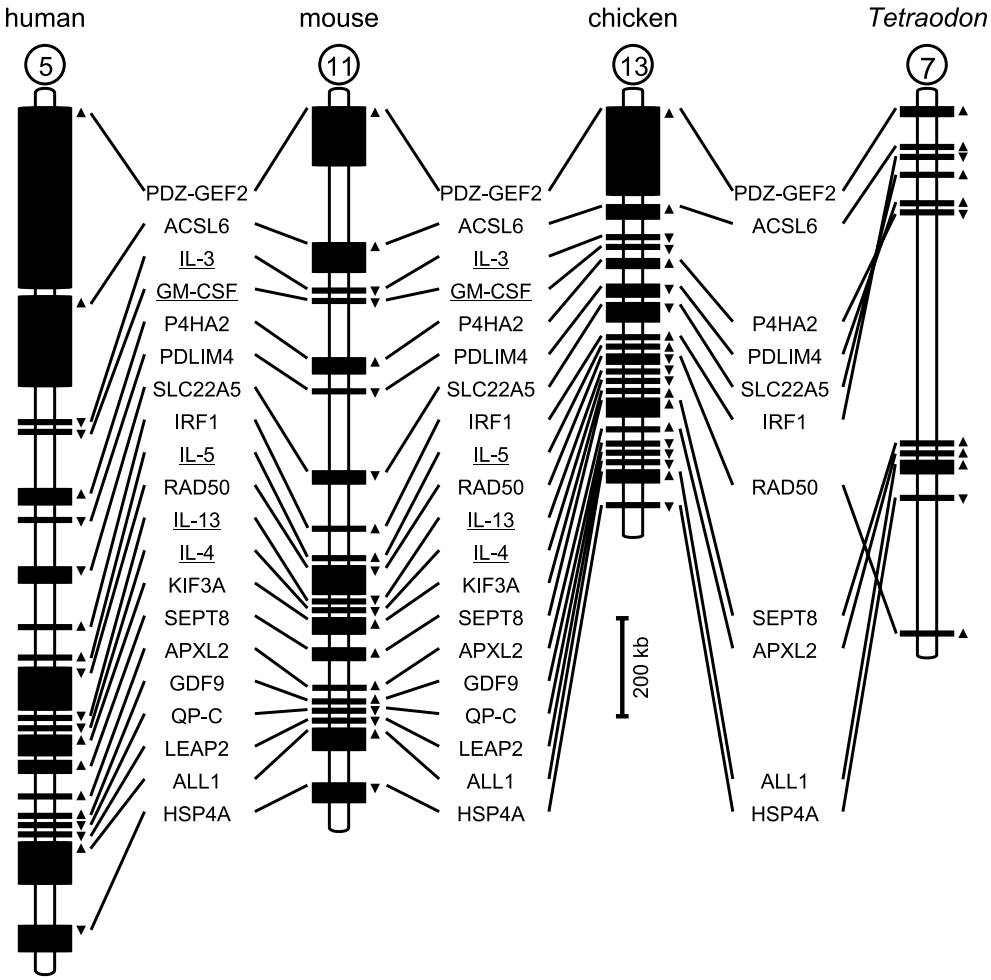


Figure 6 The synteny of the Th2 cluster is conserved in human, mouse, and chicken. A comparison of the human Th2 cluster (chromosome 5q31) with syntenic regions of mouse (11B1) and chicken (13 15·5–16·2 Mb) reveals that synteny is conserved with regard to gene order and orientation. The region of *Tetraodon* chromosome 7 that is syntenic to human 5q31 contains orthologs to 11 genes that flank the cytokine genes in human and mouse, but appears to lack cytokine genes. Genes (solid boxes) and the distance between them are drawn to scale. The arrowheads indicate the gene orientation. Cytokine genes are underlined. Chromosome numbers are indicated in the circles. PDZ-GEF2, PSD-95/DigA/20-1 (PDZ) domain containing-guanine nucleotide exchange factor-2; ACLS6, long-chain fatty acid acyl-coenzyme A (CoA) ligase 6; P4HA2, prolyl 4-hydroxylase α2 subunit precursor; PDLIM4, PDZ and LIM domain (zinc-binding domain present in Lin-11, ISI-1, Mec-3) protein-4; SCL22A5, solute carrier family 22 member 5; IRF1, IFN regulatory factor-1; RAD50, DNA repair protein RAD50; KIF3A, kinesin-like protein kinesin family member 3A; SEPT8, septin-8; APXL2, apical protein-2; GDF9, growth/differentiation factor-9 precursor; QP-C, low molecular mass ubiquinone-binding protein; LEAP2, liver-expressed antimicrobial peptide-2 precursor; ALL1, acute lymphoblastic leukemia 1 fused gene; HSP4A, heat shock 70 kDa protein-4.

Recently, the Th2 cluster of chicken was characterised and found to contain six helical cytokine genes. Five of these chicken genes were designated as orthologous to the five helical cytokines of the mammalian Th2 cluster (Avery *et al.* 2004). Since the sequence identity between the chicken and mammalian cytokines is limited and the gene structure of all genes in the Th2 cluster is similar, this designation of orthology depends in part on the conservation of synteny. The human, mouse and chicken Th2 cluster are situated at syntenic regions of chromosomes 5, 11 and 13 respectively (Fig. 6). Although the synteny of the chicken Th2 cluster is conserved with regard to gene order and orientation (Avery *et al.* 2004) (Fig. 6), the genes for chicken IL-3 and GM-CSF are reversed in the recently completed chicken genome. This difference may be attributable to a strain effect, as a BAC library of the White Leghorn strain was used in the characterisation of the chicken Th2 cluster, whereas the chicken genome was

assembled from the genetic material of red jungle fowl, the ancestor of domesticated chicken. Alternatively, the reversed gene order of IL-3 and GM-CSF may represent an artifact of the chicken genome assembly. Several differences exist between the chicken and mammalian Th2 cluster. Chicken IL-5 lacks a promoter region and is not expressed, effectively demoting it to a pseudogene in both the White Leghorn strain (Avery *et al.* 2004) and the red jungle fowl (P Kaiser, personal communication). Furthermore, the chicken Th2 cluster contains a sixth helical cytokine gene, designated KK34 (Koskela *et al.* 2004), which is probably unique to the avian lineage. Collectively, this indicates that, although the Th2 cytokine locus predates the avian–mammalian split estimated to have occurred around 310 million year ago (Hedges 2002), several differences exist between the Th2 clusters of bird and mammalian species.

Of the three fish genomes currently available, the genome assembly of *Tetraodon nigroviridis* is most complete in the region that is syntenic to the human, mouse and chicken Th2 cluster. This *Tetraodon* region is located on chromosome seven and contains orthologs to many of the genes that flank the helical cytokine genes of the Th2 cluster in mammalian and avian genomes (Fig. 6). The synteny of these genes is not completely conserved. The genes for the *Tetraodon* orthologs of P4HA2, PDLIM4, SLC22A5 and IRF1 are inversed in gene order and orientation, indicative of a block inversion event (Fig. 6). An artifactual *Tetraodon* genome assembly, which could provide an alternative explanation for the inversed gene order, is unlikely in this particular instance (Roest Crollius, personal communication). Although we identified a *Tetraodon* locus that is syntenic to the Th2 locus of humans, mice and chickens, there is as yet no evidence for any helical cytokine gene within this approximately 700 Mb region of the *Tetraodon* genome. Also the genomes of zebrafish and *Takifugu rubripes* do not reveal any of the cytokine genes of the Th2 cluster, although orthologs of several non-cytokine genes within the mammalian Th2 cluster are readily identifiable in both these fish species, as they were in *Tetraodon* (not shown). There are two explanations for the lack of identifiable fish orthologs of the mammalian Th2 cluster cytokines. The first explanation is that one or several orthologs of IL-3, -4, -5, -13 and GM-CSF are present in the fish genome, but are not retrieved in searches with the usual computational algorithms. The other explanation is that these genes are absent from fish altogether. This is in line with the lack of Th2 cytokines in the long list of cytokine and chemokine genes that were identified via the sequencing of numerous fish EST libraries, constructed via subtractive hybridisation to enrich for genes that are upregulated in leukocytes in response to stimulation. It is also striking that the five members of the chicken Th2 cluster are also the five cytokines that display the poorest sequence conservation of all chicken class-I helical cytokines described to date when compared with human orthologs (Fig. 5). This indicates that Th2 cytokines, although present in chicken, are poorly conserved and raises the possibility that fish orthologs to Th2 cytokines may be lacking altogether. However, the arguments above are merely circumstantial; it is much more difficult to definitively prove the absence rather than the presence of a gene. Nevertheless, the close association of all major Th2 cytokine genes on a single locus suggests a scenario where the appearance of the Th2 response depended on the expansion of a single locus by gene duplication. From the presence of the Th2 cluster in the chicken it follows that this expansion had already occurred prior to the avian–mammalian split. The question remains as to whether an ancestor of the Th2 cytokine cluster exists in the teleostean lineage. The identification of teleostean orthologs of the receptors of Th2 cytokines may provide us with an answer to this question. Although the extracellular domain of the Th2 cytokine receptors is not necessarily better conserved than their ligands, their overall sequences are longer and will contain a conserved intracellular domain; both are characteristics that facilitate their discovery compared with the discovery of their ligands. Through the identification of their receptors we may find valuable clues regarding the presence and number of Th2 cytokines, and consequently a Th2-like response, in early vertebrates. Indeed, although none of the cytokines of the Th2 cluster has been identified in teleosts to date, receptor molecules that have been identified in *Tetraodon nigroviridis* share similarities with human IL-2Rβ/IL-4Rα, IL-13Rα and a receptor chain similar to the β-subunits of the receptors for IL-3, IL-5 and GM-CSF (Jaillon *et al.* 2004). In contrast to the (provisional) absence of Th2 cytokines outside birds and mammals, orthologs of IL-12, IL-18 and IFNγ, the main initiators of the mammalian Th1 response, have been identified in fish (Yoshiura *et al.* 2003, Huising *et al.* 2004*a*, 2006, Jaillon *et al.* 2004, Pestka *et al.* 2004, Zou *et al.* 2004, Daisuke *et al.* 2005), suggestive of the existence of a fish Th1-like immune response. And although Th1 and Th2 are considered the Yin and Yang of mammalian immunology, they may not necessarily have developed simultaneously. A fish Th1-like response may function autonomously, without the cross-regulatory activity of Th2 cytokines. Th2 responses in mammalian species are associated with a predominantly humoral immune response elicited by extracellular pathogens. In addition, although fish can mount an antigen-specific antibody response, evidence for refined properties such as isotype switching and affinity maturation is limited, in line with the absence of lymph nodes and histologically identifiable germinal centres (Van Muiswinkel *et al.* 1991). Recently, fish orthologs of several short-chain class-I helical cytokines other than those of the Th2 cluster, such as IL-2, IL-15 and IL-21 (Jaillon *et al.* 2004, Bird *et al.* 2005*a*), have been described, indicating that short-chain class-I helical cytokines are represented within the

fish genome. From the above, it is clear that the presence or absence of the Th2 cytokine cluster in lower vertebrates needs to be addressed, as it is the last major group of cytokines that lacks representatives in lower vertebrate species.

Vertebrates outdated: insect cytokines

Most long-chain class-I helical cytokines are situated at a distinct locus, either alone or in pairs (Fig. 4). For many of these (pairs of) helical cytokines unambiguous orthologs have recently been identified in bony fish species and more are likely to follow in the near future. Under the plausible assumption that class-I helical cytokines constitute a monophyletic group, this implies that the origin of helical cytokines predates vertebrates. The draft genome of the invertebrate chordate *Ciona intestinalis* contains orthologs of JAK, STAT, SOCS and PIAS (Hino *et al.* 2003), but ascidian helical cytokine or cytokine receptor genes have not been described to date.

In *Drosophila melanogaster*, compelling evidence has emerged for the involvement of cytokine signalling in various physiological processes. A *Drosophila* JAK/STAT pathway was first identified through its role in embryonic segmentation. The *Drosophila* JAK is named hopscotch (hop) and shares 27% overall amino acid identity with human JAK2 (Binari & Perrimon 1994). The single *Drosophila* STAT identified to date is named STAT92E (or marelle) and shares 37% amino acid identity with human STAT5 (Hou *et al.* 1996, Yan *et al.* 1996). Fly embryos deficient in either hop or STAT92E gene activity are characterised by the stripe-specific loss of the expression of several pair-rule genes, leading to the selective loss of a specific subset of body segments (Binari & Perrimon 1994, Hou *et al.* 1996, Yan *et al.* 1996). The same phenotype was observed in the absence of gene activity of a third gene, unpaired (upd). *Drosophila* upd is a secreted glycoprotein of over 400 amino acids, which is associated with the extracellular matrix through association with glycosaminoglycans, effectively confining its range of actions (Harrison *et al.* 1998). The complete overlap in phenotypes displayed upon the absence of either upd, hop or STAT92E suggests that they are all components in the same pathway. Indeed, upd is capable of inducing hop phosphorylation, reminiscent of the JAK phosphorylation that follows cytokine activation in vertebrates (Harrison *et al.* 1998). The receptor that mediates the upd-induced phosphorylation of hop was later identified as domeless (dome) and belongs to the class-I helical cytokine receptor family. Extracellularly, dome has five FnIII domains, two of which form a cytokine-binding domain with an incomplete WSXWS motif that is characteristic of vertebrate class-I helical cytokine receptors (Brown *et al.* 2001, Chen *et al.* 2002, Hombria & Brown 2002). dome also has a single intracellular consensus STAT-binding motif (YXXQ). Since its discovery as important regulators of embryonic segmentation, involvement of the *Drosophila* dome/hop/STAT92E pathway has been shown in oogenesis (Ghiglione *et al.* 2002), eye development (Chen *et al.* 2002, Bach *et al.* 2003, Tsai & Sun 2004), hindgut epithelial cell rearrangement (Johansen *et al.* 2003), trachea formation (Harrison *et al.* 1998, Chen *et al.* 2002), and the fly immune response (Agaisse & Perrimon 2004). Recently, two additional *Drosophila* unpaired proteins, designated upd2 and upd3, have been discovered at the upd locus (Hombria & Brown 2002). The three *Drosophila* upd proteins differ, at least to some extent, in their involvement in the physiological processes that require dome/hop/STAT92E activation. For example, upd3, but not upd, is upregulated in fly haemocytes in response to septic injury (Agaisse *et al.* 2003, Agaisse & Perrimon 2004). Recently, the embryonic expression of upd2 was found to closely overlap with that of upd and both proteins are capable of activation of dome (Hombria *et al.* 2005). The same study revealed an interesting difference between upd and upd2 as the latter is secreted in soluble form, in contrast to upd which is associated with the extracellular matrix (Hombria *et al.* 2005). The above illustrates that (partial) redundancy also exists within the *Drosophila* upd family, similar to the redundancy that characterises the vertebrate class-I helical cytokine family. As unambiguous orthologs to the separate members of the *Drosophila* upd family have to date not been discovered in related dipteran species such as *Anopheles gambiae* (malaria mosquito), it appears that the *Drosophila* upd family arose through gene duplications that occurred relatively recently in the *Drosophila* lineage (Hombria *et al.* 2005).

Given the similarity of their downstream signalling components with the vertebrate class-I helical cytokine family, the obvious question is whether *Drosophila* upd proteins belong to the helical cytokine family. *Drosophila* upd amino acid sequences are longer than that of any mammalian helical cytokine and although predicted to be of high α-helical content (Harrison *et al.* 1998) it is not known whether these α-helices fold into a four-helix bundle motif. The predicted pI of upd is extremely basic at almost 12, a characteristic that is generally considered to be unlike any vertebrate helical cytokine (Harrison *et al.* 1998, Chen *et al.* 2002), although, for example, vertebrate IL-11 proteins have a similarly high predicted pI (Zheng *et al.* 2001, Huising *et al.* 2005). Interestingly, all introns in *Drosophila* upd, upd2 and upd3 are phase 0, in line with the consensus intron phase in vertebrate class-I helical cytokines (Fig. 4). Nevertheless, although the similarities between upd signalling and the vertebrate JAK/STAT pathway are compelling, the definitive answer to the question as to whether *Drosophila* upd proteins are members of the helical cytokine family or constitute a novel ligand family capable of activation of the JAK/STAT pathway may require the resolution of their crystal structures.

Summary and perspectives

Although the list of known fish class-I helical cytokines is certainly incomplete at this stage, the picture that emerges from the description of the individual members of the class-I helical cytokine family is that many mammalian helical cytokines have orthologs in fish. It follows that these cytokines already existed before the fish–tetrapod divergence that occurred approximately 450 million years ago (Hedges 2002). Following this split, the class-I helical cytokine family witnessed several lineage-specific expansions by gene duplications. Examples include the duplication of IL-11 and IL-12p40 genes and the presence of SL that are all specific to the teleost fish lineage. On the other hand, PLs are restricted to placental mammals and the gene duplications that have led to the occurrence of pairs or triplets of class-I helical cytokines at a single locus (LIF/OSM, 22q12·2; CNTF/CLC, 11q12–13; CT-1/NP/IL-27p28, 16p11) may also be unique to the tetrapod lineage. However, these examples of (potential) lineage-specific gene duplications do not change the view that the class-I helical cytokine family was already largely established and distributed over many loci early in the vertebrate lineage. And since the three unpaired genes, which are the only *Drosophila* potentially cytokine-like peptides discovered to date, all reside on a single locus, the resolution of the genome of species that occupy an evolutionarily intermediate position between vertebrates and insects, such as *Ciona intestinalis*, may reveal important insights into the evolution of the class-I helical cytokine family.

A number of cytokines that belong to the other cytokine families have been discovered in the last decade in teleost fish. These include pro-inflammatory cytokines such as IL-1β and TNFα as well as anti-inflammatory cytokines such as tissue growth factor-β (Hardie *et al.* 1998) and IL-10 (Savan *et al.* 2003). Representatives of several classes of chemokines are also present in fish (Huising *et al.* 2003*b*, 2004*b*, Laing & Secombes 2004), although there is compelling evidence that the chemokine repertoire of fish and mammals differs extensively due to lineage-specific gene duplications (Huising *et al.* 2003*a*). Also, IFNγ and IL-18, key cytokines that drive the immune response towards Th1 in concert with IL-12, are present in fish (Huising *et al.* 2004*a*, Pestka *et al.* 2004, Zou *et al.* 2004, Daisuke *et al.* 2005). Although the status of many individual cytokines is still uncertain, the only major group of cytokines for which not a single fish ortholog has been reported to date is the Th2 cluster. Clearly, the question as to whether orthologs of mammalian Th2 cytokines are present in early vertebrates needs to be addressed in the near future, as the answer to this question will assist us in appreciating the evolutionary significance of one of the major paradigms of the mammalian immune system. A common feature of all fish cytokines discovered to date is the low degree of primary sequence conservation they share with their mammalian orthologs. This relatively poor sequence conservation has complicated (and still complicates) the discovery of non-mammalian orthologs of cytokine genes. This extensive sequence dissimilarity also serves as a reminder that the functions of orthologous cytokines in different vertebrate classes may differ considerably. For only a very select number of fish class-I helical cytokines has their discovery been followed-up by a functional characterisation that exceeds the level of gene expression. Nevertheless, a better understanding of fish immunity as well as a more refined comparison between immune systems of different vertebrate classes will eventually require such an approach.

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