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Evolution of Leptin Structure and Function

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Leptin · Leptin receptor · Obesity · Hypothalamus · Pituitary · Evolution

Abstract
Leptin, the protein product of the *obese (ob* or *Lep)* gene, is a hormone synthesized by adipocytes that signals available energy reserves to the brain, and thereby influences development, growth, metabolism and reproduction. In mammals, leptin functions as an adiposity signal: circulating leptin fluctuates in proportion to fat mass, and it acts on the hypothalamus to suppress food intake. Orthologs of mammalian *Lep* genes were recently isolated from several fish and two amphibian species, and here we report the identification of two *Lep* genes in a reptile, the lizard *Anolis carolinensis*. While vertebrate leptins show large divergence in their primary amino acid sequence, they form similar tertiary structures, and may have similar potencies when tested in vitro on heterologous leptin receptors (LepRs). Leptin binds to LepRs on the plasma membrane, activating several intracellular signaling pathways. Vertebrate LepRs signal via the Janus kinase (Jak) and signal transducer and activator of transcription (STAT) pathway. Three tyrosine residues located within the LepR cytoplasmic domain are phosphorylated by Jak2 and are required for activation of SH2-containing tyrosine phosphatase-2, STAT5 and STAT3 signaling. These tyrosines are conserved from fishes to mammals, demonstrating their critical role in signaling by the LepR. Leptin is anorexigenic in representatives of all vertebrate classes, suggesting that its role in energy balance is ancient and has been evolutionarily conserved. In addition to its integral role as a regulator of appetite and energy balance, leptin exerts pleiotropic actions in development, physiology and behavior.

Introduction

Two mouse strains discovered at the Jackson Laboratory in 1950 and 1965 had identical phenotypes: morbid obesity, insulin resistance, infertility and lethargy [1]. The two strains were designated obese *(ob/ob)* and diabetic *(db/db)*, and were found to be due to single gene deficiencies. A series of elegant parabiosis experiments conducted by Douglas Coleman showed that the *ob/ob* strain was deficient in a blood-borne factor, while the *db/db* strain was deficient in the receptor for this factor [1–3]. Over forty years passed before the mouse *obese* gene *(ob*

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or *Lep)* was positionally cloned by Jeffrey Friedman's group and was found to encode a hormone that they named 'leptin' after the Greek word '*leptos*' for thin [4]. Leptin is a member of the type I helical cytokine family, and is related to growth hormone, prolactin and the interleukins [5]. The year after leptin was identified Tartaglia et al. [6] reported the isolation of the leptin receptor gene *(LepR)* by expression cloning. Shortly thereafter it was confirmed that the mutation in the *db/db* mouse was in the *LepR* gene [7, 8].

In mammals leptin is secreted into the bloodstream, primarily from adipocytes, and acts on the brain to regulate food intake and metabolism [4, 9]. Leptin acts on the hypothalamus to signal when the body has sufficient energy stores, thus inhibiting appetite (i.e. it functions as an 'adipostat'). The actions of leptin occur over both short and long time frames. In the short term, plasma leptin serves as a satiety signal [10], and over longer periods, daily mean plasma leptin concentration communicates long-term energy status to the brain [11]. Central leptin signaling plays a pivotal role in the regulation of metabolic activity by peripheral tissues [12–14]. The rising prevalence of human obesity and type 2 diabetes has generated intense interest in the physiological roles that leptin plays in energy balance and food intake regulation [15–19].

In mammals, leptin acts on complex neural circuitry to regulate food intake and energy metabolism [20, 21]. Leptin receptor expression is highest in neurons within nuclei of the basomedial hypothalamus that include the arcuate (ARC), dorsomedial hypothalamic and ventromedial hypothalamic nuclei [22, 23]. Primary targets for leptin action in the hypothalamus are two populations of neurons located in the ARC that project axons to the lateral hypothalamic area [21, 24]. Leptin acts on ARC proopiomelanocortin (POMC)/cocaine and amphetamine related transcript (CART) neurons to increase POMC (and CART) biosynthesis which generates an anorectic signal via alpha melanocyte-stimulating hormone (αMSH) [24, 25]. Leptin also acts on ARC neuropeptide Y (NPY)/Agouti-related protein (AgRP) neurons to inhibit expression of the orexigenic signals NPY and AgRP [24, 25]. Leptin receptors have also been reported in several extrahypothalamic sites that include the midbrain and brainstem [21, 26]. Second-order neurons that synthesize thyrotropin-releasing hormone (TRH) or corticotropin-releasing factor (CRF) located in the paraventricular nucleus are regulated indirectly by leptin targets in the ARC, and thus mediate leptin's inhibitory actions on food intake, increases in thermogenesis, and increases in pituitary hormone secretion [10]. Although much less is known about the organization and regulation of feeding control centers in the brains of nonmammalian species, the data support that the basic features of mammalian feeding control circuits are present in fishes and amphibians [27–29], and that leptin engages similar neuropeptidergic pathways in the hypothalamus/preoptic area (for frogs, see [30] [C. Li and R.J. Denver, unpublished], and for fishes, see [31, 32]).

In addition to its roles in the regulation of appetite and metabolism, leptin has pleiotropic actions in development and physiology. Some of the major actions of leptin uncovered in recent years include the promotion of linear growth through its influence on energy balance, the induction of mitosis of different cell types including chondrocytes of the epiphyseal growth plate, and the stimulation of secretion of pituitary growth hormone [33]. Leptin is permissive for the onset of puberty in mammals, perhaps acting via the cellular energy sensor mammalian target of rapamycin (mTOR) [34]. It plays critical roles in neural development. *Ob/ob* and *db/db* mice have reduced brain weight and DNA content which, in *ob/ob* mice, can be reversed by leptin administration [35]. The hormone has been shown to induce mitosis in different brain regions [36, 37] and in the hypothalamus, leptin influences the maturation of feeding control circuitry by promoting the formation of neuronal projections among hypothalamic nuclei [38]. Leptin actions also include bone development, growth and homeostasis [30, 39–42], as well as lung development and function [43], immune function [44, 45], thyroid function [46] and stress response [12]. A full discussion of the diversity of leptin actions is beyond the scope of this review. See the references cited above for detailed discussions of leptin actions.

Molecular Evolution of Vertebrate Leptin Genes

Shortly after the mouse *Lep* gene was isolated, orthologous genes were identified in human and several other mammalian species [4, 47, 48]. Many mammalian *Lep* genes have since been cloned, and molecular phylogenetic analysis shows that most taxa form distinct clades that largely agree with accepted mammalian orders (fig. 1). High rates of leptin evolution are apparent in some seals *(Halichoerus grypus* and *Leptonychotes weddellii)*, beavers *(Castor canadensis)*, pikas *(Ochotona curzoniae)*, and marmosets *(Callithrix jacchus)*. In adult seals leptin is expressed in the lung, whereas lung leptin expression in other mammals appears to occur only in the fetus [49].

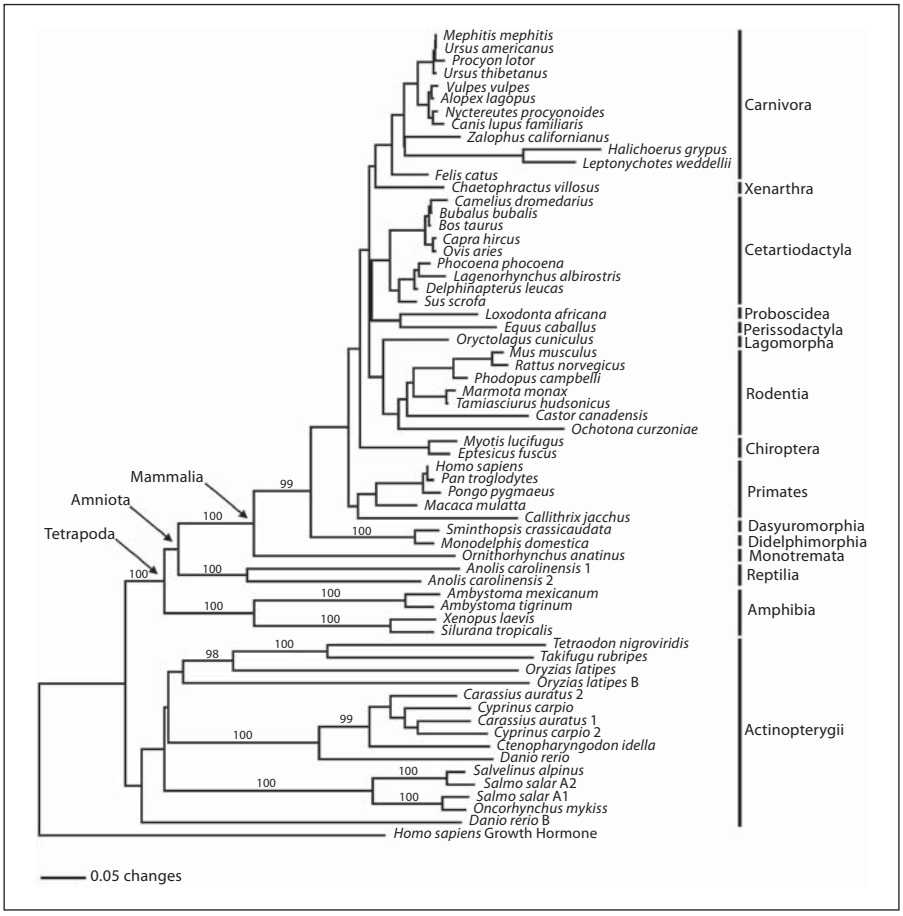
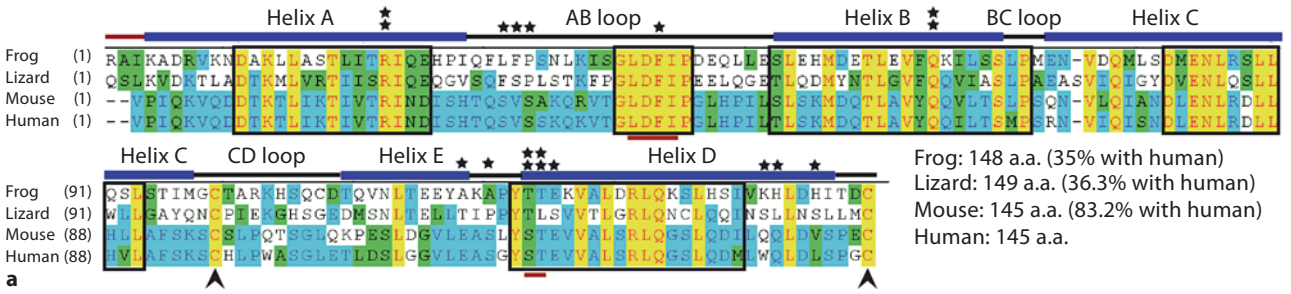


Fig. 1. Neighbor-joining phylogram of vertebrate leptins. The alignment was conducted using Clustal W2 and based on the BLOSSUM protein weight matrix. The neighbor-joining phylogram was based on uncorrected pair-wise sequence divergence of 229 amino acid positions (including gaps). Bootstrap values subtend major, well-supported nodes (≥90%) and were based on 1,000 pseudoreplicates. The tree was rooted with human growth hormone. Vertical lines on the right indicate mammalian orders and nonmammalian vertebrate classes. Due to the differential rates of leptin evolution in some mammalian lineages, taxa in the mammalian orders Carnivora, Cetartiodactyla, Rodentia, and Primates were each constrained to be monophyletic prior to the neighbor-joining analysis. Bootstrap values are not shown for the constrained nodes. Branch lengths reflect evolutionary divergence and high rates of leptin evolution are apparent in some seals *(Halichoerus grypus* and *Leptonychotes weddellii)*, beavers *(Castor canadensis)*, pikas *(Ochotona curzoniae)*, and marmosets *(Callithrix jacchus).*

Hammond et al. [49] speculated on a role for leptin in pulmonary surfactant production in seals that may be related to the unique respiratory challenges associated with diving. Yang et al. [50] proposed that in pikas, which are non-hibernating mammals that live at high elevation or high latitudes, adaptive evolution in leptin was driven by physiological adaptation to extreme cold.

When the mouse *Lep* gene was first isolated the authors concluded, based on low stringency Southern hybridization of the mouse *Lep* probe to genomic DNA isolated from chicken, eel and fruit fly, that *Lep* genes were evolutionarily conserved [4]. In the decade following the discovery of the mouse *Lep* gene, several laboratories attempted to isolate orthologous genes from nonmammalian species using nucleic acid hybridization (e.g. library screening) or RT-PCR with degenerate primers. These attempts were uniformly unsuccessful, except in the case of the chicken, where a cDNA was isolated by RT-PCR and reported to share 95% identity to the mouse gene [51, 52]. Subsequently, a putative *Lep* ortholog was isolated from turkey which also had very high sequence identity to rodent *Lep* genes [48]. The validity of these sequences has since been questioned [53–57]; but see counterpoint [58]. Searches of several EST databases and the chicken genome were unsuccessful in identifying a chicken *Lep* sequence [53, 56], and synteny analysis showed that the entire chromosomal region within which the chicken *Lep* gene should be found (on chromosome 1, which is homologous to human chromosome 7, mouse chromosome 6) is missing [56]. Neighbor joining analysis of all known vertebrate *Lep* genes showed that the chicken sequence was phylogenetically nested amongst mammals and very closely related to rodents (fig. 1; the bird sequences are not included in the tree) [55]. Sharp et al. [55] pointed out that, based on the estimated frequency of synonymous substitutions due to random mutation in genes, the likelihood that the reported chicken *Lep* gene cDNA was correct was less than one in a million.

Despite the failure to identify avian orthologs of mammalian *Lep* genes, chickens have a leptin receptor in their


Helix A
AB loop
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Lizard (1)
Mouse (1)
Human (1)
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Helix D
Frog (91)
Lizard (91)
Mouse (88)
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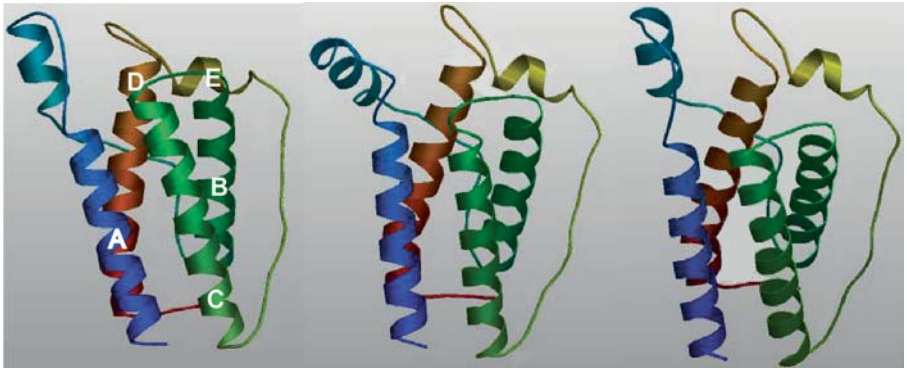
a
Frog: 148 a.a. (35% with human)
Lizard: 149 a.a. (36.3% with human)
Mouse: 145 a.a. (83.2% with human)
Human: 145 a.a.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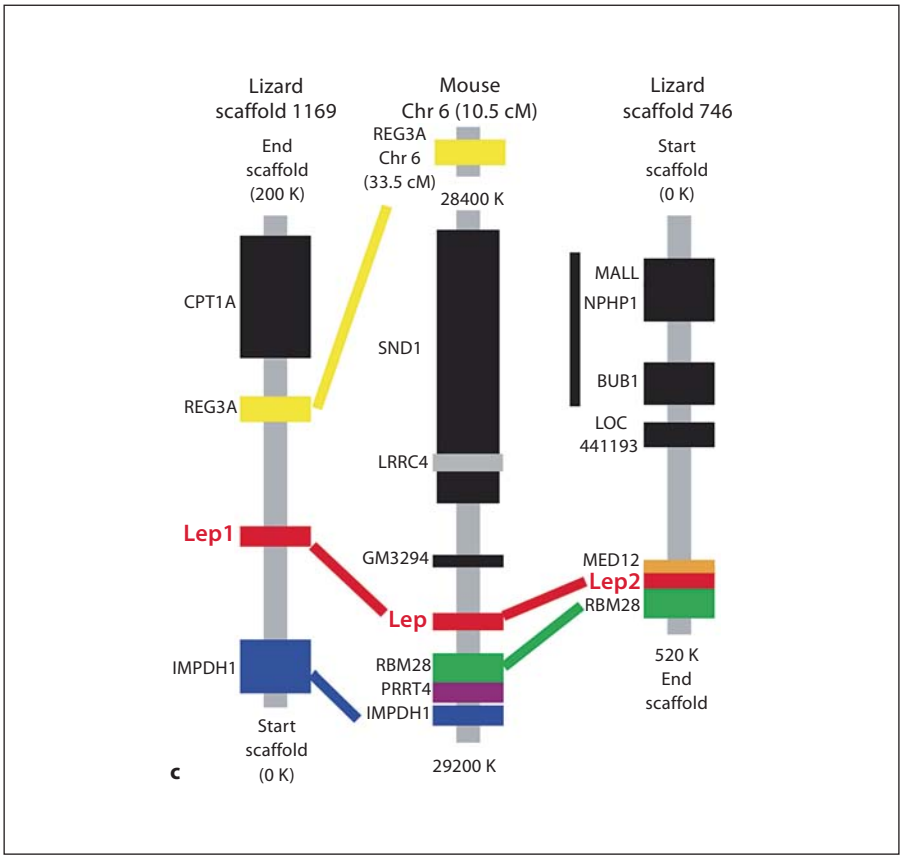
Lizard scaffold 1169
End scaffold (200 K)
CPT1A
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Lep1
IMPDH1
Start scaffold (0 K)
Mouse Chr 6 (10.5 cM)
REG3A Chr 6 (33.5 cM)
28400 K
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LRRC4
GM3294
Lep
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PRRT4
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MALL
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BUB1
LOC 441193
MED12
Lep2
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2

genome that is expressed, is activated to signal via the Janus kinase (Jak)/signal transducer and activator of transcription 3 (STAT3) pathway by human and frog leptins in vitro, and likely mediates the actions of administered leptin in vivo [59]. Pitel et al. [56] proposed that the gene for the ligand was lost in birds while the gene for the receptor was retained. It is possible that this occurred in some birds, but that leptin genes are retained in other avian lineages. Leptin genes were clearly present in the common ancestor of birds and squamate reptiles since the lizard, *Anolis carolinensis* possesses two *Lep* genes (discussed below). Using our predicted platypus *Lep* gene as the search sequence we were able to locate two candidate *Lep* genes within the lizard genome (online supplementary tables 1 and 2; for all online supplementary material, see www.karger.com/doi/10.1159/000328435); however, similar searches through the chicken and zebra finch genomes using platypus and lizard *Lep* gene sequences produced no returns. More work on other avian and reptilian species is required to determine if the *Lep* gene has been lost in Aves.

As discussed above, for a decade after the first isolation of *Lep* genes in mammals no orthologs were identified in nonmammalian vertebrates. In 2005, Kurokawa et al. [60] reported the isolation of a putative homolog of mammalian leptin in the pufferfish *Takifugu rubripes*. The deduced pufferfish protein is only 13% similar to human leptin (or to frog leptin – see below). Kurokawa et al. did not test whether the deduced protein product of the putative pufferfish *Lep* gene had biological activity commensurate with a role as a leptin.

Shortly after the publication by Kurokawa et al. [60], we reported the molecular cloning of frog *(Xenopus)* orthologs of mammalian *Lep* and *Lep* genes and conducted the first functional characterization of this ligand-receptor pair in a nonmammalian vertebrate [30]. The frog *Lep* gene encodes a predicted 16.9 kDa protein (fig. 2). The primary amino acid sequence of frog leptin is 35% similar to human, but only 13% similar to pufferfish leptin [60]. We showed that recombinant frog leptin activated the frog LepR in vitro, signaling via STAT3, and frog leptin was potently anorexigenic when injected intracerebroventricularly into juvenile frogs [30]. Boswell et al. [61] isolated a cDNA for a putative *Lep* ortholog in the tiger salamander *Ambystoma tigrinum* that shares 60% identity with frog leptin.

Sequences orthologous to mammalian *Lep* genes have been described in several fish species based on genomic analyses [60] and RT-PCR cloning [31, 32, 57, 62–64]. Deciphering the molecular phylogeny of fish *Lep* genes is complicated by the fact that multiple, and sometimes highly divergent, copies have been identified in several species. These duplicates may have resulted from historical events such as the whole-genome duplication that occurred early in the teleost lineage [65], or the additional genome duplication event that occurred later in some teleost lineages leading to tetraploidy (e.g. salmonids [66]).

Fig. 2. Comparison of the amino acid sequences and structures of some tetrapod leptins. **a** Amino acid alignment of frog (*Xenopus laevis*; AY884210), lizard (*Anolis carolinensis*; Lep1; online suppl. table 1), mouse (*Mus musculus*; AAA64564) and human (*Homo sapiens*; AAA60470) leptin. The alignment colors are as follows: red character on yellow background = consensus residue derived from a completely conserved residue at a given position; blue character on cyan background = consensus residue derived from a block of similar residues at a given position; black character on green background = consensus residue derived from the occurrence of greater than 50% of a single residue at a given position; black character on white background = non-similar residues [83]. Boxed regions are the most highly conserved sequences among tetrapod leptins. The indicated helices and loop structures are based on Zhang et al. [145]. Amino acids with two stars above them are residues in mouse leptin that when mutated lead to loss of biological activity [85]. Note that these amino acids tend to be conserved among tetrapod leptins. Amino acids with one star above are residues in mouse leptin that when mutated lead to partial loss of biological activity [85]. In most cases these amino acids show low conservation among tetrapod leptins. Red bars underneath the alignment indicate amino acids that when mutated cause the hormone to lose receptor activation function but to retain receptor binding activity, thereby generating an antagonist (LDFI – mouse a.a. 38–43; ST – mouse a.a. 120, 121) [86]. The two conserved cysteines are indicated by arrows. The alignment was conducted using the Align X module of Vector NTI Advance 11 software (Invitrogen, Carlsbad, Calif., USA). **b** Ribbon diagrams showing secondary and tertiary structures of mouse, lizard and frog leptins. Three-dimensional modeling was done using the ProModII program at the SWISS-MODEL automated protein modeling server, and was based on the human leptin (1AX8.pdb) Protein Data Bank structure file. **c** Synteny mapping of leptin genes from mouse and lizard. Gene position on the mouse chromosome was determined by NCBI sequence viewer of *Mus musculus* chromosome 6, reference assembly (C57BL/6J). Gene positions on the lizard scaffolds were determined using the UC Santa Cruz Genome Browser of *Anolis carolinensis* (Feb. 2007 AnoCar v. 1.0). Only limited gene arrangement information is available for these lizard scaffolds because of their short length. The vertical black bar to the left of the lizard scaffold 746 indicates a block of neighboring genes (MALL, NPHP1 and BUB1) found on mouse chromosome 2 (73 cM).

Differential rates of gene loss or divergence in different lineages, the limited sequence data now available, and the tenuous phylogenetic placement of divergent fish leptins are also challenging our understanding of the history of this gene in fishes. Some fishes (e.g. zebrafish and medaka) [62, 67] have two divergent types of *Lep* genes (designated A and B; fig. 1) that may have arisen from the initial whole-genome duplication event. Alternatively, these may have resulted from clade-specific gene duplications with subsequent divergence of one of the two copies. Multiple copies of 'A lineage' *Lep* genes have been isolated from Atlantic salmon, common carp, and goldfish, with minimal divergence among the paralogs and likely result from lineage-specific tetraploidizations in salmonids [62, 68] and cyprinids [57, 63, 69, 70]. *Takifugu rubripes,* a teleost species that has undergone drastic genome reduction, appears to have retained only one *Lep* gene [60].

We searched the genome of the lizard *Anolis carolinensis* and identified, for the first time, *Lep* and *LepR* genes in a reptile. *Anolis* has two putative *Lep* genes (designated *Lep1* and *Lep2*; online suppl. table 1; [71]). The predicted mature Lep1 protein of the lizard is 149 amino acids in length and shares 35.6% amino acid sequence identity with human leptin. The lizard *Lep2* gene has multiple, single nucleotide deletions at the end of exon 3, which results in a frame shift. This causes the predicted Lep2 mature protein to diverge from Lep1 after position 117; the Lep2 protein is predicted to be 192 amino acids. The two predicted lizard leptin proteins are 60.7% identical in the first 117 amino acids.

We conducted synteny mapping of genes neighboring mouse *Lep* on chromosome 6 and this supported that the two lizard *Lep* genes are orthologs of mouse *Lep* (fig. 2c). The lizard *Lep1* gene resides in a homologous genomic region to mouse *Lep*. The lizard *Lep2* gene resides in a genomic region homologous to mouse chromosome 2; the lizard *Lep2* gene likely arose through a recombination event (that included gene RBM28). Preliminary results suggest that lizard *Lep1* mRNA is expressed in several tissues but *Lep2* mRNA is not expressed [71]. The lizard Lep1 can activate LepRs: we made recombinant lizard Lep1 in *E. coli* and found that it activates mouse and frog LepRs in transient transfection assay with potency comparable to the homologous leptins [L. Lavner, A. Dziuba, G.C. Boorse and R.J. Denver, unpublished].

Prior to the isolation of nonmammalian *Lep* genes, several groups used mammalian reagents (recombinant mouse leptin, antibodies to mouse leptin or leptin receptor) to study leptin biology in nonmammalian species [27, 72–77]. In the goldfish and a lizard, injections of recombinant mammalian leptin reduced food intake, which was consistent with the existence of a leptin-like protein in nonmammalian species that functions in energy balance regulation [27, 77, 78]. However, mammalian leptin failed to affect food intake in several other fish species [27]. Some investigators have used (and continue to use) antibodies to mouse leptin to investigate the tissue distribution and expression of leptin in nonmammalian species [27, 79–81]. Given the low conservation of the primary structure of leptins from different vertebrate classes (fig. 2), we suggest that results obtained with heterologous immunological reagents should be interpreted with caution. On the other hand, despite low primary sequence identity, leptins from different species are active on heterologous receptors, although the potency varies [30, 59]; see also [82] for activity of pufferfish leptin on proliferation of BAF/3 cells stably transfected with the long form of human leptin receptor. Therefore, studies in which murine leptin was used in nonmammalian species could point to a physiological role for the endogenous, native leptins, but this must be verified once the homologous hormones become available.

Conserved Structural Features of Vertebrate Leptins Related to Their Function

When the crystal structure of human leptin was solved, it was found to have four α helix bundle folds, closely resembling the structures of other class I helical cytokines [5]. Like other class I helical cytokines, vertebrate leptins show significant divergence in their primary structures, but are nevertheless highly similar in their predicted secondary and tertiary structures (modeling based on the crystal structure of human leptin; fig. 2b) [57, 60, 63]. Natural selection tends to maintain the intrinsic stability of secondary and tertiary structures of proteins [83], and this is illustrated well by the class I helical cytokines [5].

Leptin resembles other class I helical cytokines in that it has four antiparallel helices designated A-D, but it differs in that it has a small helical segment designated helix E found in the loop linking helices C and D (fig. 2b). All vertebrate leptins have a pair of conserved cysteine residues that in human leptin have been shown to form a disulfide bridge required for full biological activity [30, 60, 84] (fig. 2a). Three receptor interacting sites on mammalian leptins have been mapped by mutational analysis [85]. Site I is located on the face of helix D, site II is on helices A and C, and site III at the N-terminus of helix D. Each of these regions shows some degree of conservation of primary amino acid sequence among vertebrate leptins

(fig. 2a). Amino acid substitutions that resulted in a significant reduction in biological activity of the hormone were likely selected against, as these positions tend to be completely or mostly conserved across tetrapods (indicated by double stars above sequences in fig. 2a).

There are other regions of vertebrate leptins located outside of the three identified receptor binding sites that show a high degree of sequence identity. For example, there is conservation in helix B and the BC loop. The most highly conserved stretch of amino acids is the six residue sequence GLDFIP (positions 38 to 43 in human leptin); this sequence is completely conserved among tetrapods (fig. 2a). Mutation of LDFI to all alanines generates an antagonist (a leptin 'mutein') that binds to the LepR but fails to activate it [86]. Although this sequence is not a part of the receptor binding sites, it is required for activation of the LepR and has apparently been subject to strong stabilizing selection. Elinav et al. [87] produced a PEGylated form of the LDFI mutein that has enhanced antagonist activity in vivo due to reduced clearance. We recently engineered a frog leptin LDFI mutein and found that it also has antagonist activity on the frog LepR when tested in vitro [C. Pelletier, A. Dziuba, M. Cui and R.J. Denver, unpublished]. The GLDFIP sequence is absent in fish leptins [85] and may indicate a different mechanism for binding to and activation of the LepR compared with tetrapods. Other amino acids that have been mutagenized to generate a leptin antagonist are the S and T residues at positions 120 and 121, respectively, of mouse leptin [86].

As mentioned above, there is considerable divergence in primary amino acid sequence among vertebrate leptins. However, their secondary and tertiary structures, and key amino acids required for biological activity, particularly among tetrapods, are evolutionarily conserved (it should be noted that the predicted structures of nonmammalian leptin are based on models of the crystal structure of human leptin). This conservation parallels the conservation of key structural elements within the two cytokine receptor homology domains (CHDs) of vertebrate LepRs [88] (discussed below; fig. 3). The significance of this conservation is highlighted by the finding that heterologous hormone-receptor pairings among tetrapod leptins and LepRs leads to productive receptor activation, often with similar potency to the homologous hormone. For example, recombinant leptins of frog and lizard activated the mouse LepR with equal potency to human leptin. Similarly, human and lizard leptins activated the frog LepR, although with lower potency than frog leptin [30] [L. Lavner, A. Dziuba, G.C. Boorse and R.J. Denver, unpublished].

Tissue Sites of Leptin Production

In mammals, the major sites of *Lep* mRNA expression are adipose tissue, stomach and liver [89–92]. *Lep* mRNA is expressed at lower levels in heart, placenta and fetal tissues [93, 94], the pituitary gland, where leptin may modulate pituitary hormone secretion [95–97], and in the brain [98] where the hormone can influence neural development [36, 99, 100] and cognitive function in the adult [101]. In nonmammalian species, *Lep* mRNA appears to be more widely expressed compared with mammals, although the major sites of expression may differ among taxa [30]. For example, in the frog, *Lep* mRNA expression levels were highest in brain, pituitary and heart [30]. The *Lep* gene is expressed throughout the frog GI tract and in the two major sites of expression in mammals, liver and fat, although the levels of expression in frog liver and fat were lower than in some other organs that express leptin. Widespread tissue expression of the *Lep* gene has also been reported in several fishes [63]. In salmon, the highest *Lep* mRNA expression (sLepA1) was found in the brain, liver, white muscle and ovary [63]. Many ectothermic species express the *Lep* gene in liver [30–32, 57, 60, 62–64], and so this organ may be a major source of circulating leptin, and a site for nutritional regulation of leptin production [57, 63].

Molecular Evolution of Vertebrate Leptin Receptor Genes

The actions of leptin are mediated by hormone binding to the LepR located in the plasma membrane. The LepR belongs to the class I helical cytokine receptor family [102] (fig. 3). These receptors all signal via the Jak/STAT pathway [103]; although other signaling pathways may be engaged by the LepR, as discussed below [104]. The Jak (Jak2) and STAT (STAT3 and STAT5) proteins important for LepR signaling (and signaling by other hormone-activated cytokine receptors) are highly conserved across vertebrate taxa, much more so than Jaks or STATs involved with immune signaling [105]. In mammals, six isoforms of the LepR (LepRa-f) generated by alternate splicing of transcripts derived from a single *LepR* gene have been identified [12]. All LepR isoforms have a common extracellular ligand binding domain. The long form of the LepR (LepRb) has an approximately 300 amino acid cytoplasmic tail that mediates intracellular signaling upon leptin binding. The LepRa, -c, -d and -f have short (~30–40) amino acid cytoplasmic extensions, while the LepRe lacks transmembrane and cyto-

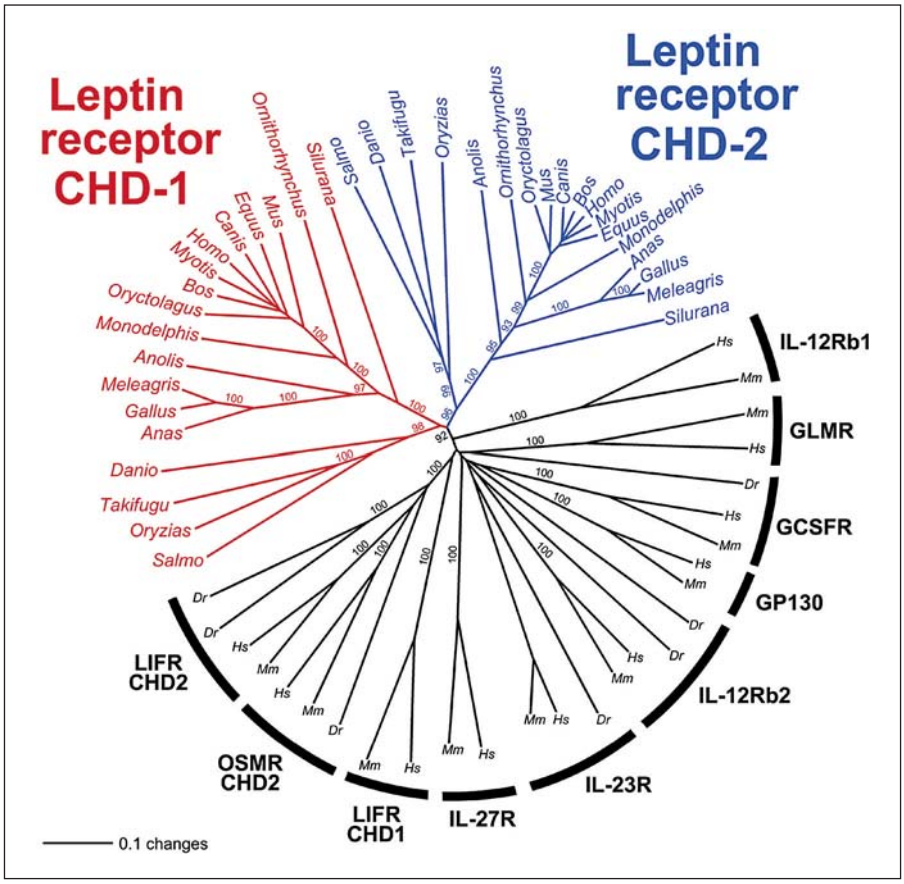


Fig. 3. Neighbor-joining phylogram of cytokine receptor homology domains (CHD) from group 2, class 1 cytokines [102]. The alignment was conducted using Clustal W2 and based on the BLOSSUM protein weight matrix. The neighbor-joining phylogram was based on uncorrected pairwise sequence divergence of 218 amino acid positions (including gaps). Bootstrap values subtend major, well-supported nodes (≥90%) and were based on 1,000 pseudoreplicates. *Homo sapiens* (Hs), *Mus musculus* (Mm), and *Danio rerio* (Dr) were used as representative taxa for glycoprotein 130 (GP130), GP130-like monocyte receptor (GLMR), granulocyte-CSF (GCSFR), interleukin receptors (ILR), leukemia inhibitory factor receptor (LIFR), and oncostatin M receptor (OSMR). The two CHDs of leptin receptors are monophyletic. Leptin receptor taxa are the same as those used for figure 4. Only single representatives were included for each of the mammalian orders. The leptin receptor CHD-1 clade is red and the leptin receptor CHD-2 clade is blue.

plasmic domains and may function as a secreted leptin binding protein. The LepRb is the only isoform that contains intracellular tyrosine residues necessary for signaling; the physiological functions, if any, for other LepR isoforms are unknown.

The leptin receptor has been isolated by molecular cloning, or predicted based on genome sequence in nine mammals, three birds, a reptile (lizard, *Anolis carolinensis*), an amphibian (frog, *Silurana (Xenopus) tropicalis*) and four fishes, although partial sequence data is available for other species (fig. 4; table 1; online suppl. table 2). Phylogenetic analysis of LepR tracks accepted vertebrate phylogeny well with respect to the monophyly of the classes and the relationships among them (fig. 4). The rate of LepR evolution (averaged across the whole gene) appears to be constant in different vertebrate lineages. LepR is a group 2, class 1, helical cytokine receptor [102]. This is based partly on the structure of its CHDs. Our phylogeny based on CHDs from a diversity of vertebrae LepRs shows that the two CHDs of LepR (CHD1 and CHD2) are each monophyletic, and are sister clades with respect to other group 2, class 1, helical cytokine receptors (fig. 3). This indicates that the CHD duplication in LepR occurred after this gene was distinct from other cytokine receptors, but prior to the divergence of fishes and tetrapods. The chicken [106] and frog [S. Grommen and R.J. Denver, unpublished] may produce truncated LepR isoforms. Atlantic salmon have five LepR isoforms, one that is similar to mammalian LepRb, one that possesses the transmembrane domain but lacks most of the cytoplasmic tail, and three that may be secreted forms [63]. More data are needed to assess the production and variation of LepR isoforms among vertebrates and to determine their biological roles.

The structural features of the extracellular ligand binding domain that govern interactions between leptin and the LepR have been and continue to be investigated due to the potential to develop selective LepR agonists and antagonists of therapeutic value [88]. Rønnestad et al. [63] recently compared the structures of the LepR extracellular domains of several vertebrate species from fish to mammal. Here we focus on structural features of the cy-

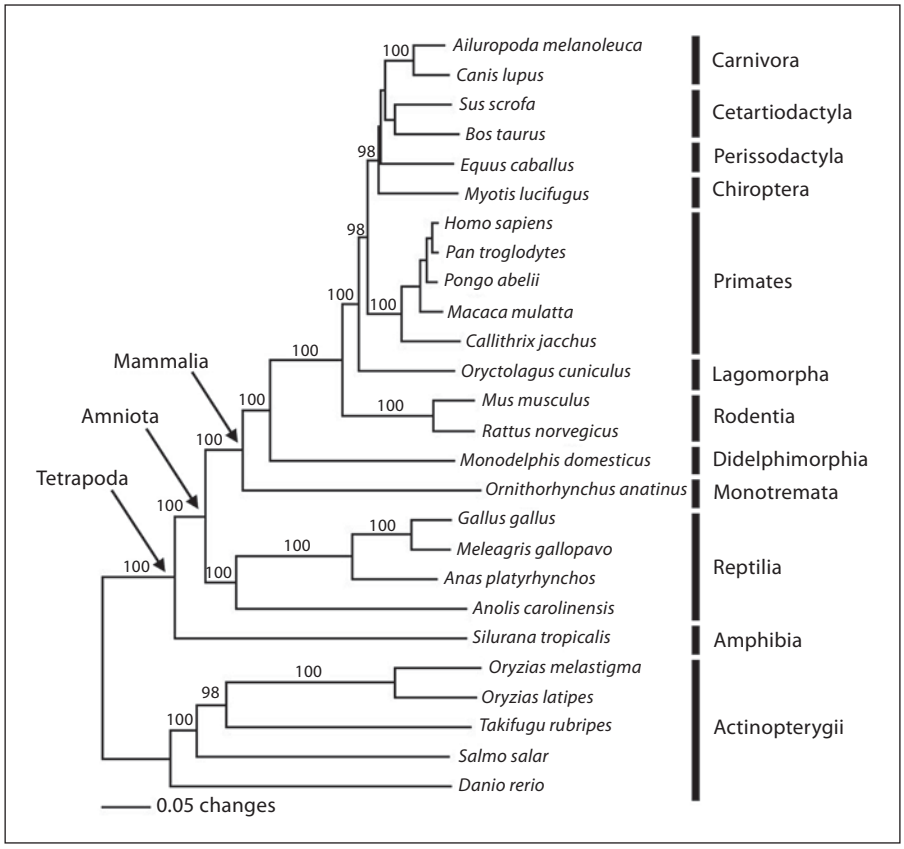


Fig. 4. Neighbor-joining phylogram of vertebrate leptin receptors. The alignment was conducted using Clustal W2 and based on the BLOSSUM protein weight matrix. The neighbor-joining phylogram was based on uncorrected pairwise sequence divergence of 1,242 amino acid positions (including gaps). Bootstrap values subtend major, well-supported nodes (≥90%) and were based on 1,000 pseudoreplicates. Vertical lines on the right indicate mammalian orders and nonmammalian vertebrate classes.

toplasmic domain of the LepR that are necessary for intracellular signaling.

Jak2 is constitutively associated with the mouse LepRb at membrane-proximal residues located within the cytoplasmic domain [107] (fig. 5). The location of these sites has been mapped by deletion analysis [107, 108]. Jak2 recruitment to LepRb depends on cytoplasmic domain amino acids PXP (a.a. 14–16) located within a region called Box 1 that shares features with other class I cytokine receptors [102, 109]. The Box 1 homology motif is highly conserved among vertebrate LepRs (fig. 6). Also necessary are amino acids 19–24 (CSWAQG), which are completely conserved among tetrapods and largely conserved among fishes; and amino acids 31–48. There is flexibility in the sequence requirements for amino acids 37–48, but the sequence of amino acids 31–36 plays a critical role in Jak2 activation [107]. The latter sequence shows some degree of conservation among vertebrates, while the former sequence is only conserved within tetrapods (fig. 6a, c). Bahrenberg et al. [108] showed that the two hydrobic residues, L and F, located at positions 36 and 37 were indispensable for receptor signaling. These two residues are completely conserved among tetrapods and largely conserved in fishes (indicated by stars above the sequences in fig. 6).

Two putative Box 2 homology motifs were proposed for the LepRb (intracellular amino acids 49–60 and 202–213 [107, 109, 110], shown in figure 6a with question marks) but were found not to be required for Jak2 signaling [107, 108]. However, the relatively high evolutionary conservation of residues within the first putative Box 2 motif (a.a. 49–60) suggests that it has some, as yet undiscovered role in LepR signaling. The second putative Box 2 motif (a.a. 202–213) shows very little sequence conservation across taxa. The Box 3 homology motif is critical for recruitment and activation of STAT3 [109, 111].

Hormone binding induces Jak2 autophosphorylation, which increases the Vmax of the kinase, and Jak2 then phosphorylates three tyrosine residues located in the cytoplasmic domain of LepRb [111] (fig. 5 and 6). These three tyrosine residues are conserved among all vertebrates that have been studied. Phosphorylation of Y^{985} in

Table 1. Species, common name, accession number, and length of vertebrate leptin (Lep) and leptin receptor (LepR) proteins used in phylogenetic analyses

Species (common name)		Lep accession No.	Lep length	LepR accession No.	LepR length
Carassius auratus (goldfish)	1	ACL68083	169	–	–
	2	ACO82076	171		
Ctenopharyngodon idella (grass carp)		ACF23048	173	–	–
Cyprinus carpio (common carp)	1	CAI30828	171	–	–
	2	CAI30827	171		
Danio rerio (zebrafish)	A	CAJ33891	166	AAY16198	989
	B	CAP15930	168		
Oncorhynchus mykiss (rainbow trout)		CAJ14971	166	–	–
Oryzias latipes (medaka)	A	BAD94448	155	NP001153915	1,074
	B	BAH24202	158		
Oryzias melastigma (Indian medaka)		–	–	ABC86922	1,109
Salmo salar (Atlantic salmon)	A1	ACZ02412	171	NP001158237	1,146
	A2	ADI77098	175		
Salvelinus alpinus (Arctic char)		BAH83535	175	–	–
Takifugu rubripes (Japanese pufferfish)		BAD94444	152	NP001124341	1,116
Tetraodon nigroviridis (spotted green pufferfish)		BAD94451	160	–	–
Ambystoma mexicanum (axolotl)		CO792338	177	–	–
Ambystoma tigrinum (tiger salamander)		CN054256	183	–	–
Silurana (Xenopus) tropicalis (Western clawed frog)		XM002931835	109	NP001037866	1,148
Xenopus laevis (African clawed frog)		AAX77665	169	–	–
Anolis carolinensis (green anole)	1	see suppl. table 1	170	see suppl. table 2	1,145
	2	see suppl. table 1	213	–	–
Anas platyrhynchos (mallard duck)		–	–	ACF17729	1,155
Gallus gallus (domestic chicken)		–	–	NP989654	1,148
Meleagris gallopavo (wild turkey)		–	–	AAG40323	1,147
Ornithorhynchus anatinus (platypus)		see suppl. table 2	167	XP001512303	1,050
Monodelphis domestica (gray short-tailed opossum)		XP001366398	167	ENSMODP00000001388	1,142
Sminthopsis crassicaudata (fat-tailed dunnart)		AAD44337	167	–	–
Bubalus bubalis (Asian water buffalo)		AAS86311	167	–	–
Bos taurus (domestic cow)		CAD54745	167	DAA31276	1,165
Camelus dromedarius (dromedarian camel)		AAO91910	109	–	–
Capra hircus (domestic goat)		CAJ38273	167	–	–
Ovis aries (domestic sheep)		Q28603	146	–	–
Sus scrofa (wild pig)		Q29406	167	ACT52815	1,165
Ailuropoda melanoleuca (giant panda)		–	–	XP002923682	1,166
Alopex lagopus (Arctic fox)		AAM21763	118	–	–
Canis lupus familiaris (domestic dog)		BAA35129	118	NP001019805	1,166
Felis catus (domestic cat)		BAA95481	167	–	–
Halichoerus grypus (gray seal)		CAF02066	167	–	–
Leptonychotes weddellii (Weddell seal)		CAJ43201	140	–	–
Mephitis mephitis (striped skunk)		AAL32137	89	–	–
Nyctereutes procyonoides (tanuki)		AAM21764	118	–	–
Procyon lotor (raccoon)		AAL32138	89	–	–
Ursus americanus (American black bear)		AAN41652	89	–	–
Ursus thibetanus (Asiatic black bear)		BAE92862	167	–	–
Vulpes vulpes (red fox)		AAM21765	118	–	–
Zalophus californianus (California sea lion)		CAJ43200	166	–	–
Delphinapterus leucas (beluga whale)		AAL32140	90	–	–
Lagenorhynchus albirostris (white-beaked dolphin)		ABK88255	122	–	–
Phocoena phocoena (harbor porpoise)		CAJ43198	138	–	–
Eptesicus fuscus (big brown bat)		AAL32139	90	–	–
Myotis lucifugus (little brown bat)		AAL16404	123	AAU47264	1,153
Oryctolagus cuniculus (European rabbit)		AAL32133	90	XP002715611	1,156

Table 1 (continued)

Species (common name)	Lep accession No.	Lep length	LepR accession No.	LepR length
Equus caballus (domestic horse)	AAR88257	145	XP001500430	1,165
Callithrix jacchus (common marmoset)	see suppl. table 2	167	XP002751006	1,153
Homo sapiens (human)	AAA60470	167	AAA93015	1,165
Macaca mulatta (rhesus macaque)	AAC50730	167	Q9MYL0	1,163
Pan troglodytes (common chimpanzee)	O02750	146	XP001161937	1,165
Pongo abelii (Sumatran orangutan)	–	–	XP002810771	1,165
Pongo pygmaeus (Bornean orangutan)	AAB17092	146	–	–
Loxodonta africana (African bush elephant)	see suppl. table 2	167	–	–
Castor canadensis (North American beaver)	AAQ10008	90	–	–
Marmota monax (groundhog)	AAQ10007	90	–	–
Mus musculus (house mouse)	AAA64564	167	NP666258	1,162
Ochotona curzoniae (black-lipped pika)	ABB90403	167	–	–
Phodopus campbelli (Campbell's dwarf hamster)	CAI99387	118	–	–
Rattus norvegicus (Norway rat)	AAC52514	154	BAA12698	1,162
Tamiasciurus hudsonicus (American red squirrel)	AAQ10009	90	–	–
Chaetophractus villosus (large hairy armadillo)	AAL32134	90	–	–

mouse LepRb leads to recruitment of SH2-containing tyrosine phosphatase-2 (SHP2; [112, 113] which then promotes activation of the extracellular signal-regulated kinase (ERK) cascade [104]. Leptin activation of the ERK cascade has been linked to phosphorylation of ribosomal protein S6 and cap-dependent protein translation [104]. The Y^{985} site also mediates feedback inhibition of the LepRb by suppressor of cytokine signaling 3 (SOCS3) [114]. The residues immediately following Y^{985} are completely conserved among tetrapods (consensus sequence YAT; fig. 6a) but not among fishes (fig. 6b).

Phosphorylation of Y^{1077} leads to recruitment and phosphorylation of STAT5 [115] (fig. 5). Mutation of Y^{1077} leads to obesity in mice, but the molecular physiological pathways regulated by STAT5 are unclear [104]. Phosphorylation of Y^{1138} leads to recruitment and phosphorylation of STAT3 [104]. The STAT3 pathway has been shown to be critical for mediating leptin actions on food intake, glucose metabolism, and weight gain, but does not influence fertility [116]. The Box 3 sequence surrounding Y^{1138} that forms the binding site for STAT3 is evolutionarily conserved from fishes to mammals (fig. 6c). The consensus STAT3 binding sequence is considered to be YXXQ; however, the complete conservation of the P in position 3, and the following two residues FQ (FR in fishes) suggests that the critical residues in the LepR are YXPQFQ/R. This site has been functionally conserved through tetrapod evolution as evidenced by the finding that the frog LepR activated STAT3 signaling when tested in transient transfection assay [30], leptin injection increased pSTAT3 immunoreactivity in frog brain [C. Hu, C. Pelletier and R.J. Denver, unpublished], and mutation of Y^{1127} of the frog LepR (homologous to Y^{1138} in the mouse LepR) abrogated STAT3 signaling [A. Dziuba and R.J. Denver, unpublished]. Activation of STAT3 leads to the upregulation of SOCS3, which binds to Y^{985} on LepRb and inhibits signaling [104]; fig. 6). The phosphoinositol 3 kinase and mammalian target of rapamycin pathways are also engaged by LepRb signaling but the mechanisms for their activation are poorly understood [104].

Tissue Sites of Leptin Receptor Expression

In mammals the LepRb is highly expressed in the hypothalamus and at lower levels in several other tissues including liver [91], kidney, lung [117], stomach [118], pancreatic β cells [119], and immune cells [120]. Leptin's role in energy balance/body weight control is mediated by LepRb expressed in the brain [104, 121].

In the few nonmammalian species that have been studied, *LepR* mRNA is as highly expressed in the brain as it is in mammals [30, 63, 122]. Liu et al. [122] reported that *LepR* mRNA expression was restricted to the adult zebrafish hindbrain and hypothalamus. Using increased pSTAT3 immunoreactivity following leptin injection we mapped biologically active LepR in frog to the anterior preoptic area (location of neurosecretory neurons in the

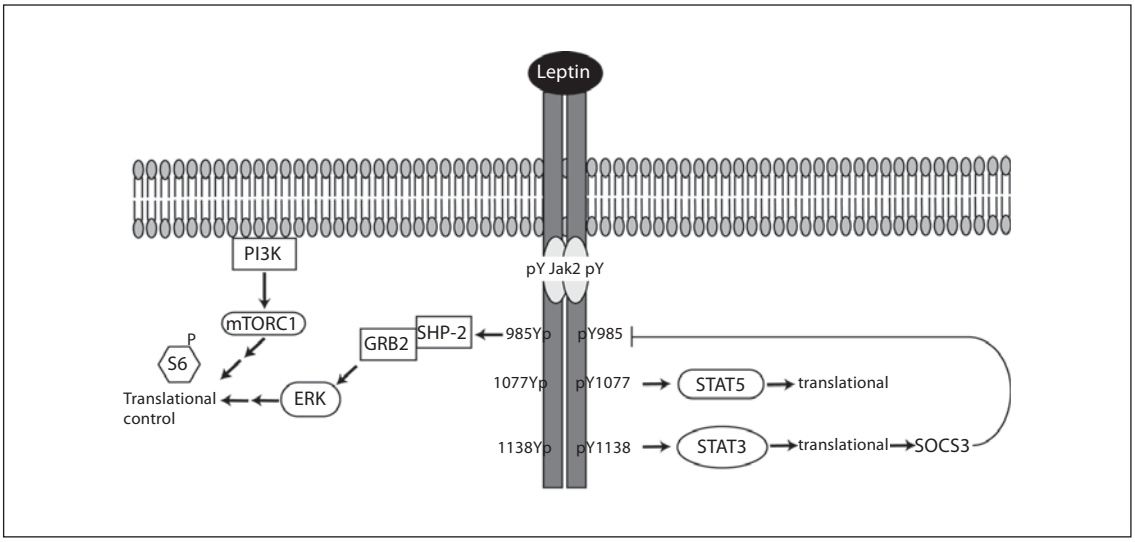
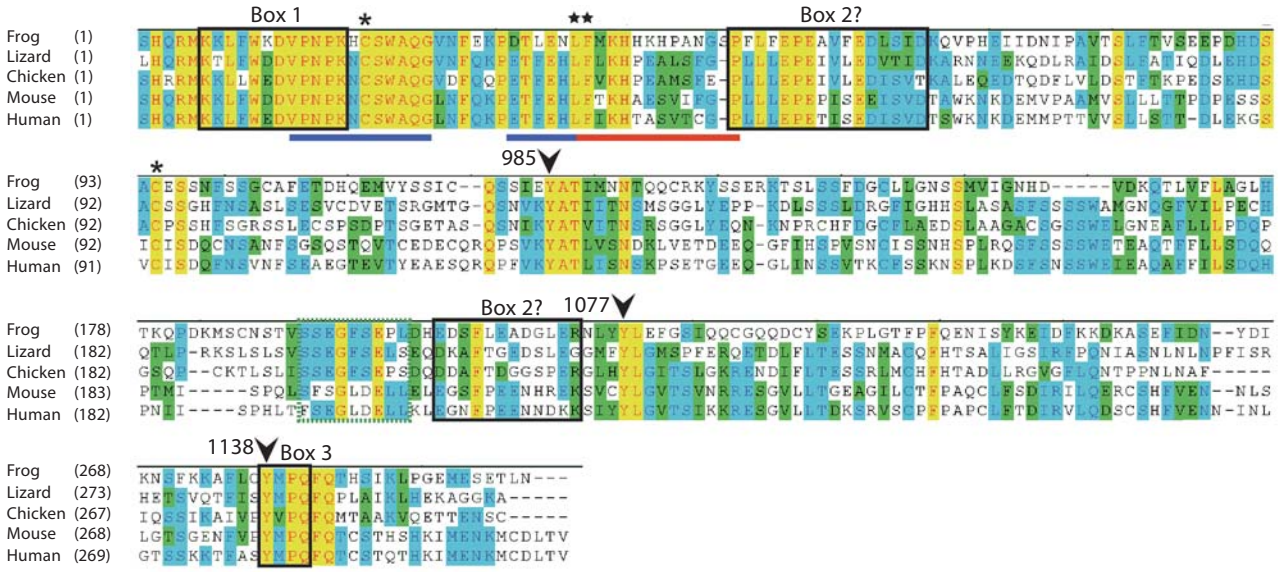


Fig. 5. Intracellular signaling pathways engaged by the leptin receptor (mouse LepRb). The LepRb forms a homodimer in the membrane. Leptin binds to the extracellular domain leading to a conformational change in the receptor, activating Janus kinase 2 (Jak2) which then phosphorylates (designated by 'p') three evolutionarily conserved tyrosine residues within the cytoplasmic domain of LepRb. The Y^{985} is required for activation of the SH2-containing tyrosine phosphatase-2 (SHP2)/extracellular signal-regulated kinase (ERK) cascade (activation of ERK via growth factor receptor binding protein 2 – GRB2) which leads to the phosphorylation of ribosomal protein S6 and increased translation. Y^{1077} is required for signaling by signal transducer and activator of transcription 5 (STAT5), while Y^{1138} mediates STAT3 signaling. STAT3 transactivates the suppressor of cytokine signaling 3 (SOCS3) gene, and SOCS3 mediates negative feedback on LepRb signaling via Y^{985}. The LepRb also activates the phosphatidylinositol 3 kinase (PI3K) and mammalian target of rapamycin 1 (mTORC1) pathways, but the mechanism is not understood. The diagram is based on figure 1 of Villanueva and Myers [104].

Fig. 6. Conserved structural features of the cytoplasmic domains of vertebrate leptin receptors. The alignment color scheme is as described in the legend of figure 2. **a** Alignment of tetrapod LepR cytoplasmic domains. Shown are frog [*Silurana (Xenopus) tropicalis*; NP001037866] lizard (*Anolis carolinensis*; online suppl. table 2), chicken (*Gallus domesticus*; NM204323), mouse (*Mus musculus*; AAC52705) and human (*Homo sapiens*; AAB09673). Box homology motifs 1, 2 and 3 are regions conserved among class I helical cytokine receptors [102]. Sequences within the Box 1 homology motif are important for Jak2 recruitment and activation [107, 108]. The existence, position and function of the putative Box 2 homology motif in vertebrate LepRs is uncertain ([107, 108]; the two predicted Box 2 homology motifs are shown with question marks). A region immediately N-terminal to the second Box 2 homology motif shows evolutionary conservation and is indicated by the dotted green box. Blue bars beneath the sequence alignments correspond to amino acids 13–24 (long bar) and 31–36 (short bar) in the mouse LepRb (numbering from the beginning of the cytoplasmic domain) that have been shown to be critical for Jak2 binding and activation [107]. The red bar corresponds to amino acids 37–48 which have also been shown to function in Jak2 activation but whose sequence requirements are more flexible than amino acids 31–36 [107]. Stars above the sequences indicate the two hydrophobic residues, leucine and phenylalanine, located at positions 36 and 37 shown by Bahrenberg et al. [108] to be indispensable for receptor signaling. The Box 3 homology motif is critical for recruitment and activation of STAT3 [109, 111]. The asterisks designate conserved cysteine residues. Arrows show conserved tyrosine residues in the mouse LepRb necessary for signaling via SH2-containing tyrosine phosphatase-2 (Y^{985} mouse; Y^{986} human; Y^{976} chicken; Y^{973} frog), STAT5 (Y^{1077} mouse; Y^{1079} human: Y^{1071} chicken; Y^{1066} frog) and STAT3 (Y^{1138} mouse; Y^{1141} human; Y^{1129} chicken; Y^{1127} frog). The comparable positions in the lizard LepR could not be fixed because the precise N-terminus of the protein has not yet been determined. **b** Alignment of three fish LepR cytoplasmic domains: salmon (*Salmo salar*; AB489201), zebrafish (*Danio rerio*; DQ007541) and pufferfish (*Takifugu rubripes*; AB385663). Shown are the predicted Box 1, 2 and 3 homology domains. The dotted green box corresponds to the conserved region in tetrapod LepRs that is N-terminal to the second putative Box 2 motif. The three conserved tyrosine residues are indicated by the arrows (with the corresponding amino acid position for the mouse LepRb). **c** Alignment of the LepR cytoplasmic domains of a representative teleost fish (zebrafish – *Danio rerio*; DQ007541), nonamniote tetrapod [amphibian – *Silurana (Xenopus) tropicalis*] and amniote tetrapod (human – *Homo sapiens*). Shown are the predicted Box 1, 2 and 3 homology domains. The dotted green box corresponds to the conserved region in tetrapod LepRs that is N-terminal to the second putative Box 2 motif. The three conserved tyrosine residues are indicated by the arrows (with the corresponding amino acid position for the mouse LepRb).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Box 2?
985
1077
Box 3
1138
Frog (1)
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Chicken (1)
Mouse (1)
Human (1)
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Lizard (92)
Chicken (92)
Mouse (92)
Human (91)
Frog (178)
Lizard (182)
Chicken (182)
Mouse (183)
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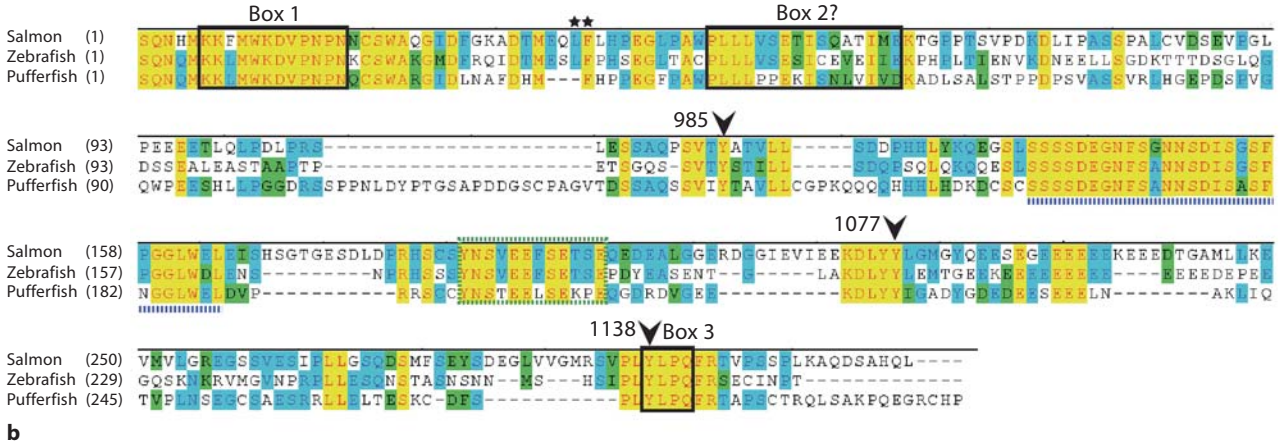
Frog (268)
Lizard (273)
Chicken (267)
Mouse (268)
Human (269)

a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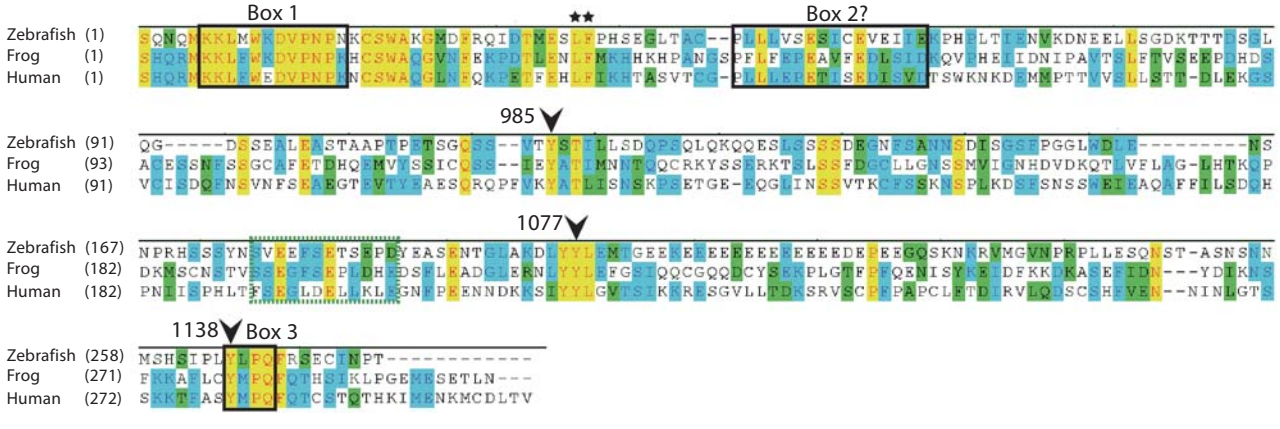
Pufferfish (245)

b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Human (182)
Zebrafish (258)
Frog (271)
Human (272)

c

frog brain), ventral hypothalamus and anterior pituitary gland [C. Hu and R.J. Denver, unpublished]. Similar to mammals, *LepR* mRNA is found in many different tissues in ectotherms [30, 63, 122] supporting that the hormone has the potential to have diverse influences on development and physiology. In the frog, *LepR* mRNA is highly expressed in brain, but of all tissues analyzed, *LepR* mRNA was highest in the pituitary gland [30], and leptin increased pSTAT3 immunoreactivity in the frog pituitary [C. Hu and R.J. Denver, unpublished]. These findings suggest that leptin could play a role in pituitary development and/or function [97].

Evolutionary Conservation of Leptin Actions on Food Intake and Metabolism

The major site of leptin action in mammals is the brain where it acts to inhibit appetite and increase energy expenditure [123, 124]. Leptin influences energy balance through its primary actions on hypothalamic feeding and autonomic control centers, and secondarily through its influence on hypothalamo-pituitary-adrenal (HPA) and hypothalamo-pituitary-thyroid (HPT) axes [46, 125, 126].

Before *Lep* genes were isolated from nonmammalian species, injections of recombinant mouse (or 'chicken') leptin into chickens was found to reduce food intake in one study [127] but not another [128]. In a lizard, injections of mouse leptin reduced food intake and increased metabolic rate [77]. Mouse leptin inhibited food intake in the goldfish [129] but not in other fishes (e.g. coho salmon, catfish, and green sunfish; [27]). Although these findings pointed to a similar role for leptin in the regulation of food intake and energy metabolism in nonmammals as in mammals, the use of heterologous hormone and the conflicting results obtained in different species prohibited definitive conclusions.

Recent comparative studies in nonmammalian species using homologous leptin preparations support an ancient role for leptin in regulating food intake and metabolism [30–32, 130]. The first demonstration of an anorexigenic effect of a homologous leptin in a nonmammalian species was shown in the frog, *Xenopus laevis* [30]. Intracerebroventricular injections of recombinant frog leptin (rxLeptin) in juvenile frogs strongly inhibited food intake, and this action developed in the tadpole during prometamorphosis. As in mammals, chronic administration of recombinant frog leptin reduced body weight of prometamorphic tadpoles [30]. Also, frog leptin injections caused food-deprived tadpoles to lose more weight than vehicle-injected controls, thus showing that leptin increases energy expenditure in prometamorphic tadpoles as it does in mammals. Murashita and colleagues [31, 130] showed that injections of recombinant rainbow trout leptin into trout decreased growth, inhibited feeding, decreased hypothalamic neuropeptide Y mRNA, and increased hypothalamic POMC mRNA. Li et al. [32] reported an anorexigenic effect of injected recombinant grass carp leptin on food intake in the carp.

Nutritional Regulation of Leptin and Leptin's Role in Body Weight Regulation

In mammals, leptin is secreted in proportion to fat stores and thus signals to the brain long-term energy balance [131, 132]. Fasting decreases plasma leptin concentration, while refeeding reverses this decline. Little information is available in nonmammalian species relating nutrition to leptin production. In carp, hepatic *Lep* mRNA levels were increased after feeding but did not change during long-term fasting [57]. The only nonmammalian species for which a leptin radioimmunoassay has been developed is the rainbow trout [133]. Paradoxically, plasma leptin concentration was elevated during fasting in rainbow trout [133]. This led the authors to conclude that the regulation of circulating leptin concentration in fish differs from mammals, and that leptin may not function as an adiposity signal in fish (at least not in salmonids). However, the fact that leptin injections inhibit appetite in nonmammals suggests that it can signal to the brain information about energy balance, and so more work needs to be done to test the hypothesis that leptin functions as an adipostat in nonmammalian species.

Summary and Directions for Future Research

Recent molecular cloning and functional studies have increased our understanding of the diversity of functions and evolutionary history of the hormone leptin. Despite low primary amino acid sequence conservation, leptins from diverse species are predicted to form similar tertiary structures and bind to the LepR, which leads to activation of common intracellular signaling pathways via highly conserved structural motifs located within the LepR cytoplasmic domain.

A major role for leptin in mammals is as an adiposity signal, acting on the hypothalamus to suppress food intake and increase metabolic energy expenditure. Leptin's actions on the brain are mediated by the long form leptin

receptor (LepRb), which leads to the activation of central melanocortin pathways that inhibit feeding. Similar actions of leptin on feeding have been discovered in nonmammalian species, although the hypothesis that leptin functions as an adiposity signal in nonmammals remains to be tested.

In mammals, leptin is an important indicator of body condition/nutritional state, signaling available energy for development, growth, and metabolism. Insufficient energy stores delay animal growth and development, and leptin's role as an adipostat suggests that it can influence the timing of energy-requiring developmental processes such as reproductive maturation (puberty in mammals) [96, 134, 135]. Females must achieve a minimum body size and body condition (i.e. fat stores) to initiate puberty. Leptin may play similar roles in nonmammalian species, signaling appropriate timing for developmental processes such as metamorphosis, a critical life history transition, and the onset of reproductive maturity, an energetically expensive process.

Findings of links between birth weight and adult onset metabolic disorders have focused on the relationships among leptin, growth and development during embryonic and fetal stages [136–140]. Circulating leptin is elevated in the human fetus during late gestation and correlates with fat mass and birth weight [141, 142]. Before the formation of adipose tissue, leptin and LepR are expressed in liver, heart, hair follicles, and primordial bone of fetal mouse [94, 143], and leptin is found in the circulation of fetal sheep [144]. Plasma leptin exhibits a surge during early postnatal development in rodents, and recent findings suggest that leptin plays an important role in controlling neurogenesis, and the maturation of feeding circuits in the hypothalamus [124]. *Lep* mRNA is expressed in frog oocytes and embryos before feeding stages and before adipose tissue formation [30]. *LepR* mRNA is expressed in tadpole hind limb, and injections of recombinant frog leptin accelerated hind limb development [30]. Thus, in addition to its integral role as a regulator of appetite and energy balance in juveniles and adults, these findings highlight the potential for important roles for leptin during early development.

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